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Xenotransplantation: Endothelial cell protection by low molecular weight dextran sulfate - mechanism of action

Master thesis

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from Fribourg

Declaration of Authorship

I herewith confirm that I wrote this thesis without external help and that I did not use any resources other than those indicated. I have clearly acknowledged all parts of the text where material from other sources has been used, either verbatim or paraphrased. I am aware that non-compliance with the above statement may lead to withdrawal of the academic title granted on the basis of this master's thesis by the Senate, according to the law governing the University of Bern.

February 2nd, 2018.

Contents

1 Abstract

Background: Low molecular weight dextran sulfate (DXS) of 5000 Da is known to be an inhibitor of the complement- and coagulation cascades by potentiation of C1 inhibitor. DXS can be used in the context of xenotransplantation to delay hyperacute rejection. It is supposed to act as a 'repair coat' by replacing the heparan sulfate proteoglycans (HSPG) that are shed from the glycocalyx of the activated endothelium. We hypothesized that DXS may protect the porcine aortic endothelial cells (PAEC) activated with normal human serum (NHS) by 'functionally replacing' the shed HSPG in our microfluidic *in vitro* model.

In the present study, the objective was to understand the mechanism of action of DXS. The shedding process of the endothelial glycocalyx (EG) was evaluated with NHS as well as the possible contribution of shear stress.

Method: We used a novel *in vitro* microfluidic model composed of vessel-like microchannels of 550 μm diameter, which were cast in a poly-dimethyl siloxane matrix. PAEC were cultivated under pulsatile flow in this microfluidic chip with the help of a peristaltic pulsatile pump, exposing the cells to a defined flow rate and shear stress. To mimic a pig-to-human xenotransplantation situation PAEC in the artificial blood vessels were exposed to NHS under different shear stress conditions. Shedding of HSPG the effect of DXS on this were analyzed by immunostaining and quantification of fluorescence intensity using a laser scanning confocal microscope.

Results: NHS induced a significant decrease of the fluorescence quantification about 28,83% of HSPG under static condition (p=0,0015). NHS induced a significant increase of E-selectin fluorescence quantification about 115,60% under static condition (p= 0,0203). NHS under flow of 10 dyn/cm2 induced a significant decrease of fluorescence quantification of HSPG about 37,59% (p=<0,0001). **Shear stress** induced a significant increase about 99,62% of the WGA-lectin/DAPI quantification under 20 dyn/cm^2 (p=0,0014) compared to static condition. Shear stress induced a significant increase of HSPG quantification about 85,63 % under 10 dyn/cm^2 (p<0,0001) compared to static condition. DXS did not produce any statistically significant results.

Conclusion: Contrary to our expectations concerning the protective role of DXS by acting as a "repair coat" in our microfluidic model, DXS did not significantly induce an increase of HSPG fluorescence quantification compared to the control microchip shed with NHS. Shear stress is inducing a significant increase of HSPG and WGA-lectin fluorescence quantification compared to static condition. Shear stress might induce an adaptation of the EG but it needs to be more in detail investigate.

2 Introduction

2.1 Xenotransplantation

Transplantation is currently the best treatment for many diseases associated with organ failure but the lack of organs, tissues and cells does not permit to fulfil the high demand. The gap between donor organ availability and the patients on the waiting list is increasing every year. (Figure1) The U.S. Department of Health reported that 20 people die each day in the U.S. while awaiting for a transplant. [93]

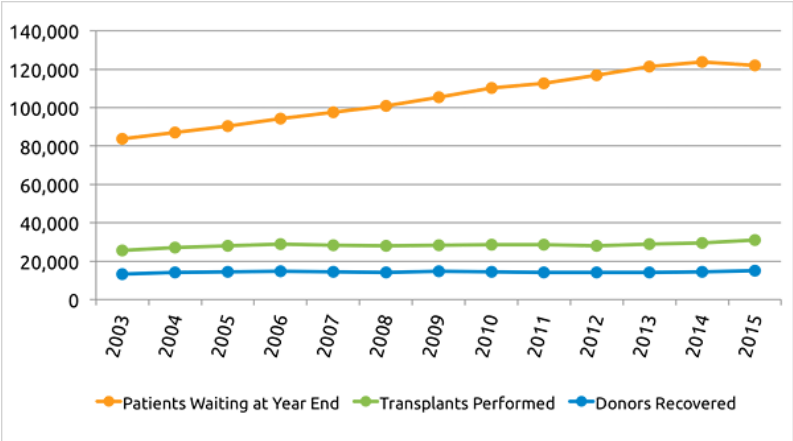


Figure 1: The organ shortage continues (U.S Department of Health & Human Services 2017)[93]

Xenotransplantation – the transplantation of living cells, tissues or organs from one species to another – could be a potential solution. The limitation is the physiological difference between the species, rejection of the graft and the risk of transmission of diseases (such as AIDS). The pig is the most suitable source of organs. It is the most relevant choice because of the short period of gestation, the reproductive capacity, the cost, the physiological and the anatomical proximity with humans.[91]

However, there are three main barriers that need to be overcome to make xenotransplantation clinically feasible: The immunological incompatibilities, the physiological barrier and the risk of zoonosis.[92]

The immunological incompatibility which leads to rejection is a major issue in xenotransplantation. There are four types of rejection: The **Hyper Acute Rejection (HAR)**, the **Acute Vascular Rejection (AVR)**, the **Acute T cell mediated** (or cellular) **rejection**, and the **Chronic Rejection**. Immunosuppressive regiments are able to modulate host response to a xenograft almost exclusively in the context of acute T cell mediated rejection and novel

strategies of immunosuppression to prevent HAR and AVR need(ed) to be developed for xenotransplantation.[14]

All the mammals except humans and Old World monkeys are expressing the **αGal** epitope on their cell surfaces. Humans, apes and old world monkeys produce **anti-αGal antibodies**. The presence of these antibodies forms an immunological barrier between species. This **αGal incompatibility** is at the basis of HAR.
Major improvements in **genetic engineering** allow for a prolongation of the survival of the xenograft in pig-to-non human primate models. These improvements comprise the production of knock-outs or gene overexpression in animal species suited for xenotransplantation. The **αGal** epitopes are produced by the glycosylation enzyme α1,3-galactosyltransferase. The knock-out of the α1,3-galactosyltransferase gene (**GTKO**) in the porcine cells made it possible to overcome the HAR.[28]

The risk of zoonosis is another reason why xenotransplantation should be approached with caution. A porcine organ can unfortunately also carry viruses, bacteria and parasites that can be potentially transmitted to humans.[69] The pig genome contains porcine endogenous retroviruses (PERV) which can be potentially transmitted to humans by mutation and possibly cause disease.[8] However, a successful removal of the PERV gene from the pig genome has been achieved.[31]

The endothelial glycocalyx is a structure where the natural antibodies are directed against it and initiating the rejection of the xenograft.[56] It's an important structure in a context of xenotransplantation.

2.2 Endothelial Glycocalyx

2.2.1 Overview

The endothelial glycocalyx (EG) is a carbohydrate-rich surface layer consisting of **glycoproteins**, **proteoglycans** and **glycosaminoglycans** (GAG**)**. The endothelial glycocalyx is mainly composed of glycoproteins bearing acidic oligosaccharides, terminal sialic acids, proteoglycans like HSPG and glycosaminoglycan side chains.[82] The EG plays many important roles in **vascular physiology**, **mechanotransduction** (shear stress and pressure sensor), **vascular permeability**, **signaling** and **blood cell – vessel interactions**. The glycocalyx and

its dysfunction are closely linked to many diseases such as diabetes, atherosclerosis, ischemia and reperfusion. This structure should not be seen as a static structure but as a dynamic one.[63]

The glycocalyx is closely linked to the endothelial cell surface with proteoglycans and glycoproteins. The network formed by this structure can contain soluble molecules. There is a steady state between the blood flow and the composition of the glycocalyx which is continuously under the influence of the vascular environment. **The EG is a dynamic structure** which responds to different stimuli like shear-stress, enzymatic stimuli, presence of inflammation, etc. It is continually shaped and turned-over by the biosynthesis of new glycans and shear-dependent shedding. The EG is a **flow-dependent structure**. The glycocalyx thickness can decrease when exposed to high flow and this can affect its properties to resist to flow and its adhesion properties to blood cells.[45,89]
The thickness of the EG can go from 0.5 µm in small muscle capillaries to 4.5 µm in carotid arteries.[48,72,73]

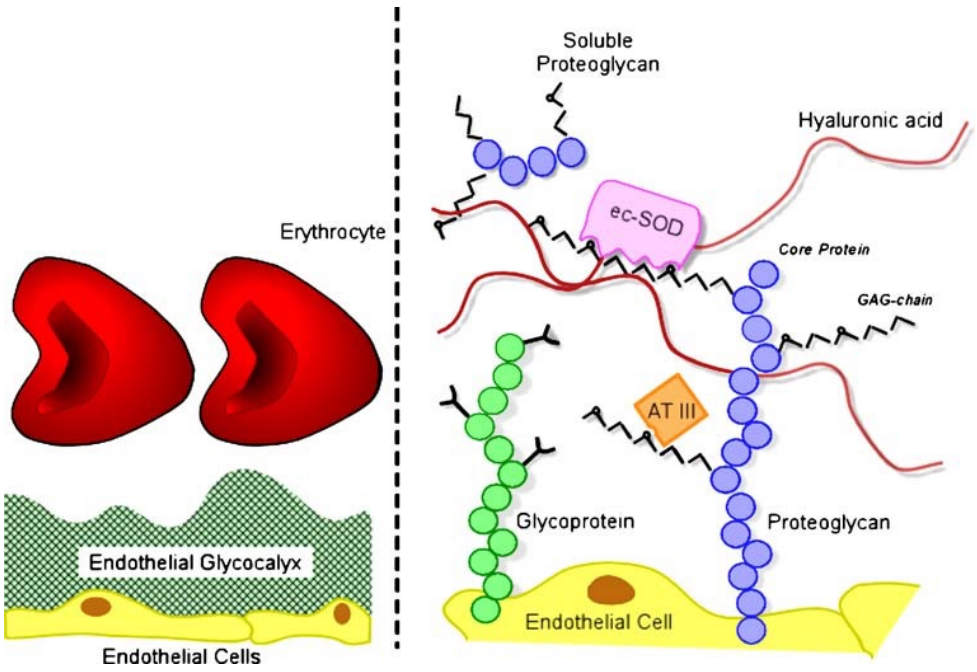


Figure 2: Schematic representation of the endothelial glycocalyx, where we can see the main components. Left: EG in contact with the blood cells on the top of the endothelial cells. EG is a membrane bound and soluble molecule structure. Right: glycosaminoglycan side chains (GAG-chain), hyaluronic acid, glycoprotein, soluble proteoglycans, superoxide dismutase (ec-SOD) and antithrombin III (AT III) are incorporated in the EG.[63]

2.2.2 Proteoglycans

The Proteoglycans form the backbone of the glycocalyx. A proteoglycan is a **core protein** to which one or more **glycosaminoglycan (GAG)** chains are connected. The most common core proteins are the **syndecans** and the **glypicans** which have a strong connection with the cell membrane.[64]

The secreted form of proteoglycans, like mimecan, perlecan and biglycan, where the GAG chain is modified, leads to soluble proteoglycans. They can stay in the glycocalyx or go in the blood flow.[36]
The proteoglycans can be linked by various types of glycosaminoglycan chains. Different stimuli and the environment can change the variety of the glycosaminoglycans.

Five distinct **GAG chains** have been identified: Heparan sulfate, chondroitin sulfate, dermatan sulfate, keratan sulfate and hyaluronan (or hyaluronic acid). They are composed of linear **disaccharide** polymers with different lengths and they can be modified by variations in sulfation and/or (de)acetylation. The disaccharides are formed by uronic acid and hexosamine.[63]

2.2.3 Glycoproteins

Glycoproteins are also influencing the connection of the glycocalyx with the endothelial cell membrane. The structure of the glycoproteins is characterized by small and branched carbohydrate side chains.
The endothelial cell adhesion molecules (**ECAMs**) are glycoproteins that are responsible for cell recruitment and cell signaling. There are three main families of ECAM in the endothelial glycocalyx, which are the **integrin** family, the **selectin** family and the **immunoglobulin** superfamily. The selectins linked with the vascular endothelium are **E-selectin** and **P-selectin**. These selectins are at the forefront of leukocyte-endothelial cell interactions.[68]
The integrins are found on many cell types such as endothelial cells, leukocytes and platelets. They play a role in the interaction with the extracellular matrix ligands; more precisely with laminin, fibronectin and collagen. Some famous members of the immunoglobulin family are the Intercellular Adhesion Molecule (ICAM), the vascular cell adhesion molecule (VCAM) and platelet/endothelial cell adhesion molecule (PECAM). These molecules are ligands for integrins on leukocytes and platelet. They are responsible for leukocyte homing and diapedesis.[53]

The EG is a **mechanotransductor sensor.** The blood flow through the vessels exposes the endothelium to a mechanical shear stress that can influence the cell behavior, morphology and function. The glycocalyx layer around endothelium plays, besides a functional role, also a major role as a sensor detecting mechanical cues.[18,21] The exact mechanism is not fully understood yet.
The endothelial cells produce nitric oxide (NO) in a context of vascular regulation when they are exposed to shear stress.[65] A treatment with heparinase can specifically destroy the heparan sulfate and this may lead to inhibition of both steady and oscillatory shear-induced NO.[25] **The glycocalyx thickness** is proportional to the shear-stress profile. The glycocalyx composition and thickness seems to be shear-dependent.[5,81,80]

The shedding of glycocalyx (heparan sulfate) is of one the main issues in xenotransplantation. This shedding and the reduction of some structure and proteins (HSPG) on the EG is triggering the defense mechanism like the complement or the coagulation pathways. Finding a way to reduce this shedding will help to delay the rejection of the xenograft.[43 57]

2.3 Heparan Sulfate & Dextran sulfate

2.3.1 Heparan sulfate

Heparan sulfate proteoglycan (HSPG) is one of the major components of the endothelial glycocalyx. The endothelial cells contain a surface layer of HSPG. HSPG is one of the components which are important to maintain the steady state environment of the endothelium with anticoagulant and anti-inflammatory substances. The endothelial cells can be activated by different stimuli that leads to the shedding of glycocalyx. This can expose the endothelium to a procoagulant and proinflammatory environment.[57]

Heparan sulfate is one of the dominant proteoglycans in the vasculature, roughly 50% to 90% in the glycocalyx. The chondroitin sulfate/dermatan sulfate is in the second most prevalent GAG in the EG.[35,60]

What could be the role of the HSPG in the endothelial cells? HSPG acts as a **barrier** for protein diffusion and cell migration. It is responsible of the **blood fluidity** by tethering and activating the antithrombin III and tissue factor pathway inhibitor. It regulates the **initiation of**

the complement system. The HSPG is also have a role in the inflammation process and immune cell activation by linking chemokines and cytokines to the endothelial surface.[58]

The HSPG plays a substantial role in the xenograft rejection process. For example, it has an important impact **on T cell activation** process which leads to the rejection of a potential xenograft.[77]

2.3.2 Dextran sulfate

Dextran sulfate (DXS) is a synthetically sulfated, linear or slightly branched polysaccharide substance. The low molecular weight (5000 Da) form of DXS has been shown to **inhibit the coagulation** and all the pathways of the **complement cascade** dose-dependently.[44] DXS could potentially play a role as an endothelial cell protector by replacing shed HSPG after traumatic events like ischemia/reperfusion injury, or xenotransplantation. DXS is supposed to act as a "repair coat" of the EG. In a study, DXS was incubated with porcine endothelial cells and it was demonstrated that DXS protects the endothelium cells by binding to it and by avoiding the complement system and NK cell defense mechanism.[44]. DXS inhibits the classical pathway of the complement system mainly by **enhancing the activity of C1-inhibitor**.[78] The alternative pathway is inhibited by DXS via its interaction with the polyanion binding site of factor H.[49] DXS could potentially delay HAR and improve the initial survival of a xenograft.[24]

2.4 Shear stress

The shear stress leads to an endothelial stress and circumferential wall stress of the blood vessels because of the blood pressure and the flow. The morphology of the vascular endothelium changes under this mechanical forces but it is also initiating biochemical and biological process. A disturbance of this mechanical stress on the blood vessel endothelium causes an imbalance in biochemical homeostasis which leads to a vascular remodeling and possible dysfunction.[46]

The EG is a signaling center from where the cells can detect the shear stress and respond by inducing the **remodeling of the glycocalyx** (heparan sulfate).[82] Shear stress is responsible for example for clustering main components of the EG like glypican-1 (HSPG).[89]The exposure of the EG to shear stress induces its remodeling. The thickness of the glycocalyx is changing to resist to the change of the shear stress. Some specific components like HSPG, chondroitin sulfate, glypicans-1 or syndecan-1 of the EG are enhanced. [89] The change in

mechanotransduced signals due to a variation of the shear stress will induce glycocalyx reorganization which might be space and time dependent.[89] As a consequence of the shear stress the actin cytoskeleton (F-actin) is adapting by an alignment in the direction of the flow.[82]

The HSPG is a key player in the mediation of the response of cell proliferation, motility and actin cytoskeleton adaptation for shear stress.[79,71,51] A shear stress of 15 dyn/cm^2 is enough to induce a change in HSPG (clustering) via glypican-1. Long-term adaptation to the shear stress of the glycocalyx is mainly associated with neo-synthesis of its components and the actin cytoskeleton.[89] The HSPG starts to decrease after 30 minutes of exposure to shear stress (clustering) and then after 24 hours it HSPG start to be restored.[89]

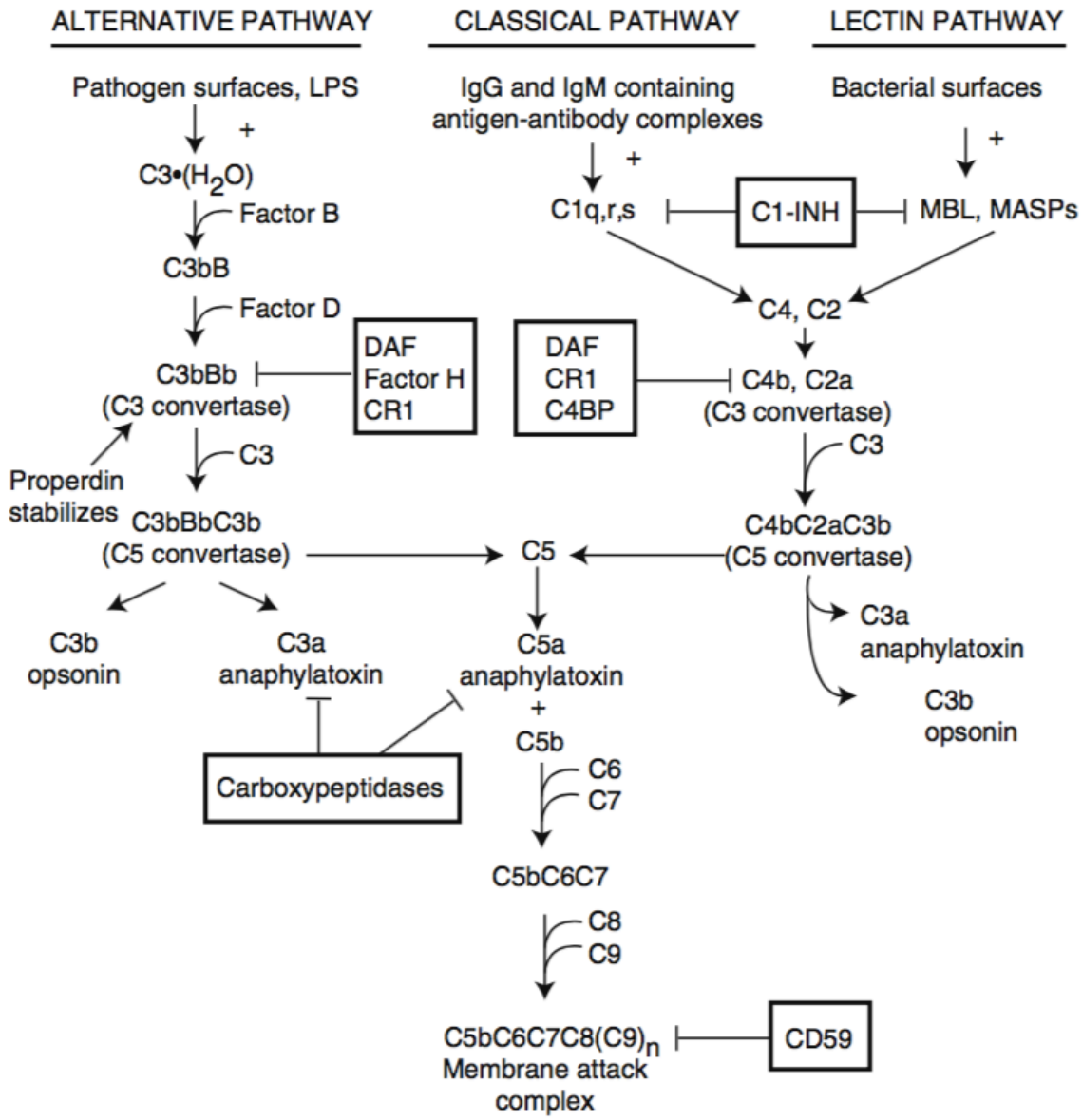
2.5 The complement system

The complement system is part of innate immunity and as such one of the defense weapons of the human body. It consists of a network of proteins that are responsible for the host defense and inflammation processes. Thanks to this tool, humans are protected against foreign noxious pathogens.[66]

The complement system involves three different pathways, the **classical**, the **alternative**, the **lectin** pathway, that are able to recognize different specific pathogens and activate the adequate processes to exterminate them. These three different pathways are responsible for **opsonization**, **phagocytosis** and **lysis** of the target cell. The complement system is closely interconnected with the **coagulation cascade.**
Regulating the complement system to tolerate xenotransplantation is crucial in order to enhance the survival time of the xenograft in the human body. This is a potentially high impact target to improve xenotransplantation. There is some possible way to balance the effect of the complement system.[84,24] For example, genetic engineering is used to express the human membrane cofactor protein (hCD46) in porcine tissue to attenuate the complement mediated rejection and to give better xenograft outcomes.[15,41]

The complement system is also an important key player in the innate and adaptive immune system. It is the bridge which connects the two parts of the immune system together.[23]

Figure 3: The three different pathways of complement activation: The alternative, the classical and the lectin pathway. The inhibiting factors are in boxes. The different stimuli that activates the different pathways is shown. The aim of the complement cascade is to produce the Membrane attack complex to eliminate the initial pathogen stimuli.[66]



2.5.1 Complement activation Pathway

The network of proteins of the complement pathway consists of approximately 30 proteins that are mainly zymogenes. All the three pathways converge at the level of the **C3 protein**. The activation of the pathway leads to the initiation of factor that leads to a cascade of enzymatic

reaction. This results in the formation of C3a and C5a. The anaphylatoxins **C3a** and **C5a** pilot an arsenal of physiological answers in order to react against the danger.[66]

2.5.1.1 Classical Pathway

The classical pathway starts with the formation of an **immune complex** with the binding of IgG or IgM to a foreign antigen. Immune complexes are then recognized and bound by C1q, leading to the C1 complex, which is composed of C1q, C1r and C1s molecules that bind to the Fc part on IgG or IgM. The activated C1s can induce the cleavage of C4 and C2, which leads to the formation of CP C3 convertase (C4bC2a). The C3 convertase is responsible of the amplification of the complement system by acting as an opsin.[66]

The classical C3 convertase C4b2a then catalyzes the proteolytic cleavage of C3 to C3b and C3a. The larger C3b fragment acts as opsonin or it can bind to the C3 convertase to form the C5 convertase. The C5 convertase cleaves C5 into C5a and C5b. C3a and C5a are called anaphylatoxins and are responsible for the recruitment of immune cells to the site of complement activation. C5b contributes to the initiation of the terminal pathway of the complement system which leads to the formation of the so-called **membrane attack complex** (C5b-9, MAC). This leads to formation of a pore in the cell membrane and contributes to the cell lysis.[66]

2.5.1.2 Alternative pathway

The alternative pathway is initiated by recognition of certain polysaccharides, lipids and proteins found on foreign pathogens.[62] The continuous low-grade hydrolyzation of C3 to form C3b-H_2O is necessary for his binding to the pathogens. Some other factors like Factor B, Factor D are also required to form the alternative C3 convertase C3bBb.[66]

2.5.1.3 Lectin pathway

The stimuli that activate the lectin pathway is mannose binding lectin (MBL) or ficolin that bind to carbohydrates on pathogens like yeast, bacteria, parasites and viruses. MBL and ficolin are traveling in the blood stream, bind to the above-mentioned activating surfaces similar to C1q-binding of immune complexes, and then lead to binding of MBL-associated proteins (MASP) which in turn activate C4 and C2 to form the C4b2a convertase of C3. There are 4 distinct specific MASP. Certain pathogens also directly bind to MASPs, induce a structural change that leads to autoactivation and the inducing of the cleavage of C4 into C4a and C4b. [66]. Lectin-pathway initiated complement activation will then feed into the classical activation route to

destroy the initial noxious stimulus. The result is the formation of the MAC complex which can destroy the cell by creating a pore in the cellular membrane.[74,70]

2.5.1.4 C3 independent pathways

Some substances can bypass the traditional complement pathway and lead to complement activation. Neutrophils and macrophages can release substances like kallikrein, plasmin and factor XIIa that lead to complement activation.[75,34] Some other factors like thrombin, which is a key player in the coagulation cascade, can lead to direct activation of C5 and generate C5a.[33]

2.6 Hemostasis & Coagulation system

The coagulation system is a key player in human physiology. It is responsible for the healing process during an injury leading to blood clot. The system is classified into two distinct pathways: the **extrinsic pathway** and the **intrinsic pathway**.[55]

The coagulation system is a cascade because of the proenzymes in the first rank are leading to the activation of other enzymes downstream. This typical way of activation amplifies the initial stimuli like the fall of “dominos”. The aim of this type of activation is to have a quick and strong response of the coagulation cascade to prevent blood loss and start the healing process.[3]

Hemostasis means the suppression of bleeding. The clot formation occurs in the coagulation process and it is under the control of inhibitors to maintain an equilibrium. The equilibrium is disturbed when an event enhances the pro-coagulants or decreases the activity of the inhibitors.

2.6.1 Primary hemostasis

2.6.1.1 Platelet plug

Primary hemostasis starts with the “platelet plug” initiation. The endothelial cells exposed to the lumen express antithrombotic factors like negatively charged heparin-like GAG, neutral phospholipids, platelet inhibitors, coagulation inhibitors and fibrinolysis activators. In opposite, the layer under the endothelial cells express thrombogenic factors like collagen, von Willebrand (vWF) factor and proteins that play a role in platelet adhesion. When this layer is exposed to the blood because of an injury, the pro-thrombotic events are initiated.[85,90]

Platelets are key players in hemostasis. They are responsible for the first step of hemostasis which brings the formation of the platelet plug. They generate from megakaryocytes. They are disk shaped and anucleated. The platelet plug gives a backbone for the concentration of activated coagulation factors that results in the formation of fibrin-platelet aggregates. The formation of this relevant structure called platelet plug go through several steps that are: **Platelet adhesion**, **secretion** and **aggregation**.

2.6.1.2 Platelet adhesion

The platelet adhesion starts after an injury in the vascular vessel wall that brings vWF factor in contact with the circulating blood and acts as a bridge between the endothelial collagen and the receptor GpIb on the platelet surface.

2.6.1.3 Platelet aggregation

The platelet aggregation is the next step. The activated platelets produce a factor called thromboxane A2 (TxA2) that enhances the platelet aggregation process. The initial platelet plug is a transient seal of the injury and it is remodeled to make the stronger and durable structure of the platelet plug.

TxA2 is working in synergy with ADP to form the platelet plug. The binding of the ADP leads to a change in the structure of the surface receptor of GPIIb/IIIa causing fibrin deposition. Thrombin cleaves fibrinogen into fibrin which is a more stable platform for the platelet plug and it is a part of the secondary hemostasis.[55]

Prostacyclin is an inhibitor of platelet aggregation. There is a balance between prostacyclin and TxA2 that is responsible for a precise, locally confined local platelet aggregation. This equilibrium is preventing the expansion of the clot further than the local injury.[90]

2.6.2 Secondary hemostasis (Coagulation cascade)

The **secondary hemostasis** where the real coagulation process occurs is divided in the extrinsic and the intrinsic pathway. These two pathways converge on the activation of the factor X, ultimately leading to the transformation of soluble fibrinogen into insoluble fibrin strands.[9]

2.6.2.1 Extrinsic pathway

The extrinsic pathway starts with **the impulse of the tissue factor** which is released by the sub-endothelial tissue because of an injury.[9] In a steady state situation, the tissue factor is

separated from the pro-coagulant substances of the blood by the vascular endothelium. It binds to and cleaves coagulation factor VII, which in turn, via FIX and IXa, leads to activation of factor X into Xa.[54]

2.6.2.2 Intrinsic pathway

The intrinsic pathway begins with **contact activation**. It is a secondary pathway for the activation of thrombin by factor XII. The complete activated enzymatic cascades result in the activation of the factor X.

2.6.3 Coagulation system and xenotransplantation

The coagulation system plays an important role in xenotransplantation. It is not only a rescue system which includes platelets and clotting factors of the injured vascular wall. There are many key players: platelets, endothelial cells, monocytes, erythrocytes and also molecular compounds like coagulation factors, inhibitors, fibrinolysis factors, cytokines, etc. This system is not only responsible to form a clot. The complement system is also involved in other physiological response like tissue repair, autoimmunity, arteriosclerosis, tumor growth and metastasis.[9]

The coagulation system can be activated by a xenograft, which represents a foreign stimulus recognized as an injury and triggers the process of hemostasis. This activation leads to the rejection process.

2.7 The rejection

The immune mechanisms of the human body contribute to xenograft rejection. Several main key players are involved in rejection mechanisms like the complement system and the coagulation system. These systems must be regulated in order to avoid rejection episodes.[92]

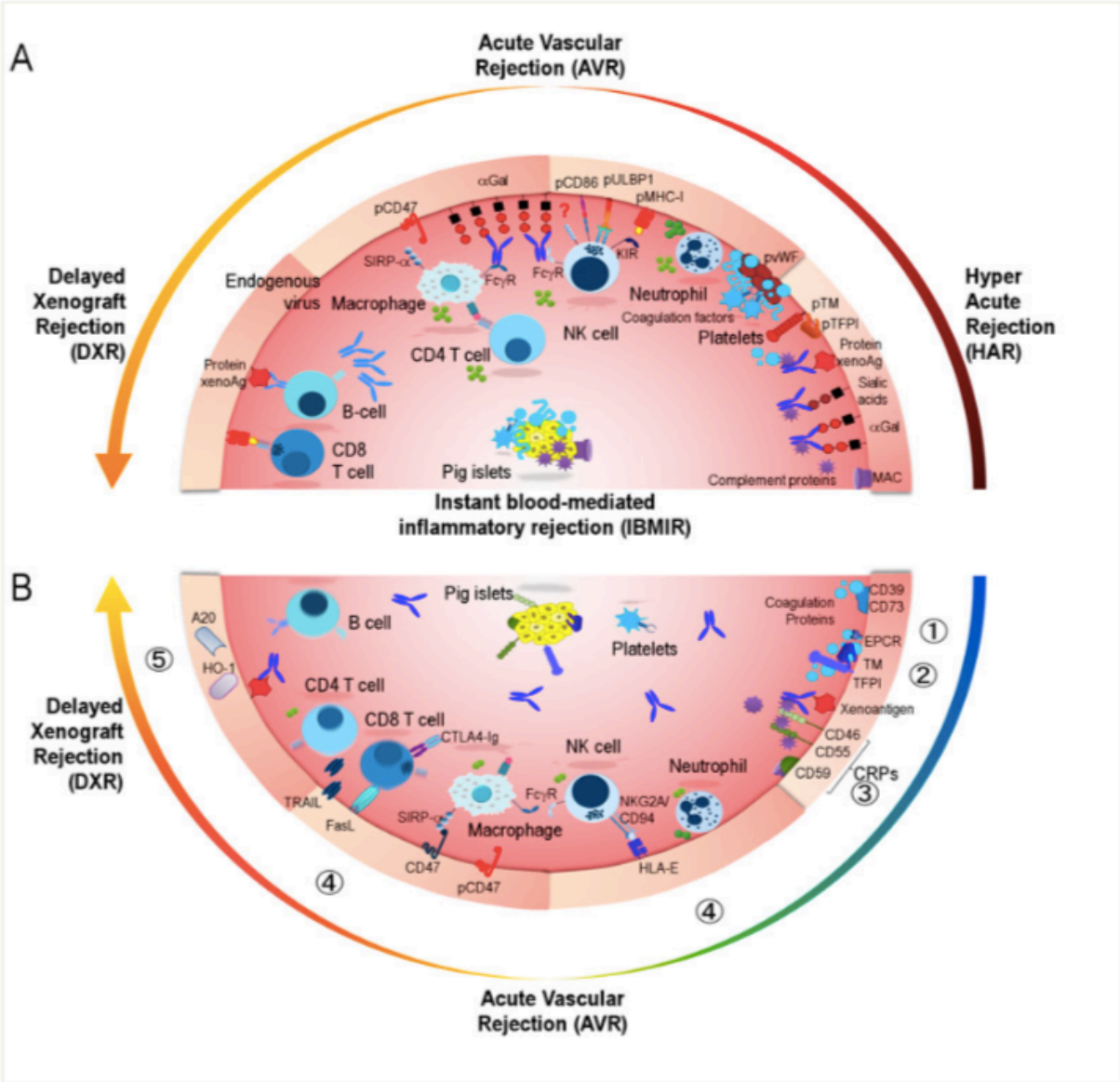


Figure 4: Immunological and coagulation hurdles in pig-to-human xenograft rejection and genetic modifications to overcome [92]

2.7.1 Immunological Barrier

The immunological barrier is responsible of the rejection of the xenograft. The delay before the rejection depends of the compatibility and tolerance of the host. There are two kinds of

xenotransplantation the one with relatively close species called **concordant** and the other one between far related species **discordant**.[47,11]

The concordant case is for example the xenotransplantation between monkey and human where the rejections occurs after several days due to the absence or low level of antibody of the host against the donor. The discordant case is for example the xenotransplantation pig-to-human or pig-to-monkey. The rejection process occurs already within minutes after the vascularization of the xenograft.[47,11]

2.7.1.1 Hyperacute rejection

Hyperacute rejection (HAR) is the first observed immunological barrier when a xenotransplantation is performed in pig-to-human model for example. The HAR is characterized by hemorrhage, edema, thrombosis and a lack of cell infiltration. There are three key players involved in this process, the natural antibodies, porcine endothelium and the activation of the complement system. The xenograft is rejected within minutes to hours.
To prevent rejection in discordant xenotransplantation, the innate and acquired immunity needs to be overcome. This is in contrast to ABO-compatible allotransplantation, where only acquired immunity needs to be overcome.[59,47]

Humans and non-human primates develop natural antibodies against the Galα(1-3)Gal (αGal) disaccharide, which is present on many bacteria, viruses and parasites. These antibodies react against the porcine endothelium which expresses αGal epitopes. The binding of natural antibodies produced by the human body activates the complement system. This leads to rejection and destruction of the xenograft. The removal of this antigen by genetic engineering improved the survival of the xenograft.[26,4,27,17]

2.7.1.2 Acute vascular rejection

If the HAR is overcome, the **acute vascular rejection** will occur after **some days to weeks**. There is an important production of **anti-Gal IgG antibody** and other anti-pig antibody specificities in a tight collaboration with immune cells like NK cells, macrophages, neutrophils and lymphocytes. The cellular components are a part of the innate immune system. The fixation of this substances on the porcine endothelium cells leads to cytotoxic cell destruction.[19,67,47] Most importantly, however, the fixation of the antibodies will activate the endothelium that will acquire a pro-coagulant state and facilitate to thrombus formation. The

activated endothelium can express some specific markers like E-selectin, P-selectin and ICAM, which will attract immune cells and accelerate the process of rejection.[38]

2.7.1.3 Delayed xenograft rejection

The delayed xenograft rejection (DXR) is an immunologic response occurring in a second phase of rejection. DXS appears in xenograft where HAR has been overcome. It's due to a recurrent activation of the barrier that has been encountered in HAR. The mechanism behind DXR involves the infiltration of activated macrophages, natural killer cells, platelet aggregation, fibrin deposition and endothelial activation. The activation of innate defense mechanisms and failure of regulatory mechanisms contribute to this form of rejection.[30]

2.7.2 Physiological Barrier

Porcine organs are physiologically and structurally slightly different from human organs. After breaching all the immune barriers, the long term compatibility of the physiology of porcine organs can be assessed. They are differences between the human and the porcine physiology like the body temperature or metabolic efficiency. For example, the heart is not only a pump but produces substances like natriuretic peptides. The natriuretic peptide is produced in the right atrium and it regulates many physiological parameters like the blood pressure. It is still unknown if the porcine natriuretic peptide can be effective for humans in a xenotransplanted heart.[87]

2.8 Genetic modification of donor pigs

Xenotransplantation is facing a new era with the discovery of precise genetic engineering tools like **CRISPR/Cas9 technology**. The generation of transgenic pigs and novel immune suppression therapies are opening a new path. The development of the above-mentioned techniques is allowing xenotransplantation to progress and to increase the survival time of xenografts. Genetic modifications are used to reduce the barrier issues between the species and to regulate the plasma cascade systems. Thanks to that xenotransplantation is becoming more and more a reality that is reachable[15]

2.8.1 Genetic engineering – Transgenes

Genetic engineering of donor pigs has helped to improve xenograft survival. Genetic overexpression of regulatory proteins and knockout of the so called xeno-antigens help to overcome some of the interspecies incompatibilities.

2.8.1.1 GTKO

The α1,3-galactosyltransferase gene-knockout (GTKO) has shown to successfully improve graft survival. This enzyme is responsible for the production of the Gal antigen on the cell surface. The knock out of this enzyme eliminates the expression of Galα(1,3)Gal.[50]
The naturally occurring **anti-αGal antibodies** in humans cannot find any binding site on GTKO pigs and this leads to the reduction of the incompatibility and a better acceptance of the graft. A study demonstrated that GTKO pig hearts transplanted into baboons substantially increased the survival of the xenograft in the host and **avoided hyperacute reaction.**[40]

2.8.1.2 hCD46 - hCD55 - hCD59

The complement regulatory proteins (CRP) are responsible for the regulation of the complement system. CD46 – CD55 – CD59 are CRP which down regulate the complement system to protect the cells from being damaged. CD46 is called membrane cofactor protein, CD55 is a decay-accelerating factor and CD59 is a MAC-inhibitory protein.[83]

CD46 is expressed ubiquitously on most of the cells and it is responsible for the protection of the cells against the complement system. Human CD46 (hCD46) has been (over-)expressed in pigs and hearts from such genetically modified pigs have been transplanted into baboons. The overexpression of hCD46 has been demonstrated to efficiently inhibit the complement pathway and overcome the hyperacute rejection.[83]

CD55 knows as “Decay acceleration factor” is responsible for the acceleration of the decay of the C3 and C5 convertases of the complement system. It is contributing to inhibit the complement pathway. Overexpression of the hCD55 is know to help against complement deposition and delay the rejection in a xenotransplantation situation.[10,93]

CD59 protein is an inhibitor of the membrane attack complex that leads to the protection against the complement system and less tissue damage.[22] Transgenic pigs which are able to express the hCD59 have been used in several studies to demonstrate that it is possible to overcome the HAR.[20]

A strong **immunosuppressive therapy** combined with transgenic pigs are essential for the xenograft to survive longer. The immunosuppressive drugs are an essential component for the acceptance of the xenograft by the human physiology. The success of xenotransplantation is related to the use of the immunosuppressive agents. This topic is briefly mentioned and not explained in detail. The survival of the host is a delicate balance between the immunosuppressive agents and transgenic modifications.[14]

2.9 Aim of the project

In this present study, we aimed to assess the **effect of low molecular weight** (5000 Da) **dextran sulfate** on the endothelial glycocalyx in a context of a xeno-environment. DXS is known to induce complement system and coagulation regulation. In a second part, we wanted to assess the effect of the **shear stress** on the EG to have a better comprehension of the HSPG adaptation response.

To perform our study, we used **a novel in-vitro microfluidics model** with porcine aortic endothelial cells (PAEC) grown in a PDMS vessel-like channel of 550 μm diameter where a pulsatile flow with a peristaltic pump was applied. All the parameters were precisely controlled such as shear stress, viscosity and flow rate to be as close as possible to the physiological in-vivo situation. The fluid which is circulating through the tubings is composed of DMEM media with 4% dextran and normal human serum (NHS). This interface between the PAEC and the circulating NHS is mimicking a pig-to-human xenotransplantation setting.
With the used microfluidic system, it is possible to assess rejection processes, the effectiveness of transgene expression and the role of drugs which are supposed to increase the acceptance of a xenograft. This model was developed to help scientists to to predict the effectiveness of drugs or other treatments with a reduced need for animal experimentation.

2.9.1 Hypothesis 1: DXS restores the HSPG layer

We hypothesized that DXS, as analog to the natural HSPG, should replace shed HSPG in the EG. The replacement of the EG with DXS may help to restore the main properties of surface regulation of the coagulation and complement systems. This is supposed to lead to an improvement of the graft survival time and delay the rejection process in a xenotransplantation situation.

2.9.2 Hypothesis 2: Shear stress

We hypothesized that different shear stress should impact the HSPG on the EG. The shear stress experiment was conducted to have a better understanding of the behavior of the EG. We believed that during an increase of the shear stress, the EG HSPG might start to shed and afterwards there could be adaptive reaction where the HSPG starts to reconstruct and to increase its thickness.

3 Materials and Methods

3.1 Cell Culture, NHS and PBS

The Pig Aortic Endothelial Cells (PAEC) were isolated by ourselves from pig aortas. PAEC were cultured in DMEM Glutamax / D-Glucose Medium (gibco) supplemented with 10% FBS (seraglob), 1 % penicillin-streptomycin (gibco) and 2 ml ECGM growth factor supplement (PromoCell). Passages 3 to 7 were used for the experiments. The medium was changed every 2 days.

To acquire Normal Human Serum (NHS), the blood was obtained from healthy voluntary donors into a 9 ml syringe (SARSTEDT S-Monovette) to activate the coagulation. The obtained blood was kept during 30 minutes at room temperature then it was centrifuged at 2000 rcf for 10 minutes. The serum was aliquoted and stored at -60°C.

To prepare the phosphate buffered saline (PBS) 1 x working buffer, pH 7.4. First the 10 x PBS was prepared for a stock of 1L. We add: 80.0 g NaCL (Merck), 2.0 g KCL (Merck), 14.2 g Na2HPO4-2H20 (Merck) and 2.0 g KH2PO4 (Merck) in 1000 ml H20 (ultra pure water). This 10 x stock solution was diluted at a ratio of 1:10 with pure water to form the PBS, 1 x working buffer. To prepare the PBS $^{++}$, 5 ml of 0.03M CaCI2 x 2H20 (Merck) and 5 ml of 0.1M MgCL2*6 H20 (Merck) were added in 1000 ml of 1 x working PBS buffer.

3.2 A novel *in vitro* microfluidic model

The cell culture in vitro assays have been the principal source of data for biomedical research for decades. However standard assays often fail to reproduce the real *in vivo* environment and this aspect can introduce a huge difference in the physiology and in the real functionality of the cells.[12] Standard cell culture based assays often failed to predict drug response in pharmacodynamics/pharmacokinetic studies and to represent the tissue function *in vivo*. A difference, for example, is that the *Petri dish* where the cells are cultivated are a 2D environment, but in the human body they are in a complex 3D environment.
The microfluidic system used in the present study aims to mimic as close as possible the real *in vivo* like environment of blood vessels.[1] This technique has also a great potential for drug discovery studies and to reduce the number of animals used for *in vivo* experimentation.

3.2.1 Advantages of microfluidics

The microfluidic in vitro system allows to work with a low number of cells. Because of the miniaturization the consumption of reagents is enormously reduced and the cost with it. It allows to perform efficient high throughput experimentation and screening which is highly reproducible because of less systematic errors due to the handling. This novel microfluidic system provides a precise control of many parameters that can be defined for each experiment.

The use of a silicon polymer called polydimethylsiloxane (**PDMS**) to produce the microchips gives an enormous flexibility to design experimental models. The advantages to use PDMS is for example: the optically clear interface for microscopic visualization, the substance is non-toxic and non-flammable. The PDMS can be polymerized and cross-linked (cured) to form a solid PDMS structure.[7]
One of the disadvantages of the PDMS surface is its hydrophobic property where water is repelled and it can absorb hydrophobic substances. Some organic solvents can diffuse into the PDMS and make it swell. To overcome this hydrophobic situation, the technique of **plasma etching** is used. The surface of the PDMS is modified to become hydrophilic, the process with the plasma oxidation changes the surface bound of silicon-oxygen. PDMS is widely used in biomedical research because of its cost-effectiveness.[7]

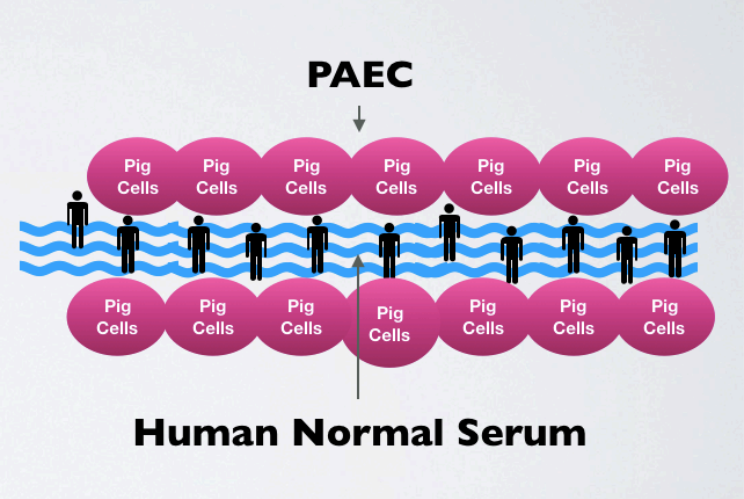


Figure 5 : PDMS microchip schematic view - interface pig-human. PAEC are coated in PDMS vessel like channel of 550 μm. NHS is circulating through the PDMS channel with the help of a peristaltic pulsatile pump. This microfluidic model aims to mimic xenotransplantation situation.

3.3 PDMS Microchip fabrication

For the fabrication of the PDMS vessel-like microchip of 550 μm diameter, the silicon elastomer PDMS (from Dow corning) was used. The first step was to mix in a ratio of 10:1 the PDMS and the curing agent (from Dow corning) into a Petri dish. Air bubbles were removed with a vacuum pump.

The second step was to place the needles after they were cleaned with isopropanol inside the liquid PDMS to mimic the shape of a vessel. The use of two kind of needles was necessary. First, two acupuncture needles of 0.12 x 30 mm (Braun) were first placed in the Petri dish, parallel to each other, with a distance of approximately 1.2 cm. These needles were used as support for four 0.55 x 25 mm needles (Seirin), which were the actual molds for the microchannels and placed at a 90 degree angle on top of the support needles, leaving about 4 mm distance between each. The liquid PDMS was then poured over the needles in the Petri dish and allowed to cure over night at 60°C.

The third step was to cut the PDMS chip out of the petri dish and to remove the needles. Holes were made with a biopsy puncher (diameter = 2mm) at both sides of the channel to allow the connection of the inlet and outlet for the peristaltic pump. Once the holes were made, each end of the holes was sealed with the PDMS-curing agent mixture and placed again overnight at 60°C to cure.

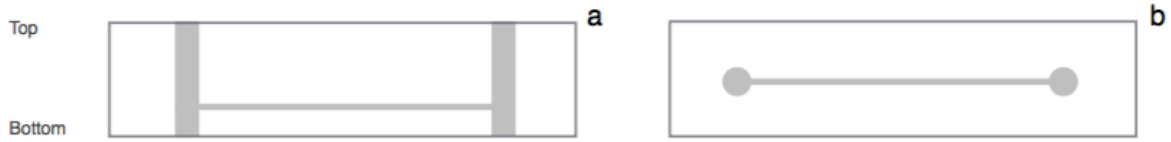


Figure 6 : a. Sideview of the microchip bearing the 500 μm-wide capillary. The two vertical holes a created with 2mm biopsy puncher from the top into the hardened PDMS. This allows us to connect to the microchip from both sides, thus we are able to create a unidirectional flow. b. top view of the microchip. Only the 2 ends are open at the top and bottom. This holes allow the connection to the peristaltic pump.

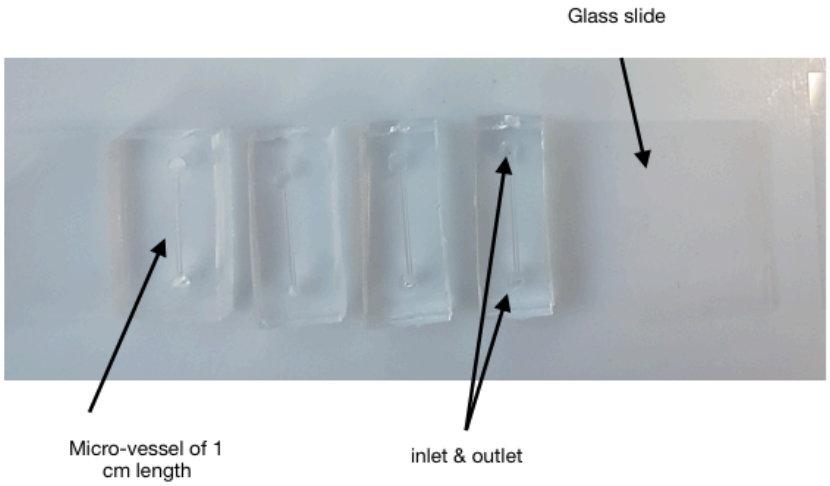


Figure 7 : PDMS microchannel sealed on a cover glass slide. Channel length of 1 cm. The inlet and the outlet are required for the peristaltic pump connection and cell seeding process

3.4 Cell Seeding Procedure on PDMS Microchip

3.4.1 PDMS – Glass bonding

The PDMS chip was bonded to the glass using the technic of plasma oxidation. This reaction is altering the surface chemistry by adding silanol (SiOH) groups. The PDMS becomes hydrophilic with this surface treatment. The microchip was cut in four channels and placed on a scotch tape with a small space between the chips. The four microchips and a glass slide (24 x 60mm #1) was placed into the plasma cleaner (HARRICK PLASMA) to be activated. The pressure dropped until 300 mTorr before turning on the oxygen valve. Then the pressured had to be around 650 mTorr or at least wait 10 minutes before turning on the plasma to high level for glass-chip bonding for 3 minutes and then the chip was taken out of the plasma cleaner

carefully. The activated chip was placed without touching it in the center of the activated glass slide to form between them silicon-oxygen bond and to be permanently sealed.

3.4.2 Covalent cross-link fibronectin and collagen-1 to PDMS

Immediately after the bonding-process, a solution of APTES (Sigma Aldrich) of 5% obtained after the dilution of the stock solution in a ratio of 1:20 in dH2O was injected into the PDMS channel, then incubated for 20 minutes and washed three times with dH2O.

The water was replaced with 0.1% glutaraldehyde (Sigma) solution obtained after the dilution of the stock solution of 25% at a ratio of 1:250 in dH2O, then incubated for 30 minutes and washed three times with dH2O.

The water was replaced with 50 µg/ml fibronectin (Merck) obtained after dilution of the stock solution of 1 mg/ml at a ratio of 1:20 in PBS. In that step the microchip was incubated at 37°C during 60 minutes. 100 µg/ml collagen (Merck) obtained after dilution of the stock solution of 5 ml/ml at a ratio of 1:50 in 0.02M of acetic acid was **directly** injected without washing steps and incubated during 90 minutes at room temperature.

Afterwards, DMEM cell culture medium was added into the chip and the whole device placed into the incubator at 37°C during at least 15 minutes or longer before the cell seeding process.

All the solutions should be really well mixed before adding to the chips.

3.4.3 Cell seeding

The PAEC cells were harvested with trypsin EDTA when they reached 80% of confluency. The PAEC are counted and resuspended at a density of 1 million cells/mL in DMEM solution with 4% of dextran (Leuconstocc spp. Mr ~70'000 – Sigma) solution obtained by 1:10 dilution of a 40% stock.
One or two drops of the cell suspension were injected each microchannel of the chip and placed in the incubator during 10-15 minutes upside down for the cell attachment on the top and then the remaining cell suspension was aspired. A new drop of the cell suspension was added and the microchip was placed into the incubator again during 10-15 minutes in normal position for the cell attachment at bottom. After that, the chip was washed really well three to five times with DMEM medium 4% dextran and one drop was left on both sides of the channel.

The chip was kept in the incubator overnight until the cells reached confluency, which happened usually the next day.

3.4.4 Pump connection

A peristaltic pump was used to apply a specific smooth, low pulse flow with a defined shear stress which was precisely calculated. The peristaltic pump was purchased from Gilson – Peristaltic pump – minipuls 3. The pump procures a highly accurate and precision. The flow rate range is between 0.3 μl /min to 30 ml/min. The head speed can be adjusted from 0 to 48 rpm by 0.01 rpm increments. The same pump can be connected to a maximum of 8 channels. Another peristaltic pump was used from ISMATEC to provide the same flow rate and shear stress as the first pump.

The tubings (1.0 x 0.5 mm – from Silicontubing) with a defined length was first placed around the rotor of the peristaltic pump and washed with three different warm autoclaved solutions: dH2O, PBS and DMEM dextran 4% with supplements. The tubings were checked to not contain any leakage. For each channel, one Falcon tube of 15 ml was filled up with 10ml of DMEM 4 % dextran supplemented solution and connected really gently with the outlet and inlet to the microchip to avoid detachment. The microchip connected to the pump was placed in the incubator with the medium reservoir, while the pump was kept outside.

The pump flow was started directly at 10 dyn/cm^2 during usually for 48 hours until the cells were well aligned and had time to adapt to the flow. After 48 hours, the microchip was ready to perform the experiments.

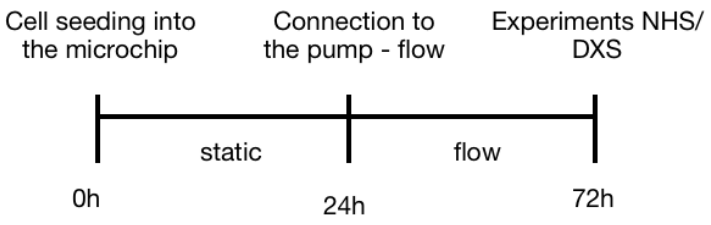


Figure 8 : Experiment protocol. The cell was seeded into the microchip at 0h. We waited 24 hours before the cells are confluent and proceeded with the connection of the peristaltic pump. After two days under flow, the experiment was performed with NHS or DXS.

3.5 Dextran sulfate experiments

After the connection of the microchips to the pump, the microchip was kept during 48 hours at 10 dyn/cm^2 before starting the actual experiment. DXS (5000 Da, IBsolvMIR, TIKOMED) was always mixed in DMEM 4% dextran without any supplement or, depending on the experimental conditions, mixed with NHS in different ratios (1:4 or 1:10).

3.5.1 Dextran sulfate with NHS

To assess the effect of low molecular dextran sulfate to restore the EG, the DXS was injected from 0.3 mg/ml to 3 mg/ml concentration in the microchip in various conditions. Normal human serum (NHS) from a single donor was used to induce a shedding of the glycocalyx and to assess the effect of DXS.

NHS was mixed with DMEM 4% dextran without supplement at a ratio of 1:10 and perfused trough the microchip during 2 hours before washing with PBS^{++} and fixing it with paraformaldehyde 4%.

DXS was mixed in pure DMEM 4% dextran without supplement. The mix was then circulated through the microchip during 2 hours before stopping the experiment by washing several times with PBS^{++}. Then, the cells were fixed with 4% paraformaldehyde to proceed with the immunostaining protocol.

DXS was mixed **with** 1:10 NHS in the DMEM 4% Dextran without supplement together before circulating trough the microchip. The same experiment was conducted without flow in **static condition** once the cells were confluent after 24h.

3.5.2 Dextran sulfate with preactivation

In order to get a stronger shedding and activation of the EG. The cells were preactivated with 1:4 diluted NHS for 30 minutes. Then DXS was circulated alone into the chip or it was injected again with 1:4 NHS for 30 minutes. Then, the microchips were washed with PBS^{++} and fixed with 4% paraformaldehyde to proceed with the immunostaining protocol.

3.6 Shear stress experiment

To investigate the impact of the shear stress on the EG. Microchip experiments were conducted at three different shear stress conditions: static (no flow), 10 dyn/cm^2 and 20 dyn/cm^2.

For the static condition, the microchips were kept at the initial condition by changing three times a day the DMEM 4% dextran with supplements during 24 hours from the cell seeding procedure before washing with PBS^{++} and fixing with 4% paraformaldehyde.

Once the cells were confluent in the microchips after 24 hours, the chip was connected to the pump and directly put at 10 dyn/cm^2 during 48 hours before stopping the experiment. For the 20 dyn/cm^2 experiment, the microchips were kept during 24h at 10 dyn/cm^2 and then it was slowly increased to 20 dyn/cm^2 in the same day. The 20 dyn/cm^2 condition was kept for 48 hours before stopping the experiment by washing with PBS^{++} and fixed with 4% paraformaldehyde.

3.7 Immunofluorescence staining

The immunostaining protocol was immediately performed after the fixation with 4% paraformaldehyde during 10 – 15 minutes. Then the microchip was washed with PBS^{++} three to five times. For intracellular antigens, the cell membrane was permeabilized with 0.5% TritonX-100 for 10 minutes on a shaker.
Then the microchips were washed three to five times with a blocking buffer solution (PBS-3% BSA) and incubated during 45 minutes at room temperature. The primary antibody was directly added to the microchip by washing it three times and incubated overnight at 4°C.
The next day, the microchip was really well washed up to five times with PBS^{++} before adding the 2^{nd} antibody with DAPI during 1 hour at room temperature on a shaker. At the end of the time, it was again washed up to five times with PBS^{++} and kept with PBS^{++} on both side of the channel. After the immunofluorescence staining process, the samples were imaged with the Zeiss Laser Scanning Microscope 710.

3.8 Confocal Microscopy Acquisition and Quantification Analysis

All samples were imaged with LCI (Live Cell Imaging) Zeiss Laser Scanning Microscope 710 Confocal. Different objectives were used to acquire the images the plan-apochromat 10x/0.3 M27/ a=2.00mm and the EC plan-neofluar 40x/1.30 Oil DIC M27/ a=0.21mm. The images were analysed with ImageJ FIJI (1.0).

The 10x objective was used to take five pictures of the whole microchip channel. The picture acquirement started on one side of the channel to the second end. The picture acquirement settings were 2048 x 2014 pixels, 12 bit and a speed of 6.

The 40x water objective was used to take pictures of the EG of the PAEC in the microchip. Five pictures were taken from different regions of the microchannel.

3.8.1 Antibodies

Antibodies were diluted in PBS – 1% BSA – 0.05% Tween 20 to obtain the right concentrations.

3.8.1.1 HPSG

To assess the heparan sulfate proteoglycan (HSPG) on the EG. The antibody 10E4 from amsbio was used which is a mouse IgM and was diluted 1:100. The 10E4 antibody reacts with the 10E4 epitopes that includes N-sulfated glucosamine residue(s). This antibody is known to bind also to DXS. This is why it is possible to assess the theoretical replacement of the HSPG by DXS. The 2nd antibody is a goat anti-mouse IgM-FITC from, which diluted 1:500.

3.8.1.2 WGA-Lectin

Wheat germ agglutinin (WGA) – Lectin from Triticum vulgaris (Sigma-Aldrich) is used to bind to **N-acetyl-D-glucosamine** and **sialic acid** component of the glycocalyx.[6] It is possible with this lectin to perform an *in vivo* staining. It was diluted in PBS^{++} at 1:200 before use.

3.8.1.3 CD31

The CD31(anti-PCD31/PECAM-1) antibody is a rat monoclonal IgG from RD SYSTEMS and diluted at 1:100 before use. The 2nd antibody is a Cy3-labeled goat anti-rat IgG from Jackson ImmunoResearch, diluted 1:500.

3.8.1.4 E-selectin

The E-selectin antibody (CD62E9) is a mouse IgG and was used at 1:50 dilution. As 2nd antibody we used a goat anti-mouse IgG, labeled with Alexa Fluor 546 from Invitrogen diluted 1:500.

3.9 Statistical analysis

Date are presented as mean ± SD obtained from an average of five representative pictures of one microchannel or five representative pictures of one microchip. All the data were analyzed with t Tests (parametric test, unpaired) and for multiple comparison with one – way ANOVA. Differences in means were considered significant if $P<0.05$. The graphs and the analyses were made with the software Prism 7 (Version 7.0c)

4 Results

The results were obtained by simulating different shear stress and by injecting different concentration of the low molecular weight dextran sulfate (DXS) in the microfluidic system. The compared shear stress was: static condition (no flow), a 10 dyn/cm^2 and 20 dyn/cm^2. The compared DXS concentration were: 0.3 mg/ml, 1 mg/ml and 3 mg/ml.

4.1 PAEC WT static

4.1.1 HSPG: Control vs NHS

In order to prove that NHS might shed the EG HSPG, NHS 1:10 diluted in DMEM 4% dextran without supplements was incubated with the PAEC microfluidic chip for 2 hours.

The measurement of the fluorescence intensity is significantly decreased in the HPSG WT PAEC + NHS compared to HSPG WT NO NHS {NO NHS: 44136164 ± 4757690, n=5, NHS: 31410716 ± 3693816, n=5, **p=0,0015**, unpaired t test}. These results suggest that NHS is shedding the HSPG **significantly** compared to the control microchip. (Figure 9)

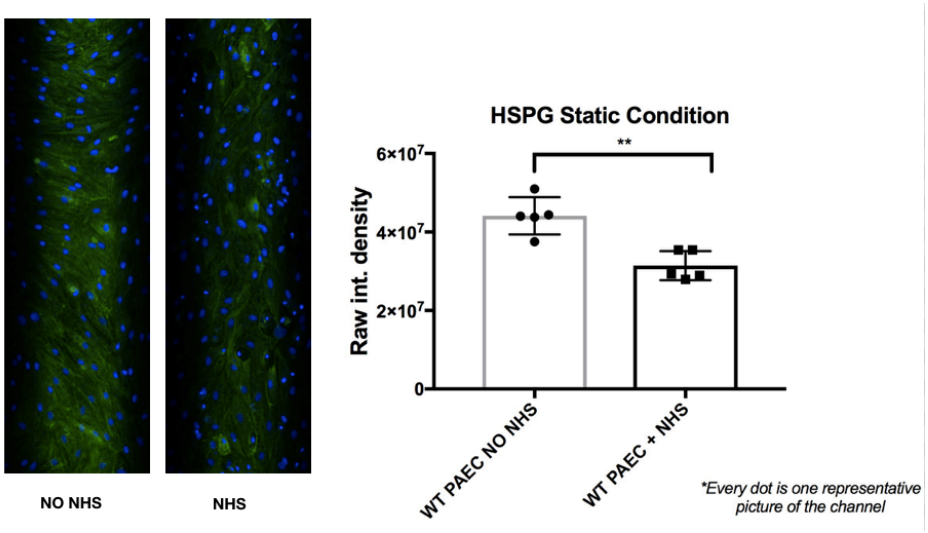


Figure 9 : 10x magnification of 500μm microchannel. Representative picture of the microchannel. Static condition. Incubation with NHS 1:10 during 2 hours. The nuclei are stained blue. The HSPG are stained green. {NO NHS: 44136164 ± 4757690, n=5, NHS: 31410716 ± 3693816, n=5, p=0,0015, unpaired t test}

4.1.2 WGA - lectin: Control vs NHS

In order to prove that NHS might shed the EG, NHS 1:10 diluted in DMEM 4% dextran without supplements was incubated with the PAEC microfluidic chip for 2 hours.

The measurement of the fluorescence intensity has **no significant** differences between the WT PAEC with and without NHS {NO NHS: 60405004 ± 12239895, n=5, NHS: 55280420 ± 4334883, n=5, **p=0,4033**, unpaired t test}. These results suggest that NHS has no tendency to shed the EG compared to the control microchip. The results were **not significant**. (Figure 10)

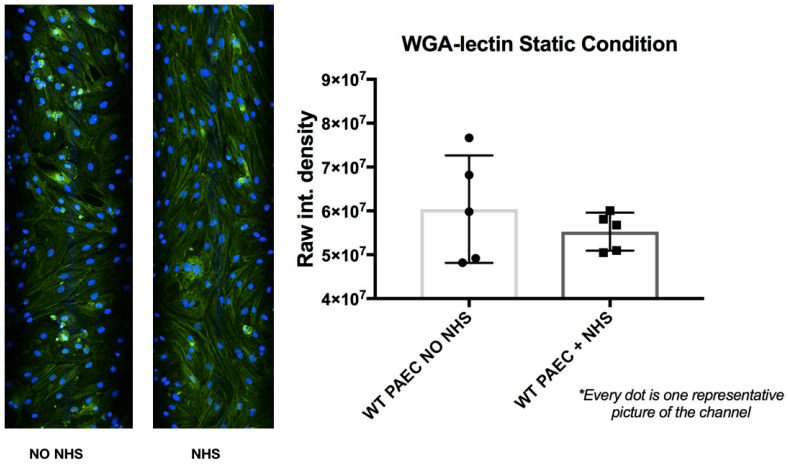


Figure 10 : 10x magnification of 500μm microchannel. Representative picture of the microchannel. Static condition. Incubation with NHS 1:10 during 2 hours. The nuclei are stained blue. The EG component stained by WGA-lectin are green. {NO NHS: 60405004 ± 12239895, n=5, NHS: 55280420 ± 4334883, n=5, p=0,4033, unpaired t test}

4.1.3 E- selectin: Control vs NHS

To assess the effect of NHS on the EG of PAEC, a surface marker E-selectin (CD62E) was stained. The NHS 1:10 diluted in DMEM 4% dextran without supplements was incubated with the PAEC microfluidic chip for 2 hours. The E-selectin is a cell adhesion molecule expressed by **activated** endothelial cells and they play a key role in the inflammation process.[52]

The measurement of the fluorescence intensity is **significantly** increased in the microchip with NHS incubation compared to the one without {NO NHS: 4861982± 3485606, n=5, NHS: 10483097 ± 2612535, n=5**, p= 0,0203**, unpaired t test}. These results suggest that NHS is significantly activating the EG of the PAEC compared to the control microchip and might induce an inflammation process. (Figure 11)

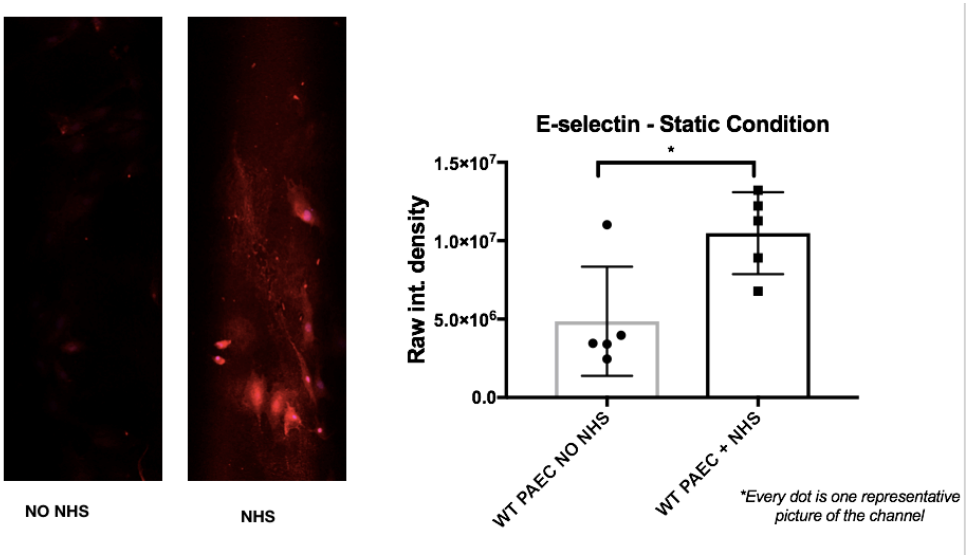


Figure 11 : 10x magnification of 500μm microchannel. Representative picture of the microchannel. Static condition. Incubation with NHS 1:10 during 2 hours. The E-selectin markers are stained red. {NO NHS: 4861982± 3485606, n=5, NHS: 10483097 ± 2612535, n=5, p= 0,0203, unpaired t test}

4.2 PAEC WT under flow

4.2.1 HSPG 10 dyn/cm^2: Control vs NHS

In order to assess the effect of EG HSPG under flow, the microfluidic PAEC microchip was perfused with a **shear stress of 10 dyn/cm^2** and perfused with NHS 1:10 diluted in DMEM 4% dextran without any supplements.

The measurement of the fluorescence intensity is **significantly decreased** in the microchip with NHS incubation {NO NHS: 63435161± 4243978, n=5, NHS: 39591280 ± 3770852, n=5, **p=<0,0001**, unpaired t test}. These results suggest that NHS is shedding the EG HSPG under a flow with a shear stress of 10 dyn/cm^2 compared to the control microchip. (Figure 12)

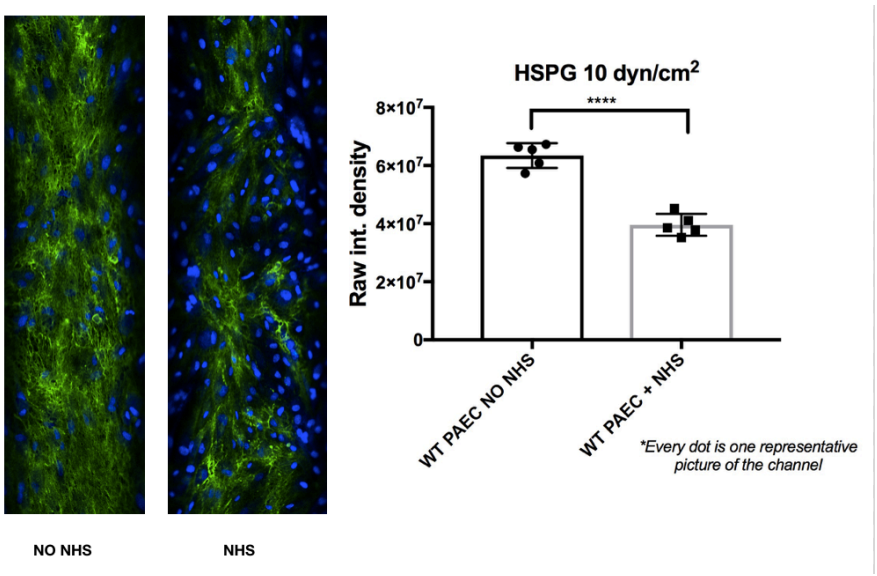


Figure 12 : 10x magnification of 500μm microchannel. Representative picture of the microchannel. Under flow with a shear stress of 10 dyn/cm^2. Incubation with NHS 1:10 during 2 hours. The nuclei are stained blue. The HSPG are stained green. {NO NHS: 63435161± 4243978, n=5, NHS: 39591280 ± 3770852, n=5, p=<0,0001, unpaired t test}

4.3 PAEC WT Shear Stress effect

4.3.1 HSPG: Shear stress

To assess the effect of shear stress on EG HSPG, the microfluidic PAEC microchip was perfused with DMEM 4% dextran with supplements with three different shear stress condition: static (no flow), 10 dyn/cm^2 and 20 dyn/cm^2. The shear stress was exposed during 48 hours before stopping the experiment.

The measurement of the fluorescence intensity shows **no significant difference** between the three shear stress condition {static: 44136164, n=1, 10 dyn/cm2: 42004358 ± 15135990, n=4, **p=0,9849**, multiple comparisons ordinary one-way ANOVA}, {static: 44136164, n=1, 20 dyn/cm2: 27879051± 3069873, n=2, **p=0,5508**, multiple comparisons ordinary one-way ANOVA}. The results for 20 dyn/cm^2 might be interpreted as a shedding of HSPG due to the high shear stress but the difference it's **not statistically significant** to make any conclusion. (Figure 13)

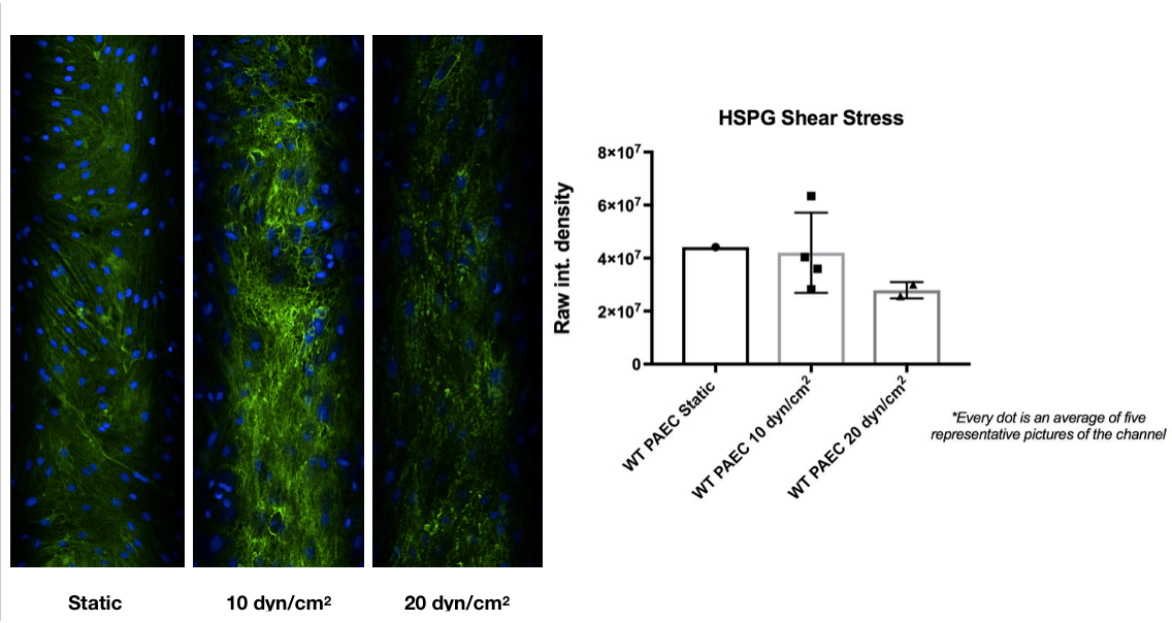


Figure 13 : 10x magnification of 500μm microchannel. Representative picture of the microchannel. Static condition (no flow). Under flow with a shear stress of 10 dyn/cm^2. Under flow with a shear stress of 20 dyn/cm^2. The nuclei are stained blue. The HSPG are stained green. {static: 44136164, n=1, 10 dyn/cm2: 42004358 ± 15135990, n=4, p=0,9849, multiple comparisons ordinary one-way ANOVA}, {static: 44136164, n=1, 20 dyn/cm2: 27879051± 3069873, n=2, p=0,5508, multiple comparisons ordinary one-way ANOVA}

After the analysis of the pictures of the microchip exposed to 20 dyn/cm2, the decrease of HSPG fluorescence might be due to **the cell loss** because of the exposition to supra-physiological high shear stress of the PAEC. The quantification of the green channel of HSPG was divided by blue channel of DAPI to take in account the loss of cell. The results show **no significant difference** between the three shear stress condition. Shear stress seems to not to induce the shedding or adaptation of HSPG {static: 1,971, n=1, 10 dyn/cm2: 2,458 ± 1,133, n=4, **p=0,8728**, multiple comparisons ordinary one-way ANOVA}, {static: 1,971, n=1, 20 dyn/cm2: 1,905 ± 0,1818, n=2, **p=0,9978**, multiple comparisons ordinary one-way ANOVA}. (Figure 14)

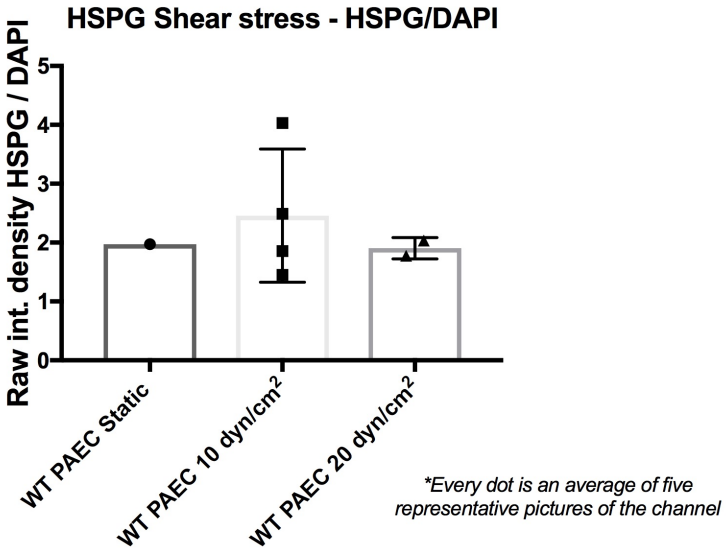


Figure 14 : Fluorescence quantification of the HSPG green channel divided by the DAPI blue channel. Static condition (no flow). Under flow with a shear stress of 10 dyn/cm^2. Under flow with a shear stress of 20 dyn/cm2. {static: 1,971, n=1, 10 dyn/cm2: 2,458 ± 1,133, n=4, p=0,8728, multiple comparisons ordinary one-way ANOVA}, {static: 1,971, n=1, 20 dyn/cm2: 1,905 ± 0,1818, n=2, p=0,9978, multiple comparisons ordinary one-way ANOVA}

4.3.2 WGA –lectin: Shear stress

To assess the effect of shear stress on EG, the microfluidic PAEC microchip was perfused with DMEM 4% dextran with supplements with three different shear stress condition: static (no flow), 10 dyn/cm^2 and 20 dyn/cm^2. The shear stress was exposed during 48 hours before stopping the experiment.

The measurement of the fluorescence intensity is **significantly decreased** in the two microchip with shear stress than the static {static: 60405004±12239895, n=5, 10 dyn/cm2: 23179889± 3510714, n=5, **p<0,0001**, multiple comparisons ordinary one-way ANOVA}, {static: 160405004±12239895, n=5, 20 dyn/cm2: 25149901± 3021023, n=13, **p<0,0001**, multiple comparisons ordinary one-way ANOVA}.

These results suggest that the shear stress is **significantly** inducing the shedding or loss of some components of the EG compared to static condition. (Figure 15) These findings might be due to **cell loss** in the microchip with higher shear stress. (Figure 16)

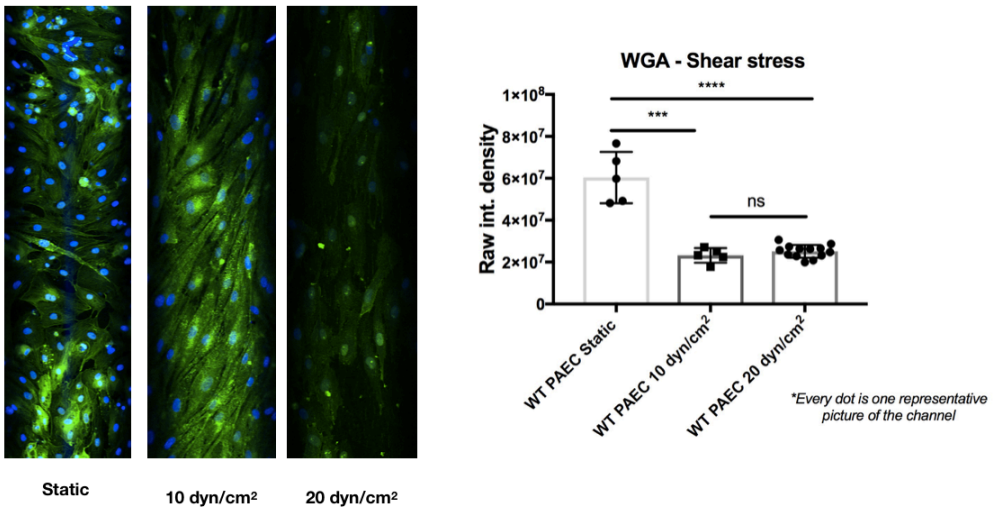


Figure 15 : 10x magnification of 500μm microchannel. Representative picture of the microchannel. Static condition (no flow). Under flow with a shear stress of 10 dyn/cm2. Under flow with a shear stress of 20 dyn/cm2. The nuclei are stained blue. The component of EG stained by WGA-lectin are green. {static: 60405004±12239895, n=5, 10 dyn/cm2: 23179889± 3510714, n=5, p<0,0001, multiple comparisons ordinary one-way ANOVA}, {static: 160405004±12239895, n=5, 20 dyn/cm2: 25149901± 3021023, n=13, p<0,0001, multiple comparisons ordinary one-way ANOVA}

After the analysis of the pictures of the microchips for the 10 dyn/cm^2 and 20 dyn/cm^2 channels, the decrease of WGA-lectin fluorescence might be due to the **cell loss** because of the exposition to supra-physiological high shear stress of the PAEC. The quantification of the green channel of WGA-lectin was divided by blue channel of DAPI to take in account the loss of cell. The results show a **significant increase** of the WGA-lectin fluorescence quantification in 20 dyn/cm^2 microchip compared to the static condition {static: 1,58±0,2115, n=5, 10 dyn/cm2: 2,514± 0,2907, n=5, **p=0,1098**, multiple comparisons ordinary one-way ANOVA}, {static: 1,58±0,2115, n=5, 20 dyn/cm2: 3,154± 0,942, n=13, **p=0,0014**, multiple comparisons ordinary one-way ANOVA}. That tendency of the 20 dyn/cm^2 microchip might indicate an adaptation of the glycocalyx to the flow by increasing his thickness or some component of the EG. (Figure 16)

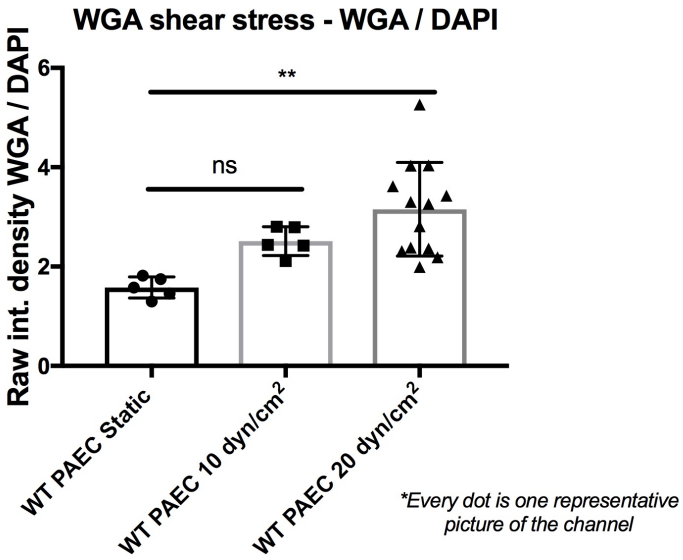


Figure 16 : Fluorescence quantification of the HSPG green channel divided by the DAPI blue channel. Static condition (no flow). Under flow with a shear stress of 10 dyn/cm^2. Under flow with a shear stress of 20 dyn/cm2. {static: 1,58±0,2115, n=5, 10 dyn/cm2: 2,514± 0,2907, n=5, p=0,1098, multiple comparisons ordinary one-way ANOVA}, {static: 1,58±0,2115, n=5, 20 dyn/cm2: 3,154± 0,942, n=13, p=0,0014, multiple comparisons ordinary one-way ANOVA}

To have a better understanding about the effect of a shear stress of 10 dyn/cm^2 compared to static condition. The 10 dyn/cm^2 shear stress microchip was exposed during 48 hours to the flow. The static microchip was kept for 24 hours. (Figure 17)

With this experiment, the quantification of fluorescence of the HSPG **is significantly increased** in microchip under a shear stress of 10 dyn/cm^2 compared to the static condition {Static NO NHS: 44136164 ± 4757690, n=5, 10 dyn/cm^2: 6343516 ± 4243978, n=5, **p=<0,0001**, unpaired t test}. That tendency of the 10 dyn/cm^2 microchip might indicate an adaptation of the EG to the flow by increasing the HSPG. (Figure 17)

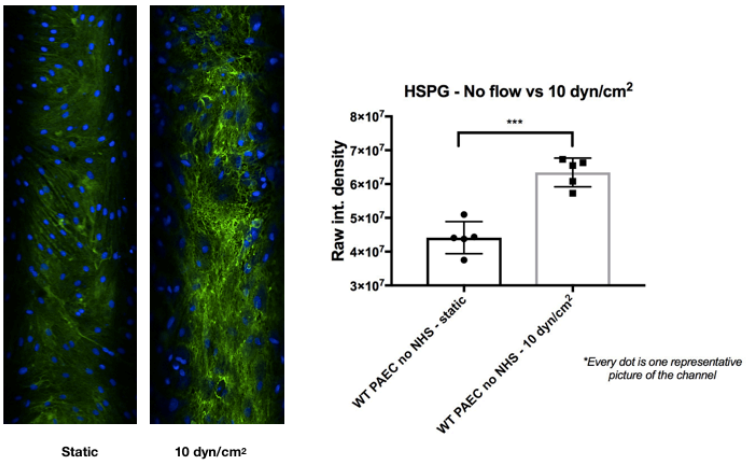


Figure 17 : 10x magnification of 500µm microchannel. Representative picture of the microchannel. Static condition (no flow). Under flow with a shear stress of 10 dyn/cm^2. Under flow with a shear stress of 20 dyn/cm^2. The nuclei are stained blue. The HSPG are stained green. {Static NO NHS: 44136164 ± 4757690, n=5, 10 dyn/cm^2: 6343516 ± 4243978, n=5, p=<0,0001, unpaired t test}

4.4 PAEC WT with low molecular dextran sulfate (5000)

The following experiments aimed to assess the mechanism and efficiency of DXS to replace the shed of HSPG induced by NHS in our microfluidic model. This is done by injecting into the circulation of the PAEC microchip the DXS with different concentration.

4.4.1 HSPG - 0.3 mg/ml dextran sulfate

This experiment was conducted to assess the potential of the DXS to restore the HSPG EG in the microfluidic PAEC model under a shear stress of 10 dyn/cm^2. The PAEC microchip was perfused with DMEM 4% dextran + **DXS 0.3 mg/ml** without any supplements for 2 hours. The results suggest that there is **no significant difference** between the control microchip of HSPG and the microchip with DXS 0.3 mg/ml + NHS 1:10 or the one with DXS 0.3 mg/ml only {10 dyn/cm^2 NO NHS: 42004358 ± 15135990, n=4, DXS 0.3 mg/ml only: 37627205, n=1, NHS 1:10 only: 35371008 ± 3184233, n=3, DXS 0.3 mg/ml + 1:10 NHS: 29796086 ± 4025858, n=2, multiple comparisons ordinary one-way ANOVA}. (Figure 18) The DXS 0.3 mg/ml do not show any significant results with this concentration to restore the shed EG HSPG. (Figure 19)

p-value	0.3 mg/ml DXS only	NHS 1:10 only	0.3 mg/ml DXS + 1:10 NHS
Control 10 dyn/cm^2	0,9683	0,7753	0,4858

Figure 18 : Fluorescence quantification of HPSG. P-value for multiple comparisons ordinary on-way ANOVA for DXS 0.3 mg/ml. Comparison of the mean of each microchip with the mean of the control 10 dyn/cm^2. Not significant

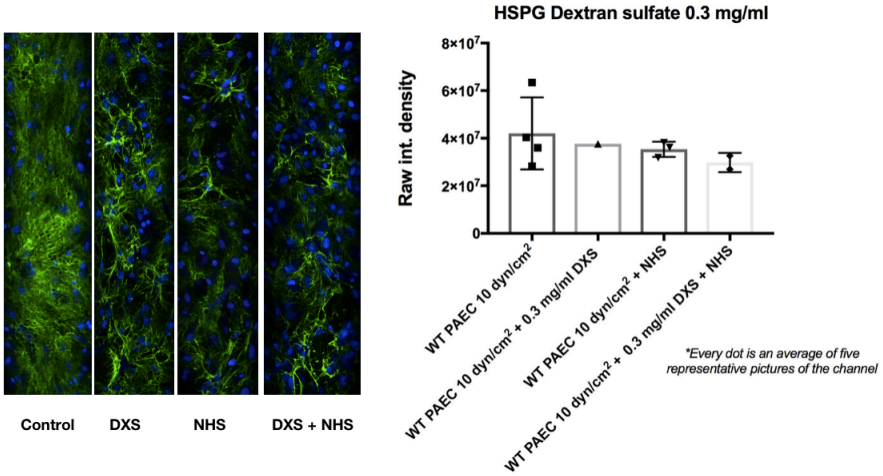


Figure 19 : 10x magnification of 500μm microchannel. Representative picture of the microchannel. All the microchip under flow with a shear stress of 10 dyn/cm^2. Control WT PAEC 10 dyn/cm^2. DXS 0.3 mg/ml only. NHS 1:10 only. DXS 0.3 mg/ml + NHS 1:10. The nuclei are stained blue. The HSPG are stained green. {10 dyn/cm^2 NO NHS: 42004358 ± 15135990, n=4, DXS 0.3 mg/ml only: 37627205, n=1, NHS 1:10 only: 35371008 ± 3184233, n=3, DXS 0.3 mg/ml + 1:10 NHS: 29796086 ± 4025858, n=2, multiple comparisons ordinary one-way ANOVA}.

This experiment was conducted to assess the potential of the DXS to restore the HSPG EG in the microfluidic PAEC model under a shear stress of 10 dyn/cm^2. The PAEC microchip was perfused with DMEM 4% dextran + **DXS 1 mg/ml** without any supplements for 2 hours. The DXS concentration is higher than the previous experiment. The results suggest that there is **no significant difference** between the control microchip of HSPG and the microchip with DXS 0.3 mg/ml + NHS 1:10 or the one with DXS 0.3 mg/ml only {10 dyn/cm^2 NO NHS: 42004358 ± 15135990, n=4, DXS 0.3 mg/ml only: 30576595 ± 685699, n=2, NHS 1:10 only: 35371008 ± 3184233, n=3, DXS 0.3 mg/ml + 1:10 NHS: 29016841 ± 3374879, n=4, multiple comparisons ordinary one-way ANOVA}. (Figure 21) The DXS 1 mg/ml do not show any significant results with this concentration to restore the shed EG HSPG. (Figure 20)

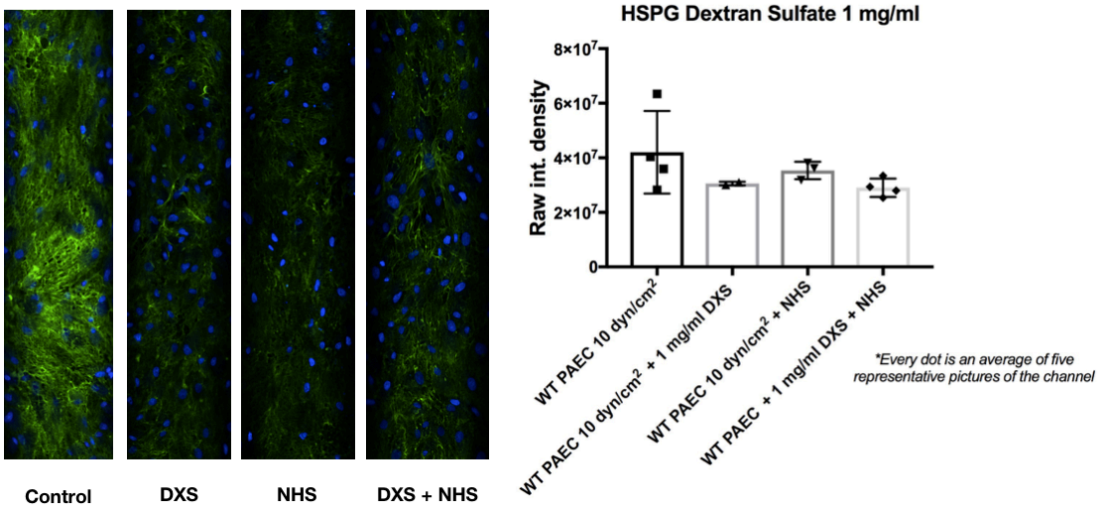


Figure 20 : 10x magnification of 500μm microchannel. Representative picture of the microchannel. All the microchip under flow with a shear stress of 10 dyn/cm^2. Control WT PAEC 10 dyn/cm^2. DXS 0.3 mg/ml only. NHS 1:10 only. DXS 0.3 mg/ml + NHS 1:10. The nuclei are stained blue. The HSPG are stained green. {10 dyn/cm^2 NO NHS: 42004358 ± 15135990, n=4, DXS 0.3 mg/ml only: 30576595 ± 685699, n=2, NHS 1:10 only: 35371008 ± 3184233, n=3, DXS 0.3 mg/ml + 1:10 NHS: 29016841 ± 3374879, n=4, multiple comparisons ordinary one-way ANOVA}.

p-value	0.3 mg/ml DXS only	NHS 1:10 only	0.3 mg/ml DXS + 1:10 NHS
Control 10 dyn/cm^2	0,3798	0,6696	0,1699

Figure 21 : Fluorescence quantification of HSPG. P-value for multiple comparisons ordinary on-way ANOVA for DXS 1 mg/ml. Comparison of the mean of each microchip with the mean of the control 10 dyn/cm^2. Not significant

4.4.2 CD31: 1 mg/ml dextran sulfate

This experiment was conducted to evaluate if there is any effect of the DXS on the EG in the microfluidic PAEC model under a shear stress of 10 dyn/cm^2. The PAEC microchip was perfused with a with DMEM 4% dextran + **DXS 1 mg/ml** without any supplements for 2 hours. **CD31** (PECAM-1) is a surface maker who plays a role as for angiogenesis**, endothelial shear stress sensor**, leucocyte trafficking and as a regulator of endothelial junctional integrity. [61] The results suggest that there is **no significant difference** between the control microchip of CD31 and the microchip with DXS 0.3 mg/ml + NHS 1:10. {10 dyn/cm^2 NO NHS: 31436608 ± 3015650, n=2, DXS 0.3 mg/ml only: 32491831, n=1, NHS 1:10 only: 3306605, n=2, DXS 0.3 mg/ml + 1:10 NHS: 36738476 ± 992070, n=2, multiple comparisons ordinary one-way ANOVA}. (Figure 23) However, DXS might maybe have an impact in the microchip exposed to NHS + DXS even if it's no significant difference the quantification of the expression of CD31 seems to be higher compared to the others microchip**.** (Figure 22)

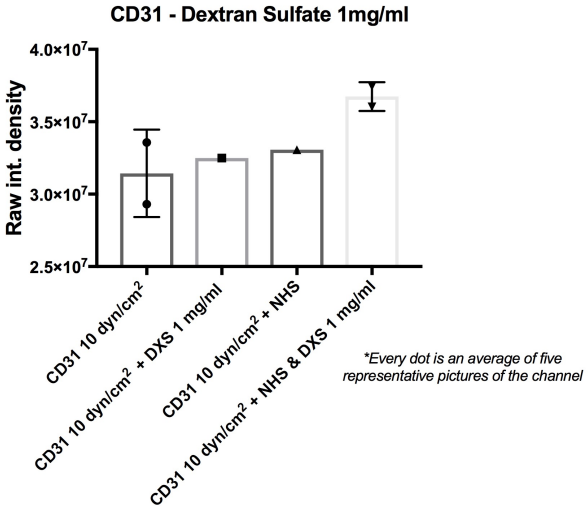


Figure 22 : Fluorescence quantification of CD31. All the microchip under flow with a shear stress of 10 dyn/cm^2. Control WT PAEC 10 dyn/cm^2. DXS 0.3 mg/ml only. NHS 1:10 only. DXS 0.3 mg/ml + NHS 1:10. {10 dyn/cm^2 NO NHS: 42004358 ± 15135990, n=4, DXS 0.3 mg/ml only: 30576595 ± 685699, n=2, NHS 1:10 only: 35371008 ± 3184233, n=3, DXS 0.3 mg/ml + 1:10 NHS: 29016841 ± 3374879, n=4, multiple comparisons ordinary one-way ANOVA}.

p-value	0.3 mg/ml DXS only	NHS 1:10 only	0.3 mg/ml DXS + 1:10 NHS
Control 10 dyn/cm^2	0,9578	0,8811	0,2613

Figure 23 : Fluorescence quantification of CD31. P-value for multiple comparisons ordinary on-way ANOVA for DXS 1 mg/ml. Comparison of the mean of each microchip with the mean of the control 10 dyn/cm^2. Not significant

4.5 PAEC WT with dextran sulfate – Preactivation with NHS

With the previous study, we concluded that the inefficacy of the DXS might be ascribed to concentration of DXS or because of the lack of activation of the endothelial cells. This experiment was conducted by activating the cells with NHS 1:30 during 30 minutes before perfusion with DXS at a higher concentration.

4.5.1 HSPG - 3 mg/ml dextran sulfate + preactivation

This experiment was conducted to assess the effect of the DXS on the EG in the microfluidic PAEC model under a shear stress of 10 dyn/cm^2. The PAEC microchip was preactivated with NHS 1:4 during 30 minutes and then it was perfused with a with DMEM 4% dextran + **DXS 3 mg/ml** and/or **NHS 1:4** without any supplements for **30 minutes** instead of 2 hours in the previous experiments. The results suggest that the DXS 3mg/ml has **no significant** impact on PAEC when it's preactivated with NHS 1:4 and perfused with NHS 1:4 {10 dyn/cm^2 NO NHS: 42004358 ± 15135990, n=4, DXS 3 mg/ml: 29718098, n=1, NHS 1:10 only: 35371008 ± 3184233, n=3, NHS 1:4 only: 39711430, n=1, DXS 3 mg/ml + 1:4 NHS: 46797957, n=1, multiple comparisons ordinary one-way ANOVA}. (Figure 25) The DXS 3 mg/ml with preactivation do not show any significant results with this concentration to restore the shed EG HSPG. (Figure 24)

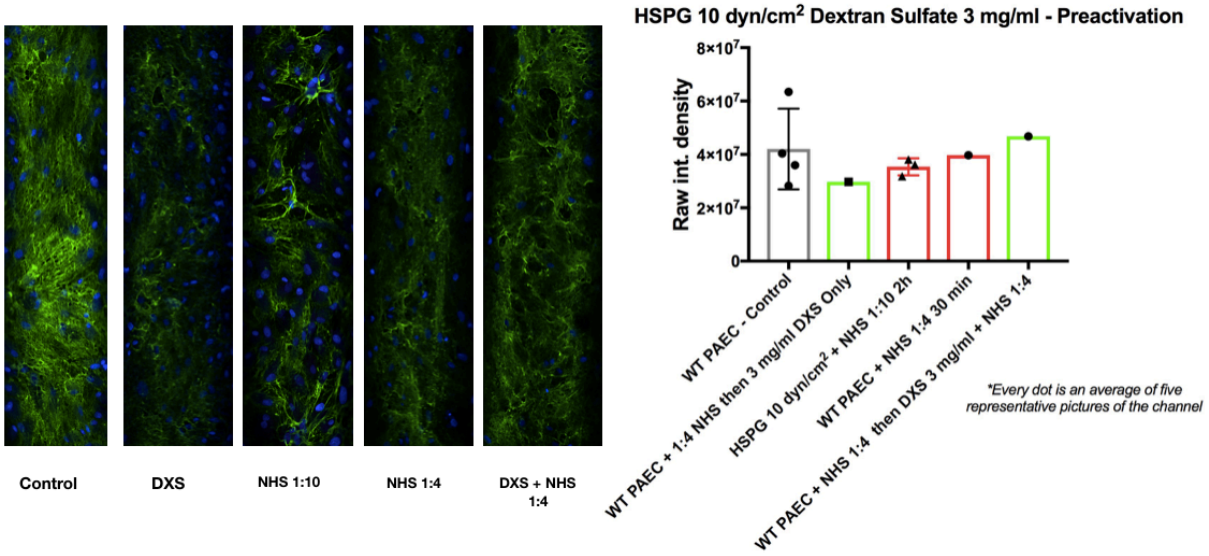


Figure 24 : 10x magnification of 500μm microchannel. Representative picture of the microchannel. All the microchip under flow with a shear stress of 10 dyn/cm^2. Control WT PAEC 10 dyn/cm^2. DXS 3 mg/ml. NHS 1:10. NHS 1:4. DXS 3 mg/ml + NHS 1:4. The nuclei are stained blue. The HSPG are stained green. {10 dyn/cm^2 NO NHS: 42004358 ± 15135990, n=4, DXS 3 mg/ml: 29718098, n=1, NHS 1:10 only: 35371008 ± 3184233, n=3, NHS 1:4 only: 39711430, n=1, DXS 3 mg/ml + 1:4 NHS: 46797957, n=1, multiple comparisons ordinary one-way ANOVA}.

p-value	3 mg/ml DXS	NHS 1:10 / NHS 1:4	3 mg/ml DXS + 1:4 NHS
Control 10 dyn/cm^2	0,7694	0,8715 / 0,9991	0,9864

Figure 25 : Fluorescence quantification of HSPG. P-value for multiple comparisons ordinary on-way ANOVA for DXS 3 mg/ml. Comparison of the mean of each microchip with the mean of the control 10 dyn/cm^2. Not significant

4.5.2 CD31 - 3 mg/ml dextran sulfate + preactivation

This experiment was conducted to assess the effect of the DXS on the EG in the microfluidic PAEC model under a shear stress of 10 dyn/cm^2. The PAEC microchip was preactivated with NHS 1:4 during 30 minutes and it was perfused with DMEM 4% dextran + **DXS 3 mg/ml** and **NHS 1:4** without any supplements for **30 minutes** instead of 2 hours in the previous experiments. The results are **not significant.** They **might** maybe suggest that the DXS 3 mg/ml has an impact in the microchip where it's exposed two times to NHS 1:4 {10 dyn/cm^2 NO NHS: 31436608 ± 3015650, n=2, DXS 3 mg/ml: 35299985, n=1, NHS 1:10 only: 33066058 ± 3184233, n=1, NHS 1:4 only: 36391802, n=1, DXS 3 mg/ml + 1:4 NHS: 40728932, n=1, multiple comparisons ordinary one-way ANOVA}. (Figure 27) The CD31 expression might be increased in the microchip with DXS 3 mg/ml exposed two times to NHS 1:4 during 30 minutes compared to the others. (Figure 26)

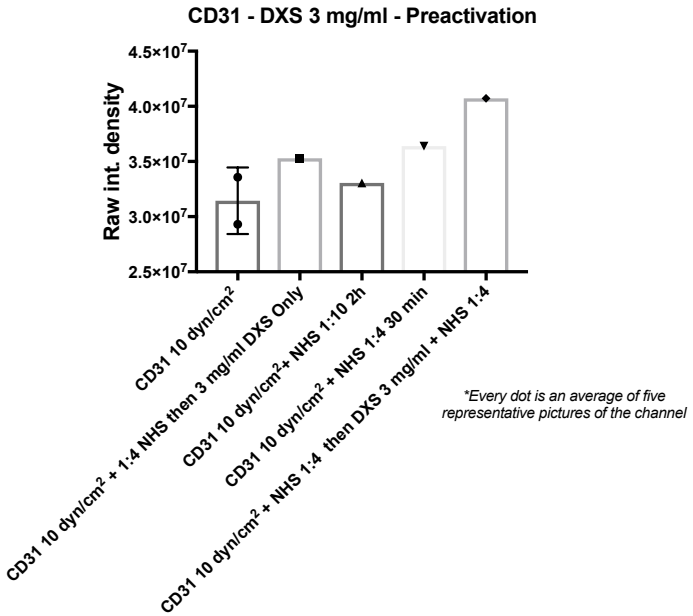


Figure 26 : Fluorescence quantification of CD31. All the microchip under flow with a shear stress of 10 dyn/cm^2. Control WT PAEC 10 dyn/cm^2. DXS 3 mg/ml. NHS 1:10. NHS 1:4. DXS 3 mg/ml + NHS 1:4. T {10 dyn/cm^2 NO NHS: 31436608 ± 3015650, n=2, DXS 3 mg/ml: 35299985, n=1, NHS 1:10 only: 33066058 ± 3184233, n=1, NHS 1:4 only: 36391802, n=1, DXS 3 mg/ml + 1:4 NHS: 40728932, n=1, multiple comparisons ordinary one-way ANOVA}.

p-value	3 mg/ml DXS	NHS 1:10 / NHS 1:4	3 mg/ml DXS + 1:4 NHS
Control 10 dyn/cm^2	0,7504	0,9618 / 0,6515	0,4038

Figure 27 : Fluorescence quantification of CD31. P-value for multiple comparisons ordinary on-way ANOVA for DXS 1 mg/ml. Comparison of the mean of each microchip with the mean of the control 10 dyn/cm^2. Not significant

5 Discussion

We showed that DXS did not act as a “repair coat” to replace the shed HSPG on the activated PAEC with NHS in our novel *in vitro* microfluidic model in a context of xenotransplantation situation. The shear stress might induce an increase of some component of the EG and HSPG exposed to shear stress when it is compared to static (no flow) condition.

Our results concerning the DXS did not verify our hypothesis (2.9.1). The fluorescence quantification of DXS should be significantly increase than the microchip exposed to NHS to conclude that DXS is effective to protect the EG by acting as a “repair coat”. Contrary to our hypothesis, we found no improvement of the HSPG layer of PAEC exposed DXS. Our results concerning the shear stress did partially verify our hypothesis (2.9.2). The microchip exposed to shear stress seems to have an increase of the component of EG and HSPG compared to static condition which might be interpreted as an increase of thickness.

Based on relative few studies of the low molecular weight dextran sulfate linked to xenotransplantation, we wanted to give a new perspective of the mechanism of DXS in the context of xenotransplantation. [24,37,42,44,78] **Low molecular DXS** is a synthetic substance which is known to induce complement inhibition dose dependently, to inhibit the coagulation pathway by potentiation of C1 inhibitor[24], to be a potential protective agent of the endothelial cells[32,44] and to play a role in modulating the immune system[44]. The hypothesis was postulated that DXS can play a role in preventing the rejection process of xenotransplantation. DXS is supposed to replace the cleaved HSPG of the glycocalyx.[44]

The shear stress is known to impact the physiology of the glycocalyx. The glycocalyx through its components is regulating many physiological parameters like the coagulation, lipid metabolism or smooth muscle.[13,16,76] The endothelium glycocalyx is a shear sensitive dynamic

structure.[82] The endothelial glycocalyx can adapt to a change in shear stress in its environment by the synthesis of new component or by remodeling of the principal component of the glycocalyx.[82] Some studies showed that shear stress is inducing synthesis of GAG, remodeling and in contrary others showed that the synthesis of GAG is inhibited by the shear stress.[5,29] According to Zeng et al. long term exposure to shear stress is inducing an **adaptive remodeling** of the EG.[89] The controversy increase or decrease of HSPG due to the shear stress might be due to the techniques of measurement, the time of exposition to the shear stress or the level of shear stress itself. According to Arisaka et al, EG needs to be exposed to shear stress to at least 24h before the GAG synthesis process occurs. According to Zeng et al. the HSPG might be clustered when it is exposed to shear stress.[81] These studies can partially explain the reason why in our experiment sometimes the HSPG did not significantly change when exposed to shear stress. It can be due to the shedding or the clustering of HSPG proposed by Zeng et al. or because the cells need more time to adapt to the environment.

In xenotransplantation, the antibodies are the main key players in the activation of the endothelial cells which leads to the initiation of the complement system.[43]
Activation of endothelial cells in xenotransplantation is mostly induced through binding of antibodies and activation of the complement system. Activated EC lose their (HSPG) layer and exhibit a procoagulant and pro-inflammatory cell surface.[43]
The heparan sulfate is shed when there is an even which disturbs the surface of the endothelium glycocalyx. The HSPG is shed for example during the activation of the complement system on the surface of the endothelial cell. This shed HSPG is going to stimulate the immune system to react.[58]

The first experiments conducted were necessary to prove that the EG HSPG is specifically shed by the NHS when circulating through the tubings. The results concerning the **static conditions** confirmed our hypothesis that the NHS is **significantly** shedding the endothelium glycocalyx HSPG specifically. (Figure 9) The results of the quantification of **WGA-lectin** can be seen as a tendency of NHS to shed the EG components but are not statistically significant. (Figure 10) The **significant** increase of **E-selectin (CD62E)** expression when the PAEC are exposed to NHS shows that the endothelial cells are activated. (Figure 11) The E-selectin is known to be express during inflammation process and shear stress. E-selectin is responsible of recruiting leukocytes on the injury place and it is known to be a good marker for endothelial activation. It has already been shown that E-selectin is expressed in case of acute rejection in allograft transplants.[52] In case of E-selectin upregulation, shedding takes place and soluble E-selectin can be detected in serum. This is proving the fact that NHS is efficient to shed the EG.

Further research could focus on the amount of endothelial stress by assessing the levels of membrane-bound and soluble E-Selectin.[39]

The second part of the experiments were conducted under a **peristaltic pulsatile flow** with a shear stress of **shear stress of 10 dyn/cm^2**, **20 dyn/cm^2** and compared to the static condition (no flow).

The aim was to assess the effect of the shear stress on the PAEC and more specifically on the HSPG. The quantification of HSPG did not induce a significant change in the 10 dyn/cm^2 and 20 dyn/cm^2 microchips compared to the static one. Shear stress seems to not to impact the HSPG. (Figure 13) The results concerning **the comparison of shear stress** between static (no flow), 1**0 dyn/cm^2 and 20 dyn/cm^2** show **no significant difference** for HSPG measurements. But, the microchip with 20 dyn/cm^2 was exposed to a supra-physiological [88] shear stress for an aortic endothelial cells and after the analysis of the pictures it might be that cells have been lost because of the high shear stress. (Figure 13) To take in account the **cell loss** in the microchip, the quantification of HSPG green channel was divided by the DAPI blue channel. (Figure 14) Here again, the results did not show any significant difference after the exposition of different state of shear stress. **Shear stress seems to not induce any effect on the HSPG component of the glycocalyx.**

With this experiment, the quantification of fluorescence of the HSPG is significantly increased in microchip under a shear stress of 10 dyn/cm^2 compared to the static condition. This tendency seen in the 10 dyn/cm^2 microchip might indicate an adaptation of the EG to the flow by increasing the HSPG. (Figure 17) But it is rather not clear, the reason why sometimes we obtained a significant increase of HPSG under shear stress and sometimes no significant change.

The shear stress experiment was repeated but this time a staining for **WGA-lectin** was performed. The quantification of the WGA-lectin showed that compared to static condition, the PAEC exposed to a shear stress of **10 dyn/cm^2 and 20 dyn/cm^2** has a **significant decrease** of WGA-lectin binding. (Figure 15) **The results can be interpreted as a shedding due to the shear stress**. But after the analysis of the pictures, the significant decrease for WGA-lectin might be due to the cell loss in the microchip. To take in account the **cell loss** in the microchip, the quantification of WGA-lectin green channel was divided by the DAPI blue channel. (Figure 16). Here, the results show the opposite tendency. There is a **significant increase of WGA-lectin** fluorescence in 20 dyn/cm^2 microchip compared to the static one.

Our findings about the comparison of shear stress for WGA-lectin 20 dyn/cm^2 compared to static condition (Figure 16) and for HSPG 10 dyn/cm^2 compared to static condition (Figure 14) suggest that there might be an increase of the EG exposed to shear stress. However, the mechanism and the exact timing of this adaptation is not clear. This process might maybe start

with a shedding before having an increase of the glycocalyx or no shedding at all**. Here, the results suggest that exposed to high shear stress the EG might adapt to the flow by increasing his component.** (Figure 15) (Figure 16) (Figure 17) This has been demonstrated that the EG can adapt to long-term shear stress and also that shear stress is inducing remodeling of the principal component of the EG.[89,82]

The third part of the experiments were conducted under a peristaltic pulsatile flow of 10 dyn/cm^2. The aim was to assess the potential of the **low molecular weight DXS** to restore the HSPG EG in the microfluidic PAEC model. Three different concentrations were tested: 0.3 mg/ml, 1 mg/ml and 3 mg/ml with preactivation.

The results concerning **0.3 mg/ml DXS** showed **no significant difference** between the control microchip of HSPG 10 dyn/cm^2 and the one with DXS 0.3 mg/ml + NHS. We expected that the measurement of the fluorescence intensity of the microchip treated with DXS + NHS should be significantly higher than the microchip treated with NHS only. The inefficacy of the DXS could be due to an inadequate concentration. Thus, the experiment was reproduced with higher concentration. (Figure 19)

The results concerning **1 mg/ml DXS** showed **no significant difference** between the control microchip HSPG 10 dyn/cm^2 and the one with DXS 1 mg/ml + NHS. We expected that the HSPG level should be significantly higher than the microchip with NHS only. (Figure 20) **The measurement of CD31** showed that DXS 1 mg/ml + NHS might maybe induce an increase of the CD31 compared to the control chip CD31 10 dyn/cm^2 but the results are **not significant** and it is purely speculative. (Figure 22)

The inefficacy of the DXS could be due to an inadequate concentration or because of not adequate activation of the cells, or also the restoration can require a longer period than the two hours exposition to DXS like our experiments. But despite that the DXS is not inducing a restoration of the HSPG in 2 hours, the DXS might induce a **change in the expression of CD31** but it is not significant compared to the control. (Figure 22) These findings indicate that there are maybe some underlying processes occurring and DXS might need more time to be effective.

The fourth part of the experiments were conducted with an extra step was included which is the **preactivation** of the microchip with **NHS 1:4 for 30 minutes** and the **DXS 3 mg/ml** was perfused during again 30 minutes with or without NHS 1:4. The preactivation of the cells should induce more damage to the HSPG EG and strongly activate the cells. **The quantification of HSPG** shows **no differences** between the other microchips. Only one experiment with 3 mg/ml DXS was done. The microchip exposed two times to NHS 1:4 with DXS might have a higher amount of HSPG than the other microchips but it is not significant. (Figure 24) **The measurement of CD31** shows the same pattern as HSPG quantification. We supposed that

the level of expression of CD31 might be high in the microchip exposed to 1:4 NHS two times compared to the other microchips but the results were not significant. (Figure 26)

The microfluidics is targeting to be a better predictive model than the standard classical cell culture assay. As the microfluidics is taking into account more physiological parameters like mechanical shear stress, pulsatile flow, 3D environment or cell to cell contact, this technic is believed to be close to the *in-vivo* situation.[2,7] When a porcine tissue is transplanted into a human host, the human blood which contains the proteins from the coagulation and complement system is circulating through the vessels of the porcine heart leading to the rejection process of the xenograft. With our microfluidics model, this is precisely that situation which is mimicked

Our findings about the effect of shear stress on the EG suggest that exposition to a shear stress it might somehow induce a remodeling of the glycocalyx main component and produce a thicker layer to resist to the flow.[81,5,82] (Figure 16) (Figure 17)

Since we did not obtain a significant difference in DXS inducing replacing the shed HSPG layer, it is a little delicate to draw conclusion. This study's findings suggest that with our microfluidic in-vivo like model we were not able to prove that the DXS is significantly restoring the shed HSPG layer. We could not confirm the data from Laumonier et al. that propose that DXS is acting as a "repair coat" for the endothelium glycocalyx. One of the reason could be the difference of concentration of DXS used or also the method of activation and shedding of the glycocalyx that we used.[44]

Limitations of our study would have been that the PAEC did not well elongate and and align in the direction of the flow in response to the shear stress and the flow rate every time. This issue with the PAEC compared to other bovine or rat cells has already been described in some studies [5] This difference of alignment and elongation between the microchip can be an issue of reproducibility between the experiments. The cells sometimes do not resist to the flow and start to detach. This is one of the major issue which is time consuming and need to be improved by understanding why exactly the cells can not sometimes adapt to the flow. To take into account the potential loss of cell, the fluorescence quantification of HSPG or WGA-lectin was divided by DAPI quantification but it maybe not the adequate method to standardize the potential cell loss between the microchips.

Further experiments should be done to focus on the mechanism of DXS by assessing relevant surface markers in details like P-selectin, E-selectin, CD31 and to more to fully understand its

effect on the glycocalyx and its interaction with HSPG. The shear stress should not be neglect and neither his important interplay with the EG. The understanding of the mechanism of shear stress and of the DXS could play an essential role in the future of xenotransplantation.

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