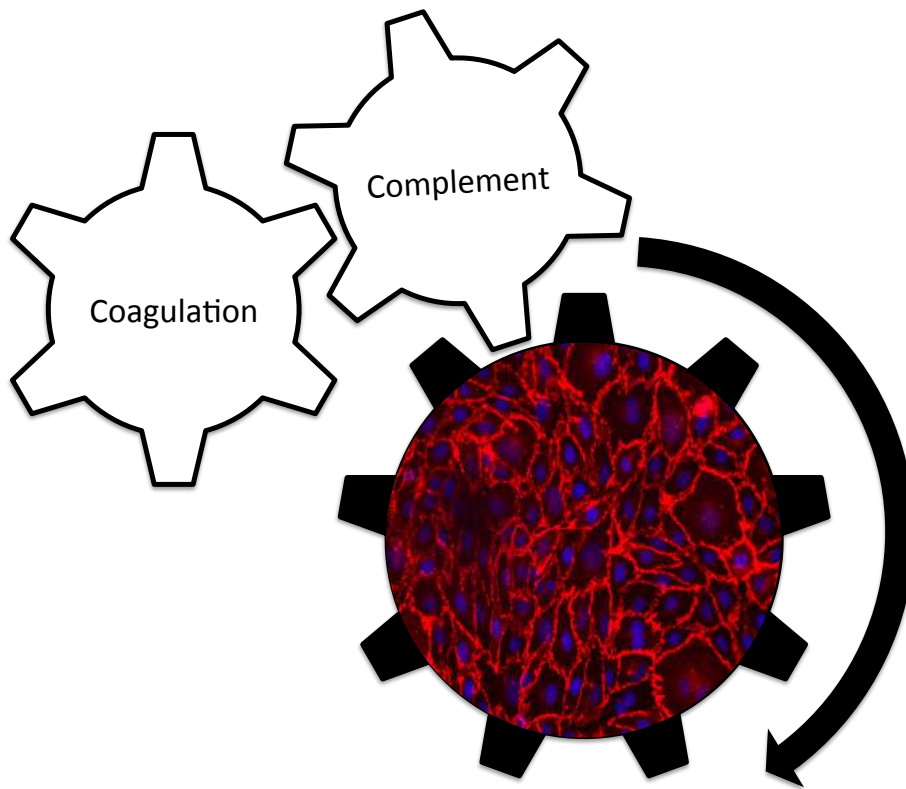


Role of complement and coagulation in ischemia/reperfusion injury



PhD Thesis

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Role of Complement and Coagulation in Ischemia/Reperfusion Injury

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الحمد لله الذي تتم بنعمته الصالحات

إلى من نذرت عمرها لي
إلى من علمني العزيمة و الإصرار
إلى شريك حياتي و نصفى الآخر

Thank Allah for blessing my life

To my mother

To my father

To my husband

Preface

Ischemia/reperfusion injury is involved in all the clinical conditions where the blood supply is cut for a minimum length of time in certain tissues or organs and followed by recirculation of the blood. The ischemia/reperfusion injury is a highly complicated inflammatory condition. Although it was described over 50 years ago, its exact mechanism is still unclear. As a consequence, there is no approved medication that is used for its prevention.

The aim of my thesis was to study in-depth the role of complement and coagulation and their interaction with the endothelial surface during ischemia/reperfusion injury, namely in the context of the heart and skeletal muscle. Porcine models of skeletal muscle and heart ischemia/reperfusion injury were used. Furthermore, a detailed protocol of the porcine closed chest myocardial infarction model was provided in order to increase the reproducibility of the experiments between different laboratories.

In the current thesis, I have included one published article, one submitted manuscript and another three manuscripts that are in preparation for submission to different journals. I am the first author on four papers and I contributed as co-author to the last paper. The first three papers focused on ischemia/reperfusion injury in the skeletal muscle and heart. The fourth paper is a methodological paper about the porcine myocardial infarction model. The final one investigated the fine-specificities of naturally occurring anti-carbohydrate antibodies in the context of the pathogenesis of Primary Antibody Deficiency Syndromes. This thesis ends with an overall conclusion, acknowledgements and my curriculum vitae.

At the end of this preface, I would like to express my deep gratitude to the amazing and great support and guidance provided by my supervisor Prof. Dr. Robert Rieben, who gave me the chance to do my PhD with his team. I also would like to thank all our team members for their direct or indirect role in my thesis. Last but not least, I would love to thank my husband Dr. med. Ahmed Shaker for his support and encouragement, my beloved daughters Khadija and Rokaia, as well as my parents Mona Metwally and Moustafa Hafez.

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Abstract

Ischemia reperfusion injury was discovered and defined since more than half a century. Nonetheless to date, there is no approved treatment that shows an efficacy to prevent it. Ischemia reperfusion injury is a complex disease that can occur in all tissues or organs in the body. Its complexity is due to the participation of several damage mechanisms involving activation of the vascular endothelium, complement, coagulation and kinin systems as well as oxidative stress, cellular ion imbalance and mitochondrial damage. The contribution of the above-mentioned mechanisms is different between different organs, which makes it even more difficult to find a suitable treatment. In the last few decades translational research on reperfusion injury faced the problems that a number of novel molecules that showed a highly promising effect in preventing ischemia reperfusion injury in small animal models failed to show success in clinical trials. The anatomical and physiological similarity between humans and pigs might increase the success of translational medicine especially with regard to novel and promising drugs. In addition, the availability of transgenic pigs gives the scientific community the opportunity to confirm the pathophysiological mechanisms of ischemia reperfusion injury clinically relevant animal models.

In this thesis, a study was performed which aimed to prove the efficacy of human C1-esterase inhibitor in reducing ischemia reperfusion injury in a pig model using an amputated limb and ex-vivo reperfusion. Transgenic pigs have also been used to investigate in-depth the role of complement and other plasma cascades in a model of myocardial ischemia reperfusion injury. Finally, a refinement of the myocardial ischemia reperfusion injury model has been described, aiming to increase the reproducibility of this model between labs.

In conclusion, C1-esterase inhibitor is a promising candidate for reducing skeletal muscle reperfusion injury. The next step will be to use this drug in clinical trials for elective surgical interventions involving skeletal muscle ischemia followed by reperfusion. Moreover, myocardial ischemia reperfusion injury was greatly reduced when human complement regulatory proteins were overexpressed in pigs, underlining the importance of complement activation in this model. The use of membrane-targeted complement inhibitory drugs might therefore be promising to prevent myocardial ischemia reperfusion injury.

Introduction

The humoral innate immunity

The innate immune system is part of the body's defense system and differs from the adaptive immune system in that it provides a basic and quick response to eliminate invading pathogens. It does not have a memory [1]. Over the years, several mechanisms have been proposed to explain how the innate immunity is initiated. The self-nonsel, the infectious nonself and most recently the danger signal mechanisms explained many of the immune responses but not all [2]. The innate immune system does not only protect the body against microbial invasion and stimulate the adaptive immune system but also eliminates damaged and host dead cells. Stressed and ischemic self cells express specific signals, that can be explained by the danger model, and trigger the innate immune system [3]. The innate immune system works mainly via stimulating the inflammatory response and antiviral defenses [4]. Inflammation normally involves the recruitment of leukocytes and activation of the plasma cascades (complement, coagulation and kinin systems) at the site of injury.

The complement system

In the late the nineteenth century, the term complement was first mentioned by Paul Ehrlich in 1899, although several other authors also contributed to the actual scientific discovery of the complement system [5]. The complement system is the first line of defense against the invading pathogen. It also works against damaged host cells to facilitate their removal. The complement proteins are produced by the liver and a wide variety of other cells that are stimulated by different cytokines or hormones. The complement system is composed of several plasma proteins that circulate as inactive precursors and are activated at the site of injury. Anaphylatoxins, opsonins and the terminal attack complex are three major components that are generated upon activation of the complement system [6] as illustrated in table (1).

As reviewed by Ricklin [7], the complement system can be activated through three main known pathways: The classical, the alternative and the lectin pathway as shown in figure (1). The classical and the lectin pathways are similar

and share similar downstream complement activation molecules starting from activation of C4 and C2.

Table (1): Active components of complement

Effectors	Name	Function
Anaphylatoxins	C3a and C5a	Highly pro-inflammatory. Attract and activate white blood cells
Opsonins	C3b, iC3b and C3d	Deposit on the target cell to facilitate its elimination
Terminal membrane attack complex (MAC)	C5b-9	Forms a hole on cell surfaces and lyses the pathogen or damaged cell

The classical pathway requires immunoglobulin binding on cell surfaces (either pathogen or host expressing DAMPs) followed by binding to C1q and a tetramer containing 2 of each C1r and C1s, which are proteases. The complex C1r₂s₂ activates C4 protein to generate C4b. The cell bound C4b then binds to the next complement protein C2 and C1s break it down into small C2b and larger C2a. The complex C4b2a is now formed on the cell surface and known as C3 convertase. The lectin pathway generates the C3 convertase via a similar mechanism, varying in the fact that the sequence starts when lectins, namely mannose binding lectin (MBL) or ficolins, attach to the cell surface (pathogen or damaged cell) in the absence of antibodies or in the presence of IgM. The lectins are associated with the MBL-associated serine proteases (MASPs), which hydrolyze C4 and C2 to form C3 convertase (C4b2a) in the same way as in the classical pathway. Both classical and lectin pathways share the same downstream cascade. The C3 convertase splits C3 into soluble C3a and C3b that covalently binds to the cell surface. One molecule of C3 convertase can cleave thousands of C3 molecules and the amplification of the complement cascade occurs at this step. Then C3b binds to C4b2a (C3 convertase) to form C4b2a3b (C5 convertase) to start the late steps in the complement cascade by cleaving C5 into soluble C5a and cell-bound C5b. This late sequence of the complement cascade requires two C5b molecules to be deposited on the cell surface, which then bind to the next proteins C6, C7 and C8. C5b-8 has a side chain that is inserted into the lipid cell membrane but not yet capable of lysing the cell. C9, the last protein in the complement cascade, polymerizes at the site of C5b-8 to form

the membrane attack complex (MAC, C5b-9), which can make pores in the cell membrane leading to osmotic swelling and cell rupture.

The alternative pathway has a unique feature compared to the other pathways. It is always active in the serum generating low concentrations of soluble C3a and C3b. If the C3b produced remains in the fluid phase, then it will be hydrolyzed and the complement cascade will not start. If the C3b produced covalently binds to cell surface, factor B will recognize C3b and the bound factor B will be cleaved by factor D into small Ba which will be released into the fluid phase and large Bb which will remain attached to C3b to form C3bBb (C3 convertase of the alternative pathway). C3bBb will then act on more C3 to produce more C3b and a positive feedback loop will start. It is also important to know that bound C3b on the cell surface from the classical and lectin pathways can be bound to factor B and form more C3bBb. The amplification loop will lead to more complement activation and down steaming to form MAC. The newly formed C3b will bind to C3bBb to form C3bBbC3b (C5 convertase) and cleave C5. All the three complement pathways share the same downstream sequence from C5b down to the formation of MAC which will cause cell lyses [4], [6] (figure 1).

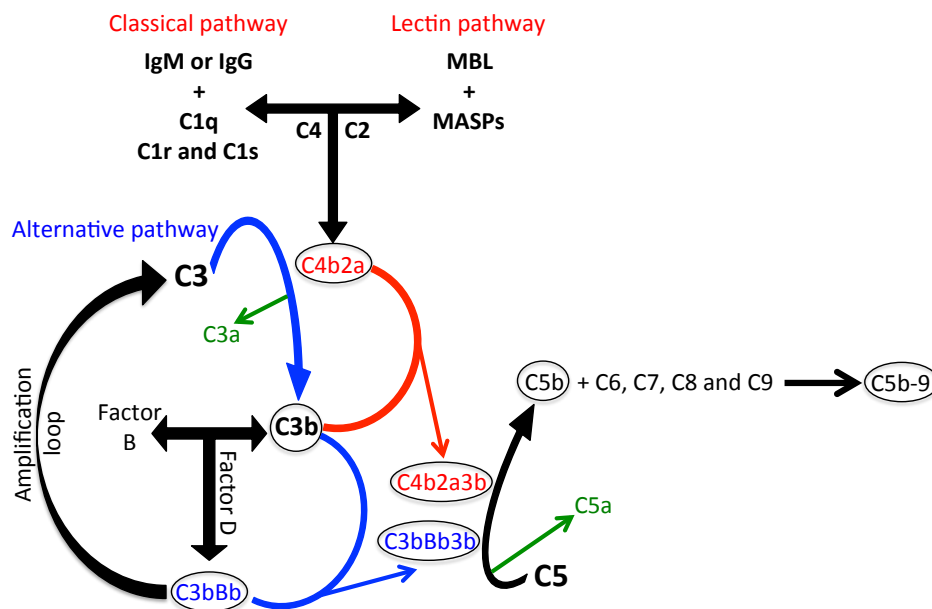


Figure (1): The complement pathway. The encircled proteins are deposited on the cell surface. C3a and C5a are anaphylatoxins. C4b2a and C3bBb are C3 convertases. C4b2a3b and C3bBb3b are C5 convertases. C3b formed from the classical and lectin pathway can be amplified through the alternative pathway amplification loop.

As the complement components are widely distributed in the body and can be activated by the host's signals and some of them, namely C3, is continuously cleaved into its active C3a and C3b components, our body also has a variety of complement regulatory proteins:

- C1-inhibitor (C1-INH), present in the plasma, irreversibly inactivates C1s and C1r of the classical pathway as well as MASP-1 and 2 of the lectin pathway and it is also known to be a serine protease inhibitor [8].
- C4 binding protein, a fluid phase regulatory protein, inactivates the classical and lectin pathways by binding C4 and displacing C2 so that it deactivates C3 convertase [9]. It also inhibits the alternative pathway by acting as cofactor for factor I to inhibit C3b [10], [11].
- Factor I, present in the plasma, inhibits C3b to inactive C3b (iC3b) as well as C4 only in the presence of cofactor proteins membrane cofactor protein (CD46), type 1 complement receptor (CR1), factor H or C4-binding protein [12], [13].
- Factor H, present in the plasma, works as cofactor for factor I to inactivate conversion of C3b into iC3b. It also regulates the alternative pathway by binding C3b and competing with factor B as well as facilitating C3bBb dissociation [14]. Factor H also has a high binding affinity for the endothelial glycocalyx so that it works on both the fluid phase and on cell surfaces [15], [16].
- Membrane cofactor protein (MCP, CD46) is a membrane bound complement regulatory protein. It acts as cofactor for inactivation of both C3b and C4b by factor I [17].
- Decay accelerating factor (DAF, CD55) is also a membrane bound regulatory protein that is expressed on endothelial cells and erythrocytes. It accelerates dissociation of C3 convertase of all complement pathways but with different affinity [18].
- Type 1 complement receptor (CR1), a membrane bound protein, acts as a cofactor for inhibition of C3b by factor I [19].
- MAC inhibitory protein (MAC-IP, CD59), a membrane bound protein, prevents the formation of MAC by competing with C9 and preventing its incorporation into C5b-8 [20], [21].

- S-protein, present in the fluid phase, binds the soluble C5b, C6 and C7 complexes and prevents their insertion into the cell membrane [22].
- Thrombomodulin, a membrane bound protein, increases factor H cofactor activity. It also acts as an important cofactor for activation of thrombin activatable fibrinolysis inhibitor (TAFI) that inactivates C3a and C5a [23].

The coagulation system

Hemostasis is a state of equilibrium between the vascular endothelium, platelet recruitment, and coagulation. It prevents spontaneous coagulation in the blood stream or excessive bleeding at the site of a trauma [24]. Davie and his colleagues were the first to describe the coagulation cascade and its downstream enzymatic effects [25].

The coagulation cascade is classically divided into the intrinsic and extrinsic pathways depending on the starting point of coagulation. As described in a review by Adams and Bird, the intrinsic pathway starts by contact activation of factor XII by negatively charged surfaces (high molecular weight kininogen, HMWK and prekallikrein, PK) into active factor XIIa, then factor XIIa activates factor XI in the presence of HMWK and calcium ions into active factor XIa followed by activation of factors IX and X in the presence of factor VIIIa and calcium ions to form active factor Xa. Factor Xa is common to both the extrinsic and intrinsic pathways. The extrinsic pathway starts in the presence of trauma and activated endothelial cells that express tissue factor that interacts with factor VIIa in the presence of the platelet to activate factor X into active factor Xa. The tissue factor-factor VIIa complex also activates factors XI and IX in the intrinsic pathway resulting in a positive feedback amplification loop. Both intrinsic and extrinsic pathways share a common pathway that starts with active factor Xa. Factor Xa in the presence of platelets, calcium ions and factor Va converts prothrombin into thrombin, which converts soluble fibrinogen into insoluble fibrin resulting in clot formation [26] (figure 2).

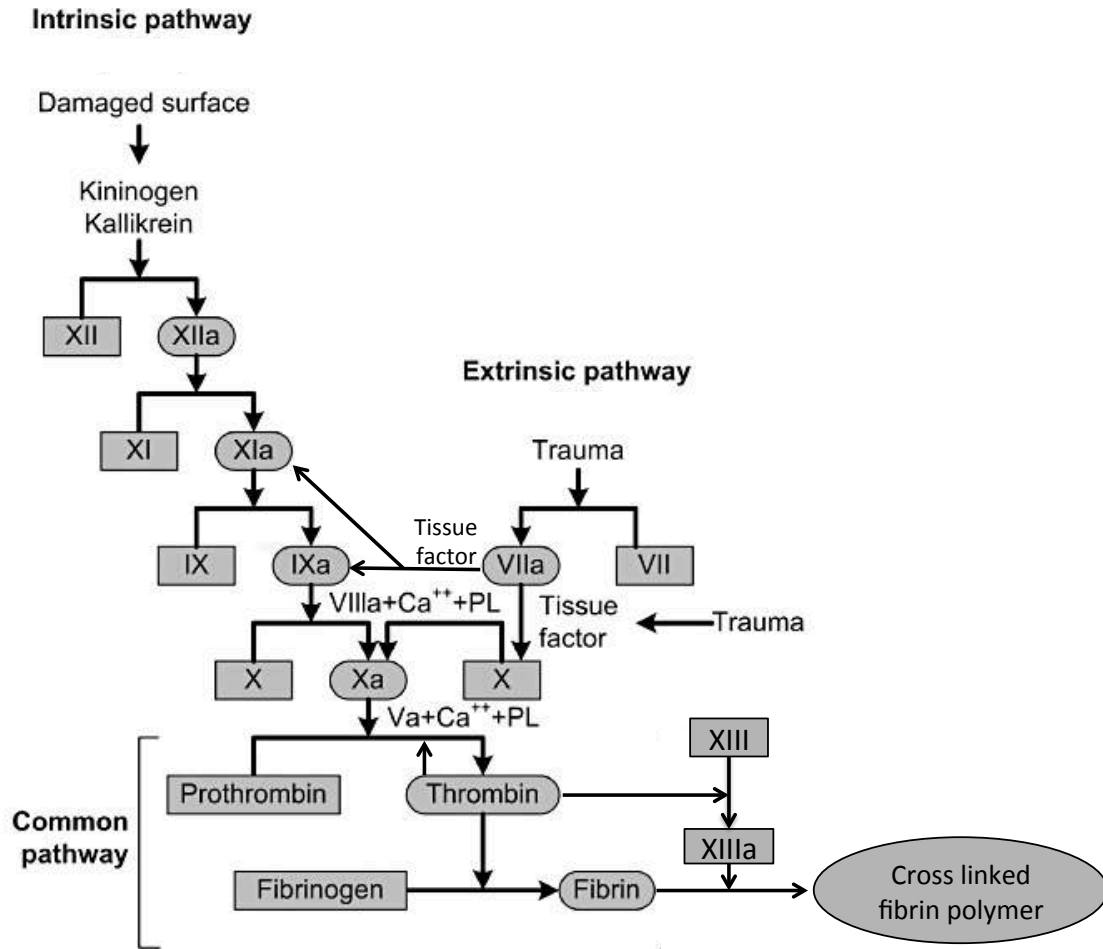


Figure (2): The coagulation cascades. The intrinsic, extrinsic and common coagulation pathways. Adapted from [26].

The vascular endothelium plays an important role to maintain hemostasis as it has anti-coagulant properties via:

- Glycocalyx layer, covers the endothelial cell surface and reduces endothelial-platelet interaction.
- Antithrombin III
- Expression of tissue factor pathway inhibitor, which deactivates the biological activity of the tissue factor/VIIa/Xa complex through binding of factor Xa [27].
- Expression of thrombomodulin (TM) which interacts with thrombin to activate the anti-coagulant activated protein C (APC). APC inactivates factor V, VIII and fibrinogen. TM also interacts and inactivates factor Xa [28].
- Expression of Protein S, a cofactor for APC activity [29].

- APC receptors expression that regulate the TM-thrombin-APC pathway [30].

The kinin system

The kinin system (plasma kallikrein-kinin system) is one of the plasma cascades involved in the innate immune system of the body. It consists of the complex of prekallikrein (PK) and high molecular weight kininogen (HMWK) and coagulation factor XII (FXII). Bradykinin, a strong pro-inflammatory peptide, is generated when PK and HMWK are activated in the plasma [31]. C1-inhibitor and anti-thrombin III are important regulators of the kinin system [32].

The plasma kallikrein-kinin system has several biological functions: (1) vasodilation and control of vasodepressor substances of the cardiovascular system as well as increasing vascular permeability, (2) a central role in vascular inflammation and expression of vascular endothelial growth factor, (3) converting the anti-inflammatory state of the endothelium into pro-inflammatory and anti-fibrinolytic state via stimulating endothelial cells to express tissue factor and plasminogen activator inhibitor-1 [33].

Bradykinin receptors are classified into two types, bradykinin receptor B1 and B2. Bradykinin receptor B2 is continuously expressed on most tissues while B1 is only expressed in the pro-inflammatory state [34].

Overview of the interactions between the plasma cascade systems

The soluble components of the innate immune system (complement, coagulation and kinin systems) interact with each other and with the vascular endothelial layer. It is important to explore and understand this communication in order to restore the normal physiological condition and treat any pathological conditions affecting endothelial cells and plasma cascades. Both the complement and the coagulation cascade are initiated by expression of altered self (damage-associated molecular patterns, DAMPs) or pathogens and activation occurs in close contact with the endothelial surface. Moreover, both systems have common regulatory proteins that are involved in amplification as well as positive or negative feedback regulations as reviewed by Oikonomopoulou [35].

Proteins of the coagulation and kinin systems can activate the complement system on the level of C3 and downstream. Wiggins et al. and Discipio et al. reported the activation of both C5, C3 and factor B by kallikrein [36]-[38]. Activated factors FIX, FX and FXI as well as thrombin and plasmin can generate active C3b, C3a, C5b and C5a in-vitro, the latter process has been defined as the extrinsic complement pathway [39]. Thrombin-mediated C5 cleavage is particularly important for activation of the terminal complement pathway in case of a C3 deficiency [40]. Moreover, active FXII can activate C1 and lead to classical pathway complement activation even in the absence of antigen-antibody complexes [41]. Recently, it was confirmed in-vitro that both fibrin and fibrinogen can activate ficolins and MBL in the lectin pathway [42]. Furthermore, activated platelets up-regulate the expression of P-selectin which in turn acts as a binding site for C3b and stimulates more C3 activation [43]. On the other hand, coagulation proteins can also inhibit the complement activation as thrombin enhances the expression of the complement regulatory protein decay-accelerating factor (CD55) through a negative regulatory feedback mechanism on the cell surfaces [44], and thrombin-activatable fibrinolysis inhibitor (TAFI), which is activated by thrombin-thrombomodulin interaction, can inactivate the complement anaphylatoxins C3a and C5a [44]. Also, tissue factor pathway inhibitor (TFPI) inhibits the lectin pathway through inhibition of MBL-associated serine protease inhibitors 2 [45].

The complement proteins intercommunicate with the coagulation pathways in different ways. MBL-associated serine protease inhibitors 1 and 2 (MASP-1 and MASP-2) have the capability to deposit fibrin at the site of activation. In an in-vitro study it has been shown that MASP-1 has thrombin like activity that can activate FXIII and convert fibrinogen into fibrin [46]. While MASP-2 has a direct prothrombinase activity that can generate thrombin from prothrombin when MASP-2 is bound to ficolins or MBL leading to deposition of fibrin at the site of inflammation [47]. Moreover, MASP-1 cleaves high molecular weight kininogen to produce bradykinin [48].

The endothelial cell layer is an important surface for the development of inflammation during I/R injury. The plasma cascades interact closely with the endothelial cell layer and the affected endothelium produces several molecules

that positively and negatively affect the progress of the inflammatory reaction. The binding of the up-stream complement protein C1q to the endothelium leads to its activation via nuclear factor κ B [49]. Further details are given in the next chapter on the endothelial glycocalyx.

The endothelium and its glycocalyx

Although the endothelial glycocalyx was first described and visualized by Luft in 1966 [50] using electron microscopy, its functional importance was only described by Pries et al in 2000 [51]. The endothelial glycocalyx is composed of a carbohydrate-rich layer that is connected to the endothelium through a proteoglycan and glycoprotein backbone. The proteoglycan is a core protein linked to one or more glycosaminoglycan chains. The core proteins, which are strongly connected to the cell membrane, can be syndecans or glypicans [52]. In addition, there are soluble proteoglycan proteins namely perlecan, versican, decorin, biglycan and mimecan, which are diffused in the blood stream as soon as they are formed, only to stick back to the endothelial glycocalyx as soon as they have bound to plasma proteins [53], [54]. As reviewed by Reitsma et al 2007 there are five types of glycosaminoglycan chain that are linked to the core protein. The different glycosaminoglycan chains are named heparan sulfate, chondroitin sulfate, dermatan sulfate, keratan sulfate, and hyaluronan, which are linear polymers of disaccharides with variable lengths. The length of glycosaminoglycan chain can be modified by the variable extent of sulfation and/or (de) acetylation [55]. About 50-90% of the vascular glycocalyx is composed of heparan sulfate proteoglycan and the second abundant is the chondroitin sulfate/ dermatan sulfate chains [56]. The second backbone consists of glycoprotein molecules that are characteristically smaller sugar residues and also incorporate endothelial cell adhesion molecules. The selectin family consists of both P and E-selectin and plays an important role in leukocyte-endothelial cell interaction during the inflammation process [57]. The integrin family is a heterogeneous glycoprotein group that is expressed on many cell surfaces, not only endothelial cells, and plays an important role in platelet adhesion and interaction with other molecules such as fibronectin and collagen [58]. Finally the platelet endothelial adhesion molecule-1, the vascular endothelial adhesion molecule-1 and the intracellular adhesion molecule 1 and 2 are all members of an immunoglobulin superfamily (glycoprotein molecules) that are expressed on endothelial cell surfaces and either up or down regulated during inflammation. In addition to adhesion molecules, the glycoprotein Ib-IX-V complex, which interact with both von Willebrand factor (vWF) and P-selectin, is involved in

forming the balance between coagulation and fibrinolysis. It enhances platelet endothelial interaction via P-selectin. Moreover, it works as a receptor for vWF [59].

In addition to the above-mentioned complex structure of the glycocalyx, a group of several soluble molecules are integrated into the glycocalyx mesh. The soluble molecules play an important role in the functional importance of the glycocalyx layer and its components are either produced by the endothelium or captured from the blood stream[55]. It is important to realize that the glycocalyx is a dynamic layer that undergoes continuous changes due to several reasons: (1) the continuous enzymatic or mechanical shedding of the glycocalyx and the balance with its continuous synthesis (2) the equilibrium state between the soluble molecules and the blood components [60]. Furthermore the glycocalyx is considered as a fine, sensitive complex layer such that the loss of only one component might lead to a switch from the normally anti-inflammatory and anti-coagulant function to a pro-inflammatory and pro-coagulant one [61].

The glycocalyx plays an important role in controlling vascular permeability as it allows the passage of small molecules and hinders large molecules. This function works through steric hindrance and electrostatic charges [62]. In a rat model of myocardial infarction it has been proven that the glycocalyx shedding leads to increased vascular permeability and edema [63]. In addition to controlling the vascular permeability, the endothelial glycocalyx affects the blood cell-vascular wall interaction. The glycocalyx layer protects the vascular endothelium from interaction with all blood cell types. The glycoprotein backbone of the glycocalyx contains the adhesion molecules that allow leukocyte rolling only after endothelial cell activation as reviewed by Reitsma [55]. Recently it has been confirmed that the glycocalyx forms an important connection between shear stress and production of biochemically active molecules such as nitric oxide, which is a potent in-vivo vasodilator. The glycocalyx is important in the conversion of the physical signal (shear stress) into biochemical signals (i.e. the production of nitric oxide) [64]. The endothelial glycocalyx does not only harbor the plasma-derived molecules but also facilitates the interaction with their ligands.

Reitsma and his colleagues reviewed a number of plasma derived molecules and showed that their interaction is required to be within the glycocalyx layer and plays an important role in controlling (1) coagulation through anti-thrombin III, heparin cofactor II and tissue factor pathway inhibitor (2) inflammation via modulation and attenuation of different cytokines on the vascular endothelial cells (3) oxidative stress by superoxide dismutase scavenging reactive oxygen species and maintaining the nitric oxide efficacy (4) the concentration gradient of growth factors to keep up its repair effect and finally (5) lipolytic system via interaction between lipoprotein lipase and low density lipoprotein[55].

Ischemia reperfusion injury

Ischemia reperfusion (I/R) injury is a pathological sterile inflammatory condition that can occur in almost all tissues after they were exposed to a certain of time ischemia [65], [66], followed by reperfusion. Cutting of the blood supply is an emergency state that will eventually lead to severe tissue or organ damage and even death. Restoration of the blood supply is essential as soon as possible to save the ischemic tissue or organ but, nevertheless reperfusion injury always develops [67].

I/R injury occurs in a broad range of clinical conditions such as myocardial infarction, stroke, and acute kidney disease as well as surgical procedures such as organ transplantation, limb replantation or even when a tourniquet is needed such as in total knee replacement surgery (table 2).

Table 2: Most reported clinical conditions that lead to I/R injury

Affected organ	Clinical condition
Brain	Stroke[68]
Heart	Myocardial infarction[69] Heart transplantation[70] Cardioplegic arrest from various cardiac surgeries[71]
Intestine	Abdominal and thoracic vascular surgery Small bowel transplantation Hemorrhagic shock[72]
Kidney	Infarction Sepsis Kidney transplantation[73]
Liver	Prolonged surgical liver resection Reduced liver perfusion due to heart or respiratory failure, shock, sepsis, trauma or hemorrhage Liver transplantation[74]
Lung	Atherosclerosis Pulmonary embolism Trauma[75] Cardiac bypass surgery[76] Lung transplantation[77]
Skeletal muscle	Limb replantation or transplantation Tourniquet use during surgery [78]

I/R injury can increase the morbidity and mortality of the patients. I/R injury contributes to a worsening of clinical condition, for example it causes bigger infarctions in both the brain and the heart, with the latter resulting in acute or chronic heart failure. It can also lead to acute kidney or lung injury. I/R injury can also contribute to acute and early graft rejection after organ transplantation [66].

The injury that occurs due to I/R injury can be divided into two phases.

Ischemic injury

The first phase is the injury due to ischemia where no O₂ is supplied to the tissue and the main source of energy generation ATP synthesis and oxidative phosphorylation is stopped. The cells will then shift to anaerobic metabolism resulting in a drop in pH followed by activation of the Na⁺/H⁺ pump and accumulation of Na⁺ in the cells. As a result all the ion homeostasis is disrupted resulting in Na⁺ and Ca⁺⁺ accumulation in the cytosol, inactivated ATPase and increased mitochondrial permeability leading to further impairment of ATP production [79], [80].

The length of ischemia and the temperature are two important parameters that affect the degree of reperfusion injury in different organs. Also different organs respond differently to ischemic- as well as reperfusion-mediated injury [80]. For example, brain is very sensitive to ischemia due to its high energy requirement. An irreversible cell death occurs within few minutes of warm ischemia [68]. Other organs are less susceptible to ischemia such as the lung and liver due to their dual blood supply but they lose this advantage during lung or liver transplantation because they are exposed to global ischemia [74], [77]. A vascularized composite tissue such as a limb is more resistant to ischemia, with ischemic tolerance ranging from 4 hours for skeletal muscle, 8 hours for the nerves, 24 hours for the skin and up to 4 days for bones [81].

The ischemic phase is also characterized by formation and release of damage associated molecular patterns (DAMPs) and neo-epitopes, which can be recognized by naturally occurring antibodies (namely IgM) and activate the innate immune system as well as trigger a sterile inflammatory response[82]. Several molecules that are expressed and released in response to ischemic injury have been identified as DAMPs and include non-muscle myosin heavy chain II type A and C (NMHC II) [83], Annexin IV [84], [85] and different phospholipids moieties [86].

Reperfusion injury

Effect of ischemia reperfusion injury on the vascular endothelium

The endothelium is the first structure that comes into contact with the blood during the reperfusion phase. Vascular endothelial cells act as a protective layer that prevents intravascular leukocyte adhesion and platelet activation. The pH change, ion homeostasis disturbances, stress and the neo-epitope expression are all hallmarks of ischemia that induce endothelial activation, dysfunction and damage including shedding of the glycocalyx layer [87], [88].

Effect of ischemia reperfusion injury on endothelial glycocalyx

It has been reported that I/R injury leads to a reduced glycocalyx layer thickness and shedding during the reperfusion phase in an intestinal I/R injury rat model [89], a myocardial I/R guinea pig model [90] as well as in a clinical trial for patients subjected to global or regional ischemia during major vascular surgery [87]. Taking all the previously mentioned research into consideration, the glycocalyx shedding and damage appears to cause an increase in the negative effects of the oxidative stress on the endothelial cells as well as allowing the blood components (cells and plasma) to interact with the endothelium that leads to an increase in the inflammation and tissue damage.

Activation of endothelial cells during ischemia reperfusion injury

The endothelial cells are the inner lining of the vasculature. They have anti-coagulant and anti-inflammatory properties. The activation of the endothelial cells during I/R injury converts endothelial cells into a pro-coagulant and pro-inflammatory state and allows the endothelial cells to localize the inflammation at the site of injury.

The early stage of endothelial cell activation is characterized by shedding of the glycocalyx layer and release of von Willebrand factor and IL-8 within the first minutes following the reperfusion injury [91]-[93]. The later stage involves expression of different cell adhesion molecules leading to endothelial cell leukocyte and platelet adhesion as well as activation of the plasma cascade systems which means initiation of sterile inflammation and activation of the innate immune system [66]. Moreover, the activated endothelial cells up-regulate several pro-coagulant and pro-inflammatory proteins such as tissue

factor, plasminogen activator inhibitor-1 (PAI-1), fibrinogen like protein 2 (FGL-2) and different cytokines [94]-[96]. From this point a vicious circle starts where more inflammation leads to more endothelial cell activation and more plasma cascade activation that ends with tissue injury and cell death.

Role of natural antibodies and complement in ischemia reperfusion injury

I/R injury triggers the change of the cellular epitopes which can be recognized by natural antibodies circulating in the blood [97], [98]. The innate immune system recognizes these neoepitopes as DAMPs and enhances the inflammation process. There are several neoepitopes that have been recognized in the context of reperfusion injury such as non muscle myosin heavy chain type II A and C, phospholipids, β 2 glycoprotein [83], C-reactive protein and high mobility group box-1 (HMGB-1) [99]. The expressed neo-epitopes interact with preformed natural antibodies in the blood. The inflammatory response during I/R injury does not relate only to the cellular leukocyte infiltration to the site of injury but also to the soluble innate response involving natural antibodies and the plasma cascades [100].

In the early 1970's, Hill and his colleagues described the role of complement in a myocardial I/R injury model [100]. Since then several animal models have confirmed the role of the complement system in I/R injury in different organs. It has been demonstrated that all complement pathways are activated to various degrees in different models of I/R injury. The use of different complement regulatory protein or complement protein knock out animals has successfully reduced or inhibited the tissue damage associated with different animal models of I/R injury. The study of complement in I/R injury in humans has mainly concentrated on the myocardial setting, and suggested that mostly the classical and lectin pathways are involved in the pathogenesis of I/R injury [101]. It is still controversial which complement pathways participate in the pathogenesis of I/R injury and how important the respective activation pathways are with respect to the pathophysiology. All complement pathways can be initiated in I/R injury. Complement pathways involvement is organ- and species dependent as reviewed earlier [102], [103]. Moreover, several studies focused on the novel role of lectin pathway over the antibody dependent

classical pathway [102]. The important role of lectin pathway was confirmed in both myocardial and gastrointestinal mouse models of I/R injury [104]. In addition, in our own recent study on skeletal muscle I/R injury in a pig model, activation of the lectin pathway was also proven [105].

Role of coagulation in ischemia reperfusion injury

I/R injury can initiate coagulation via activation of the vascular endothelium as well as via the complement cascade. Activated vascular endothelium loses its protective vascular glycocalyx layer and increases the expression of adhesion molecules leading to increased platelet endothelial adhesion incidence and activation of the coagulation cascade [106]. Tissue factor, an important activator of the extrinsic pathway, can be released from both activated vascular endothelium as well as neutrophils that recruited at site of I/R injury [107]. Inhibition of tissue factor has been shown to reduce renal injury in a model of kidney I/R injury [108]. Tissue expression of FGL-2, which has a direct prothrombinase activity, has been shown to initiate inflammation and coagulation in both myocardial and hepatic models of I/R injury [96], [109]. In a lung I/R injury model, Lau and his colleagues showed that the plasminogen activator inhibitor-1 knockout mice showed less histological lung injury as well as lower plasma levels of inflammatory cytokines [110]. The activation of the coagulation cascade during I/R injury leads to formation of thrombin that activates more endothelial cells to produce more tissue factor leading to a positive feed back loop resulting in more injury (figure 3) [111]. In a mouse model of myocardial I/R injury, it has been shown that the use of anti-thrombin III can reduce myocardial I/R injury but the required dose was high so that it increased the risk of bleeding [112]. The fibrinolysis also plays an important role in I/R injury, but is still controversial today. Healthy endothelial cells constantly express tissue plasminogen activator (tPA) as an important pro-fibrinolytic molecule. In I/R injury the activated and damaged endothelial cell reduce tPA expression and increase the formation and secretion of plasminogen activator inhibitor-1 (PAI-1), leading to reduction in the fibrinolytic property of the vasculature [110], [113]. In contrast, a recent ex-situ study of human liver I/R injury showed activation of fibrinolysis and upregulation of both tPA and PAI-1

[114]. Moreover, a tPA knockout mouse model of brain and kidney I/R injury showed less brain infarct size and kidney injury than wildtype controls [115], [116].

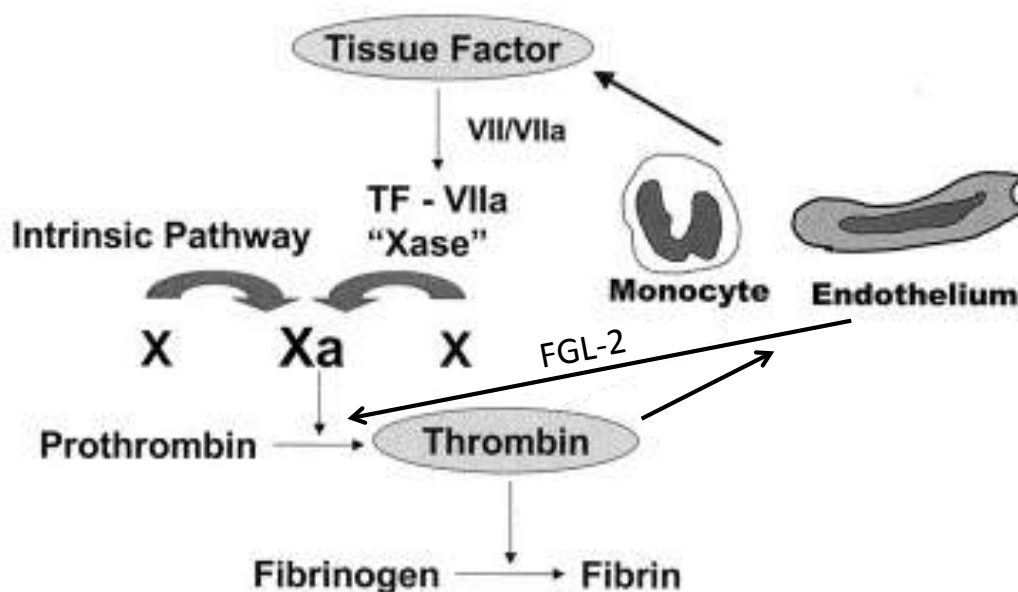


Figure (3): The interaction between endothelium and coagulation pathway. FGL-2: fibrinogen like receptor-2. Adapted from [110]

Role of bradykinin receptors in ischemia reperfusion injury

The role of the bradykinin receptor in the I/R injury is still controversial. In a rat model of brain I/R injury, the bradykinin receptors B1 and B2 were upregulated but interestingly the bradykinin receptor B1 antagonist showed a protective effect and improved neurological function of the rat as well as reducing the gene expression of the pro-inflammatory cytokines while the bradykinin receptor B2 antagonist worsened the neurological functions [117]. In contrast, data from a mouse model of renal I/R injury, where the mice were deficient in both bradykinin receptor B1 and B2, showed that the reduced B1 and B2 expression had a protective effect in minimizing the injury [118]. Similarly, a model of rat lung transplantation and lung I/R injury showed a strong upregulation of bradykinin receptor-2 during I/R injury, which could be reduced by administration of bradykinin receptor-2 antagonist in the presence of DPP-4 inhibitor [119]. Research from our laboratory showed that the both bradykinin receptors B1 and B2 are upregulated in both rat and porcine models for skeletal muscle I/R injury [105], [120].

Skeletal muscle ischemia reperfusion injury

Skeletal muscle I/R injury occurs as a result of partially or totally cutting the blood supply to the limbs (leg or arm, whole or part) followed by restoration of the supply during the reperfusion process. The cut to the blood supply can be total when replanting an amputated limb or partial in the case of vascular injury. Irreversible skeletal muscle damage starts after 3 and is complete within 6 hours under normothermic condition [81].

Mechanisms of skeletal muscle I/R injury

Endothelial cell activation

Our research group has demonstrated the damage to the vascular endothelium within the skeletal muscle as a result of rat hind limb I/R injury. The non-treated group had shed heparan sulfate proteoglycan (HSPG), indicating a damaged endothelium [120]. Later, in an amputated and ex-vivo porcine limb I/R injury model, the endothelial damage was supported by the loss of the endothelial markers CD31, VE-cadherin as well as shedding of HSPG, in addition to increased expression of the adhesion molecule E-selectin. The endothelial cells are not only damaged, but they also increase the expression of FGL-2 which activates the coagulation via activation of prothrombin into thrombin [105].

Complement activation

The reperfused tissue expresses neo-epitopes that are exposed to natural antibodies in the plasma. The deposition of immunoglobulin has been shown in several models of skeletal muscle I/R injury. The latter led to activation of the classical and lectin complement pathways, which indicates their involvement in the inflammatory progression of the injury. The formation of MAC as the final down-stream product of the complement system leads to cell lysis. More inflammatory cytokines are released from neutrophils that are recruited to the site of the injury and produce more reactive oxygen species (ROS), leading to propagation of the injury [121].

Oxidative stress and production of reactive oxygen species ROS

The production of ATP is dramatically reduced during the time of ischemia as a result of oxygen deficiency leading to accumulation of intracellular Na^+ and Ca^{++} , increased glycolysis and a reduction of pH. Next, during early reperfusion, a rapid elevation of pH leads to increased mitochondrial permeability which activates cell apoptosis [122]. In addition to loss of mitochondrial function, xanthine dehydrogenase, an enzyme found attached to the endothelial glycocalyx layer, is converted to xanthine oxidase which upon reperfusion leads to ROS formation [123].

ROS are strong oxidizing agents that can react with the cell membrane leading to cell destruction and lysis. The major source of ROS during I/R injury are the neutrophils recruited to the site of injury with the help of pro-inflammatory cytokines [124].

No-reflow phenomenon

The no-reflow phenomenon refers to the failure of reperfusion after ischemia due to massive capillary and endothelia dysfunction. Formation of microthrombi and severe edema in the surrounding tissue can worsen the injury by a complete breakdown of microvascular perfusion. No-reflow leads to a prolonged ischemia time and total necrosis of the ischemic tissue [125], [126].

Management of skeletal muscle ischemia reperfusion injury

To date there is no effective treatment which prevents or reduces skeletal muscle injury in the clinical setting. However, the use of high dose vitamin E, administrated daily for 8 days before surgery, was successful in reducing oxidative stress and histological damage in lower limb skeletal muscles in patients who underwent visceral surgery [127]. In addition to this – as far as I can see single clinical study – there are some candidates that may have promising effects on the translation of animal experiments to clinical trials.

Cyclosporine A showed a protective effect in a rat model of hind limb I/R injury and managed to reduce skeletal muscle as well as distal organ damage via enhancing the mitochondrial integrity and reduction of microvascular damage [128]. On the other hand, a similar study using elderly rats showed no beneficial effect of cyclosporine A [129].

Another study in a rat hind limb model of skeletal muscle I/R injury used dexmedetomidine and vitamin E. Dexmedetomidine is a potent α_2 adrenoreceptor agonist and known to have an anti-inflammatory and antioxidant effects. Vitamin E is also known to have strong anti-oxidant properties. Both dexmedetomidine and vitamin E reduced oxidative stress in the skeletal muscle when administered during ischemia (1 hour before reperfusion, each treatment alone) and the author considered them as effective and promising agents for preventing skeletal muscle I/R injury [130]. Several other antioxidant substances have shown protective effectiveness against skeletal muscle I/R injury in rat models including curcumin [131], hesperidin and ellagic acid [132], caffeic acid phenethyl ester [133] and *N*-acetylcysteine [134].

The inhibition of the complement cascade at different levels has also been shown to have a protective effect against I/R injury. In a mouse [135] and a rat [136] hind limb model, a soluble recombinant form of complement receptor 1, which promotes the breakdown of complement C3b and C4b, was effective in reducing vascular permeability, recruitment of neutrophils at the site of injury and complement activation. The use of a C5a receptor antagonist reduced both the local and remote injury in a rat hind limb model. The C5a receptor antagonist significantly reduced skeletal muscle edema, the serum level of the skeletal muscle damage markers, serum creatine kinase and lactate dehydrogenase, as well as liver damage markers alanine transaminase and aspartate aminotransferase. It also reduced neutrophil infiltration in muscle, liver and lung [137]. In our laboratory, we used a C1-INH in a rat hind limb model which revealed that it is an effective molecule in reducing muscle edema and remote organ damage, namely lung. The histological evaluation also showed a reduced complement activation in skeletal muscle tissue [120]. In addition, C1-INH was effective in reducing plasma cascade activation and protecting the vascular endothelium against I/R injury in a porcine model of limb amputation and ex-vivo reperfusion [105].

C1-esterase inhibitor

C1-INH is a soluble acute phase protein that is synthesized by a wide variety of cells including endothelial cells, fibroblasts, macrophages, monocytes

and others in response to interferon gamma (INF- γ) and to a lesser extent tumor necrosis factor alpha and other cytokines [138]. C1-INH is a serine protease inhibitor that consists of two domains, the carboxylic domain and N-terminal domain that bind together with covalent bond [139]. Regarding the complement system inhibition, C1-INH is the only known inhibitor of the classical pathway proteases proteins C1r and C1s. C1-INH can bind either the free form of C1s and C1r or the bound form with C1q that leads to formation of the inactive complex C1-INH-C1r-C1s-C1-INH tetramer. Moreover, C1-INH inhibits MASP-1 and MASP-2 leading to inhibition of the lectin pathway [138]. In the contact phase of coagulation (intrinsic pathway), C1-INH inactivates the active FXIIa [140], FXIa [141] as well as kallikrein. C1-INH is involved in tissue plasminogen activator (tPA) inhibition only if there is an increased expression of tPA [142]. C1-INH also works on T lymphocytes and inhibits their activation by specific breaks of the major histocompatibility complex class 1 [143] and altering the T-lymphocyte production of cytokines INF- γ , IL-10 and IL-12 and negatively regulating IL-2 and IL-4 production [144].

Myocardial ischemia reperfusion injury

Acute myocardial infarction (AMI) is still one of the main causes of death worldwide [145]. The only way to save the life of a patient suffering from AMI is to reopen the closed coronary by percutaneous coronary intervention (PCI) or thrombolytic therapy. The shorter the time, the smaller the infarct size and the better the prognosis for the patient and his/her quality of life [146]. However, the effect of re-establishing the blood the supply after a certain time of ischemia is a double-edged sword. An important life-saving effect is the reintroduction of oxygen and nutrients to the affected myocardium and removal of metabolic products. The unwanted effect, which was first described by Jennings in a canine model of AMI [147], is called ischemia reperfusion injury which is responsible for almost one third of the final infarct size of the myocardium.

Mechanisms of myocardial ischemia reperfusion injury

The underlying mechanisms of myocardial I/R injury are complicated and not fully understood. It involves activation of innate immunity with all its plasma cascades, inflammatory mediators, leukocyte infiltration, oxidative stress, intracellular calcium ion overload, mitochondrial dysfunction and cell death [102], [148]-[150].

Innate immunity

The role of the complement in myocardial I/R injury was first described by Hill and Ward [100] when they linked C3 cleavage with chemotaxis and leukocyte activation. Not only the classical and alternative pathways are involved in the pathophysiology of myocardial I/R injury [151], but also the lectin pathway plays an important role [152]. Indeed, in a clinical study it was also shown that the MASP-2 levels were reduced in patients that suffered from AMI [153]. The process of complement activation and formation of MAC leads to propagation of the inflammatory processes through production of ROS, recruitment of leukocytes to the site of injury via anaphylatoxins C3a and C5a as well as activation of the endothelial and inflammatory cells to produce pro-inflammatory cytokines [154]. The activated endothelial cells shed their protective glycocalyx layer and its surface is converted from an anti-inflammatory and anti-coagulant state into a pro-inflammatory and pro-

coagulant one [155]. The endothelium expresses a number of surface proteins that play an important role in the progression of myocardial I/R injury:

- Tissue factor, which will activate the extrinsic coagulation pathway [156]
- FGL-2, a direct prothrombinase that converts prothrombin into thrombin. Thrombin will not only lead to fibrin deposition but also activates C5 complement protein [109], [157].
- Adhesive molecules will increase the rolling of leukocyte, especially neutrophils, on the endothelial cell surface which lead to ROS formation, cellular damage and cell death [149]

Metabolic changes during myocardial ischemia reperfusion injury

Myocardial stunning refers to the failure of the myocardium to regain its contractile function after reperfusion although the oxidative phosphorylation of the mitochondria is regained within seconds of reperfusion[158] . This may be due to the rapid restoration of the intracellular pH via activation of the Na⁺/H⁺ pump that moves intracellular Na⁺ and extracellular H⁺. This is followed by activation of the Na⁺/Ca⁺⁺ pump that leads to Ca⁺⁺ overload and cell death [159]. Moreover, during reperfusion there is an increase in mitochondrial permeability followed by mitochondrial dysfunction resulting in ATP depletion and cell death[160]. In addition to the role of MAC and neutrophils in the production of ROS, which have been mentioned above, the electrolyte imbalance and mitochondrial dysfunction leads to more ROS formation in the myocardium. ROS directly damage cell membranes, cellular DNA, proteins and lipids in the cells in a process known as oxidative stress [150], [161].

No-reflow phenomenon

The expression 'no-reflow' is meant to describe microvascular occlusion and reduction of myocardial perfusion after a successful opening of an occluded coronary and it affects up to 50% of AMI treated patients [162].

I/R injury is one of the main causes of myocardial no-reflow, where activation of the endothelial cells, massive infiltration of neutrophils and platelets, accumulation of antibody complexes and production of ROS leads to vascular dysfunction as well as the collapse or obstruction of the vascular wall. The complex interaction between plasma cascades during I/R injury is another

risk factor of myocardial edema that adds more pressure to the microvascular wall that leads to its obstruction. The no-reflow phenomenon eventually leads to prolonged ischemia in the myocardium and irreversible damage of a larger area of the myocardium [163].

Management of myocardial I/R injury

It has been over half a century since the first description of the myocardial I/R injury and yet still no effective treatment is established against myocardial I/R injury in the clinical settings. However, some promising targeted drugs have shown an excellent effect in preventing reperfusion injury in animal models [164]. The aim of the interventions is to reduce the final infarct size in order to improve the patient prognosis and reduce morbidity and mortality rates [165].

Pharmacological intervention

Several complement inhibitor treatments have been tried in both animal experiments, where a high success rate was obtained, and human phase II and III trials, where only very limited success was obtained. The use of the anti-complement C5 monoclonal antibody was not effective in reducing infarct size or plasma levels of creatine kinase (CK-MB) in AMI patients treated with thrombolytic therapy or in patients treated with angioplasty [166], [167]. Although again no reduction of infarct size has been achieved, there was a reduction of the mortality rate in the high risk surgical patients when monoclonal C5 antibody was used [168], [169]. A recent study showed that the use of coversin, which blocks C5 cleavage, was effective in reducing infarct size and improving ventricular function in a porcine model of AMI [170].

The soluble recombinant complement receptor type 1 (sCR1) that binds covalently to C3b and mediates its interaction with factor I to inhibit complement cascade activation succeeded in reducing myocardial I/R in a rat model of AMI [171] and also in a porcine model [172], but when translated into human trials, sCR1 failed to reduce infarct size, while still achieving a lower mortality outcome in high risk patients [173].

C1-INH showed efficacy in reducing myocardial I/R injury in three small randomized blinded clinical trials (40, 57 and 80 patients). Patients who were

treated with C1-INH had lower CK-MB or cardiac troponin I (cTnI) levels and better ventricular function compared to non-treated patients [174]-[176]. This observation needs to be translated into a larger clinical trial for the drug to be approved for AMI.

Targeting the lectin pathway could be another therapeutic option to inhibit or reduce myocardial ischemia reperfusion injury. A monoclonal antibody against MBL reduced myocardial I/R injury in rat model when administered before onset of ischemia [177]. Moreover, the use of the endogenous MBL inhibitor (MAP-1) managed to inhibit complement activation, reduce infarct size and prevent thrombogenesis in a mice model of AMI [178]. As mentioned above, the use of C1-INH, which also inhibits the lectin pathway, was effective in reducing myocardial I/R injury.

Two large clinical studies have proven that the use of a Na⁺/H⁺ exchange inhibitor failed to reduce myocardial I/R injury when administered after the ischemia onset but they were effective when given prior to the onset of ischemia (in the case of elective heart surgery) [179], [180].

A calcium channel blocker has been used to reduce intracellular Ca⁺⁺ overload and led to less myocardium stunning and better left ventricular function [181] while the use of magnesium ion salt as a calcium blocker did not show any improvement of cardiac function after AMI treatment [182].

Adenosine is a pharmacological substance that shows multiple mechanisms that can protect against myocardium I/R injury including an anti-inflammatory effect, inhibition of leukocyte activation and ROS formation, protection of vascular endothelium as well as preservation of microvascular flow. The clinical trials reviewed by Dirksen and his colleagues showed diverse results and did not succeed in preventing the myocardial I/R injury when it was administered after the onset of ischemia. However, it showed positive results in preventing the no-reflow effect and successfully reduced the infarct size when administered before ischemia (for planned surgery not emergency AMI) [164]. Pharmacological interventions that focused on inhibition of neutrophils and oxidative stress failed to obtain any inhibition or reduction of the myocardial I/R injury and recent studies are specifically targeting the mitochondria[150].

Mechanical intervention

Postconditioning has been considered as a mechanical intervention to reduce myocardial I/R injury and was first described in a canine AMI model in 2003 [183]. Several human clinical trials reviewed by Frank and her colleagues demonstrated a long-term effect of a reduction of infarct size after myocardial I/R injury by almost 35% and a lower mortality rate. A large multi-center trial is required before the approval of the postconditioning technique in the AMI setting[165].

Failure of the translation of animal experiments into clinical trials

The reasons for the high rate of failure of the translation of the animal experiments into the clinical setting are multi-factorial. The animals used are usually young and healthy while the humans are not. Most humans that experience myocardial I/R injury are loaded with many drugs and it is not easy to discover the possible interactions with the target drug. In addition, the severity and degree of the ischemia and reperfusion are more widely variable in the human setting as compared with animal trials. Moreover, the timing of the drug administration can be a limiting factor in the success of the trial. Many of the promising drugs showed only effective results when administrated in the pre ischemic phase while the patients with AMI reach the hospital during the ischemic phase. The route of administration can be another limiting factor as well as the calculation of the dose. It is also important to take into consideration that most of the animal experiments that showed negative results are not published which leads to a biased impression. The move from small animal studies to large animal experiments is highly recommended before starting the translation to the human setting. This could be a step towards improving translation[164]. The recent production of large transgenic animals (pigs) will give researchers more opportunities to design large animals experiments using specific transgenes to understand pathophysiologies as well as to set up chronic disease models similar to the clinic situation [184].

Transgenic pigs

As a result of a shortage of organ donors for different organ transplantations, the idea of xenotransplantation has been raised. Development of transgenic pigs was first used to overcome the immunological rejection response that occurs upon xenotransplantation of pig organs into baboon or ex-vivo perfusion of the pig organ with human heparinized whole blood [185]. The availability of transgenic pig models encouraged the researchers to use them in order to improve translational medicine. Pigs are genetically closer to the humans compared to mice [186] and they also show anatomical, physiological and immunological similarities with the humans. The development of novel therapeutic drugs has quickly declined in the last decades due to the failure of translation between animal experiments and clinical trials [187] and the use of transgenic pig models might help to improve this situation.

Several transgenic pig models have been produced for biomedical research, in order to increase the success rate of translational medicine, in the field of neurodegenerative diseases [188]-[190], cardiovascular diseases [191], cystic fibrosis [192], diabetes mellitus [193], [194] and cancer research [195].

The use of transgenic animals in I/R injury research is not yet widely undertaken. It was only reported once that the use of CD39, ectonucleoside triphosphate diphosphohydrolase-1, which is an important enzyme responsible for hydrolysis of ATP/ADP into AMP and then into adenosine, in a transgenic pig model of myocardial I/R injury. The CD39 transgenic hearts suffered less infarct size [196].

Membrane cofactor protein CD46

CD46, a type I transmembrane protein, is expressed on all nucleated cells. CD46 is considered as a single associated molecular pattern that controls host cells from being attacked by the innate immune system and is known to be a 'don't eat me' signal mediator [197]. CD46 consists of four isoforms that contain a terminal binding site for C3b and C4b [198]. CD46 is known as a multitasker protein because it does not only inhibit complement activation but also links adaptive and innate immune systems [199]. In order to inhibit complement pathways (classical, lectin and alternative), CD46 works as a cofactor for C3b or

C4b and binding factor I. Factor I breaks alpha chains of C3b and C4b to produce iC3b, C3f and iC4b and C4f (figure 4). The latter proteins prevent the production of C3 convertases (C3bBb and C4b2a respectively) [198]. Moreover, CD46 regulates the T cell mediated inflammatory reaction and bridges the adaptive and innate immune reaction via mediating T-cytotoxic activity, stimulating IL-2 and IL-10 production and down regulating production of IL-12 on macrophages [200].

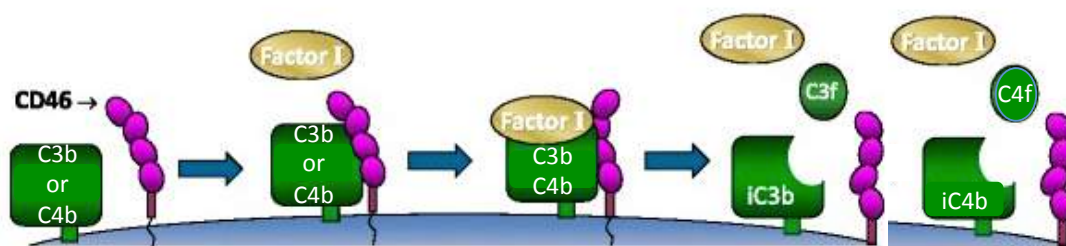


Figure (4): Role of CD46 in classical, lectin or alternative pathways inhibition. Adapted from [201]

In a mouse model of renal of I/R injury it was found that the reduction of complement receptor 1-related protein y (CD46 is the human functional analog), makes the mice more susceptible to renal I/R injury [202]. Validation of the previous results in a clinically related model (pigs) would help the development of novel therapeutics against I/R injury.

The primary aim of producing the hCD46 transgenic pigs was to reduce the acute rejection in the xenotransplantation model [203]. Acute rejection and I/R injury share the complement and plasma cascade activation pathophysiology. In our laboratory, it has been founded that hCD46 was not only effective in the reduction of the complement cascade activation in the xenogeneic situation but also in the autologous situation [204].

Thrombomodulin TM

Thrombomodulin is a membrane bound single chain type-1 glycoprotein which consist of five extracellular domains that are expressed on the endothelial cell surface [205]. TM is an important factor in the prevention of intravascular thrombus formation. In a mouse model with deleted TM expression a spontaneous fatal thrombus was formed [206]. TM binds to thrombin and

inhibits its activity on the soluble fibrinogen and, as a consequence, it inhibits fibrin deposition [207]. The Thrombin-TM complex is also important for enhancing the thrombin anti-thrombin interaction, for thrombin deactivation as well as activation of protein C. The active protein C and protein S, as cofactor, are proteolytic inhibitors of factors FVa and FVIIIa and serve as a negative feedback for thrombin activation [208], [209]. TM bridges anticoagulant and anti-inflammatory properties. Activated protein C, mediated by TM, inhibits nuclear factor κ B expression and prevents inflammatory cytokine translocation and production [210], [211]. Thrombin-TM complex activate thrombin activatable fibrinolysis inhibitor, the latter then inhibits C3a, C5a [212] and bradykinin [213], which are strong inflammatory mediators. In addition, it inhibits fibrin degradation as well as plasminogen activation to maintain hemostasis with the fibrinolysis activity of the activated protein C [214] (figure 5).

Several studies have shown that administration of human recombinant TM could attenuate I/R injury as shown in a mouse model of hepatic I/R injury via its anti-inflammatory and anti-coagulant properties. More recent studies showed similar effects in a mouse liver transplantation model and revealed the effect of TM on the inhibition of the pro-inflammatory high mobility box-1 (HMGB) protein [215] or on the HMGB-1/ toll like receptor-4 axis [216]. Moreover, the use of the N-terminal lectin like domain of TM showed a protective effect against I/R injury in a mouse model of in-situ lung I/R injury [217]. Pretreatment and secondary treatment with soluble TM protects kidneys from I/R injury in a rat partial arterial clamp model [218]. Recently, red blood cell fused TM also showed positive effects on a mouse model of brain I/R injury [219].

Porcine human TM transgenic pigs were first produced in 2009 to overcome the incompatibility between the porcine TM and the human coagulation system [220]. While porcine endothelial cells activate thrombin formation, the porcine TM fails to activate human protein C that, resulting in thrombus formations in the xenograft [221].

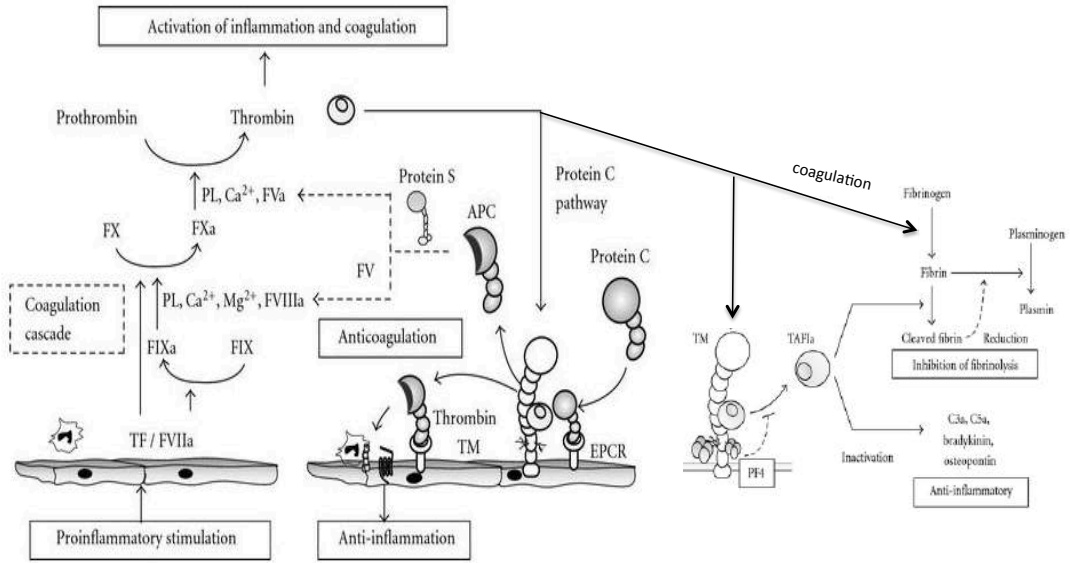


Figure (5): Thrombomodulin mechanisms of action. TF: tissue factor, APC: activated protein C, TM: thrombomodulin, EPCR: endothelial protein C receptor, PF4: platelet factor 4, TAFIa: activated thrombin activatable fibrinolysis inhibitor. Adapted from [222]

Objectives

In order to find a good clinical prevention or treatment of ischemia reperfusion injury, there are two main points that have to be taken into consideration: First, the in depth understanding of the mechanisms of ischemia reperfusion injury in each organ. Second, the success of the promising drug candidates in large animal models, especially pigs, before moving on to the clinics. Both of the above considerations have been addressed in the current thesis.

The first aim was to evaluate the effect of C1-INH in reducing skeletal muscle I/R injury in a pig model that simulates a traumatic amputation and replantation. This model was used to study the role of the plasma cascades and endothelial cell activation in skeletal muscle ischemia reperfusion injury.

The second aim was to investigate the role of CD46 overexpression on skeletal muscle I/R injury using an amputated porcine limb reperfused ex-vivo with autologous blood.

The third aim was to study in vivo the role of hCD46 and hTM expression in a pig model of myocardial I/R injury in order to improve our understanding of the pathophysiology of AMI in pigs.

Reproducibility is a main concern in the scientific community so the fourth aim of the present thesis was to provide a well detailed and clearly described methodology for the porcine closed chest model of myocardial I/R injury. The method provided here allows not only the reproducibility of the surgical model but also the reproducibility of tissue and blood sampling for investigating the underlining mechanisms of myocardial ischemia reperfusion injury.

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Results

Paper I: Effect of C1-INH on ischemia/reperfusion injury in a porcine limb ex vivo perfusion model

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Background: C1-INH provides protection against skeletal muscle ischemia reperfusion injury in rat hind-limb models. Validation of the results in larger animal models such as pigs is essential before starting clinical trials.

Aim: To study the role of plasma cascades and the vascular endothelium as well as to investigate the effect of C1-INH in a pig model of skeletal muscle ischemia reperfusion injury.

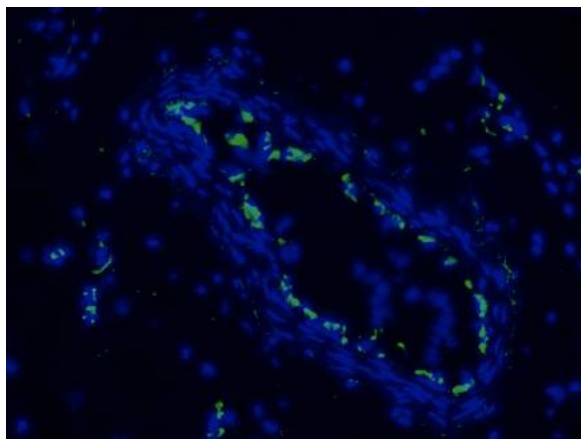


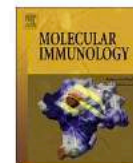
Figure: The effect of C1-INH in preserving the endothelial surface after skeletal muscle I/R injury. Blue: vessel wall nucleus. Green: healthy endothelial cell layer

Conclusion: Skeletal muscle ischemia reperfusion injury is a complex disease that involves activation of all plasma cascades as well as the vascular endothelial layer. The plasma cascades intercommunicate with each other and with the vascular endothelium in several amplification positive feedback loops that makes them difficult to stop once initiated. C1-INH succeeded in reducing immunoglobulin, complement and fibrin deposition on the affected tissue as well as protecting the endothelium from damage and activation.



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Effect of C1-INH on ischemia/reperfusion injury in a porcine limb ex vivo perfusion model



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ABSTRACT

Revascularization of an amputated limb within 4–6 h is essential to avoid extensive ischemia/reperfusion (I/R) injury leading to vascular leakage, edema and tissue necrosis. I/R injury is a pathological inflammatory condition that occurs during reperfusion of an organ or tissue after prolonged ischemia. It is characterized by a complex crosstalk between endothelial cell activation and the activation of plasma cascades. Vasculoprotective pharmacological intervention to prevent I/R injury might be an option to prolong the time window between limb amputation and successful replantation. We used C1-esterase inhibitor (C1-INH) in this study because of its known inhibitory effects on the activation of the complement, coagulation and kinin cascades. Forelimbs of 8 large white pigs were amputated, subjected to ischemia, and then reperfused with autologous whole blood. All limbs were exposed to 9 h of cold ischemia at 4 °C. After 2 h of cold ischemia the limbs were either perfused with C1-INH (1U/ml in hydroxyethyl starch, n = 8) or hydroxyethyl starch alone (n = 7). After completion of the 9-h ischemia period, all limbs were ex vivo perfused with heparinized autologous whole blood for 12 h using a pediatric heart lung machine to simulate in vivo revascularization. Our results show that I/R injury in the control group led to a significant elevation of tissue deposition of IgG and IgM, complement C3b/c, C5b-9 and MBL. Also, activation of the kinin system was significantly increased, namely bradykinin in plasma, and expression of bradykinin receptors 1 and 2 in tissue. In addition, markers for endothelial integrity like expression of CD31, VE-cadherin and heparan sulfate proteoglycans were decreased in reperfused tissue. Limb I/R injury also led to activation of the coagulation cascade with a significant elevation of fibrin and thrombin deposition and increased fibrinogen-like protein-2 expression. C1-INH treated limbs showed much less activation of plasma cascades and better protection of endothelial integrity compared to the reperfused control limbs. In conclusion, the use of the cytoprotective drug C1-INH significantly reduced I/R injury by protecting the vascular endothelium as well as the muscle tissue from deposition of immunoglobulins, complement and fibrin.

1. Introduction

Re-establishment of blood circulation after prolonged ischemia leads to an inflammatory reaction in the affected organ or tissue, the so-called ischemia reperfusion (I/R) injury (Arumugam et al., 2006). Endothelial cells (EC) of the vasculature within the limb skeletal muscle are highly susceptible to I/R injury, which leads to a high degree of EC activation. Activated EC lose their glycocalyx layer and – by presenting ‘danger’ signals – become susceptible to attack by the innate immune

system and different inflammatory mediators (Rehm et al., 2007; van GolenRowan et al., 2014; Wang et al., 2007). Activation of plasma cascades including complement, coagulation and kinin was previously demonstrated in different animal models as well as clinical studies of I/R injury (Chappell et al., 2014; Duehrkop et al., 2012; Gillani et al., 2012).

One of the major causes of limb amputation is trauma. In most cases the amputee is young with a long life expectancy. The limb loss is considered to have social and economical consequences as well as a

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negative impact on quality of life (Chalya et al., 2012). The best way to restore the function of an amputated extremity is to surgically replant it (Yueh et al., 2011). Replantation of whole limbs has become well established in the last few decades. However, the success rate is multifactorial (Dagum et al., 2007) and the ischemia time before replantation is a major limiting step for a successful procedure (Siemionow and Arslan, 2004). Replantation within 6 h of amputation is essential to save the limb. However, traumatized amputee patients often present with other life threatening conditions that need rapid intervention before saving the limb – ‘life before limb’ – (Chim et al., 2013; Märdian et al., 2015), and these interventions may delay surgical limb replantation.

Ex vivo perfusion of the amputated limb using a heart lung machine is one of the interventions that has been suggested to extend the time window to replantation (Constantinescu et al., 2011; Müller et al., 2013). Pharmacological interventions have also been used for the same purpose. Several drugs were shown to protect against I/R injury, such as Nicorandil in reconstructive surgery (Cahoon et al., 2013), C1-INH in a rat hind limb (Duehrkop et al., 2012) or mouse stroke model (Storini et al., 2005). However, currently there are no drugs available which are approved for clinical use to prevent or reduce I/R injury (Banz and Rieben, 2011).

In the current study, we used a C1-esterase inhibitor (C1-INH) as a possible prophylactic treatment against skeletal muscle I/R injury in a porcine limb amputation model, because of its known inhibitory effects on the activation of the complement, coagulation and kinin cascades. C1-INH is a serine protease inhibitor that targets multiple enzymes in different plasma cascades. In the contact and coagulation systems it inhibits FXIIa and FXIa, kallikrein in the kinin system, plasmin in the fibrinolytic system, C1s and C1r in the classical pathway of complement, and mannose-binding lectin (MBL) proteases (MASP-1 and MASP-2) in the lectin complement pathway (Caliezi et al., 2000).

The aim of the current study was to investigate the molecular mechanisms of the innate immune response during I/R injury as well as the links between EC activation and the different plasma cascades (complement, coagulation and kinin). Moreover, the study investigated the prophylactic potential of C1-INH in reducing I/R injury of amputated limbs exposed to prolonged cold ischemia (9 h, 4 °C) followed by 12 h of ex vivo reperfusion.

2. Animals and methods

2.1. Animals

All animal experiments were performed according to the NIH guidelines for the care and use of laboratory animals (Institute for Laboratory Animal Research (US), 2010), the Swiss national laws and guidelines and the ARRIVE guidelines (<https://www.nc3rs.org.uk/arrive-guidelines>). The local animal experimentation committee of the Canton of Bern approved the study (permission no. BE 79/14). We used 8 animals for this study of which both forelimbs were used, one with C1-INH and the other without (control). A formal power calculation to establish the size of the experimental groups was not possible because of lacking variability data. We therefore chose to use 8 animals with the aim to have at least 6 valid experiments per group, considering that a non-significant outcome with $n = 6$ would suggest that the treatment has no potential clinical impact.

2.2. Surgical procedure

Forelimbs of eight large white pigs of both sexes (body weight 40 kg) were amputated as described previously (Müller et al., 2013). In brief, Pigs were pretreated with ketamine (20 mg/kg) and xylazine (2 mg/kg), as well as intramuscular atropine (0,05 mg/kg) and midazolam (0,5 mg/kg) before intubation. Anesthesia was induced by isoflurane (1.5% in 1:3 O₂/air) inhalation in addition to Fentanyl (20 µg/

kg/h). After dissection of the axillary neurovascular bundle, the artery and two veins were cannulated using 10–14 French catheters according to the vessel size. All limbs were weighed and passively perfused with 500 ml hydroxyethyl starch solution (HAES, Voluven Fresenius, Bad Homburg, Germany) just after being amputated. All the limbs were exposed to cold ischemia at 4 °C for 9 h. The pigs were anticoagulated by giving 5000 IU heparin immediately after the first forelimb amputation and another 2500 IU was administered 2 h later. After amputation of both limbs, blood was collected into 2 blood collection bags containing 10000 IU heparin. 600 ml of blood were collected in each bag and stored at 4 °C. The animals were then euthanized by intravenous injection of 20 ml of 20% KCl.

2.3. Ex vivo perfusion setting

After 2 h of cold ischemia the limbs of the treatment group were passively perfused with 500 ml HAES containing C1-INH (1 U/ml, treatment group, $n = 8$), while the non-treated limbs (control group, $n = 7$) were rinsed passively with the same amount of HAES without C1-INH, and cold storage at 4 °C continued up to a total of 9 h. In six experiments treated and control limbs were from the same pig. We then lost one treated limb in experiment no. 7 due to technical problems with the heart lung machine. Both limbs were therefore treated with C1-INH in experiment no. 8.

A pediatric heart lung machine was used for ex vivo perfusion of the limbs and was primed with 150 ml HAES to ensure complete removal of air bubbles from the system. Autologous, heparinized blood was then added to the system and replaced the HAES. The amputated limbs were taken out of the refrigerator at the end of the 9-h cold storage period and connected to the perfusion system. Perfusion rate was set between 100 and 200 ml/minute using a turbine pump (MEDOS DataStream blood pump, model DP2; Medos Medizintechnik AG, Stolberg, Germany) and the temperature maintained at 32 °C. Oxygen concentration was adjusted to 21% using a membrane oxygenator (MEDOS Hillite 800 LT; Medos Medizintechnik AG, Stolberg, Germany). One hour after starting the perfusion, 40 mg of methylprednisolone (Solu-Medrol; Pfizer, Zürich, Switzerland) was administered to the perfusion circuit to decrease capillary leakage and inflammatory cytokine response. Hourly, blood gas analyses (GEM premier 3000; Instrumentation Laboratory, Lexington, MA, USA) were performed to control glucose, pH and potassium levels. Insulin (Novorapid, 100 U/ml, Novo Nordisk Pharma AG) with or without 40% glucose and/or sodium bicarbonate were administered to the perfusion line to maintain physiological glucose, potassium and lactate levels for as long as possible.

2.4. Blood and tissue samples

EDTA and citrate plasma samples were collected at baseline (just after anaesthetizing the animal) and 10 min after heparinization of the animals as well as 10 min, 2 h, 6 h and at end of perfusion from each limb. All blood samples were centrifuged at 2000 × g for 15 min at 4 °C, aliquoted and the plasma or serum stored at –80 °C until analysis. Biopsies of skeletal muscle were taken at the baseline and at the end of the perfusion from the *biceps brachii* of each limb and stored either in Tissue-Tek (OCT compound, Sakura Finetek Europe B.V., Netherlands) at –80 °C for immunofluorescence staining or in 4% paraformaldehyde at room temperature for standard histological evaluation.

2.5. Histology

Paraformaldehyde-fixed and paraffin-embedded muscle biopsies were cut into 3 µm thick sections and stained with hematoxylin-eosin (H & E). The samples were blindly evaluated for nuclear changes such as pyknosis, karyorrhexis, or karyolysis and hypereosinophilia as markers of muscle necrosis. Muscle edema, hemorrhage and neutrophil

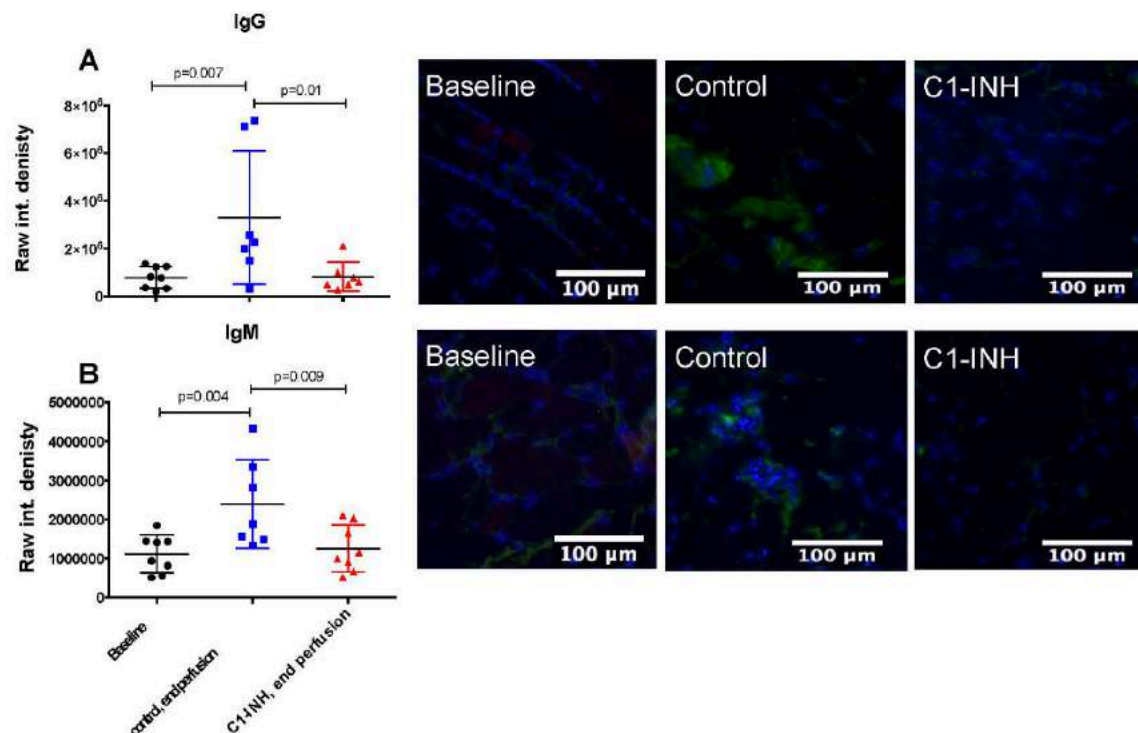


Fig. 1. Tissue deposition of IgG and IgM. Tissue deposition of IgG (A) and IgM (B) was assessed by quantitative analysis of immunofluorescence stained cryosections of muscle tissue. Diagrams on the left side show the values at baseline and end of perfusion of the control- and the C1-INH treated group. Values are shown as dots for each individual experiment with indication of mean \pm SD. P-values were calculated by ANOVA with Fisher's LSD post test. Representative pictures of tissue from controls and C1-INH treated animals, respectively, are shown on the right side.

infiltration were also evaluated as signs of vascular leakage. H & E samples were scored from 0 to 3; 3 = maximum presence, 2 = intermediate, 1 = minimal presence and 0 = absence of injury (Bongoni et al., 2014).

2.6. Immunofluorescence

Cryopreserved samples were cut into 5 μ m thick sections, air dried for 30–60 min, fixed with acetone at -20°C for 10 min, rehydrated with TBS solution for 5 min at room temperature, demarcated with Dako pen (Dako Denmark A/S, Glostrup, Denmark), and then blocked using TBS containing 3% BSA for 60 min at room temperature. The primary antibodies used were directly labeled goat anti-human C3b/c-FITC (Dako, F0201), goat anti-pig IgG-FITC (Southern Biotech, 6050-02) and rabbit anti-human fibrinogen-FITC (Dako, F0111). Unlabeled mouse anti-human C5b-9 (Antibody Shop, DIA 011-01), mouse anti-human MBL-C (Santa Cruz Biotechnology, sc-80598), goat anti-pig IgM (AbD Serotec, AAI39), rabbit anti-human bradykinin receptor-1 and 2 (Alomone, ABR-011 and ABR-012 respectively), goat anti-human VE cadherin (Santa Cruz Biotechnology, sc-6458), rat anti-pig CD31 (R & D systems, MAB33871), rat anti-heparan sulfate proteoglycan (Abcam, ab2501), mouse anti-human CD62E (Sigma, S9555), goat anti-human tPA (Abcam, ab14198), mouse anti-PAI-1 (Hycult, HM2181), sheep anti-human tissue factor (Affinity Biological, SATF-JG), rabbit anti-human FGL-2 (Aviva Systems Biology, ARP52235_p050), rabbit anti-human thrombin (abcam, ab92621). Cross-reactivity of the polyclonal anti-human antibodies with the respective porcine antigens was either tested by the manufacturer or by ourselves (Bongoni et al., 2014, 2015).

The following secondary antibodies were used for the indirect immunofluorescence technique: sheep anti-mouse Cy3 (Sigma, C2181),

donkey anti-goat Alexa Fluor 488 (Molecular Probes, A11055), sheep anti-rabbit Cy3 (Sigma, C2306), goat anti-rat Cy3 (Jackson ImmunoResearch, 112-166-003), goat anti-mouse Alexa Fluor 546 (Invitrogen, A11030). All antibodies were diluted in TBS-PBS-1%BSA. Primary antibodies were incubated overnight at 4°C , followed by a triple washing step. Secondary antibodies were incubated for 60–90 min at room temperature. Glycergel (Dako, C0563) was used as mounting medium. A fluorescence microscope (DMI4000; Leica, Wetzlar, Germany) was used to analyze the samples. All the pictures were quantified using Image J software, version 64.4.

2.7. ELISA

Using commercially available ELISA kits and following the instructions by the manufacturers the following parameters were measured in EDTA plasma samples: D-dimer (Elabscience, E-EL-P2237) and tissue plasminogen activator-/plasminogen activator inhibitor-1 (tPA-PAI-1) complexes (Molecular Innovations, POPAIPAKT-COM).

2.8. Statistics

Data are presented as mean \pm standard deviation (SD). Statistical analyses were performed using GraphPad Prism 6 software (GraphPad, San Diego, CA, USA). For tissue samples one-way analysis of variance (ANOVA) followed by Fisher's LSD post hoc test was used based on the fact that the analyzed samples are not dependent on each other (Dallal, 2007). For plasma samples a Student *t*-test was used. P values < 0.05 were considered statistically significant.

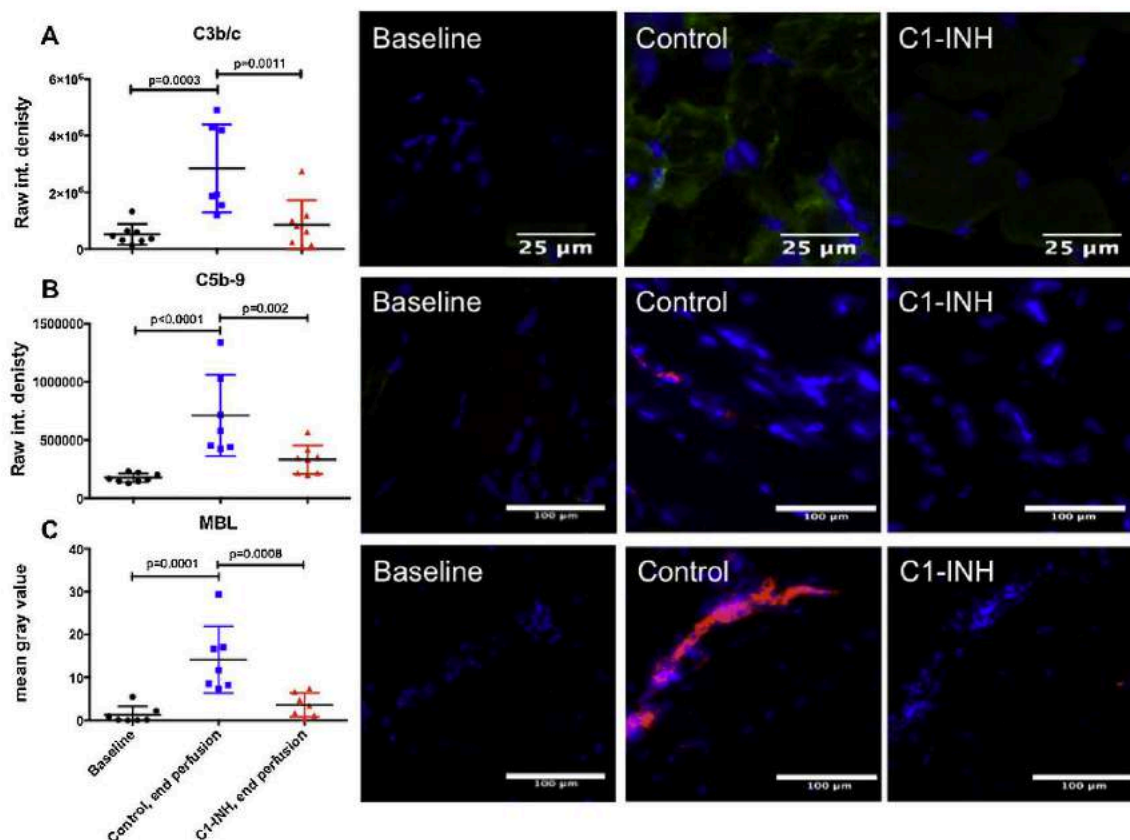


Fig. 2. Complement deposition. Tissue deposition of C3b/c (A) C5b-9 (B) MBL (C) was assessed by quantitative analysis of immunofluorescence stained cryosections of muscle tissue. Diagrams on the left side show the values at baseline and end of perfusion of the control- and the C1-INH treated group. Values are shown as dots for each individual experiment with indication of mean \pm SD. P-values were calculated by ANOVA with Fisher's LSD post test. Representative pictures of tissue from controls and C1-INH treated animals, respectively, are shown on the right side.

3. Results

3.1. C1-INH prevents tissue deposition of antibodies and complement

Immunofluorescence analysis of muscle tissue of porcine limbs exposed to 9 h of cold ischemia (4 °C) followed by 12 h of ex vivo perfusion with autologous, heparinized whole blood showed a significant reduction in IgM ($p = 0.01$) and IgG ($p = 0.009$) deposition in the C1-INH treated limb tissue compared to the control limbs. In control limbs, but not in the C1-INH treated ones, reperfusion led to a significant increase of IgM ($p = 0.004$) and IgG ($p = 0.007$) deposition compared to baseline (Fig. 1).

The use of C1-INH also led to a significant reduction of complement deposition in the tissue at the end of reperfusion as compared to controls. This was the case for all analyzed complement proteins: C3b/c ($p = 0.0011$, C1-INH vs. controls), C5b-9 ($p = 0.0059$) and MBL ($p = 0.0008$). A significant increase of complement deposition at the end of reperfusion as compared with baseline was seen in the control limbs, but not in the C1-INH group: C3b/c ($p = 0.0003$, end of perfusion vs. baseline), C5b-9 ($p \leq 0.0001$) and MBL ($p = 0.0001$, Fig. 2). In contrast, plasma levels of the soluble complement activation markers C3a, C5a and sC5b-9 clearly increased during perfusion as compared with baseline in both groups, but the differences between C1-INH treated limbs and controls was not significant. Data are shown in Supplementary Fig. 1.

3.2. C1-INH preserves endothelial cell integrity and reduces endothelial activation

As shown in Fig. 3, endothelial expressions of CD31 (PECAM, $p = 0.0006$) and VE-cadherin ($p = 0.006$) were significantly higher in the C1-INH treated limbs compared to controls. At the end of the 12-h ex vivo reperfusion, staining for CD31 ($p \leq 0.0001$ vs. baseline) and VE-cadherin ($p = 0.003$) were significantly reduced as compared with baseline in controls, whereas no such reduction was seen in the C1-INH group. Heparan sulfate proteoglycan (HSPG) expression was preserved on the vascular endothelial cells of the C1-INH treated limbs, whereas this was not the case in tissue of the control limbs ($p = 0.01$). In addition, an increased expression of the endothelial cell activation marker CD62E (E-selectin) was found in the control limbs as compared to C1-INH treated limbs ($p = 0.046$).

3.3. Plasma levels of bradykinin and expression of bradykinin receptors in tissue

In the control group, the production of bradykinin in the plasma was significantly increased compared to baseline after 2 h of ex vivo perfusion ($p = 0.02$ by ANOVA and Dunn's post test) while this was not the case in samples of the C1-INH treated group. A non-significant trend for increased bradykinin values was also observed at 6 h of perfusion in the control group ($p = 0.07$, Supplementary Fig. 2).

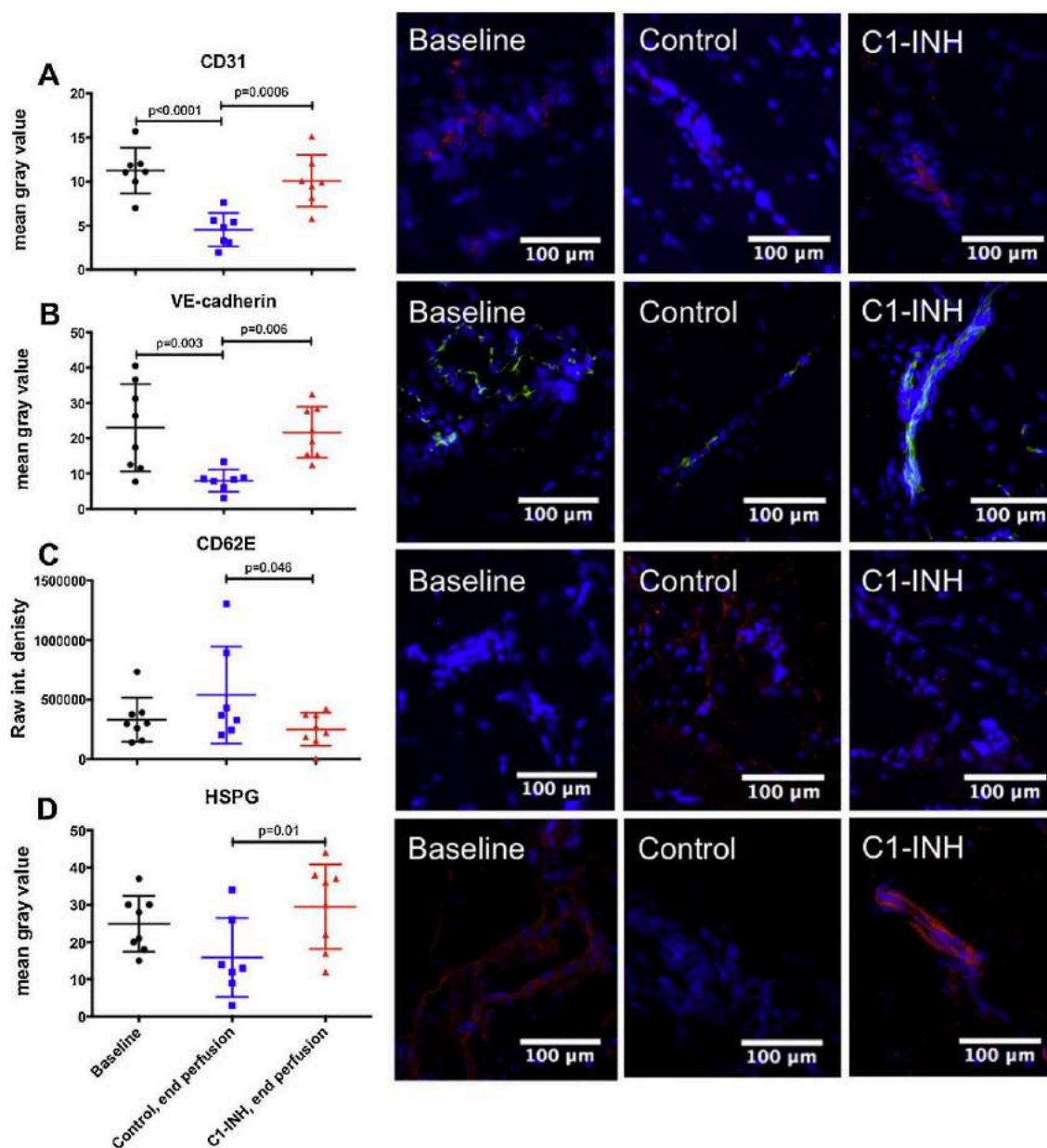


Fig. 3. Endothelial cells markers. Expression of (A) CD31 (B) VE-Cadherin and (C) E-selectin as well as (D) the extracellular matrix HSPG was assessed by quantitative analysis of immunofluorescence stained cryosections of muscle tissue. Diagrams on the left side show the values at baseline and end of perfusion of the control- and the C1-INH treated group. Values are shown as dots for each individual experiment with indication of mean \pm SD. P-values were calculated by ANOVA with Fishers' LSD post test. Representative pictures of tissue from controls and C1-INH treated animals, respectively, are shown on the right side.

A similar pattern was seen by immunofluorescence staining of bradykinin receptor-1 expression, which was increased compared with baseline after 12 h of perfusion in the control group ($p = 0.01$) but not in the C1-INH treatment group. For bradykinin receptor-2 a trend to increased expression was found in the control group ($p = 0.059$) and much less so in the C1-INH treated limbs ($p = 0.3$, Fig. 4).

3.4. Effect of C1-INH on activation of the coagulation and fibrinolytic cascades

A quantitative analysis of immunofluorescence stained pictures of fibrin and thrombin deposition as well as endothelial expression of fibrinogen like protein-2 (FGL-2), tissue plasminogen activator (tPA), plasminogen activator inhibitor-1 (PAI-1) and tissue factor (TF) was performed. There was a significant reduction of fibrin deposition

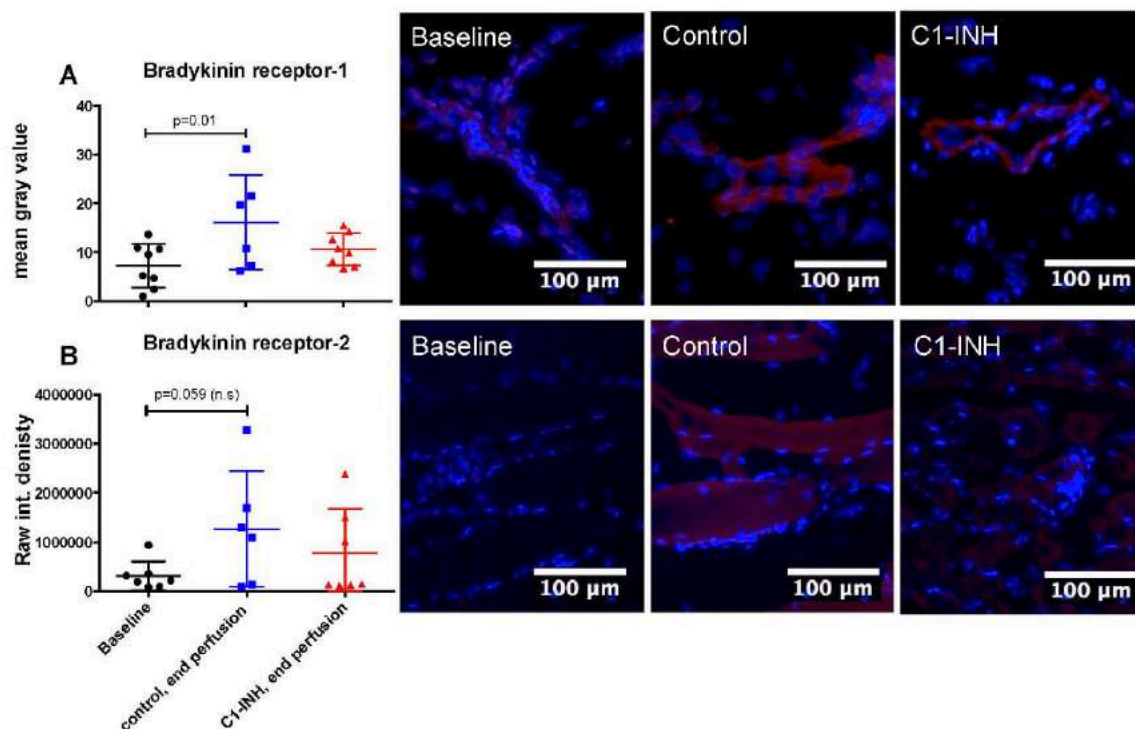


Fig. 4. Bradykinin. Tissue expression of bradykinin receptor 1 (A) and bradykinin receptor 2 (B) was assessed by quantitative analysis of immunofluorescence stained cryosections of muscle tissue. Diagrams on the left side show the values at baseline and end of perfusion of the control- and the C1-INH treated group. Values are shown as dots for each individual experiment with indication of mean \pm SD. P-values were calculated by ANOVA with Fisher's LSD post test. Representative pictures of tissue from controls and C1-INH treated animals, respectively, are shown on the right side.

($p \leq 0.0001$) and FGL-2 ($p = 0.019$) expression in the C1-INH treated group compared to the control group. As compared with baseline values, a significant increase was observed in the control, but not the C1-INH, group for fibrin ($p \leq 0.0001$) and thrombin deposition ($p = 0.049$) as well as FGL-2 expression ($p = 0.005$) at the end of perfusion. In contrast, muscle tissue of C1-INH treated limbs showed a significant increase in tPA ($p = 0.013$) and PAI-1 ($p = 0.01$) expression at end of perfusion compared with baseline. This was not seen in the control limbs. There was no significant difference between the groups and between baseline and end of perfusion regarding TF expression (Fig. 5A–F).

Analysis of EDTA plasma showed a trend for an increase in tPA-PAI-1 complexes at the end of perfusion in the C1-INH treated group compared to controls ($p = 0.1$). In the C1-INH group a trend for a decrease in D-dimers was observed at 10 min ($p = 0.1$) and 2 h of perfusion ($p = 0.08$) compared to controls (Fig. 5G and H).

3.5. Evaluation of muscle damage by histology and creatine kinase-MM

Hematoxylin-eosin stained muscle tissue samples were scored for muscle necrosis, hemorrhage and leukocyte infiltration. Only minimal muscle damage was observed in the samples at the end of perfusion, with significantly increased tissue necrosis scores in controls compared to baseline but not in the C1-INH treatment group. Inter-group differences between C1-INH treatment and controls were not significant (Supplementary Fig. 3). In particular, no hemorrhage or leukocyte infiltration was observed in the tissue of either group.

Muscle tissue necrosis was also assessed by measuring the plasma concentrations of creatine kinase-MM (CK-MM). A significant increase of CK-MM was observed as compared with baseline from 2 h of

perfusion onwards in both of the groups. No significant inter-group differences were seen (Supplementary Fig. 4).

4. Discussion

In the present study we used an ex vivo perfusion setting to mimic replantation of limbs after prolonged ischemia due to amputation. This allowed us to study activation of innate immunity and in particular the plasma cascade systems in skeletal muscle I/R injury. We confirmed that 9 h of cold ischemia followed by 12 h of ex vivo reperfusion led to severe reperfusion injury, including endothelial cell damage and activation of the plasma cascade systems. Moreover, the current study also highlighted the protective effect of C1-INH on vascular endothelial cells.

Our study demonstrated that there is a significant amount of IgG and IgM deposition in the skeletal muscle after I/R injury. This is in line with previous data from a humanized mouse (Sheu et al., 2009), as well as a rat hind limb I/R injury model (Duehrkop et al., 2012), where immunoglobulin deposition was significantly elevated after reperfusion. The use of C1-INH resulted in a significantly reduced immunoglobulin deposition after reperfusion in our study. It is known that C1-INH has an inhibitory effect on the plasma cascade systems, but inhibition of antibody-antigen binding has never been described for C1-INH. We assume, therefore, that the reduction of IgM- and IgG-binding by C1-INH is due to reduced cellular damage and thus a reduced expression of neopeptides which are the targets of natural antibodies (Zhang and Carroll, 2007).

The deposition of immunoglobulins on myocytes and endothelial cells has been described to activate both the classical and lectin pathway of the complement system in I/R injury models (Zhang and

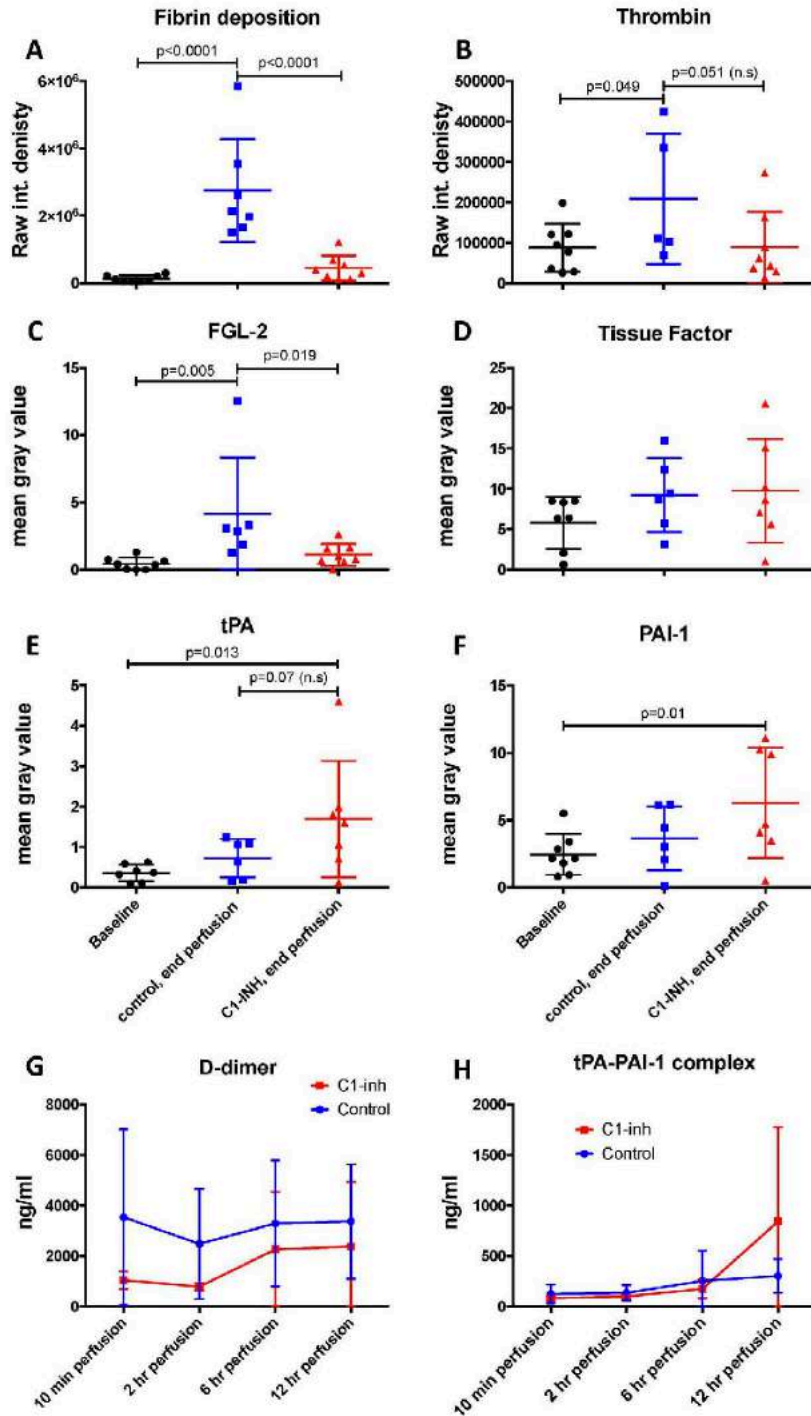


Fig. 5. Coagulation and fibrinolysis markers in tissue and plasma. Tissue deposition of fibrin (A) thrombin (B) as well as tissue expression of FGL-2 (C) TF (D) tPA (E) PAI-1 (F) was assessed by quantitative analysis of immunofluorescence stained cryosections of muscle tissue. Diagrams on the left side show the values at baseline and end of perfusion of the control- and the C1-INH treated group. Values are shown as dots for each individual experiment with indication of mean \pm SD. P-values were calculated by ANOVA with Fishers' LSD post test. D-dimer (G) tPA-PAI-1 complexes (H) were measured by competitive and sandwich ELISA respectively in EDTA plasma at different time points during ex vivo perfusion. Values are shown as a plot summary of the control- and the C1-INH treated group with indication of mean \pm SD. Student t-test was used to calculated P-value. *P-value < 0.05 is consider statistical significant.

Carroll, 2007). Indeed, in our study we found that deposition of C3b/c, C5b-9 and MBL were significantly elevated after reperfusion in the control limbs. However, when the limbs were pre-treated with C1-INH, deposition of C3b/c and C5b-9 was markedly reduced and MBL also

followed the same pattern. This is in line with an earlier study on renal I/R injury, which showed that anti-MBL treatment could reduce I/R injury (van der Pol et al., 2012). Analysis of the soluble complement activation markers C3a, C5a and sC5b-9 in plasma showed a significant

elevation compared to baseline in both the C1-INH and the control group (Supplementary Fig. 3). In contrast to tissue deposition of complement the measurement of the soluble complement activation markers did not reveal differences between controls and the C1-INH treatment group. It is known that ex vivo perfusion using a pediatric cardiopulmonary circuit activates the complement system (Chello et al., 1997; Chenoweth et al., 1981; Wan et al., 1997). The observed generation of C3a, C5a and sC5b-9 may therefore be due to complement activation in the perfusion circuit, bypassing the pathways which are inhibited by C1-INH or overwhelming its inhibitory capacity in the soluble phase.

Vascular EC in the skeletal muscle are the first cells that come in contact with the blood during reperfusion. Healthy EC provide protection against inflammation and spontaneous activation of the plasma cascades. In our model I/R injury led to shedding of HSPG from affected blood vessels and pre-treatment of the limbs with C1-INH succeeded to prevent this shedding. That C1-INH indeed had a vasculoprotective effect was further supported by the finding that endothelial expression of VE-cadherin and CD31 was preserved in limbs treated with this inhibitor and also by a decreased expression of E-selectin in C1-INH treated limbs at the end of reperfusion. Our data on specific protection of endothelial damage by C1-INH are in line with previous studies in a mouse stroke model in which binding of recombinant human C1-INH was shown (Gesueti et al., 2009).

Bradykinin, as a hormonal peptide, plays an important role in inflammatory reactions through bradykinin receptor-1 and -2. Various effects of bradykinin have been described in different models of I/R injury. In lung and intestine, blocking of bradykinin receptor 2 led to decreased I/R injury (Araoka et al., 2005), while in brain and heart upregulation of bradykinin receptors might have a beneficial effect on I/R injury (Nemethova et al., 2016; Sang et al., 2016; Yoshida et al., 2000). In the current study both bradykinin and the bradykinin receptors were elevated or showed a tendency for elevation in controls, but not in the C1-INH group.

Further, our data show that the coagulation cascade is also activated in skeletal muscle I/R injury. Fibrin and thrombin deposition were significantly elevated compared to baseline in controls, but not in the C1-INH group. D-dimer, a product of fibrin degradation, was also lower in the C1-INH group compared to the controls at early reperfusion time points (10 min and 2 h) and it remained lower in the C1-INH group during the rest of reperfusion.

In contrast to renal (Matsuyama et al., 2003; Ushigome et al., 2002) and cardiac (Mackman, 2003) I/R injury, we did not observe an upregulation of TF, suggesting that coagulation is not initiated via the extrinsic pathway in skeletal muscle I/R injury. However, we found an upregulation of FGL-2 on EC of control limbs and significantly less so in C1-INH treated limbs. FGL-2 is a prothrombinase produced by activated EC (Selzner et al., 2012). In our model of skeletal muscle I/R injury thrombin may therefore become directly activated by upregulation of endothelial FGL-2 expression. This was also previously shown in myocardial I/R injury (Jia et al., 2013).

It has also been shown before that a reduced activity of the fibrinolytic pathway by endothelial PAI-1 expression contributes to I/R injury (Denorme et al., 2016). However, fibrinolysis itself, mediated by tPA, was shown to exacerbate I/R injury of the brain (Yip et al., 2016), kidney (Roelofs et al., 2006) and lung (Fujita et al., 2001) in mouse models. In our porcine model of skeletal muscle I/R injury neither tPA nor PAI-1 were increased in controls, whereas C1-INH treatment led to upregulation of both, tPA and PAI-1, resulting in an increase of tPA-PAI-1 complexes in the plasma at the end of reperfusion ($p = 0.1$). Inhibition of fibrinolysis by endothelial PAI-1 expression may thus not be a major cause of I/R injury in our model. Despite an increased PAI-1 expression, C1-INH treatment did not lead to increased fibrin deposition and therefore did not inhibit fibrinolysis, probably due to a concomitant increase of endothelial tPA expression.

We could not observe a reduction of the myocyte injury marker CK-

MM in the plasma of C1-INH treated limbs as compared with controls. We assume that this is caused by the use of an ex vivo perfusion setting, which is a limitation of the study. It was published before that the increase of CK-MM does not always represent the degree of muscle damage, particularly if the rate of production is exceeding the elimination rate (Komulainen et al., 2007; Van der Meulen et al., 1991). The latter is clearly the case in our ex vivo study.

In conclusion, skeletal muscle reperfusion injury after 9 h of cold ischemia leads to severe damage that was seen as high levels of immunoglobulin deposition, EC damage, and activation of the complement, kinin and coagulation systems. The use of the cytoprotective drug C1-INH provided protection to the vascular endothelium and the muscle tissue against deposition of immunoglobulins, complement and fibrin. C1-INH might therefore be useful to reduce reperfusion injury after replantation of traumatically amputated limbs. However, C1-INH had no significant effect on CK-MM (Supplementary Fig. 3), a marker of myocyte damage, and the presence of C5a and sC5b-9. This might be due to complement activation in the ex vivo perfusion circuit containing plastic tubing, filters from the oxygenator, and the absence of a liver.

Disclosures or conflict of interest

The authors have no conflicts to report.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.molimm.2017.06.021>.

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Paper II: Investigation of the protective role of human CD46 expression on plasma cascade and endothelial cell activation in the context of autologous ex-vivo perfusion of pig limbs

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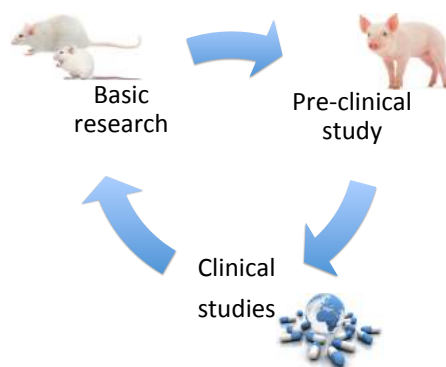
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Status: Addition of in vitro experiments and analysis of more complement markers. In preparation for submission to Nature Scientific Reports

Contribution: All experiments and graphs were performed and made by Mai M. Abdelhafez

Background: The production of pigs transgenic for human genes such as human CD46 provides a great opportunity to study the role of innate immunity in various clinically important disease models, such as ischemia reperfusion injury, and allows for better translation of the animal experimentation data to the clinical setting.

Aim: To study the effect of hCD46 on the plasma cascades and inflammation in a pig model of limb amputation and ex-vivo perfusion.



Conclusion: Expression of hCD46 on porcine cells provided protection against coagulation activation as well as inflammation in porcine skeletal muscle. It also stimulated the endothelium to express fibrinolytic protective molecules.

Investigation of the protective role of human CD46 expression on plasma cascade and endothelial cell activation in the context of autologous ex-vivo perfusion of pig limbs

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Abstract

The human membrane cofactor protein, hCD46, was introduced into porcine cells to overcome hyperacute rejection in pig-to-primate xenotransplantation. It was already shown in our group that the expression of hCD46, in the xeno setting, reduces the complement and coagulation activation as well as protects the endothelial surface. We hypothesized that the CD46 transgene might also prevent activation of the plasma cascade systems and endothelial cell activation in an extracorporeal perfusion setting. Here we tested whether transgenic expression of human CD46 could attenuate complement deposition as well as activation of the coagulation system in an autologous perfusion setting.

Human CD46/HLA-E double transgenic as well as wild type pig forelimbs were ex-vivo perfused with autologous, heparinized whole blood for 12 hours. The ischemia times of the limbs ranged from 20 to 30 minutes. Cryosections of muscle tissue from baseline and at the end of perfusion were stained for deposition of porcine C3c, C4c and C5b-9 as complement markers as well as IgG and IgM. Expression of the non-muscular myosin heavy chain type IIc peptide (NMHC IIc) was assessed as a putative marker of ischemic stress. Tissue plasminogen activator (tPA), fibrinogen-like protein-2 (FGL-2) and tissue factor (TF) were stained as coagulation markers. Baseline and end of perfusion plasma samples were analyzed for the presence of inflammatory cytokines and growth factors (IL-1 β , IL-6, IL-10, TNF- α , MCP-1, VEGF and bFGF) as well as complement (sC5b-9 and C5a) and coagulation markers (D-dimers, prothrombin fragments 1+2 [F 1+2] and tPA-PAI-1 complexes).

Fluorescence intensities for fibrin deposition showed a significant increase after 12 hours of ex vivo perfusion in the wild type pigs compared to the transgenic pigs. Moreover, expression of the pro-fibrinolytic protein tPA showed a significant increase after 12 hours of autologous blood perfusion in transgenic as compared to wild type pigs. In line with this finding FGL-2 and TF levels at end of perfusion were significantly increased compared to baseline in wild type pigs, but not in the hCD46 transgenic animals. D-dimer levels showed an increase, although not statistically significant, after 12 hours of ex-vivo perfusion in both

wild type and transgenic groups compared to baseline, but there were no differences between groups at the end of perfusion. Prothrombin F 1+2 showed a significant increase between baseline and end of perfusion in wild type, while there was no difference in transgenic pigs. The levels of tPA-PAI-1 complexes were significantly increase at the end of the perfusion compared to baseline and no difference between wild type and hCD46 transgenic limbs was found. Concentrations of cytokines, growth factors and complement activation markers showed no differences between wild type and transgenic pigs at the end of perfusion, except for IL-8 and MCP-1. C3b/c, C4b/c, C5b-9, IgM and IgG deposition showed no differences between wild type and transgenic pig limbs as well as between baseline and end of perfusion. Expression of NMHC IIc showed no significant difference either. Based on staining for CD31 and VE-cadherin, no endothelial cell (EC) damage occurred in this model.

The present data for the coagulation markers suggest that indeed the EC of the hCD46 transgenic pigs might be less activated and therefore express higher levels of tPA, similarly to healthy pro-fibrinolytic EC, and show significantly lower levels of fibrin deposition. Moreover, plasma levels of the pro-inflammatory cytokines MCP-1 and IL-8 were lower in the hCD46 transgenic animals. Furthermore, the hCD46 transgenic limbs have a trend of lower TF and FGL-2 expression, F1+2 and IL-1 β production as well as lower C5a. There was no statistically significant difference regarding complement activation between the wild type and transgenic pigs. This could be due to the short ischemia time (~20 min), which leads to only minimal IRI.

Key words: endothelial cells, human CD46, transgenic pig, plasma cascade

Introduction

The shortage of available organs for transplantation and the increased gap between the required and available organs needs an out of the box solution ¹. The short term solution was to use donation after circulatory death (DCD) and not only donation after brain death or live donation. However, the DCD solution incurs several problems including the uncontrolled condition of the organ as well as ischemia/reperfusion (I/R) injury. Even after the acceptance and introduction of DCD, the gap between the available donors and the patients on the waiting list is still large ². The long-term solution was to consider xenotransplantation from animals to humans. The pigs met the ethical requirements and have fewer anatomical differences in comparison to non-human primates ³. As a consequence, the production of transgenic pigs was introduced to the scientific research community in order to overcome the immunological barriers between humans and pigs in the context of xenotransplantation ⁴.

The human membrane cofactor protein (CD46) was introduced into pigs in order to overcome the hyper acute rejection that is mediated by the antibody-complement activation and ends with damage to the endothelium ⁵. Both xenotransplantation and I/R injury trigger the innate immunity system which is mediated by the activation of all the plasma cascades. In both cases there is activation and damage of the vascular endothelium ^{6,7}. The human CD46 expressed on the cells of the transgenic pigs inhibits the human complement system in the context of xenotransplantation as well as inhibiting the porcine complement in the context of I/R injury based on the fact that CD46 analogues cross react between different species ⁸.

During organ transplantation, the organs are exposed to unavoidable I/R injury, which increases the damage to the transplanted organ and triggers the activation of the innate immune system as well as causing microvascular damage and in severe cases it ends with graft failure ^{9,10}. I/R injury is a complex inflammatory condition that has no available treatment in the clinical setting ^{11,12}. The proposed mechanisms of the I/R injury are divided into two phases. First, ischemic injury includes expression of neo-epitopes, ion homeostasis imbalance, mitochondrial damage and a shift toward anaerobic metabolism ¹³⁻¹⁵. This is

followed by the second phase, which starts as soon as the reperfusion occurs, and is characterized by activation of all plasma cascades, activation and damage of endothelial cells, oxidative stress and recruitment of leukocytes to the site of injury^{7,16-20}.

The use of hCD46 transgenic animals interestingly showed a protective effect against complement activation in a porcine model of ex-vivo limb reperfusion with autologous blood²¹. The production of pig organs that tolerate I/R injury during organ transplantation will improve the outcome and enhance the prognosis of the patients.

In the current study we decided to investigate closely the effect of hCD46 expression on the complement, coagulation and endothelial cells in a porcine model of ex-vivo limb reperfusion with autologous blood.

Animals and methods

Animals

All animal experiments were performed according to the Swiss national laws and guidelines and the ARRIVE guidelines (<https://www.nc3rs.org.uk/arrive-guidelines>). The local animal experimentation committee of the Canton of Bern approved the study (permission no. BE 45/11). We used 12 animals for this study of which one forelimb was used for the autologous ex-vivo perfusion. Two groups of animals were used: Human CD46/HLA-E double transgenic, α 1-3-galactosyltransferase knockout heterozygous pigs (Group 1, n=6) and wild type (Group 2, n=6). A formal power calculation to establish the size of the experimental groups was not possible because of a lack of variability data. We therefore chose to use 12 animals with the aim to have 6 valid experiments per group.

Surgical procedure

Forelimbs of twelve large white pigs of both sexes (body weight 39.3 ± 4.1 kg) were amputated as described previously²². In brief, pigs were pretreated with ketamine (20 mg/kg) and xylazine (2 mg/kg), as well as intramuscular atropine (0,05 mg/kg) and midazolam (0,5 mg/kg) before intubation. Anesthesia was induced by isoflurane (1.5% in 1:3 O₂/air) inhalation in addition to Fentanyl (20 μ g/kg/h). After dissection of the axillary neurovascular bundle, the artery and two veins were cannulated using 10-14 French catheters according to the vessel size. The pigs were anticoagulated with 5000 IU-heparin immediately after the first forelimb amputation and another 2500 IU was administrated 2 hours later. After amputation of both limbs, blood was collected into a blood bag containing 10000 IU-heparin (Liquemin; Drossapharm AG/SA, Basel, Switzerland). 500 ml of blood was collected. The animals were then euthanized by intravenous injection of 20 ml of 20% KCl.

Ex vivo reperfusion setting

A pediatric heart lung machine was used for ex vivo perfusion of the limbs and was primed with 150 ml HAES to ensure complete removal of air bubbles from the system. The limbs were washed for 5 minutes using HAES to wash out

any metabolic products accumulated during the amputation process. Autologous, heparinized blood was then added to the system and replaced the HAES. The perfusion lasted for 12 hours. The perfusion rate was set between 100-150 ml/minute using a turbine pump (MEDOS DataStream blood pump, model DP2; Medos Medizintechnik AG, Stolberg, Germany) and the temperature maintained at 32°C. Oxygen concentration was adjusted to 21% using a membrane oxygenator (MEDOS Hilite 800 LT; Medos Medizintechnik AG, Stolberg, Germany). Hourly, blood gas analyses (GEM premier 3000; Instrumentation Laboratory, Lexington, MA, USA) were performed to control glucose, pH and potassium levels. Insulin (Novorapid, 100 U/ml, Novo Nordisk Pharma AG) with or without 40% glucose and/or sodium bicarbonate were administrated to the perfusion line to maintain physiological glucose, potassium and lactate levels for as long as possible.

Blood and tissue samples

EDTA and citrate plasma samples were collected at baseline (from the blood bag before starting the perfusion) as well as 10 minutes, 2 hours, 6 hours and at the end of perfusion from each limb. All blood samples were centrifuged at 2000 x g for 15 minutes at 4°C, aliquoted and the plasma or serum stored at -80°C until analyzed. Biopsies of skeletal muscle were taken at the baseline and at the end of the perfusion from the porcine triceps muscle of each limb and stored in Tissue-Tek (OCT compound, Sakura Finetek Europe B.V., Netherlands) at -80°C for immunofluorescence staining.

Immunofluorescence

Cryopreserved samples were cut into 5 µm thick sections, air dried for 30-60 minutes, fixed with acetone at room temperature for 5 minutes, rehydrated with TBS solution for 10 minutes at room temperature and demarcated with a Dako pen (Dako Denmark A/S, Glostrup, Denmark). The primary antibodies used were directly labeled goat anti-human C3b/c-FITC (Dako, F0201), goat anti-human C4b/c-FITC (Dako, F0169), goat anti-pig IgG-FITC (Southern Biotec, 6050-02) and rabbit anti-human fibrinogen-FITC (Dako, F0111), unlabeled mouse anti-human C5b-9 (Antibody Shop, DIA 011-01), goat anti-pig IgM (AbD

Serotec, AAI39), goat anti-human VE cadherin (Santa Cruz Biotechnology, sc-6458), rat anti-pig CD31 (R&D systems, MAB33871), mouse anti-human CD62E (Sigma, S9555), goat anti-human tPA (Abcam, ab14198), sheep anti-human tissue factor (Affinity Biological, SATF-IG) and rabbit anti-human FGL-2 (Aviva Systems Biology, ARP52235_p050). Cross-reactivity of the polyclonal anti-human antibodies with the respective porcine antigens was either tested by the manufacturer or by ourselves^{22,23}.

The following secondary antibodies were used for the indirect immunofluorescence technique: sheep anti-mouse Cy3 (Sigma, C2181), donkey anti-goat Alexa Fluor 488 (Molecular Probes, A11055), sheep anti-rabbit Cy3 (Sigma, C2306), goat anti-rat Cy3 (Jackson ImmunoResearch, 112-166-003), goat anti-mouse Alexa Fluor 546 (Invitrogen, A11030) and donkey anti-sheep Alexa Fluor 488 (Life Technology, A11015). All antibodies were diluted in TBS-PBS-1%BSA. Primary antibodies were incubated overnight at 4°C, followed by a triple washing step. Secondary antibodies were incubated for 60-90 minutes at room temperature. Glycergel (Dako, C0563) was used as the mounting medium. A fluorescence microscope (DMI4000; Leica, Wetzlar, Germany) was used to analyze the samples. All the pictures were quantified using Image J software, version 64.4.

ELISA and multiplex suspension array

Using commercially available ELISA kits and following the instruction from the manufacturer, the following parameters were measured in EDTA plasma samples: D-dimer (Elabscience[®], E-EL-P2237), tissue plasminogen activator- plasminogen activator inhibitor-1 (tPA-PAI-1) complex (Molecular Innovations, POPAITPAKT-COM) and prothrombin F1+2 (Molecular Innovations, MBS267478). Inflammatory markers IL-1 β , IL-6, IL-8, IL-10, MCP-1 and TNF- α , growth factor markers VEGF, bDNF and bFGF as well as complement component C5a and sC5b-9 were measured in EDTA plasma samples using a multiplex suspension array (Bio-Plex) as previously described in²⁴. Briefly, micro beads (Bio-Rad) carrying a specific fluorochrome code were coupled, using a Bio-Plex amine coupling kit (Bio-Rad, 171-406001), to capture the antibody. The bead antibody complex was detected by a biotinylated antibody followed by

Streptavidin-PE (Qiagen, Venlo, The Netherlands). Fluorescence was measured using a Bio-Plex 100 system and the concentration was calculated with Bio-Plex Manager 4.1 software (Bio-Rad)

Statistics

Data are presented as mean \pm standard deviation (SD). Statistical analyses were performed using GraphPad Prism 6 software (GraphPad, San Diego, CA, USA). For tissue and plasma samples one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test was used. P values <0.05 were considered statistically significant.

Results

Overexpression of hCD46 on porcine endothelial cells reduces activation of the coagulation cascade in autologously ex vivo perfused porcine limbs.

Immunofluorescence analysis of muscle tissue from porcine limbs ex vivo perfused for 12 hours with autologous, heparinized whole blood revealed a significant reduction in fibrin deposition ($p = 0.03$) in hCD46 transgenic compared to wild type limbs. There was also a significant increase in fibrin deposition at the end of ex-vivo perfusion ($p = 0.0006$) in comparison to baseline but not in hCD46 transgenic limbs (fig 1-A).

The hCD46 transgenic limbs showed significantly higher expression levels of the pro-fibrinolytic protein tPA as compared with wild type limbs at the end of ex-vivo perfusion with autologous blood ($p = 0.009$). Moreover, there was a significant increase in tPA expression at 12 hours of ex-vivo perfusion compared to baseline ($p = 0.01$) in the hCD46, but not in wild type limbs (fig 1-B).

With respect to pro-coagulant markers, wild type limbs showed a significant increase in FGL-2 and TF expression at the end of ex-vivo perfusion as compared to baseline ($P = 0.04$ and 0.03), but this was not seen in the hCD46 transgenic limbs. There were no statistically significant differences at the end of ex-vivo perfusion between wild type and hCD46 transgenic limbs, but there was a trend of lower FGL-2 and TF expression in the hCD46 limbs (fig 1-C and D).

Effect of hCD46 overexpression on coagulation and fibrinolysis markers in plasma

D-dimers showed a trend to increase at the end of perfusion compared to baseline in wild type limbs ($p = 0.06$) while perfusion of hCD46 limbs did not show the same response. D-dimer formation in the hCD46 transgenic limbs showed, although non-significantly, lower values compared to wild type limbs (fig 2-A). Prothrombin F(1+2) showed a significant increase at the end of perfusion ($P = 0.004$) compared to baseline samples in wild type limbs but not in hCD46 transgenic limbs (fig 2-B).

tPA-PAI-1 complexes were significantly increased at the end of perfusion compared to baseline in both wild type and hCD46 transgenic limbs (fig 2-C).

Inflammatory and growth factor markers

IL-1 β , IL-6, IL-8, IL-10, MCP-1 and TNF- α were measured at baseline and at the end of perfusion in plasma of both wild type and hCD46 transgenic limbs. At the end of perfusion, IL-8 levels showed a significant increase in the wild type compared to the hCD46 group ($p = 0.03$). IL-8 levels were also significantly increased compared to baseline in plasma from wild type ($p = 0.01$) but not from hCD46 transgenic limb perfusions (fig 3-A). IL-1 β levels were also significantly increased at the end of perfusion compared to baseline in the wild type but not in the hCD46 group ($p = 0.04$). Moreover, IL-1 β showed a trend towards higher expression in the wild type compared to the hCD46 transgenic group (fig 3-B). At the end of perfusion MCP-1 was significantly increased in the wild type ($p = 0.01$) compared to the hCD46 group and significantly increased compared to baseline in wild type ($p = 0.001$) but not in the CD46 transgenic perfusions (fig 3-C). IL-6 showed a significant increase at the end of perfusion in both wild type and CD46 groups compared to baseline ($p = 0.0024$ and 0.0018 , respectively) with no difference between the two groups at the end of perfusion. In contrast, IL-10 levels showed a significant decrease at the end of perfusion in both wild type and CD46 groups compared to baseline ($p = 0.0027$ and 0.0003 respectively), with no difference between the two groups at the end of perfusion (fig 3-D and E). No significant changes with regard to TNF- α , bFGF and VEGF were observed intra between groups and between baseline and end of perfusion (fig 3-F, 4-A and B).

Autologous perfusions did not affect complement and immunoglobulin deposition or NMHC IIc expression

Quantitative analysis of immunofluorescence stained skeletal muscle sections of both wild type and hCD46 transgenic limbs at baseline and at the end of perfusion (12 hours perfusion) showed no statistical differences regarding NMHC IIc expression (fig 5-A) as well as immunoglobulin (IgM and IgG, fig 5-B and C) and complement (C3b/c, C4b/c and C5b-9, fig 6-A, B and F) deposition. In contrast, analysis of C3a, C5a and soluble C5b-9 in the blood showed a significant increase at the end of perfusion in both wild type and hCD46 transgenic limb perfusions. A non-significant trend towards lower C5a generation was observed

at the end of perfusion in the CD46 transgenic compared to the wild type group (fig 6-C, D and E).

No loss of CD31 and VE-cadherin and no increased E-selectin expression in autologous pig limb perfusion

Immunofluorescence analysis of muscle tissue of porcine limbs ex-vivo perfused for 12 hours with autologous, heparinized whole blood showed no changes in the expression of the endothelial cell markers CD31 and VE-cadherin either within the groups between baseline and end of perfusion or between them (fig 7-A and B). Similarly, expression of the endothelial cell activation marker E-selectin was not increased in either group from baseline to the end of perfusion (fig 7-C).

Discussion

Ex-vivo reperfusion of amputated porcine limbs was initially established as a method of studying possible preservation techniques for amputated limbs before their replantation ²⁵. Later, the same technique was used in our research group to study hyperacute xenograft rejection in pig-to-human xenotransplantation. Human CD46/HLA-E double transgenic, α 1-3-galactosyltransferase knockout heterozygous pigs were used to prevent plasma cascade activation and to protect endothelial cells during the acute rejection episode when the limb was ex-vivo perfused with human heparinized whole blood. In the same context autologous perfusions were performed to serve as a controls. Surprisingly, the transgenic animals showed less inflammatory reaction compared to wild type animals ²¹.

The current study was therefore designed to investigate the effect of hCD46 expression on porcine cells on the plasma cascade systems and the endothelium, which might be activated due to ex-vivo perfusion as previously shown by Constantinescu ²⁵. Analysis of coagulation and fibrinolysis markers indeed showed significantly elevated fibrin deposition at the end of perfusion in the wild type limbs compared to baseline. Moreover, the hCD46 transgenic expression protected the skeletal muscle from fibrin deposition when compared to wild type tissue. Fibrin deposition can be explained by endothelial expression of TF and FGL-2. Both of these pro-coagulant molecules were significantly upregulated at the end of ex-vivo perfusion of wild type but not hCD46 transgenic limbs when compared to their respective baselines. TF activates the extrinsic coagulation pathway ²⁶ and FGL-2 has a direct prothrombinase activity for the conversion of prothrombin into thrombin ²⁷. The latter observation indicates endothelial cell activation in the wild type but not in the hCD46 transgenic limbs. Moreover, the pro-fibrinolytic and therefore endothelial-protective molecule tPA, was significantly upregulated at the end of perfusion in the hCD46 transgenic but not in the wild type limbs.

D-dimer, is an indicator of the breakdown of previously formed fibrin clots and therefore essentially activation of the coagulation system ²⁸. D-dimer levels were non-significantly increased at the end of perfusion compared to baseline in both wild type and hCD46 groups. There was no difference at the end

of perfusion regarding D-dimer concentrations in the plasma between wild type and hCD46 transgenic groups. In addition, the wild limbs showed increased prothrombin fragment (1+2) production at the end of reperfusion compared to baseline. Also in the transgenic group the respective values were clearly elevated after 12h of perfusion as compared to baseline, even if statistical testing did not reveal significance for this increase. Finally, plasma levels of tPA-PAI-1 complexes were used to assess activation of the fibrinolytic system. tPA-PAI-1 complexes were significantly increased as compared to respective baseline levels in both groups at the end of reperfusion, with no differences between the groups. Taken together, the analysis of these plasma markers linked to activation of the coagulation and fibrinolytic system show that the respective plasma cascades are indeed activated and the this activation is independent of the presence or absence of the hCD46 transgene. Most probably, therefore, activation of the coagulation system is due to the presence of the oxygenator, tubings and the centrifugal pump in the extracorporeal membrane oxygenation (ECMO) system used to perform the perfusions. Activation of the clotting system by ECMO is a well-known phenomenon and has been reviewed earlier ²⁹.

Cytokine levels were measured in plasma at baseline and at the end of perfusion to assess the inflammatory reaction caused by the limb perfusions. IL-8 and IL-6, both produced by endothelial cells and leukocytes, IL1- β , produced by leukocytes during the inflammatory process ³⁰⁻³², and MCP-1, which interacts with the upregulated CCR-2 on endothelial cells during inflammation ³³, were all significantly increased at the end of perfusion in the wild type limbs. The latter observation has also been seen in previous studies of autologous ex-vivo perfusion of porcine limbs ³⁴. IL-8 and MCP-1 levels were decreased by the expression of hCD46 on the porcine cells compared to wild type limbs, but this was not the case for IL-6 and IL1- β . Additionally, the anti-inflammatory cytokine IL-10, that has a protective effect against inflammation ³⁵, was dramatically reduced at the end of perfusion compared to baseline in both groups. Other inflammatory cytokines and growth factor markers (TNF- α , bFGF and VEGF) did not show any differences even with the baseline.

Interestingly, non-muscle myosin heavy chain IIc, a damage-associated molecule, which is increased as a response to tissue ischemia and forms a target

for natural antibody deposition ³⁶, as well as IgM and IgG deposition in the limb tissue, did not show any changes during ex-vivo perfusion of the porcine limbs. Furthermore, deposition of the complement components C3b/c, C4b/c and C5b-9 were not influenced by the perfusion and did not show group-specific differences. This is in contrast to the results obtained previously using a similar ex-vivo perfusion model ²⁵. However, in the current model the limbs were exposed only to a minimal warm ischemia time (less than 30 minutes) during the amputation and preparation of the ex-vivo perfusion. This ischemia time may not be enough for the skeletal muscle to upregulate damage-associated patterns like NMHC IIc, so that subsequent antibody- and complement deposition, resulting in ischemia/reperfusion injury, will not occur. This is also supported by the limited (non-significant) increase of the bFGF and VEGF, growth factors which typically are upregulated as a result of tissue ischemia.

In contrast to complement deposition in tissue, the concentration of the soluble complement components sC5b-9 and C3a were significantly increased at the end of perfusion compared to baseline in both wild type and hCD46 transgenic limbs. The pattern for C5a was less clear with a non-significant trend for increased levels in the wild type but not in the transgenic group. Similar to the soluble coagulation activation markers also these complement activation products may arise as an effect of the ECMO itself rather than being formed as a consequence of tissue inflammation due to the extracorporeal perfusion ³⁷. In line with the above assumption, the endothelial cells were not damaged or activated as stainings for VE-cadherin, CD31 and E-selectin showed no differences before and after ex-vivo perfusion in both groups.

In conclusion, the expression of hCD46 indeed seems to protect the porcine endothelial cells. The expression of procoagulant endothelial markers was reduced in the hCD46 transgenic limbs, suggesting that the transgene helps to maintain the natural anticoagulant, pro-fibrinolytic and anti-inflammatory endothelial surface.

Acknowledgments

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Figure legends

Figure 1: Effect of hCD46 expression on coagulation markers in skeletal muscle tissue. Cryosections of skeletal muscle from porcine limbs ex-vivo reperfused with autologous blood were stained by immunofluorescence. Quantitative analysis of (A) fibrin, (B) tissue plasminogen activator, (C) fibrinogen like protein 2, FGL-2 and (D) tissue factor, TF are shown on the left panel. Representative pictures of the respective immunofluorescence stains are shown to the right. Samples were taken at the end of reperfusion from both wild type and transgenic limbs. Image analysis was done by Image J software. Data are shown as dot plots with indication of mean \pm standard deviation. Statistical analysis was done using one-way ANOVA with Bonferroni post hoc testing.

Figure 2: hCD46 expression effect on plasma coagulation and fibrinolysis markers. EDTA plasma samples were collected at baseline and end of ex-vivo reperfusion. Quantitative analysis of (A) D-dimer, (B) prothrombin fragment (1+2) and (C) tPA-PAI-1 complex were measured by commercially available ELISA kit. Data are shown as dot plots with indication of mean \pm standard deviation. Statistical analysis was done using one-way ANOVA with Bonferroni post hoc testing.

Figure 3: effect of hCD46 expression on the pro-inflammatory cytokines production. EDTA plasma samples were collected at baseline and end of ex-vivo reperfusion. Quantitative analyses of (A) IL-8, (B) IL-1 β , (C) MCP-1, (D) IL-6, (E) IL-10 and (F) TNF- α were measured by custom made porcine multiplex suspension array. Data are shown as dot plots with indication of mean \pm standard deviation. Statistical analysis was done using one-way ANOVA with Bonferroni post hoc testing.

Figure 4: effect of hCD46 expression on the production of growth factors. EDTA plasma samples were collected at baseline and end of ex-vivo reperfusion. Quantitative analysis of (A) basic fibroblast growth factor (bFGF) and (B) vascular endothelial growth factor (VEGF) were measured by custom made

porcine multiplex suspension array. Data are shown as dot plots with indication of mean \pm standard deviation.

Figure 5: effect of ex-vivo perfusion on the expression of neo epitopes and immunoglobulin deposition. Cryo-sections of skeletal muscle from porcine limbs ex-vivo reperfused with autologous blood were stained with immunofluorescence. Quantitative analysis of (A) non-muscle myosin heavy chain IIc (NMHC IIc), (B) IgM and (C) IgG were measured using Image J software. Data are shown as dot plots with indication of mean \pm standard deviation.

Figure 6: effect of ex-vivo perfusion on complement activation. Cryo-sections of skeletal muscle from porcine limbs ex-vivo reperfused with autologous blood were stained with immunofluorescence. Quantitative analysis of (A) C3b/c and (B) C4b/c were measured using Image J software. EDTA plasma samples were collected at baseline and end of ex-vivo reperfusion. Quantitative analyses of (C) C3a, (D) C5a and (E) sC5b-9 were measured by custom made porcine ELISA for C3a or multiplex suspension array for C5a and sC5b-9. Quantitative analysis of (F) C5b-9 was done on tissue section of the skeletal muscle and was measured using Image J software. Data are shown as dot plots with indication of mean \pm standard deviation. Statistical analysis was done using one-way ANOVA with Bonferroni post hoc testing.

Figure 7: effect of ex-vivo perfusion on endothelial cell activation. Cryo-sections of skeletal muscle from porcine limbs ex-vivo reperfused with autologous blood were stained with immunofluorescence. Quantitative analysis of (A) VE-cadherin, (B) CD31 and (C) E-selectin were measured using Image J software. Data are shown as dot plots with indication of mean \pm standard deviation.

Figure 1:

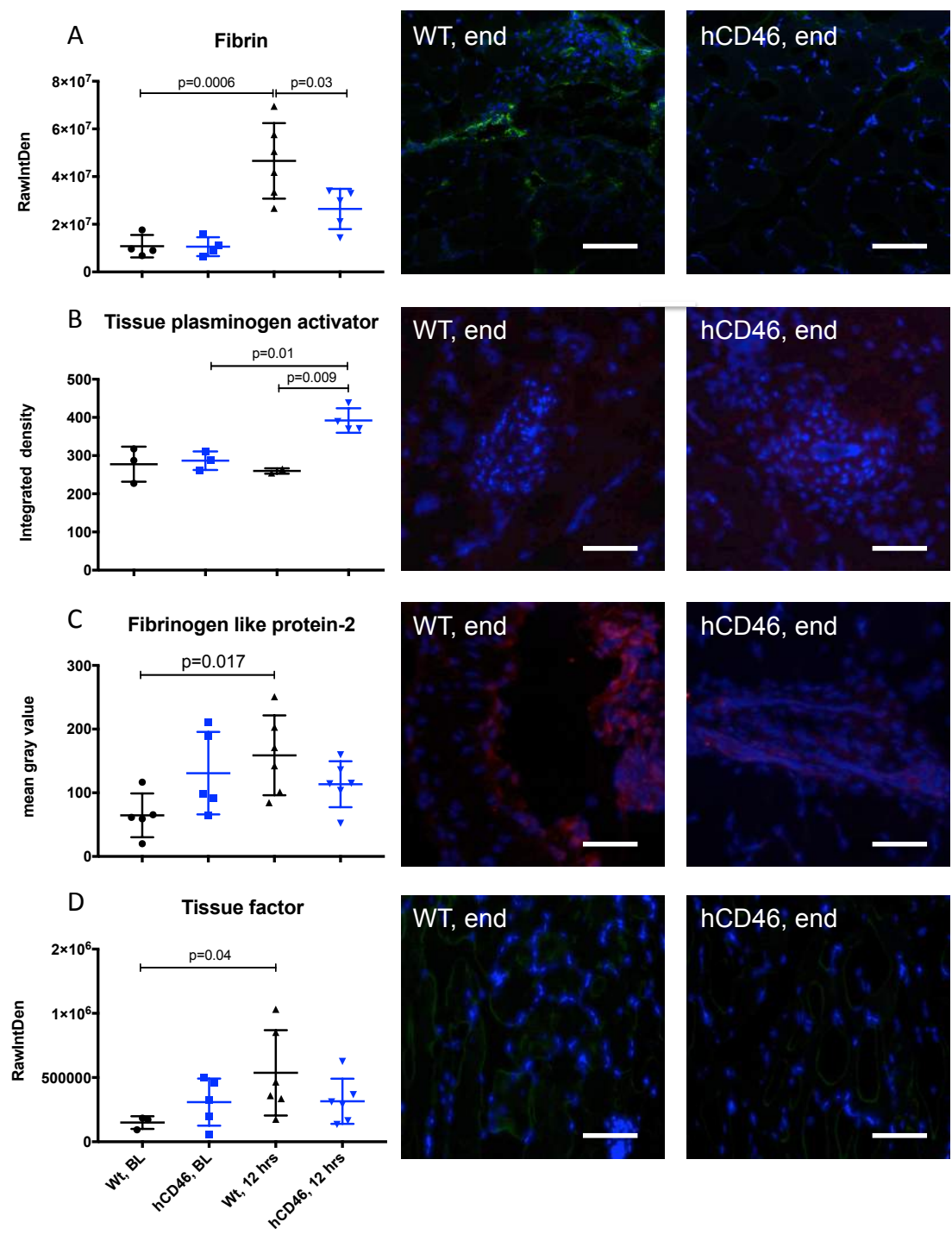


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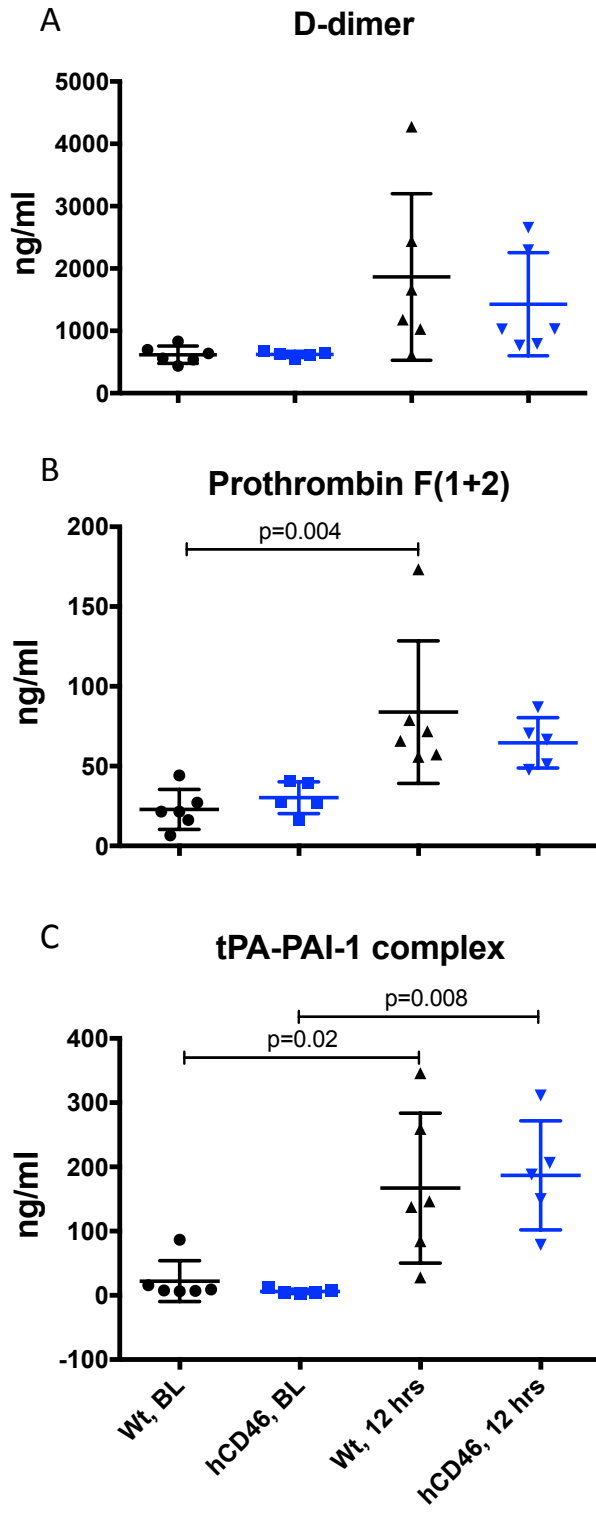


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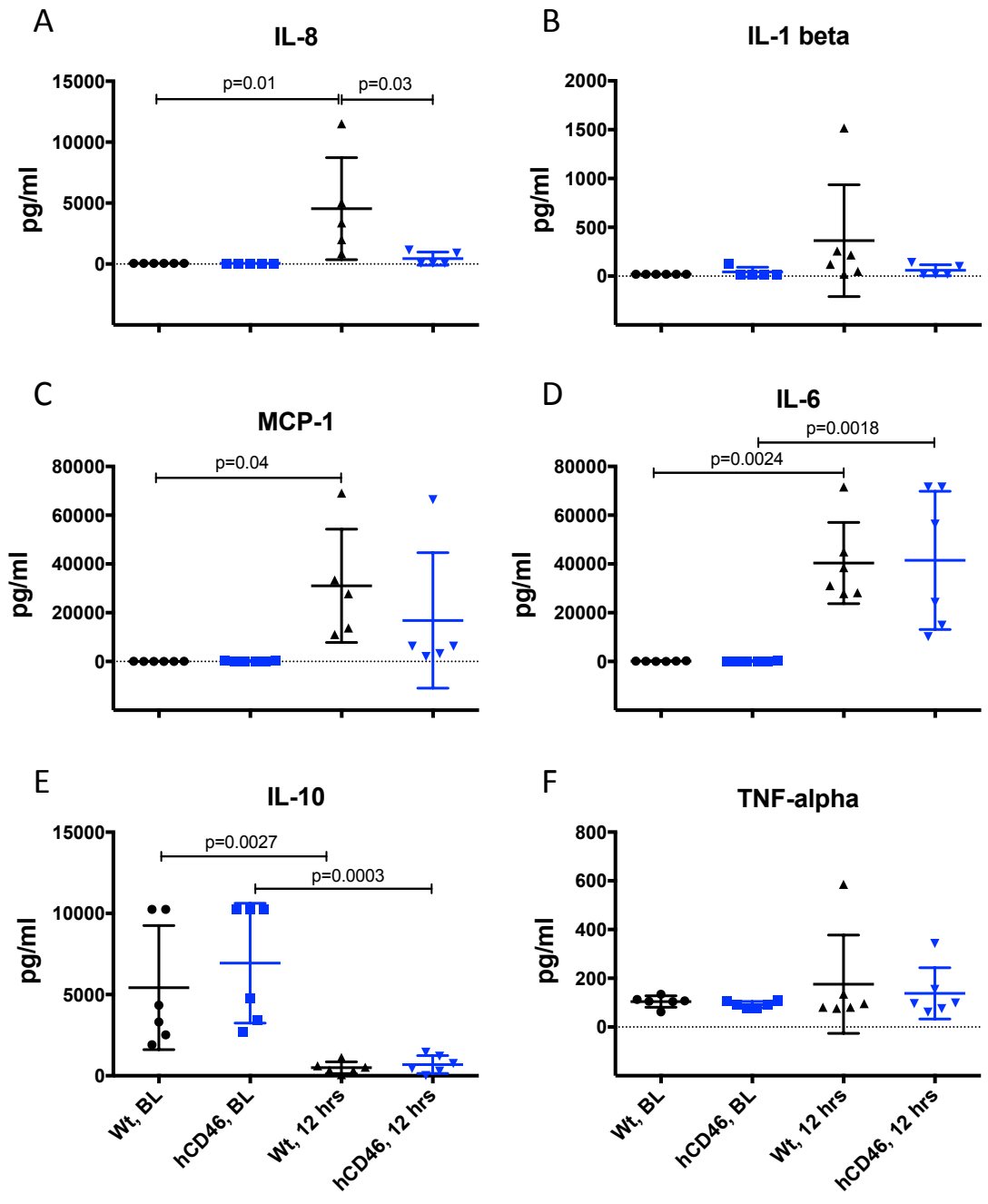


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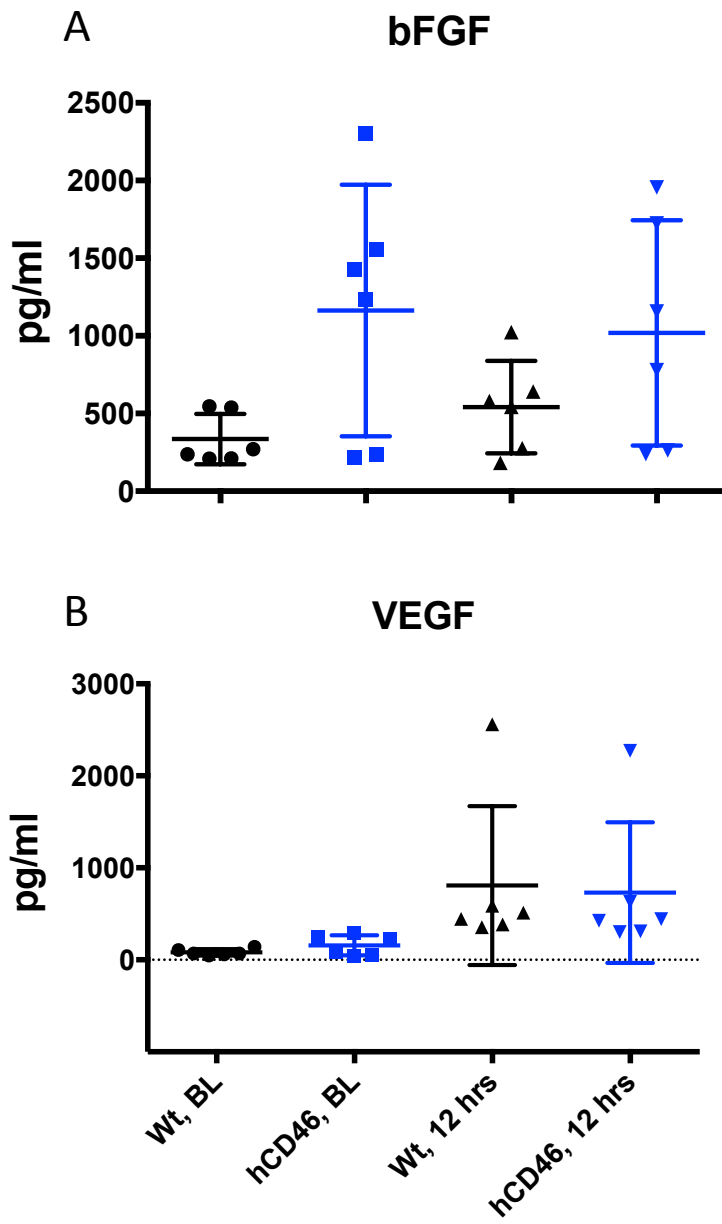


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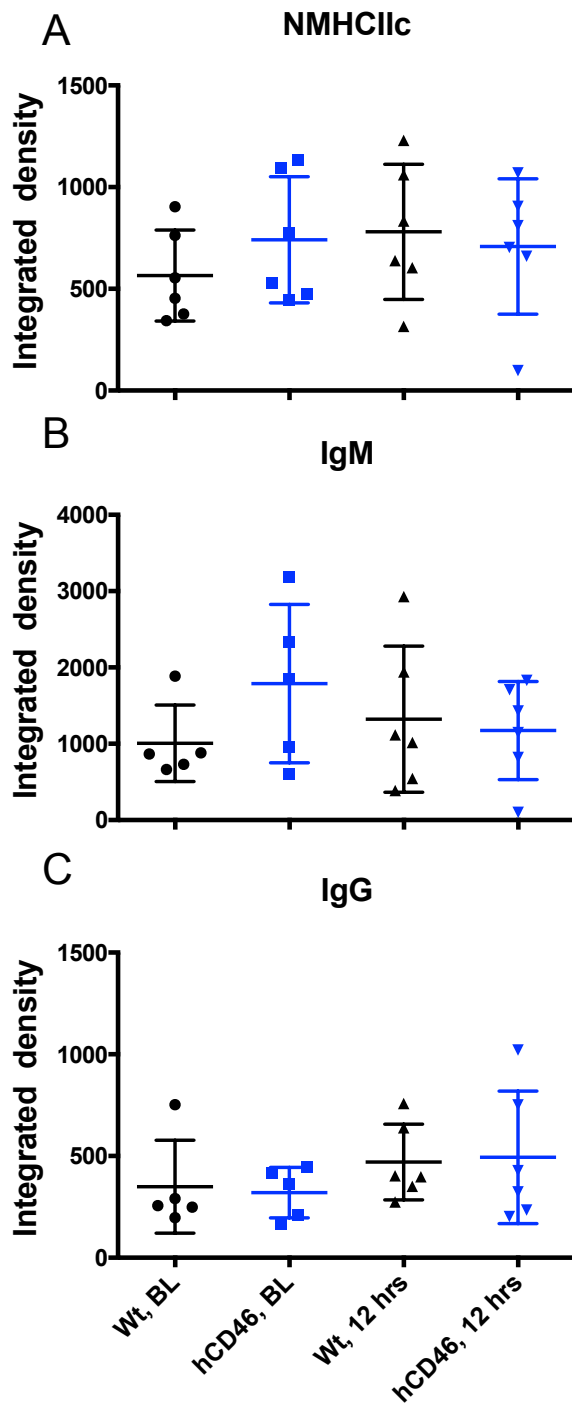


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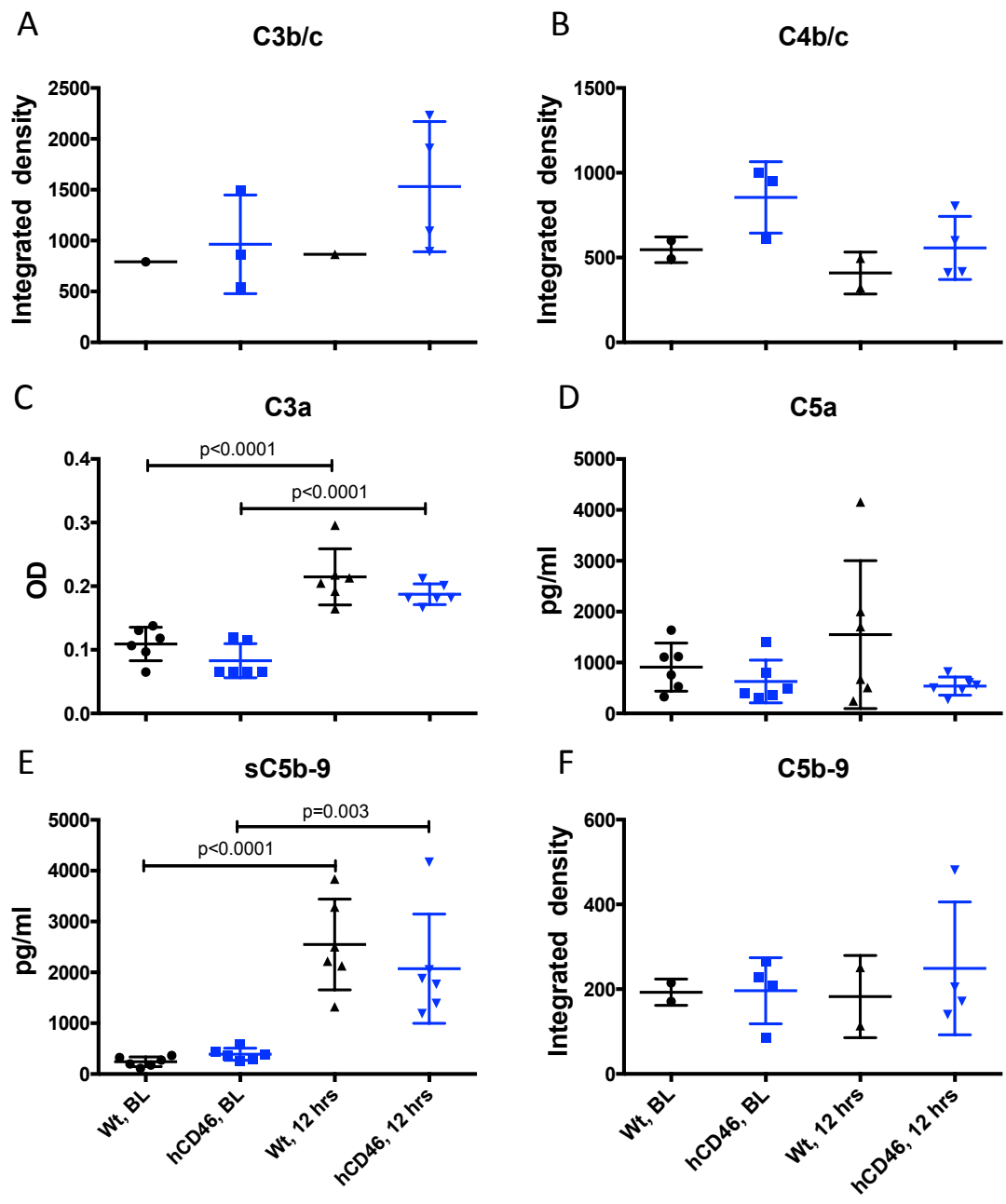
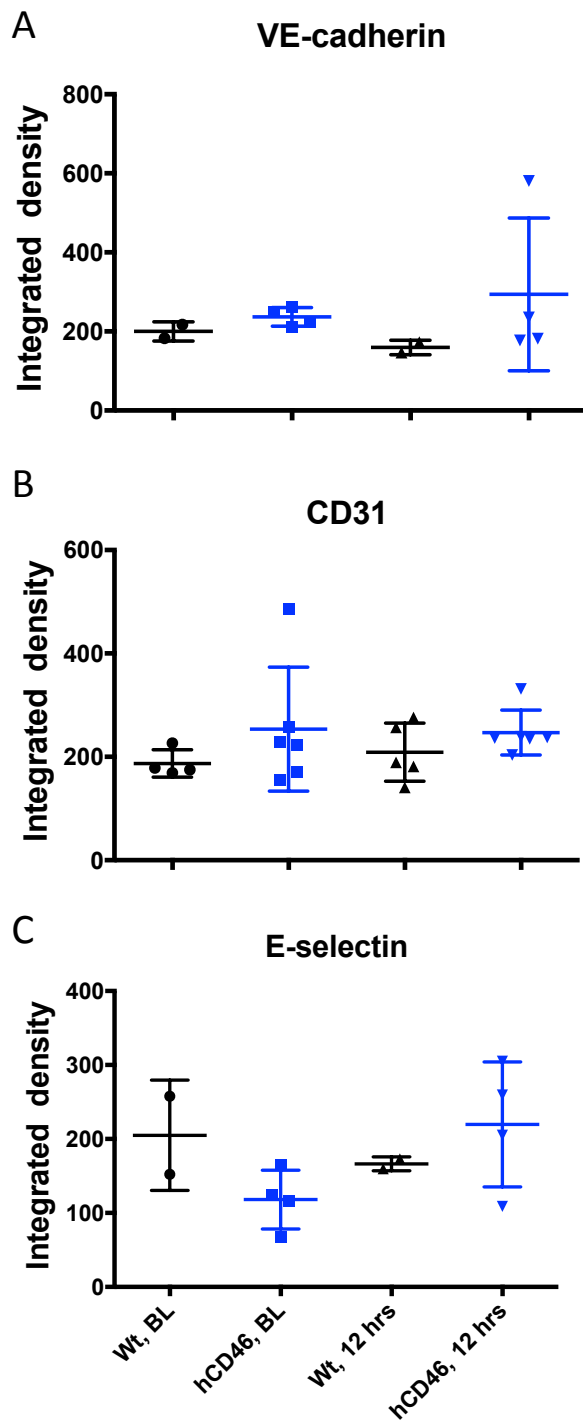


Figure 7:



Paper III: Reduction of myocardial ischemia reperfusion injury in pigs by overexpression of human membrane co-factor protein

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Status: In preparation for submission to Nature Communications

Contribution: All experiments- but not the balloon catheter intervention- and graphs were performed and made by Mai M. Abdelhafez

Background: Acute myocardial infarction is still one of the leading causes of death. Myocardial ischemia/reperfusion (I/R) injury contributes to at least one third of the infarct size. Understanding the exact mechanisms of I/R injury is critical to develop new drugs to prevent or reduce it.

Aim: The availability of transgenic pigs overexpressing hCD46 and hTM allows an in-depth study of the role of the plasma cascades (complement, coagulation and kinin systems) and endothelial cells in myocardial I/R injury in a clinically relevant model.

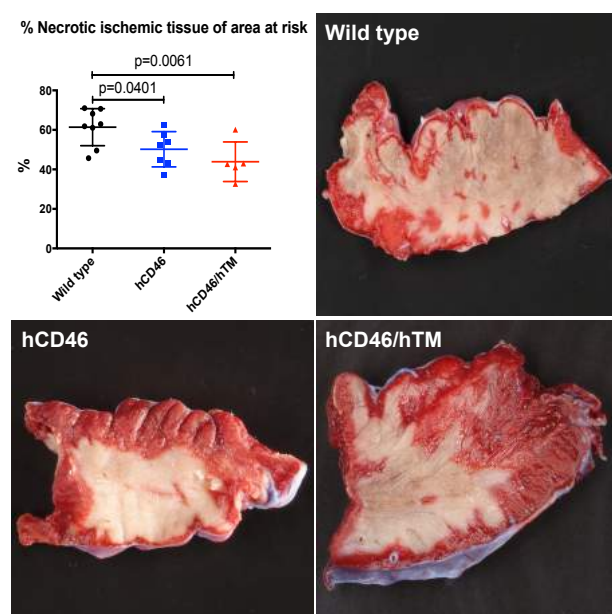


Figure: The graph shows the effect of single hCD46 and double hCD46/hTM transgenes on the infarct size in a porcine model of myocardial ischemia/reperfusion injury. One representative picture is shown for each group.

Summary: Overexpression of the human, membrane-bound complement inhibitory protein CD46 decreased the infarct size after myocardial infarction in pigs. Developing novel drugs that protect the endothelial surface and reduce plasma cascade activation may help in prevention or reduction of myocardial I/R injury that follows myocardial infarction.

Reduction of myocardial ischemia reperfusion injury in pigs by overexpression of human membrane co-factor protein

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Myocardial ischemia reperfusion (I/R) injury contributes to up to half of the infarct size after myocardial infarction (MI). Prevention or reduction of myocardial I/R injury can improve heart function after MI. This study investigates the role of the plasma cascades in myocardial I/R injury in transgenic pigs using a closed chest model of acute MI. Single human membrane co-factor protein (hCD46) transgenic and double hCD46/human thrombomodulin (hTM) transgenic animals developed a smaller infarct size compared to wild type animals with a trend towards lower cardiac troponin-I release. Overexpression of hCD46 reduced the plasma cascade activation in the infarct area compared to the wild type group. It also provided protection against endothelial cell damage and activation. Developing therapeutic options that enhance the effect of CD46 or provide membrane-targeted inhibition of the plasma cascades might be an option to reduce myocardial I/R injury in MI treated by percutaneous coronary intervention.

Percutaneous coronary intervention is the treatment of choice for myocardial infarction (MI). Rapid and early opening of the occluded coronaries is essential to reduce infarct size and save the life of the patient. Reduction of the infarct size greatly improves patient prognosis after MI ¹. Nevertheless, re-establishment of coronary perfusion is responsible for one third of the infarct size due to myocardial ischemia / reperfusion (I/R) injury ². I/R injury is a complex inflammatory condition to which several mechanisms contribute, including activation of the plasma cascade systems and formation of reactive oxygen species ³. Although myocardial I/R injury was first described in 1960 ⁴ in a canine model of MI, the pathophysiology of myocardial I/R injury is still not fully understood. Data from large animal studies and clinical trials suggest that the endothelium and the plasma cascade systems are involved in the pathophysiology of myocardial I/R injury. Activation and damage of endothelial cells (EC) via shedding of the glycocalyx, production and expression of pro-inflammatory and pro-coagulant proteins, followed by expression of neo-epitopes ^{5,6} and activation of complement pathways ⁷ have all been shown. Different plasma cascades cross react with each other as well as with the endothelium so that activation of any one of them results in all of them being activated, making it difficult to prevent or treat multifactorial pathophysiologies like I/R injury.

So far, the translation of promising drug candidates for I/R injury into clinical trials faced many difficulties with a high rate of failure. Validation of results in large animal models is therefore essential before starting clinical trials in order to save time and money ⁸.

Human membrane co-factor protein (hCD46) and human thrombomodulin (hTM) transgenic pigs were developed for xenotransplantation research in order to prevent complement-mediated xenograft rejection ^{9,10}. CD46 is known to inhibit all complement activation pathways ¹¹. It inhibits the activation of C3 as well as the formation of anaphylatoxins C3a and C5a ¹²⁻¹⁴. Thrombomodulin is expressed on EC and plays an anti-coagulant and anti-inflammatory role. Moreover, it negatively regulates the pro-inflammatory effect of complement by increasing the clearance of C3a and C5a ¹⁵⁻¹⁷. Human CD46 and hTM, alone or in combination, are known to preserve a healthy, anticoagulant and anti-

inflammatory state of the endothelium and to delay transition into a procoagulant and anti-fibrinolytic state in xenotransplantation models¹⁸. Both in xenotransplantation and in I/R injury EC become activated via complement, coagulation, and kallikrein/kinin, the so-called plasma cascade systems. These similarities between the pathophysiologies of acute vascular xenograft rejection and I/R injury motivated us to study the effect of the genetic modifications made for xenotransplantation in a clinically relevant model of acute myocardial I/R injury.

Results

Expression of hCD46, with or without hTM, on porcine myocardium reduced infarct size after acute MI

Complement activation plays an important role in the pathophysiology of I/R injury¹⁹. We hypothesized that expression of human complement inhibitory protein CD46, with or without concomitant expression of hTM, would reduce myocardial I/R injury in a porcine model of acute myocardial infarction. Tissue expression of hCD46 on porcine myocardium was confirmed by immunofluorescence staining as shown in figure (1.A-C). Additionally, expression of hTM was confirmed using in vitro staining of the corresponding isolated cells from the aorta of the animals. A positive control, human EC, was used (figure 1-D). Wild type aortic EC did not express any hTM while the double hCD46/hTM transgenic animals expressed hTM (figure 1-E and F).

Large white wild type pigs as well as single hCD46 and double hCD46/hTM transgenic pigs were used in a closed chest model of MI. There were no differences between groups regarding the ischemic area (area at risk, AAR, figure 1.G-H). In contrast, the infarcted area (necrotic ischemic tissue, NIT as percentage of AAR) was significantly reduced in both single hCD46 ($p = 0.04$) and double hCD46/hTM ($P = 0.006$) transgenic animals as shown in figure 1.I-L. The single hCD46 transgenic animals were slightly heavier than the wild type animals ($p = 0.049$), but otherwise there were no differences between the two groups regarding heart weight. There were also no significant differences between double hCD46/hTM transgenic animals and wild type or single hCD46

transgenic ones regarding both animal and heart weight. In addition, there were no differences at baseline between all the groups regarding left ventricular function, including left ventricle work, the dp/dt maximum reflecting contractility of left ventricle as well as the dp/dt minimum reflecting the left ventricle's ability to relax. The measurement of activated partial thromboplastin time (aPTT) showed a significant reduction in the double hCD46/hTM transgenic animals compared to wild type only ($p = 0.049$) but not in the single hCD46 transgenic animals (table 1).

Table (1): Demographic and baseline data

Parameter	Wild type	hCD46	hCD46/hTM	Significance
Animal weight (kg)	38.1 ± 3.7	44.7 ± 4.2*	45 ± 6	$p = 0.049$ wt vs. hCD46
Heart weight (mg)	215.9 ± 60	190 ± 26	207 ± 17	$p > 0.05$
LVW (mmHg*beat/minute)	7569 ± 1869	8515 ± 2774	5939 ± 1298	$p > 0.05$
dP/dt max (mmHg/second)	1648 ± 378	2111 ± 240	1699 ± 54	$p > 0.05$
dP/dt min (mmHg/second)	-1669 ± 469	-3018 ± 1487	-1887 ± 529	$p > 0.05$
aPTT (seconds)	12.11 ± 2.3	10.18 ± 1	9.5 ± 1.2*	$p = 0.049$ wt vs. hCD46/hTM

LVW: left ventricular work, dP/dt max: represent contractility function of the left ventricle, dP/dt min: represent relaxation function of the left ventricle, aPTT: activated partial thromboplastin time.

Transgenic expression of hCD46 and hCD46/hTM protected the endothelial cells from activation and damage

Myocardial I/R injury causes damage to EC present in the ischemic part of the heart. (Over)expression of hCD46 or hCD46/hTM protected the EC layer from activation and damage. Platelet endothelial cell adhesion molecule (PECAM-1, CD31) is normally expressed on the healthy EC surface and the expression is

lost when EC are damaged. The wild type animals significantly lost the expression of CD31 in the necrotic and viable ischemic tissue (NIT and VIT, $p = 0.0003$ and 0.0009 , respectively) compared to the area not at risk (ANR), while both single hCD46 and double hCD46/hTM transgenic animals showed no changes between the ANR, NIT and VIT. Moreover, there is a significant reduction in CD31 expression in NIT and VIT in the wild type animals ($p = 0.01$ for both) compared to double transgenic animals and a trend of lower expression in NIT of wild type animals ($p = 0.08$) compared to single transgenic animals (figure 2-A).

Heparan sulfate proteoglycan (HSPG) is an important protective layer expressed on healthy EC. HSPG tends to be shed from the EC surface in NIT and VIT compared to the ANR in the wild type animals but not the transgenic animals. HSPG was significantly preserved in NIT of single hCD46 transgenic pigs ($p = 0.03$) as compared to wild type animals and a trend to increased HSPG expression as compared to wild type was seen in NIT of double transgenic animals as well as in VIT for both single- and double transgenic pigs (figure 2-B).

The EC activation marker E-selectin, CD62E, showed a significant elevation in the wild type NIT ($p = 0.01$) compared to the ANR, but not in hCD46 or hCD46/hTM transgenic animals. There was no statistically significant difference in the NIT between all the groups. However, there was a trend towards lower E-selectin expression in the single hCD46 transgenic NIT ($p = 0.059$) compared to wild type pigs, but not the double hCD46/hTM animals (figure 2-C).

hCD46 or hCD46/hTM expression did not protect the myocardium from antibody deposition in myocardial I/R experiments

All the groups (wild type, single hCD46 transgenic and double hCD46/hTM transgenic) showed an increase in both IgG ($p = 0.001$, 0.02 and 0.058) and IgM ($p = 0.009$, 0.03 and 0.01) in the NIT compared to the ANR, respectively. The immunoglobulin deposition did not show differences between the ANR and VIT for all the groups. Furthermore, there was no significant difference between wild type and single hCD46 or double hCD46/hTM

transgenic animals in NIT regarding both IgG and IgM deposition as shown in figure 3.A-B.

Myocardial I/R injury involves activation of complement through the classical, alternative and lectin pathways and transgenic expression of hCD46 or hCD46/hTM in the myocardium reduced complement activation

The complement system can be activated through three different pathways but all of them share the activation of C3 and the downstream pathway to the membrane attack complex (C5b-9). The present study showed that the role of the alternative and lectin pathways were dominant over the classical pathway in the porcine myocardial I/R injury model. Immunofluorescence staining for C1q (the first complement moiety that starts the classical pathway) showed no significant elevation in the NIT of all groups compared to the respective ANR. There were also no differences in C1q deposition in the NIT between groups (fig 4-A). With respect to deposition of the downstream activation product of the classical and lectin pathways, C4b/c, there was no difference between the ANR and NIT of either single or double transgenic pigs, while in wild type pigs there was a significant increase in C4b/c deposition in NIT when compared to the ANR ($p = 0.01$). A comparison of C4b/c deposition in NIT between groups revealed that there was a significant reduction in both the single hCD46 ($p = 0.02$) and double hCD46/hTM ($p = 0.004$) transgenic animals compared to wild type (fig 4-B). Deposition of the central complement component C3, detected as C3b/c, did not show a significant increase in the NIT compared to the ANR in all groups. However, C3b/c showed a trend to increase in the NIT of wild type animals as compared with hCD46 group (figure 4-C).

Factor B (FB), a marker for the alternative pathway as well as the amplification loop of C3 activation, was significantly elevated ($p = 0.0003$) in wild type NIT compared to ANR. As compared with wild type pigs, there was a significant reduction of FB deposition in the NIT of the double hCD46/hTM transgenic animals ($p = 0.0007$) and a trend of reduction in the single hCD46 transgenic animals ($p = 0.06$, fig 4-D).

Mannose binding lectin (MBL) is a known initiator of the lectin pathway²⁰. Myocardial MBL deposition was significantly elevated in the NIT compared to

the ANR in the wild type, single hCD46 and double hCD46/hTM transgenic groups ($p = 0.0006$, 0.02 and 0.02 respectively). When comparing MBL deposition in the NIT between groups, there was a significant reduction in tissue of double hCD46/hTM transgenic ($p = 0.01$) and a trend to lower deposition in the single hCD46 transgenic animals ($p = 0.06$), both compared to wild type (figure 4-E).

Regarding the fluid phase, both soluble C5b-9 and the anaphylatoxin C3a showed no difference before and after myocardial I/R injury in all groups (S 1-A and B). The anaphylatoxin C5a showed a slight difference compared to sC5b-9 and C3a in that there was a trend to increased levels of C5a in wild type animals after 1 hour and 2 hours of reperfusion ($p = 0.06$ and 0.1 , respectively) compared to values at the end of ischemia, while there were no differences in both single hCD46 and double hCD46/hTM transgenic animals before and after reperfusion. A trend to lower C5a production can be seen at 2 hours of reperfusion in the single hCD46 transgenic compared to wild type ($p = 0.1$) and at 1 hour of reperfusion in the double hCD46/hTM transgenic animals ($p = 0.07$) compared to wild type animals (S 1-C).

Reduction of complement deposition in the transgenic animals was accompanied by a reduction of the bradykinin receptor expression

Upregulation of bradykinin receptors indicates an inflammatory process and has been previously described in I/R injury. Bradykinin receptor-1 was significantly upregulated in the vasculature of NIT of wild type and single hCD46 transgenic animals ($p = 0.0005$, 0.03 respectively) compared to the ANR, while there was no statistically significant increase in the double hCD46/hTM transgenic animals. Between-group comparisons revealed significantly lower bradykinin receptor-1 expression in NIT of both hCD46 and hCD46/hTM transgenic animals ($p = 0.02$ and 0.006 , respectively) as compared to wild type. The same pattern, but only as a non-significant trend for the wild type-to-hCD46 single transgene comparison, was seen in VIT. With respect to upregulation of bradykinin receptor-2 there were no significant differences in all the groups between the ANR and NIT. However, similar to bradykinin receptor-1, inter-group comparison within the NIT revealed a significant reduction in the single

hCD46 transgenic ($p = 0.002$) and a tendency to reduced expression in the double hCD46/hTM transgenic ($p = 0.09$) animals, both compared to wild type (figure 5-A and B).

Endothelial cells in myocardium of transgenic animals showed less coagulation activation

Fibrin deposition occurred during myocardial I/R injury as a result of the coagulation activation. Fibrin deposition in the wild type NIT was significantly elevated compared to levels in the ANR ($p = 0.0002$) but this was not the case in either the single or double transgenic NIT. In addition, fibrin deposition was significantly reduced in both single hCD46 and double hCD46/hTM transgenic NIT compared to wild type NIT ($p = 0.04$ and 0.001 , respectively) (figure 6-A).

Fibrinogen like protein-2 (FGL-2), which is expressed on the activated EC and enhances fibrin deposition via thrombin generation, showed no differences between ANR and NIT on EC of both single and double transgenic animals but wild type EC showed a significant increase in FGL-2 expression in the NIT compared to the ANR ($p = 0.002$). Moreover, there was significantly lower FGL-2 expression in the single hCD46 and double hCD46/hTM transgenic NIT vasculature compared to the wild type NIT vasculature ($p = 0.003$ and 0.001 , respectively) as shown in figure 6-B.

The plasma glycoprotein von Willebrand factor (vWF), which is synthesized and stored in EC, is released upon EC activation and enhances platelet aggregation and coagulation activation. There was a significant elevation in myocardial vWF staining in the wild type NIT compared to the ANR ($p = 0.002$). The representative pictures (figure 6-C) show the increased release of vWF into the surrounding tissue in the NIT and VIT compared to ANR. Additionally, there was a trend to lower vWF release in the double hCD46/hTM transgenic NIT ($p = 0.06$) compared to wild type as well in the single hCD46 transgenic NIT compared to wild type. Furthermore, the representative pictures show less vWF release from the endothelium to the surrounding myocardium in the transgenic animals as compared to the wild type ones (figure 6-C).

Effect of single hCD46 and double hCD46/hTM on coagulation

The double transgenic hCD46/hTM showed a significantly shorter aPTT at baseline compared to wild type (9.5 ± 1.2 vs. 12.1 ± 2.2 seconds, $p = 0.039$, S 2-A). These animals required less heparin (244 ± 30 IU/kg) compared to the wild type (296 ± 43 IU/kg) animals over the whole experiment to keep the activated clotting time above 180 seconds (S 2-B). Moreover, the aPTT of the double hCD46/hTM transgenic animals became elevated over the wild type at the end of reperfusion (34 ± 17 and 26 ± 8 seconds respectively, S 2-C). Additionally, tissue factor (TF), which is expressed by EC and involved in the extrinsic coagulation pathway activation, showed a non-significant elevation ($p = 0.06$) in the double transgenic ANR vasculature compared to wild type (S 2-D). The single hCD46 transgenic animals showed only a trend to a lower aPTT at baseline (10.2 ± 0.9 vs. 12.1 ± 2.2 seconds, $p = 0.07$) and a significant elevation of TF in the ANR ($p = 0.004$) compared to wild type animals (S 2-A and D).

Discussion

Validation of the results obtained from small animals experiments in large animal models is important in view of translation into clinical trials⁸. The availability of transgenic pigs is believed to increase the success rate of translational medicine and reduce the number of failed clinical trials²¹. The role of complement and coagulation has been extensively studied in different animal models of myocardial I/R injury²². However, the current study is the first to use transgenic pigs to prove that inhibition of complement activation, with or without concomitant inhibition of coagulation, is sufficient to reduce myocardial I/R injury.

Inhibition of the central complement components C3b and C4b, which bind to the target cell membrane and may lead to its activation, occurs naturally in the body via factor I in the presence of cofactor CD46, complement receptor type 1, C4 binding protein and factor H^{23,24}. As the first tissue to be in contact with blood at the onset of reperfusion, protection of endothelium is crucial to protect against myocardial I/R injury²⁵. In the present study, overexpression of hCD46 with or without hTM preserved a healthy endothelium, while wild type animals, which suffered from severe myocardial I/R injury, lost the healthy endothelial layer in the myocardium. Moreover, EC of transgenic animals presented with preserved HSPG in the glycocalyx, whereas staining for HSPG was significantly lower in wild type EC. This is in line with a previous study of our group using low molecular weight dextran sulfate that was injected intracoronarily and led to a reduced infarct area as well as preservation of endothelial HSPG compared to non-treated animals²⁶. Multimeric tyrosine sulfate, a cytoprotective agent that works similarly to dextran sulfate, but has only minimal anti-coagulant property, also reduced myocardial I/R injury in a porcine model²⁷. Moreover, protection of the blood brain barrier in a mouse model of brain I/R injury via inhibition of Rho-kinase activity led to reduced cerebral reperfusion injury compared to the control group²⁸. The pathological role of E-selectin expression in a mouse model of myocardial as well as brain I/R injury has been previously clarified²⁹. In our current study, the transgenic expression of hCD46 on the porcine cells also inhibited the activation of the EC and reduced E-selectin expression as compared to the wild type animals.

Deposition of natural antibodies in the context of I/R injury has been shown in different animal models and different organs. It was first described in a mouse model of skeletal muscle I/R injury, mice deficient in natural antibodies showed less reperfusion injury⁵. The presence of antibodies in reperfused tissue was also proven in a porcine model of skeletal muscle I/R injury published by our group^{30,31}. In the present study, the NIT of all the animals who underwent myocardial I/R injury showed a significant increase in the deposition of both IgM and IgG. The transgenic expression of the hCD46 with or without hTM did not prevent antibody deposition on the affected myocardium. This finding is in line with mouse model of myocardial I/R injury, which showed a deposition of IgM in the affected myocardium³². Moreover, xenotransplantation of hCD46 transgenic pig hearts into baboon did not reduced IgM and IgG deposition although it reduced the complement activation³³.

The lectin pathway is clearly activated in the current model along with the amplification loop of the complement system as illustrated by the significant increase in C4b/c, factor B and MBL deposition in the affected myocardium. The transgenic hCD46, with or without hTM, pigs showed less complement activation compared with wild type pigs. This is in line with a previous study that succeeded to reduce myocardial I/R by using anti rat MBL antibody in a rat model of myocardial infarction³⁴ and similar effect shown in a MBL knockout mouse model of myocardial I/R injury³⁵. Recently, the involvement of factor B was proven to contribute to myocardial I/R injury in a factor B knockout mouse model as well as in the global I/R injury of the human hearts³⁶. Moreover, activation of lectin pathway that is further augmented by factor B amplification loop was shown in the clinical setting of global ischemia in the heart. The authors showed this effect in an early phase of complement activation postoperatively³⁷. This effect is not yet proven in the clinical setting of regional ischemia (acute myocardial infarction). The classical complement upstream protein C1q did not show differences between the groups and role of classical pathway is therefore not clear in the current model. Indeed the classical pathway showed minimum role in a C1q knockout mouse model of myocardial infarction. The knockout mice were not protected against myocardial I/R injury³⁸. However, in one of our

previous porcine myocardial infarction studies we found a significant increase of C1q deposition in the myocardium following I/R injury ²⁶.

In contrast to complement components deposited in the tissue, plasma concentrations of the soluble complement activation markers C3a, C5a and sC5b-9 showed no significant differences between hCD46, with or without hTM, transgenic animals and wild type controls. A non-significant trend for both an increase of C5a during the reperfusion phase and a lower concentration of C5a in the transgenic animals were found. Most probably, complement activation occurs only locally in the reperfused myocardium and the locally produced activation markers are then released into the systemic circulation. In this model, the extent of reperfusion injury is too low to have systemic consequences via for example C5a, but this is well known from other models as well as from the clinical situation ^{39,40}.

The role of bradykinin receptors in the pathophysiology of myocardial I/R injury is still unclear. In the current study, first time to be shown in a pig model, the bradykinin receptor-1 was upregulated in response to myocardial I/R injury and its expression level was significantly reduced in both the single hCD46 and double hCD46/hTM transgenic hearts. Expression levels of the bradykinin receptor-2 seem to be much less affected by myocardial reperfusion injury in our model. Upregulation of bradykinin receptors-1 and 2 has already been shown in a rat model of myocardial infarction, and blocking bradykinin receptor-1 reduced myocardial I/R injury ⁴¹. In contrast, in a bradykinin receptor-1 knockout mouse model of myocardial I/R injury, blocking of the bradykinin receptor-2 resulted in a significant reduction of left ventricle functions and this could be prevented by the presence of functioning one of the two receptors ^{42,43}.

The coagulation cascade is activated in the context of myocardial I/R as a response to the transformation of the normally anti-inflammatory, anti-coagulant and pro-fibrinolytic EC into a pro-inflammatory, pro-coagulant and anti-fibrinolytic state, as shown in both rabbit and rat models ^{44, 45}. The current study showed that the wild type heart vasculature was indeed activated and showed a significant increase in fibrin deposition, FGL-2 expression and vWF release. This is contrasted by the relative preservation of the anticoagulant and pro-fibrinolytic state of the endothelium in both single and double transgenic

hearts. Our data therefore confirm the observations made in xenotransplantation models, in which hCD46 expression leads to a reduction of fibrin deposition as well as FGL-2 expression ⁴⁶.

In conclusion, the transgenic overexpression of the complement-regulatory protein hCD46 on porcine cardiac EC, with or without the concomitant expression of the anti-coagulant protein hTM, protected the myocardium from I/R injury in the context of acute infarction and reperfusion in a clinically related large animal (pig) model. Targeting CD46 might be a novel promising option for prevention or reduction of I/R injury.

Animals and methods

Animals

Animal experiments were conducted according to the Swiss national laws and guidelines. The local animal experimentation committee approved the current study (permission 25/16). Nineteen large white pigs of both sexes were used in the study. Three groups of animals were used: wild type pigs (n=8), human CD46 single transgenic, α 1-3-galactosyltransferase knockout pigs (n=6) and human CD46/ human TM double transgenic, α 1-3-galactosyltransferase knockout pigs (n=5). The surgical procedure and the determination of the infarct size were performed blindly for the type of transgene.

Surgical procedure

All animals were fasted for 12 hours before the start of the experiments with free access to water. The experiments were described before ²⁶, briefly, the animals were pretreated with an intramuscular injection of 20 mg/kg Ketamine and 2 mg/kg Xylazine. The anesthesia was maintained with 1.5% Sevoflurane in 1:3 oxygen:air using a Dräger Fabius respirator. Additionally a bolus dose of 250 μ g Fentanyl analgesic was injected intravenously followed by a continuous infusion of 250 μ g/hour during the whole experiment. Two central arterial lines and one venous line were cannulated. Heparin (Liquemin) 125 IU/kg was administered at baseline. A Millar catheter (MIKRO-TIP, SPR-350, reference 840-8166, 5F, 120 cm) was inserted into the left ventricle for the measurement of left ventricle functions. Baseline values were recorded for 10 minutes.

A percutaneous coronary dilation catheter (Cordis EMPIRA, USA, balloon diameters 3, 3.5 and 4 mm) was used to block the left anterior descending artery (LAD) after the first or second diagonal branch (to achieve a 35-50% ischemic area of the left ventricle). The experimental setting was as follows: One hour of ischemia followed by two hours of reperfusion. A biphasic defibrillator was used to reverse the ventricular fibrillation that might occur during the ischemia time. The blocking and opening of the LAD was controlled using a C-arm angiography setting. At the end of the reperfusion, the LAD was re-occluded and 100 ml of 2% Evans Blue (Sigma, E2129) was injected in the venous central line. As soon as the

pig turned blue, it was euthanized using 30 ml of 15% KCl. The heart was extracted and washed with room temperature saline before starting the analysis.

Infarct size

The left ventricle (LV) was dissected from the heart, cut into 3-5 mm sections and weighed. This was followed by dissection of the Evans Blue stained LV, which is the area not at risk (ANR), from the Evans Blue negative tissue, which is the area at risk (AAR) and these were then weighed. The percentage of the ischemic area in the left ventricle was calculated using the following formula: $(\text{AAR (in gm)}/\text{LV (in gm)}) \times 100$.

The AAR sections were then incubated in freshly prepared 1% triphenyl tetrazolium chloride (TTC, sigma, T8877) solution, for 20 minutes at 37°C. The TTC was used to stain the viable ischemic tissue (VIT) red. The necrotic ischemic tissue (NIT), representing the infarct area, remained unstained. The stained AAR sections were then incubated in 4% formaldehyde at room temperature for 24 hours followed by imaging of both sides. Both images were then analyzed using ImageJ software to calculate the surface area (cm²) of AAR and the NIT (white, figure 1-J, K and L). The infarct size as a percentage of the AAR was calculated using the following formula: $\text{NIT (cm}^2\text{)}/\text{AAR (cm}^2\text{)} \times 100$.

Blood and tissue sampling

Venous blood samples from the pigs were collected into commercially available EDTA and citrated tubes at baseline, end of ischemia, 10, 30, 60 and 120 minutes during reperfusion. The blood samples were directly centrifuged at 2000 x g for 15 minutes at 4°C and stored at -80°C for further analysis. Other samples were withdrawn to measure active clotting time (ACT) using Medtronic ACT plus and LR-ACT cartridges (Medtronic, USA, 402-01).

Approximately 0.3 cm³ of the ANR, NIT and VIT tissue was sampled for Tissue-Tek embedding (O.T.C compound, SARUKA, 4583) and stored at -80°C for further analysis.

Left ventricle functions

The Millar catheter, which was inserted into the left ventricle, was connected to the acquisition system PowerLab 4/35 to record left ventricular

pressure. The acquired data was analyzed using Lab Chart software to calculate left ventricle work (LVW = developed pressure X heart rate), dP/dt maximum (contractility of left ventricle) and dP/dt minimum (relaxation of left ventricle).

Activated partial thromboplastin time

An automated blood coagulation analyzer (Sysmex CA-7000 or BCS XP system) was used to measure the activated partial thromboplastin time (aPTT). Duplicates of the citrated porcine plasma samples were measured using standard reagents according to the manufacturer's instructions (Siemens Healthcare, Germany). The average of the results was calculated for all the experimental animals.

Immunofluorescence staining

Tissue-Tek embedded samples were cut into 5 μ m sections and air-dried at room temperature for 60 minutes. The sections were fixed with cold acetone at -20°C, hydrated with TBS and incubated in TBS-3% BSA for 60 minutes at room temperature to block non-specific binding. The immunofluorescence staining was done using either direct or indirect techniques. In direct staining, the following antibodies were used: goat anti-human C3b/c-FITC (Dako, F0201), goat anti-human C4b/c-FITC (Dako, F0169), goat anti-pig IgG-FITC (Southern Biotech, 6050-02) and rabbit anti-human fibrinogen-FITC (Dako, F0111). The following primary antibodies were used for the indirect technique: mouse anti human CD46 (Hycult biotech, HM2103), mouse anti human TM (abcam, ab6980), rat anti-pig CD31 (R&D systems, MAB33871), rat anti-heparan sulfate proteoglycan (Abcam, ab2501), mouse anti-human CD62E (Sigma, S9555), goat anti pig IgM (AbD Serotec, AAI39), rabbit anti- human C1q (Dako, A0136), goat anti human factor B (Merck Millipore, 341272), mouse anti-human MBL-C (Santa Cruz Biotechnology, sc-80598), rabbit anti-human bradykinin receptor-1 and 2 (Alomone, ABR-011 and ABR-012 respectively), rabbit anti-human FGL-2 (Aviva Systems Biology, ARP52235_p050), rabbit anti human vWF (Dako, A0082), sheep anti-human tissue factor (Affinity Biological, SATF-IG). The following corresponding secondary antibodies were used: goat anti-mouse Alexa Fluor 546 (Invitrogen, A11030), goat anti-rat Cy3 (Jackson ImmunoResearch, 112-166-003), sheep anti-mouse Cy3 (Sigma, C2181), donkey anti-goat Alexa Fluor 488

(Molecular Probes, A11055), sheep anti-rabbit Cy3 (Sigma, C2306), donkey anti Sheep IgG Alexa Flour 488 (life technology, A11015). The primary and secondary antibodies were incubated at room temperature for 60 minutes followed by TBS washing steps. The slides were then dried, mounted using glycerol (Dako, C0563) and visualized with an immunofluorescence microscope (Leica DMI4000). The cross reactivity of the previously mention primary antibodies with the porcine tissue were test in our lab⁴⁶⁻⁴⁸.

Multiplex suspension array (Bio-Plex) and ELISA

The complement soluble markers C5a and sC5b-9 were measured using a custom made multiplex suspension array technique ⁴⁹. In brief, using a Bio-Plex amine-coupling kit (Bio-Rad, 171-406001), the fluorochrome micro-beads were couple with the following capture antibodies: C5a (MBM, T13/9) and sC5b-9 (Diatec, 5010). After incubation with the samples, the bead-antibody-antigen complexes were detected by a biotinylated detection antibody followed by Streptavidin-PE (Qiagen, Venlo, The Netherlands). The Bio-Plex 100 system was used to measure the fluorescence and the concentration was calculated with Bio-Plex Manager 4.1 software (Bio-Rad).

The complement marker C3a was detected in the EDTA plasma samples using a home made ELISA. The Maxisorp ELISA plate was coated with mouse anti porcine C3a (Z22/8, MBM) in a carbonated buffer pH=9.6 overnight at 4°C. The plate was blocked using PBS-1%BSA for 1 hour at 37°C. The samples were incubated for another 1 hour at 37°C. C3a was detected by incubation with a biotinylated mouse anti porcine C3a antibody (MBM, K5/4) for 1 hour at 37°C followed by Streptavidin-alkaline phosphatase (RPN1234V, GH healthcare, Ohio) for 45 minutes at 37°C. The color developed after the addition of p-nitrophenyl phosphate substrate (Sigma, S0942-200) in diethanolamine buffer. A Tecan plate reader, Infinite M1000, was used to measure the optical density at the wavelengths 405/490 nm.

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Figure legends

Figure 1: hCD46 and hTM expression on porcine cells and its effect on infarct size. Representative pictures of hCD46 stained myocardium cryo-section of (A) wild type, (B) single hCD46 transgenic and (C) double hCD46/hTM transgenic group. Representative pictures of hTM stained endothelial cells from (D) human, positive control, (E) wild type and (F) hTM transgenic group. (G) Area at risk as percentage of left ventricle, values are shown as mean \pm SD. (H) Representative picture of AAR in-situ before heart extraction. (I) Necrotic ischemic tissues as percentage of area at risk, data are represented as mean \pm SD, p-value calculated using the Mann-Whitney test, wild type n = 8, single hCD46 transgenic n = 6, double hCD46/hTM transgenic n = 5. Representative images of area at risk with TTC stain, Red: viable ischemic tissue and White: necrotic ischemic tissue, (J) wild type, (K) single hCD46 transgenic and (L) double hCD46/hTM transgenic group. Scale bars are 0.5, 0.4 and 0.4 cm respectively.

Figure 2: Endothelial cells markers. The left panel shows a quantitative analysis of immunofluorescence stained myocardium cryosections of (A) CD31 expression on the myocardium vasculature, (B) heparan sulfate proteoglycan (HSPG) as part of glycocalyx on the endothelium vasculature, (C) E-selectin, an endothelial cell activation marker. Wild type n = 8, single hCD46 transgenic n = 6, double hCD46/hTM transgenic n = 5. The data are represented as dots for each individual animal and mean \pm SD, p-value was calculated using the non parametric Mann-Whitney test for non normally distributed data or unpaired Student t-test for normally distributed data between different groups and the non parametric Kruskal-Wallis test for data that are not normally distributed or one way ANOVA and Bonferroni post hoc tests for normally distributed data in the same group. ANR: area not at risk, NIT: necrotic ischemic tissue and VIT: viable ischemic tissue. The right panel shows representative images of each group taken with a Leica DMI4000 camera. Blue: DAPI staining, Green: CD31 or HSPG and Red: E-selectin. Scale bar is 100 μ m.

Figure 3: Antibody deposition. The upper panel shows a quantitative analysis of immunofluorescence stained myocardium cryosections of (A) IgM and (B) IgG deposition on the myocardium. Wild type n = 8, single hCD46 transgenic n = 6,

double hCD46/hTM transgenic n = 5. The data are represented as dots for each individual animal and mean \pm SD, p-value was calculated using the non parametric Mann-Whitney test for non normally distributed data or the unpaired Student t-test for normally distributed data between different groups and the non parametric Kruskal-Wallis test for data that are not normally distributed or the one way ANOVA and Bonferroni post hoc tests for normally distributed data in the same group. ANR: area not at risk, NIT: necrotic ischemic tissue and VIT: viable ischemic tissue. The lower panel shows representative images of each group taken with a Leica DMI4000 camera. Blue: DAPI staining and Green: IgM or IgG staining. Scale bar is 100 μ m.

Figure 4: Complement markers. The left panel shows a quantitative analysis of immunofluorescence stained myocardium cryosections of (A) C1q, (B) C4b/c, (C) C3b/c, (D) factor B (FB) and (E) mannose binding lectin (MBL) deposition on the myocardium. Wild type n = 8, single hCD46 transgenic n = 6, double hCD46/hTM transgenic n = 5. The data are represented as dots for each individual animal and mean \pm SD, p-value was calculated using the non parametric Mann-Whitney test for non normally distributed data or the unpaired Student t-test for normally distributed data between different groups and the non parametric Kruskal-Wallis test for data that are not normally distributed or the one way ANOVA and Bonferroni post hoc tests for normally distributed data in the same group. ANR: area not at risk, NIT: necrotic ischemic tissue and VIT: viable ischemic tissue. The right panel shows representative images of each group taken with a Leica DMI4000 camera. Blue: DAPI staining, Green: C4b/c, C3b/c or FB and red: MBL. Scale bar is 100 μ m.

Figure 5: Bradykinin receptors 1 and 2. The left panel shows a quantitative analysis of immunofluorescence stained myocardium cryosections of (A) bradykinin receptor-1 and (B) bradykinin receptor-2 expressed on the myocardium vasculature. Wild type n = 8, single hCD46 transgenic n = 6, double hCD46/hTM transgenic n = 5. The data are represented as dots for each individual animal and mean \pm SD, p-value was calculated using the non parametric Mann-Whitney test for non normally distributed data or unpaired Student t-test for normally distributed data between different groups and the

non parametric Kruskal-Wallis test for data that are not normally distributed or the one way ANOVA and Bonferroni post hoc tests for normally distributed data in the same group. ANR: area not at risk, NIT: necrotic ischemic tissue and VIT: viable ischemic tissue. The right panel shows representative images of each group taken with a Leica DMI4000 camera. Blue: DAPI staining, and Red: Bradykinin receptor 1 or 2. Scale bar is 100 μ m.

Figure 6: Coagulation markers. The left panel shows a quantitative analysis of immunofluorescence stained myocardium cryosections of (A) fibrin deposition on the myocardium, (B) fibrinogen like protein-2 (FGL-2) and (C) Von Willebrand factor (vWF) on the myocardium. Wild type n = 8, single hCD46 transgenic n = 6, double hCD46/hTM transgenic n = 5. The data are represented as dots for each individual animal and mean \pm SD, p-value was calculated using the non-parametric Mann-Whitney test for non-normally distributed data or unpaired Student t-test for normally distributed data between different groups and the non-parametric Kruskal-Wallis test for data that are not normally distributed or the one way ANOVA and Bonferroni post hoc tests for normally distributed data in the same group. ANR: area not at risk, NIT: necrotic ischemic tissue and VIT: viable ischemic tissue. The right panel shows representative images of each group taken with a Leica DMI4000 camera. Blue: DAPI staining, Green: fibrin and Red: FGL-2 or vWF. Scale bar is 100 μ m.

Figure 1

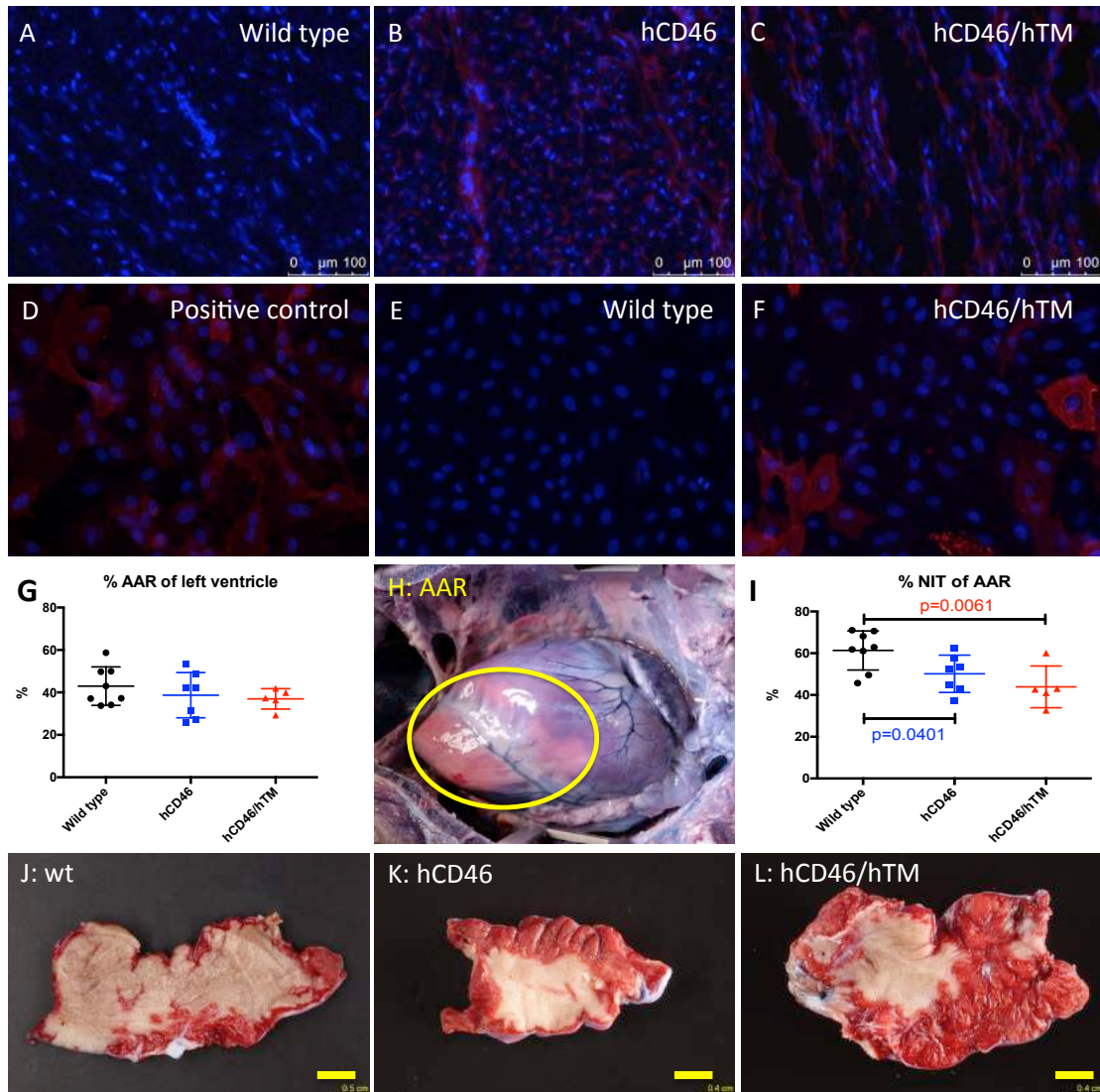


Figure 2

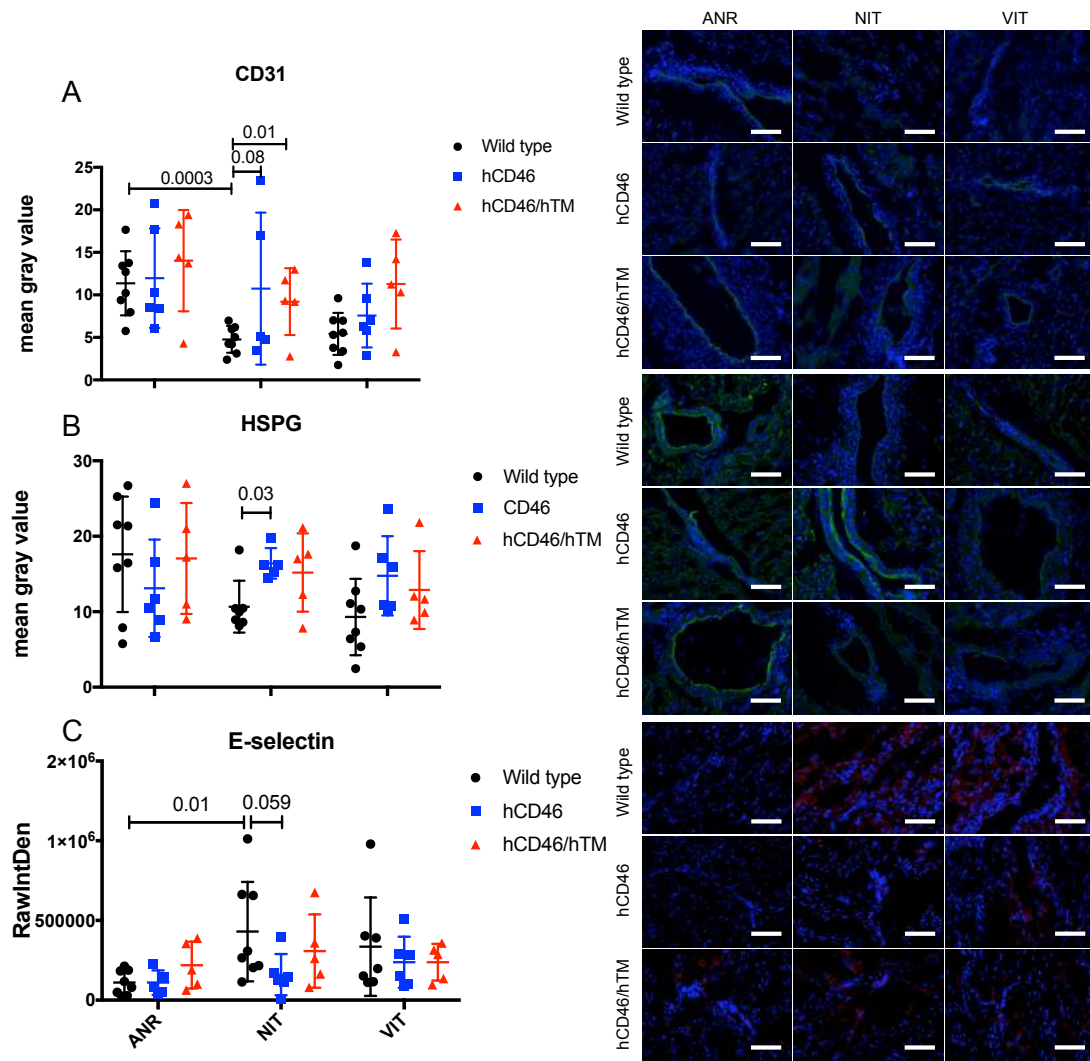


Figure 3

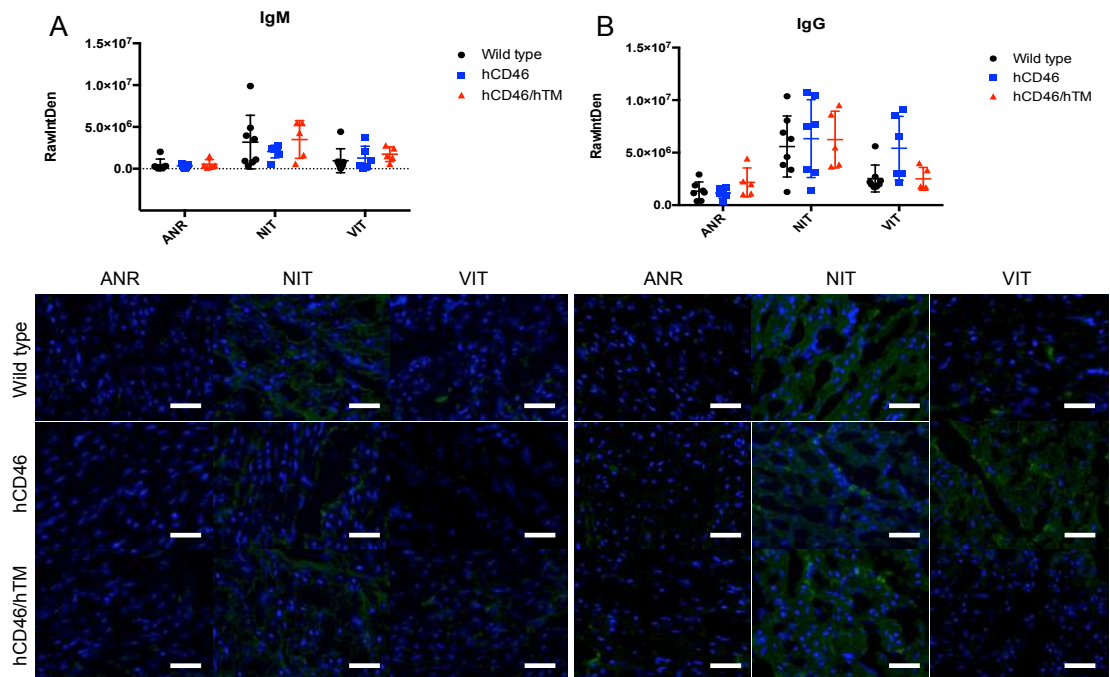


Figure 4

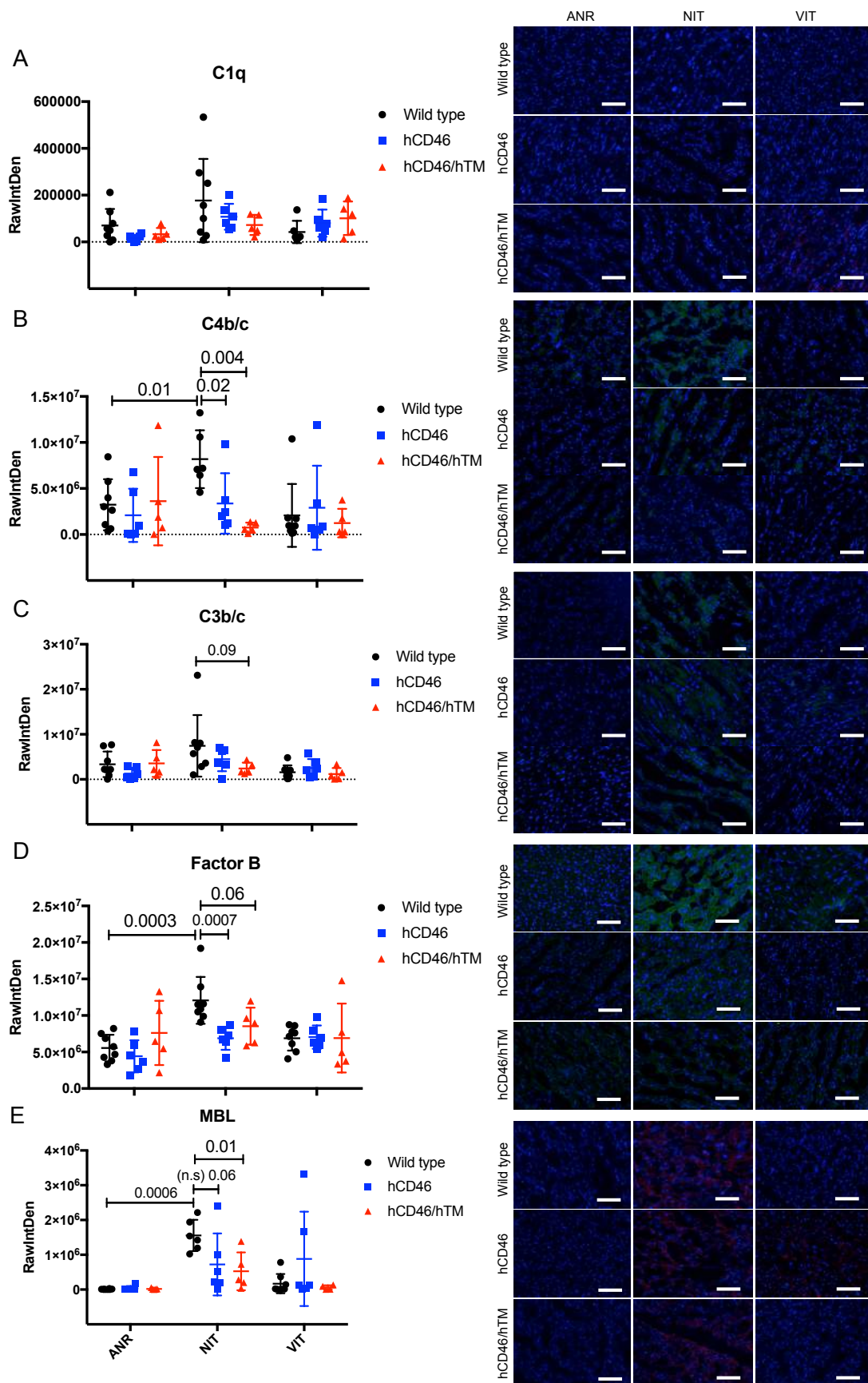


Figure 5

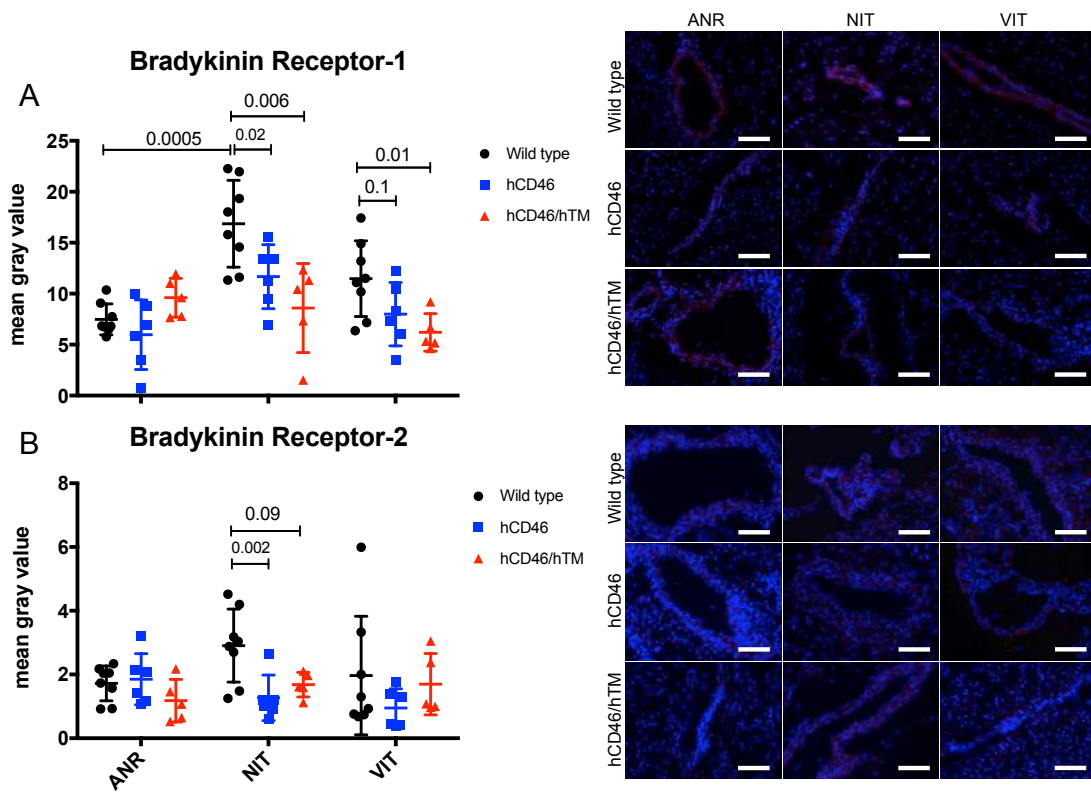
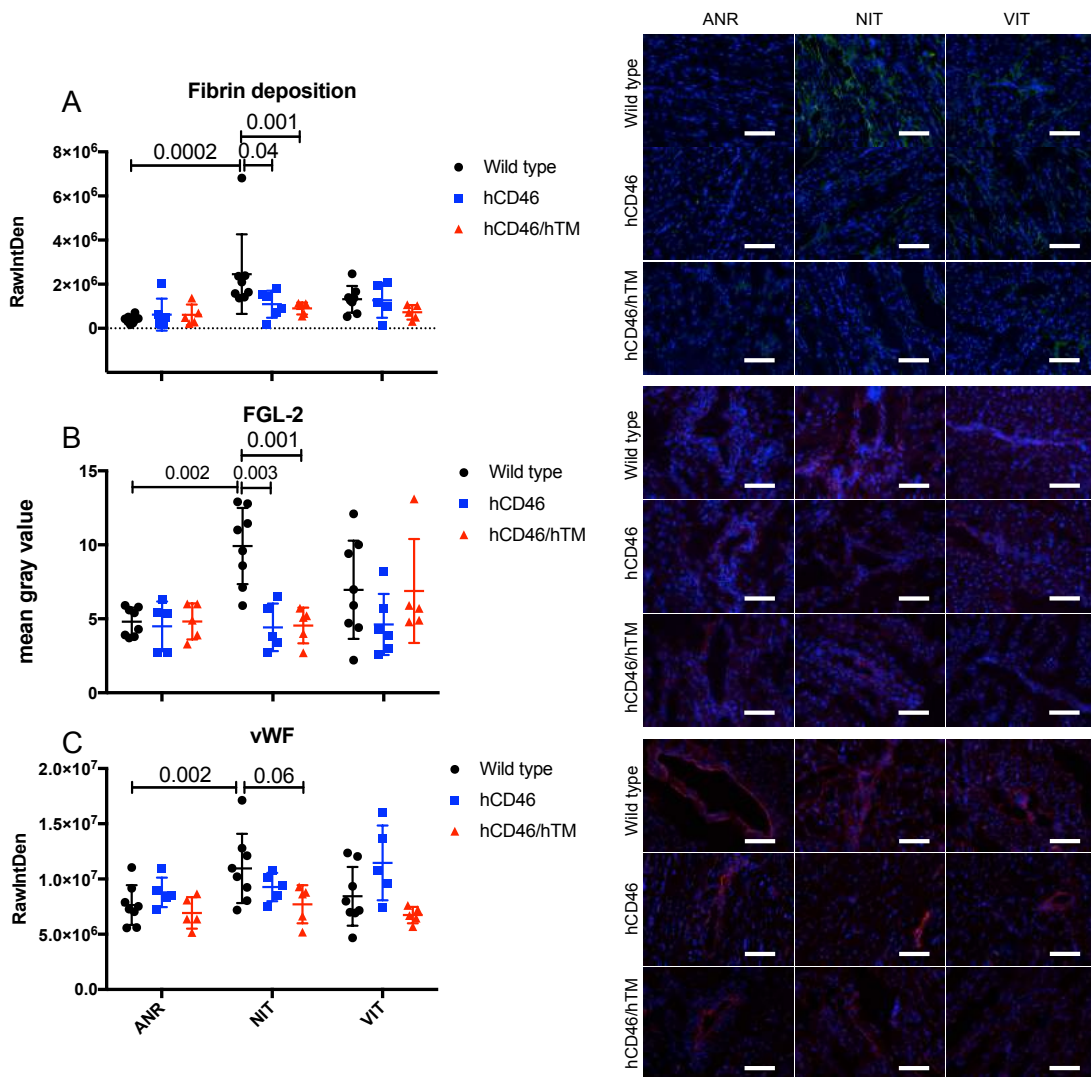
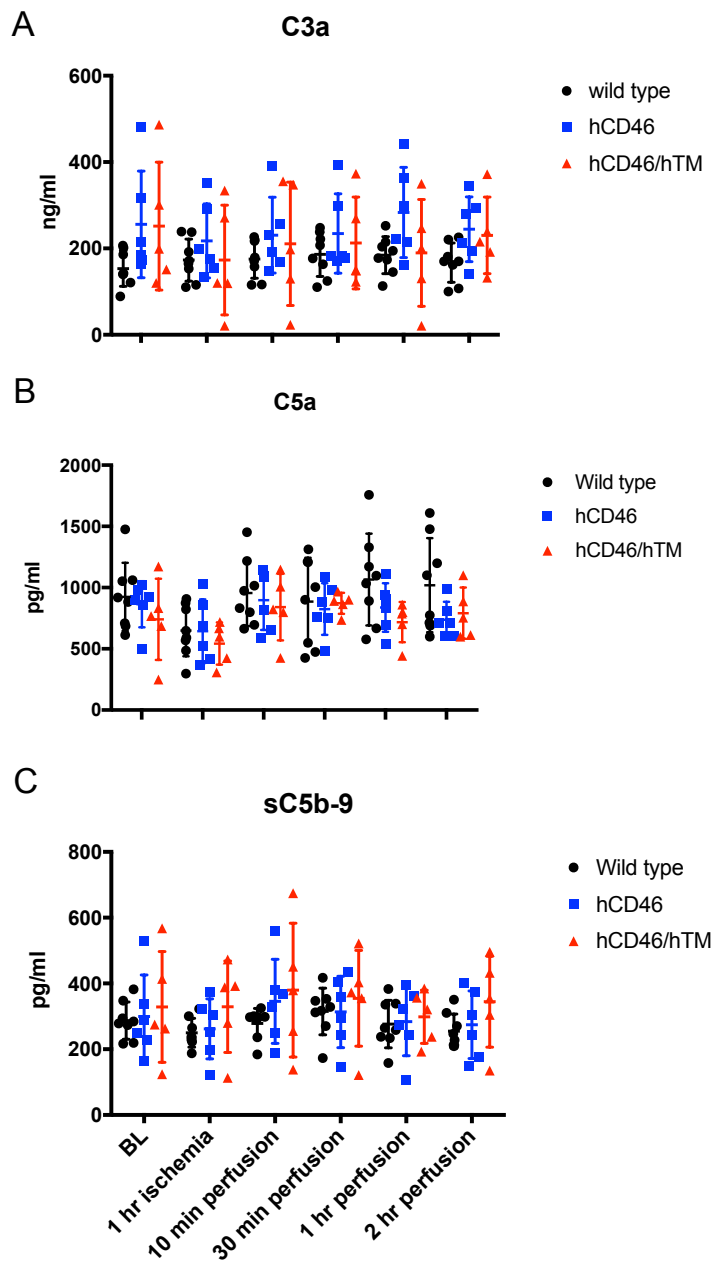


Figure 6

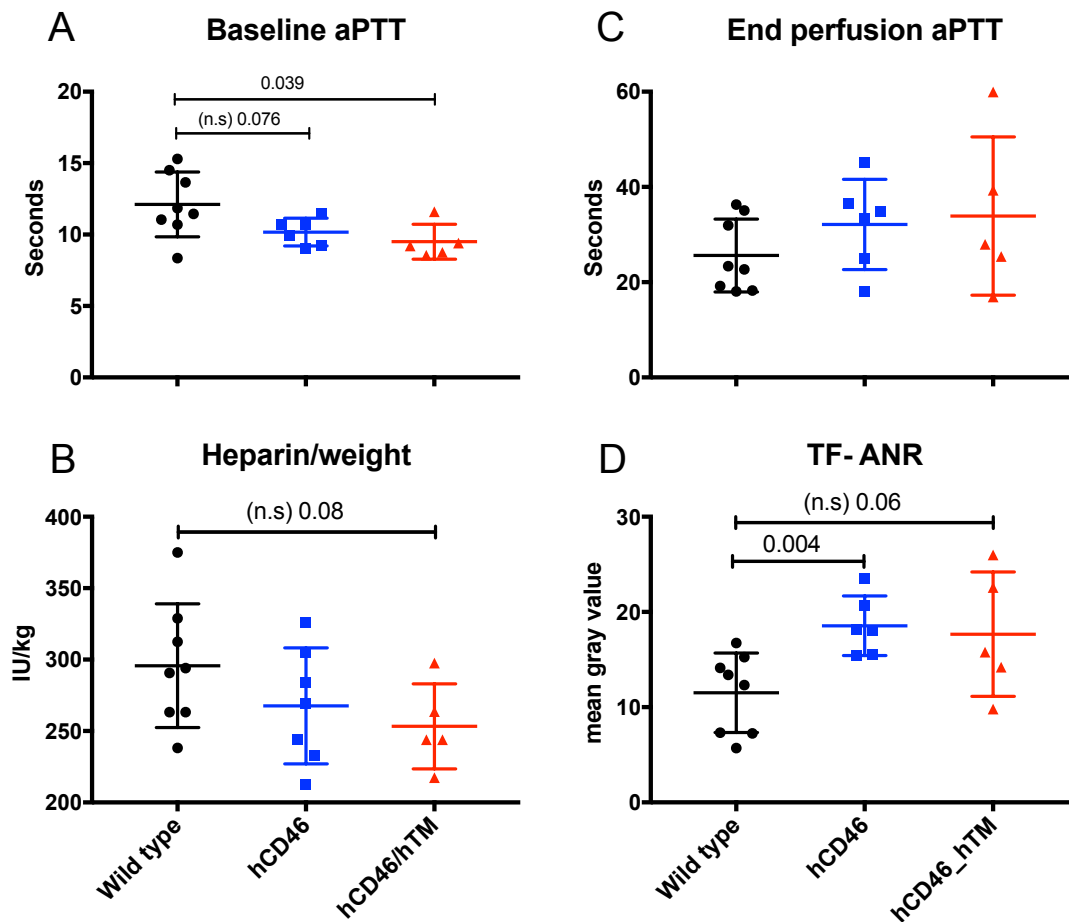


Supplementary figure 1



Supplementary figure 1: Complement activation markers. A quantitative analysis of (A) sC5b-9 (B) C3a and (C) C5a measured in EDTA plasma samples at baseline and/or different time points during experiment. The data are represented as dots for each individual animal and mean \pm SD, p-value (≤ 0.05 is significant) was calculated using non parametric Mann-Whitney test for non-normally distributed data or unpaired Student t-test for normally distributed data between different groups and using non parametric Kruskal-Wallis test for data that are not normally distributed or one way ANOVA and Bonferroni post hoc test for normally distributed data in between the same group.

Supplementary figure 2



Supplementary figure 2: coagulation markers. (A) Activated partial thromboplastin time (aPTT) measured at baseline in citrated plasma. (B) Heparin dosage required during the whole experiment and measured as IU/kg. (C) Measurement of aPTT at the end of reperfusion in citrated plasma. (D) Quantitative analysis of myocardium vasculature cryosections stained with immunofluorescence against tissue factor (TF) in area not at risk (ANR) and analyzed by image J. Wild type $n = 8$, single hCD46 transgenic $n = 6$, double hCD46/hTM transgenic $n = 5$. The data are represented as dots for each individual animal and mean \pm SD, p-value (≤ 0.05 is significant) was calculated using non parametric Mann-Whitney test for non normally distributed data or unpaired Student t-test for normally distributed data between different groups and using non parametric Kruskal-Wallis test for data that are not normally distributed or one way ANOVA and Bonferroni post hoc test for normally distributed data in between the same group.

Paper IV: Improvement of a closed chest porcine myocardial infarction model by standardization of tissue and blood sampling procedures

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Background: Pig models are very useful to increase the success rate of translational medicine and to validate results obtained from small animal models before moving on to the clinical trials. Reproducibility is still a main concern in the scientific community.

Aim: To provide a well detailed model for closed chest myocardial infarction and ischemia reperfusion injury as well as a procedure for sampling standardization and to increase reproducibility of model in the context of molecular and cellular analysis.

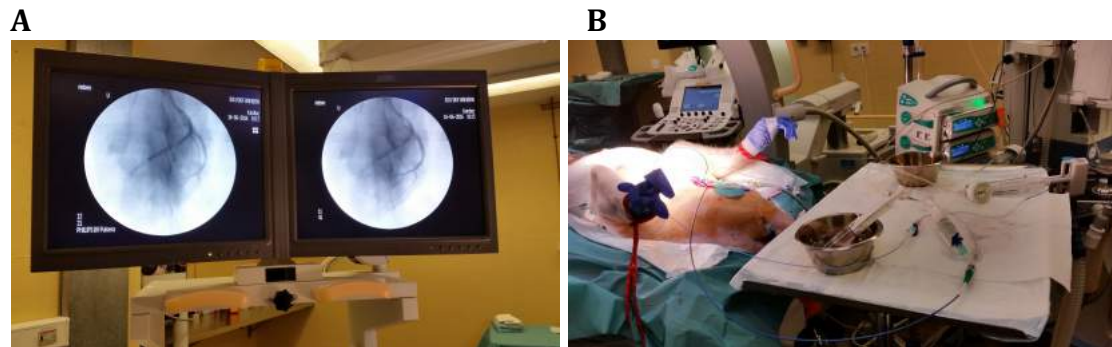


Figure: (A) Fluoroscopic view of the pig heart during experiment. (B) porcine closed chest model of acute myocardial infarction.

Summary: The current paper visualizes detailed important steps that are required to increase reproducibility of investigating the pathophysiology of myocardial ischemia reperfusion injury in a closed chest porcine model.

Improvement of a closed chest porcine myocardial infarction model by standardization of tissue and blood sampling procedures

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KEYWORDS:

Myocardial infarction, closed chest model, ischemia/reperfusion injury, sampling techniques

SHORT ABSTRACT:

In order to increase its translational value to understand the pathophysiology of myocardial ischemia/reperfusion injury and to test novel drug candidates, a porcine myocardial infarction model was refined, in particular with respect to standardization of the sampling procedure.

LONG ABSTRACT:

A porcine closed chest model of myocardial ischemia/reperfusion (M-I/R) injury was refined, in particular with respect to sampling technique, to improve its value to study pathophysiology and novel treatment options. To mimic the clinical situation of M-I/R injury, a balloon catheter is inserted into the left anterior descending coronary artery (LAD) of an anesthetized pig. The balloon is inflated to totally block the LAD for one hour and then deflated for 2 hours of reperfusion. At the end of reperfusion, the balloon is re-inflated and Evans Blue injected into the jugular vein to differentiate between the non-stained ischemic area at risk (AAR) and blue colored area not at risk (ANR). The animal is then euthanized and the heart excised. Within a maximum of 40 minutes, the left ventricle is sliced perpendicularly to the long axis and AAR and ANR are separated. Samples of the ANR are snap-frozen in Tissue-Tek and stored at -80°C as well as in 4% formaldehyde at room temperature for histological analysis. Triphenyl tetrazolium chloride is then applied to slices of the AAR to determine vital (VIT) and necrotic (NIT) ischemic tissue. Samples of NIT and VIT are cryopreserved for further analysis. The remaining AAR slices are formaldehyde-fixed, photographed 24 hours later and percent NIT within the AAR calculated. During the animal experiment, blood samples are taken at baseline, end of ischemia and defined time points during reperfusion. The blood is immediately centrifuged at 4°C, the plasma aliquoted and stored at -80°C. This model mimics acute myocardial infarction and PCI treatment in humans with the possibility of accurately determining AAR, NIT and VIT. Here it was used to investigate the effect of a bi-cyclic peptide inhibitor of FXIIa. The model can also be modified to allow longer reperfusion times to study later effects of myocardial infarction.

INTRODUCTION:

Ischemic heart disease, in particular acute myocardial infarction (MI), is the main cause of death in developed countries ¹. Today, the standard treatment of MI is percutaneous coronary intervention (PCI), the balloon catheter treatment. One of the critical factors that affect quality of life and prognosis of patients after PCI-treated acute MI is the infarction size. The reduction of the size can have a great impact on patient survival and prognosis². Myocardial ischemia/reperfusion (M-I/R) injury has a significant influence on the infarction size so one of the main aims in cardiovascular research is to prevent or reduce M-I/R injury ³. The exact mechanisms of I/R injury are still under investigation ⁴. Activation of the plasma cascades and endothelial cells are hallmarks of I/R injury ⁵. Activation of the coagulation system is clearly involved ^{6,7}. Recently, the role of FXII, as an early upstream peptide involved in contact phase activation of the coagulation cascade, has been shown in a FXII knock out rat model of cerebral I/R injury ⁸. Validation of these results in a porcine model is an important step into clinical translation. Therefore, we are testing a novel bicyclic FXIIa inhibitor in the context of M-I/R injury in a pilot study.

Animal models which mimic the clinical situation of acute MI and PCI treatment are essential to improve our understanding of the pathophysiology of M-I/R injury and to test novel treatment options. Pigs represent a good animal model for clinical M-I/R injury. This is not only because their hearts are very similar to human hearts with respect to anatomy and coronary circulation, but they also show similar pathophysiological responses to myocardial ischemia and reperfusion ^{9,10}. Other models such as rats and mice do not fulfill these criteria and show considerable differences when compared to human hearts ^{11,12}, whereas dogs for example have much more collateral coronary vessels as compared with humans ¹³.

The porcine acute myocardial infarction model has been widely used in cardiovascular research to investigate ischemic heart disease including M-I/R injury ¹⁴⁻¹⁷. The latter is an inflammatory condition because of which minimizing

the inflammatory reaction related to sternotomy or thoracotomy used in open-chest surgery is essential. The closed chest model using a clinical C-arm angiography setting overcomes this problem. Furthermore, one of the most important points is that our protocol provides an accurate distinction between ischemic (area at risk, AAR) and non-ischemic areas of the left ventricle (area not at risk, ANR) so that the infarct size (necrotic ischemic tissue, NIT) can be accurately determined. Our aim for this paper is to clearly define a reproducible methodology of a porcine M-I/R injury model, in particular with respect to myocardial tissue sampling, which will allow for a more precise analysis of the molecular mechanisms of I/R injury and a clearer readout of the effects of novel drug treatments.

PROTOCOL:

1. Animals:

All animals were treated according to the guidelines of the Swiss national laws. The study has been approved by the local animal experimentation committee of the Canton of Bern (permission no. BE 25/16).

Seven large white pigs of both sexes were used in the study. The animals were blindly divided into two groups, one group receiving a bicyclic peptide inhibitor of FXIIa and the other an inactive control peptide. One animal died prematurely before administration of FXIIa inhibitor or control peptide due to a technical error (sudden drop of blood pressure during ischemia time, before addition of test substance). One animal was excluded from the FXIIa inhibitor group because no ischemia/reperfusion injury was observed due to abnormal anatomy of the left anterior descending artery (LAD). A large part of the left ventricle, including the apex, was perfused by the circumflex artery in this animal. The animals included in the final analysis were $n = 2$ in the bicyclic FXIIa peptide inhibitor group (mean weight of 27.5 ± 2.5 kg) and $n = 3$ receiving an inactive bicyclic control peptide (mean weight of 29 ± 0.8 kg).

2. Surgical procedure (figure 1)

2.1 Anesthesia and preparation of the animal:

- 2.1.1 Animals should fast for 12 h before starting the experiment.
- 2.1.2 Pre-medicate the animal with 20 mg/kg Ketamine and 2 mg/kg Xylazine via intramuscular injection, using a 10 mL syringe, into the neck. Record animal weight and sex.
- 2.1.3 Induce anesthesia by injecting 0.5 mg/kg Midazolam and 0.05 mg/kg Atropin into the auricular vein, then intubate the animal with an endotracheal tube.
- 2.1.4 Maintain the anesthesia by mechanical ventilation using a Respirator (O₂/air 1:3, Sevoflurane 1.5%), a 7 – 8 mm airway tube and a filter. Adjust the fraction of inspired oxygen (FiO₂) to 35% and the tidal volume to 6-10 mL/kg.
- 2.1.5 Confirm sufficient depths of anesthesia by pinching the nasal septum using a clamp.
- 2.1.6 Dissect free, as previously described by Koudstaal and his colleagues steps 3-1 to 3-3¹⁸, the carotid arteries on both sides and cannulate them with a 7F sheath. Cannulate the left jugular vein with a 7F sheath for venous blood sampling.
- 2.1.7 Administer a bolus dose of 250 µg Fentanyl analgesic through the central venous line followed by 250 µg/h as a continuous intravenous infusion using an infusion pump. Monitor body temperature, heart rate, 3-lead electrocardiogram (ECG), arterial and central venous pressure during the whole experiment.
- 2.1.8 Using standard blood collecting tubes, withdraw the following baseline blood samples from the venous line: 5 mL citrated plasma and 2.9 mL EDTA plasma into the respective tubes and centrifuge immediately at 2000 x g for 15 minutes at 4 °C. Take 2.9 mL blood into a serum tube and allow to coagulate for 30 minutes at room temperature before centrifuging as described above.

- 2.1.9 Aliquot 200 μ L of plasma or serum into 500 μ L tubes and store all samples at -80 °C for further analysis. In addition to 0.5 mL of arterial blood for blood gas analysis (BGA), using special syringes for the BGA machine, take 0.5 mL of venous blood for measuring the activated clotting time (ACT), figure 2.
- 2.1.10 Administer 5000 IU unfractionated heparin using a 2 mL syringe into the venous line and allow the animal to stabilize for 20 minutes before starting the MI experiment.
- 2.1.11 Monitor ACT every 30-45 min. Withdraw 0.5 mL blood from the venous line using a standard 2 mL syringe and immediately transfer the blood into the ACT cartridge using a 30 G needle. Insert the filled cartridge into the ACT machine to measure clotting time. Inject 2500 IU unfractionated heparin intravenously if ACT is < 180 s.

2.2 Assembly of the balloon catheter

- 2.2.1 Insert the coronary guidewire (F 014/J, 175 cm) into the lumen of PCI dilatation catheter (diameter 3-4 mm, length 10-15 mm). Connect the PCI catheter with the inflation device pre-filled with contrast medium.

Note: choose the balloon catheter diameter based on baseline angiogram (2.3.2).

2.3 Myocardial infarction experiment

- 2.3.1 Use fluoroscopic guidance to insert a pressure catheter (5F, 120 cm) via the previously placed sheath in the left carotid artery. Advance it into the left ventricle using fluoroscopic guidance. The pressure catheter is connected to an acquisition system to record left ventricular pressure. The acquisition system continuously calculates and records heart rate, developed pressure, dP/dt maximum (contractility of left ventricle) and dP/dt minimum (relaxation of left ventricle) during the entire experiment. Allow the baseline to record for 10 minutes.

- 2.3.2 Insert a 6F (100 cm, EB3.75) guiding catheter via the previously placed sheath in the right carotid artery. Advance it into the left coronary artery to reach the LAD under X-ray guidance. Perform a baseline coronary angiography.
- 2.3.3 Insert the assembled system from 2.2 into the lumen of the guiding catheter. Advance the guidewire into the LAD until it reaches beyond the second diagonal branch of the LAD.
- 2.3.4 Use fluoroscopic guidance to advance the PCI catheter until it reaches about the middle of the LAD. Choose the LAD blocking site depending on the anatomy of the coronaries, usually after the second, sometimes after the first diagonal branch (figure 3) in order to have similar percentages of the AAR of the left ventricle (LV).

Note: The choice of the blocking site depends on the length of the diagonal branches and thus the area of tissue which is supplied by blood via the respective branch. In case of a long, bifurcated first diagonal branch the blocking site will be just after this. In case of a shorter first diagonal branch, the blocking is done after the second diagonal.

- 2.3.5 Remove the guidewire and then increase the pressure in the inflation device to 7-10 bar to inflate the balloon and induce myocardial ischemia for 1 hour. Gradually increase the FiO₂ to 50-60% between 15 and 40 min of ischemia. Keep tidal volume at 6-10 mL/kg.

Note: This procedure will reduce the occurrence of extrasystolies and decrease the frequency of ventricular fibrillations.

- 2.3.6 Record a 5-10 s video sequence while injecting contrast medium through the guiding catheter to have an angiogram of the balloon catheter in place; repeat after 10 minutes of ischemia to verify complete occlusion of the LAD distal to the balloon.
- 2.3.7 Monitor the animal closely to immediately detect and treat (2.3.8) cardiac arrhythmias. Extrasystolies usually occur and increase in frequency (>3

per minute) between 20 and 40 minutes after induction of myocardial ischemia. If this occurs, gently massage the neck on both sides just below the cheek. In most cases this will be sufficient to re-establish a regular heartbeat, probably by stimulation of the vagal nerve baroreceptors located on the common carotid artery.

- 2.3.8 If cardiac arrhythmias progress into ventricular fibrillation, use an external, biphasic defibrillator to re-establish a sinus rhythm. Apply 5-10 chest compressions using the defibrillator pads immediately before applying the shock in order to fill the coronaries with oxygenated blood and then shock with 150 J (for 30 kg animals).
- 2.3.9 Repeat if necessary and increase the energy to 175 J after the 3rd shock. Use higher energy settings for heavier animals.
- 2.3.10 Five minutes before the end of the ischemia time repeat the blood sampling mentioned in 2.1.8-2.1.9. Inject the test substance (either the bicyclic FXIIa inhibitor or control 4 mg/kg, this was done blindly) intravenously through the central venous line and flush the line with 20 mL saline.
- 2.3.11 Perform an angiogram (2.3.6) to confirm LAD occlusion, then deflate the balloon and remove the PCI catheter from the guiding catheter. Confirm perfusion of the LAD distal of the occlusion site by angiogram immediately after deflation and removal of the balloon, 10 min thereafter, whenever signs for myocardial ischemia were visible by ECG for more than 5 min, and immediately before re-occlusion of the LAD (2.3.13).
- 2.3.12 Allow reperfusion of the ischemic myocardium for 2 h. Take blood samples at 10, 30, 60 and 120 min of reperfusion.
- 2.3.13 Reinsert the PCI catheter together with the guidewire; see 2.2.1 and 2.3.4, to exactly the same position as used for the ischemia. Inflate the balloon as before and confirm LAD occlusion by angiogram (2.3.6). Remove the pressure catheter from the left ventricle and stop recording.
- 2.3.14 Inject 100 mL 2% Evans Blue in phosphate buffered saline (PBS, pH 7.4) into the central venous line. About 30 s later, when the whole animal turns blue, inject 40 mL 20% KCl to euthanize the animal.

2.4 Extracting, dissecting and sampling the heart (figure 4)

- 2.4.1 Perform a sternotomy to expose the heart. Follow the protocol previously described by Koudstaal and colleagues, steps 8-2 and 8-3¹⁸. Cut open the pericardium while inspecting for abnormalities, which might stem from earlier pericarditis and preclude further evaluation of the respective animal.
- 2.4.2 Deflate and remove the PCI- as well as the guiding catheter. Excise the heart for further analysis. Cut the vena cava and remove blood using a suction pump, then cut all the large vessels connecting the heart with the body.
- 2.4.3 Rinse the heart inside and out with saline at room temperature. Weigh the whole heart.
- 2.4.4 Within 30-40 min, cut the heart into slices of about 3-5 mm from the apex to the *Chordae tendinae* of the mitral valve, perpendicular to the long axis using a sharp knife.
- 2.4.5 Be careful to place the heart always in the same orientation with the ventral side facing up in order to keep the orientation of the cut samples (figure 5).
- 2.4.6 Photograph the slices of the heart.
- 2.4.7 Cut away the right ventricle (discard as not needed). Photograph again and weigh all the slices for the total weight of the left ventricle.
- 2.4.8 Differentiate between the Evans Blue positive and Evans Blue negative tissue in all the sections. Cut the slices to separate the ischemic (Evans Blue negative) from the non-ischemic tissue (Evans Blue positive) using a scalpel.
- 2.4.9 First analyze the Evans Blue negative sections (the ischemic area at risk, AAR). Weigh them all and put them all into a plastic container.
- 2.4.10 Cover the slices entirely with 100-150 mL (according to the heart size) triphenyl tetrazolium chloride solution (2 g TTC, 16 g Dextran, molecular weight 48000-90000, in 200 mL PBS, freshly prepared) so that the heart pieces can move freely inside the solution. Cover the container and incubate for 20 min at 37 °C while gently shaking.

- 2.4.11 During this 20 min incubation time weigh the Evans Blue positive pieces (area not at risk, ANR), take samples for Tissue-Tek embedding (choose the most distal part from the injury) and store at -80 °C for further analysis. Transfer the rest into 4% formaldehyde solution and store at room temperature for histology sections.
- 2.4.12 Remove the pieces of the AAR from the TTC solution. The red stained tissue is viable ischemic tissue (VIT) and the non-stained tissue is necrotic ischemic tissue (NIT). Cut 2 small pieces (blocks of 2-3 mm) from the NIT and VIT each. These samples should have the same weight.
- 2.4.13 Fix the rest of the pieces (all slices made from the AAR) by pinning them down in a Styrofoam container and covering completely with 4% formaldehyde solution for 24 h at room temperature in a fume hood. The pieces should stay flat for the photographic documentation in the next step.
- 2.4.14 The next day photograph both sides of the pieces with a high-resolution camera with the same zoom setting and distance from the tissue (same magnification). Add automatic scale bars to all pictures. All bars will have the same length.

2.5 Calculation of the AAR and the infarct size

- 2.5.1 $\%AAR \text{ of left ventricle} = (\text{weight of AAR in g} / \text{weight of left ventricle in g}) * 100.$
- 2.5.2 Use ImageJ software to calculate the total surface area of both the AAR and NIT (both sides of each piece) based on the photographs.
- 2.5.3 Adjust the scale bar by selecting the scale bar length using the straight line from the angle tool. Choose from the menu Analyze > Set Scale and insert the known distance and unit of the scale bar. Choose “global” so the same scale will be applied to all pictures.
- 2.5.4 Mark the whole surface area of the tissue using the free hand selection tool to calculate AAR. Be careful not to include the side (height) of the tissue and/or the fatty tissue (figure 6-C).

- 2.5.5 Set the measurement by choosing “area” and “display label” from Analyze > Set Measurements menu. Measure the surface area from Analyze > Measure.
- 2.5.6 Repeat step 2.5.4 to measure NIT (non-stained tissue). Note: Don’t include the fatty tissue (figure 6-D) in the NIT calculation. Repeat the step on the other side of the tissue.
- 2.5.7 Calculate average AAR and NIT for each piece of tissue.
- 2.5.8 Use the values obtained from 2.5.7 to calculate overall as follows:
%NIT of AAR = $(\Sigma \text{ average surface area of NIT in cm}^2 / \Sigma \text{ average surface area of AAR in cm}^2) * 100$.
- 2.5.9 Two different investigators should repeat the above method. The acceptable margin of difference is < 10%.

2.6 Ischemia markers

- 2.6.1 The level of cardiac troponin-I was measured using a single-plex Luminex-type assay as previously described ¹⁹.

REPRESENTATIVE RESULTS:

X-ray video imaging / coronary angiography of the pig heart is used to visualize the position of the pressure catheter and to decide where to block the LAD (figure 3-A). Figure 3-B shows the catheter position, blocking the blood flow distal to the second diagonal branch. Comparison of figures 3-A and 3-B also allows to estimate which part of the LAD-supplied myocardium will be ischemic. At the end of the 2 h reperfusion period the balloon catheter is reintroduced and inflated at the same position as it was during ischemia. Evans Blue is then injected intravenously to accurately determine the AAR (figure 5-A). After excision of the heart, the left ventricle is sliced into 3-5 mm thick sections from the apex up to the mitral valve, perpendicular to the long axis. AAR and ANR are clearly demarcated by Evans Blue staining on the slices. AAR and ANR sampling areas are shown in figure 5-B.

The AAR, expressed as percentage of the LV, shows no statistically significant differences between the FXIIa treated group and the control group (figure 6-A). The infarct size (NIT/AAR) shows no differences between the groups either (using non-parametric Mann-Whitney test, $p > 0.05$, figure 6-B). These data suggest that FXIIa inhibitor alone, at the used concentration and duration of application, could not protect the heart from M-I/R injury. Figures 6-C and D show how to mark the AAR and NIT borders in order to accurately and reproducibility measure the respective surface areas.

The used blood sampling strategy allows the release of the cardiac muscle damage marker cardiac troponin-I to be monitored over time. There is almost no difference after one hour of ischemia with the baseline while after reperfusion there is a continuous increase over time as shown in figure 7. Also for troponin-I inter-group differences were not significant in these experiments.

FIGURE AND TABLE LEGENDS:

Figure 1: Overview of the experimental timeline. Schematic timeline for the important steps in the myocardial ischemia/reperfusion injury model. Baseline coronary visualization, starting ischemia, monitor cardiac arrhythmias and injecting the test substance are important steps in the experiments. The use of exact timing in all the experiments ensures reproducibility of the experiments. Killing the animal and excision of the heart should be done within 15-20 min after completion of the 2 h reperfusion phase. KCl: potassium chloride.

Figure 2. Timeline of blood sampling and analysis. Time points for blood sampling are indicated together with type of anticoagulant used. Additional samples can be taken according to the experiment and analytes to be detected. ACT: activated clotting time, BGA: blood gas analysis, RT: room temperature.

Figure 3: Coronary angiography. Fluoroscopic view of (A) the left coronaries at baseline, the yellow arrows point to the first and the second diagonal branches, the white arrow points to the heart apex (B) the occluded LAD showing the no

flow area of the left ventricle (LV), the red arrow points to the PTCA balloon (C) re-closure of the LAD at the end of the reperfusion with the balloon re-inserted at the same site in the LAD as during ischemia. CX: circumflex coronary artery, LAD: left anterior descending coronary artery, MC: Millar catheter, inserted in the left ventricle.

Figure 4: Schematic chart of tissue sampling. Exact timing of heart dissection and sampling of the different areas for further analysis. The timeline starts at 205 min after beginning of ischemia, 20 min after termination of the animal experiment. It is important to incubate the tissue sections in TTC within a maximum of 40 min after euthanizing the animal. Sampling of ANR, VIT and NIT is indicated as white squares. Incubating the AAR in 4% formaldehyde allows clear distinction between NIT and VIT for accurate determination of the infarct size. AAR: area at risk, ANR: area not at risk, LV: left ventricle, NIT: necrotic ischemic tissue, OTC: Tissue-Tek, RV: right ventricle, TTC: triphenyl tetrazolium Chloride, VIT: viable ischemic tissue.

Figure 5: In-situ differentiation between area at risk (AAR) and area not at risk ANR. (A) Representative picture of the whole heart just after sternotomy at the end of the experiment. (B) Representative picture showing the 3-5 mm thick left ventricle slices after dissection. AAR and ANR are clearly defined, indicated by yellow arrows, and the white arrow shows the ANR sampling area.

Figure 6: Ischemia and infarct size. (A) The percentage weight of the AAR of the left ventricle (LV). (B) The percentage surface area of the NIT of the AAR. (C) A representative picture of the AAR calculation. (D) A representative picture of the NIT calculation. The white arrow shows the VIT sampling area and the black arrow shows the NIT sampling area. Data were calculated using ImageJ software. Values are shown as dots for each individual experiment with indication of mean \pm SD. Control group, n = 3 and FXIIa treated group, n = 2.

Figure 7: Cardiac muscle damage marker. Cardiac troponin-I concentration over time in pg/ml of both the control and FXIIa inhibitor treated group. Blood

was collected from the jugular vein at baseline, end of ischemia and several time points during reperfusion into EDTA plasma tubes and cardiac troponin-I was measured by single-plex suspension array (Bio-Plex). Data are shown as dots for each individual experiment with indication of mean \pm SD. Control group, n = 3 and FXIIa treated group, n = 2.

DISCUSSION:

M-I/R injury has a significant effect on the final infarct size which is directly translated into patient prognosis after acute myocardial infarction ³. Understanding the pathophysiology of M-I/R injury is the first step to reduce or prevent it. M-I/R injury is an acute condition that occurs directly after reperfusion of the occluded vessels. I/R injury leads to activation of the innate immune response and cellular damage occurs at the site of reperfusion and the surrounding tissues ²⁰. A recent study showed an improvement in the neurological outcome in a rat model of brain I/R injury when treated with FXIIa inhibitor ²¹. However, in the current pilot study we found no effect of the bicyclic FXIIa inhibitor on M-I/R injury. The used peptide is novel and its pharmacokinetics in pigs is not known yet. Therefore, the observed lack of effect might be caused by inappropriate dosing or application. This needs to be sorted out in follow-up studies.

Standardizing an animal model is essential to investigate in depth the pathophysiology of M-I/R injury and to bring suitable solution into clinics. Investigating the pathophysiology of M-I/R injury requires good and representative sampling in order to study the cellular mechanisms underlying M-I/R injury. The porcine closed chest M-I/R injury model provides a reproducible and a close preclinical model that is useful to understand the cellular mechanisms and test novel new therapeutics. Variants of the present model have been described before for the above mentioned purposes ^{14,17,18}.

Our protocol of acute myocardial infarction in pigs does not need pre-treatment with amiodarone as previously described ^{18,22}. We use carotid sinus massage to

reduce cardiac arrhythmias and a biphasic defibrillator for cardioconversion in case of ventricular fibrillation. The use of carotid sinus massage is clinically known to influence atrial fibrillation ²³, but so far it has not been described to prevent or delay the onset of ventricular fibrillation in MI, neither in humans nor in pig models. Moreover, the use of Sevoflurane helps to reduce ventricular arrhythmias as well as mortality rate of porcine model of acute myocardial infarction ²⁴.

To ensure reproducibility and reduce the risk of thrombosis during the experiment, multiple doses of heparin were injected based on the repeated measurement of ACT, rather than using fixed heparin doses as described for example by Koudstaal et al ¹⁸. A controlled amount of heparin administration helps to investigate the coagulation cascade in the context of I/R injury. Evans Blue allows accurate determination of AAR/LV. The intravenous injection of the Evans Blue after re-occlusion of the LAD at the exact site during ischemia induction with the fluoroscopic guidance leads to blue staining of the whole pig including the non-ischemic part of the heart with minimum effect on the ANR myocardium and vasculature. Evans Blue is a known cytotoxic substance ²⁵. In the current experiments it is crucial to maintain the viability of the endothelial cell layer in ANR in the heart vasculature in order to use it as an intra individual control so 100 mL Evans Blue is injected systemically and diluted with the whole blood reducing its toxicity. Previously in a similar setting, 50 ml 2% Evans Blue was injected directly into the coronaries increasing the risk of its cytotoxicity to cardiac cells ²⁶. The next important step is to dissect the heart directly into 3-5 mm slices from the apex up to the mitral valve (the exact position in every animal) and using this method to make an accurate calculation of the AAR as a percentage of the left ventricle.

The current description of the method provides finer details that have not previously been described. Incubating TTC stained section in 4% formaldehyde for 24 hours provides a clear distinction between viable (red) and necrotic (white) tissue, which finally increases the reproducibility of the sampling for further molecular staining. The Blood sampling strategy over 2 h of reperfusion

enables the detection of newly expressed molecules in very early (10 and 30 min) reperfusion as well as later (60 and 120 min). The correct blood and tissue sampling and storage are also crucial for the analysis of plasma cascade markers such as the expression of complement and coagulation proteins.

In summary the current method provides detailed important steps required to increase the reproducibility of the porcine closed chest M-I/R injury model when the intended use of the model is to study the cellular and molecular changes in the context of studying M-I/R injury pathophysiology or studying novel therapeutic options.

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DISCLOSURES:

The authors declare no conflict of interest.

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Figure 1

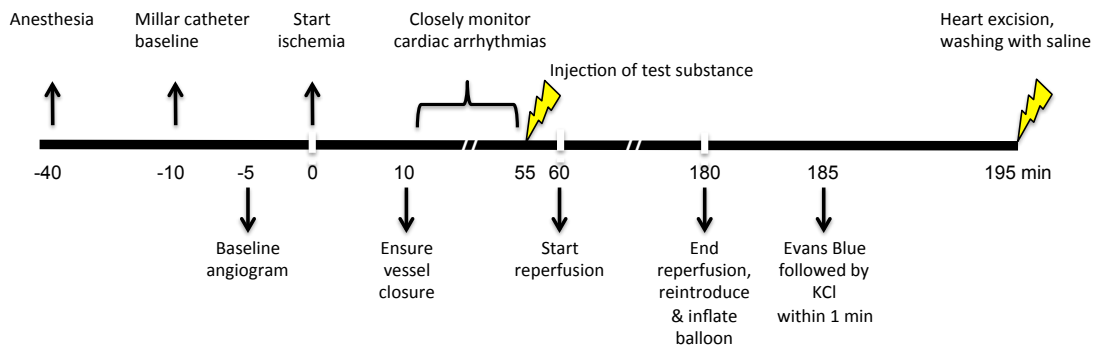
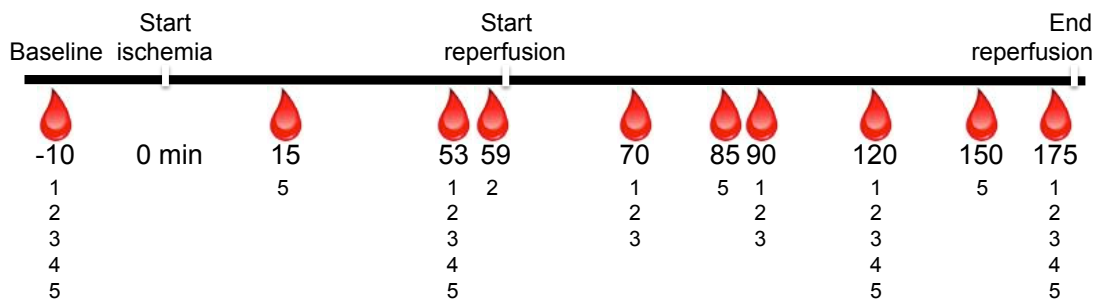
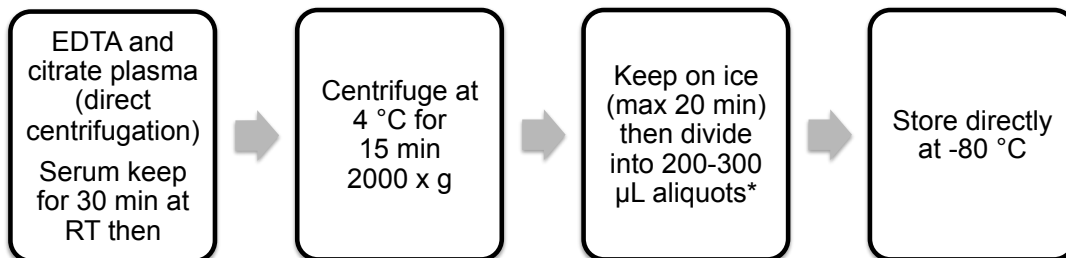


Figure 2



1 EDTA plasma, 2 Citrate plasma, 3 Serum, 4 BGA, 5 ACT



* Number of the aliquots depends on the number of markers of interest.

Figure 3

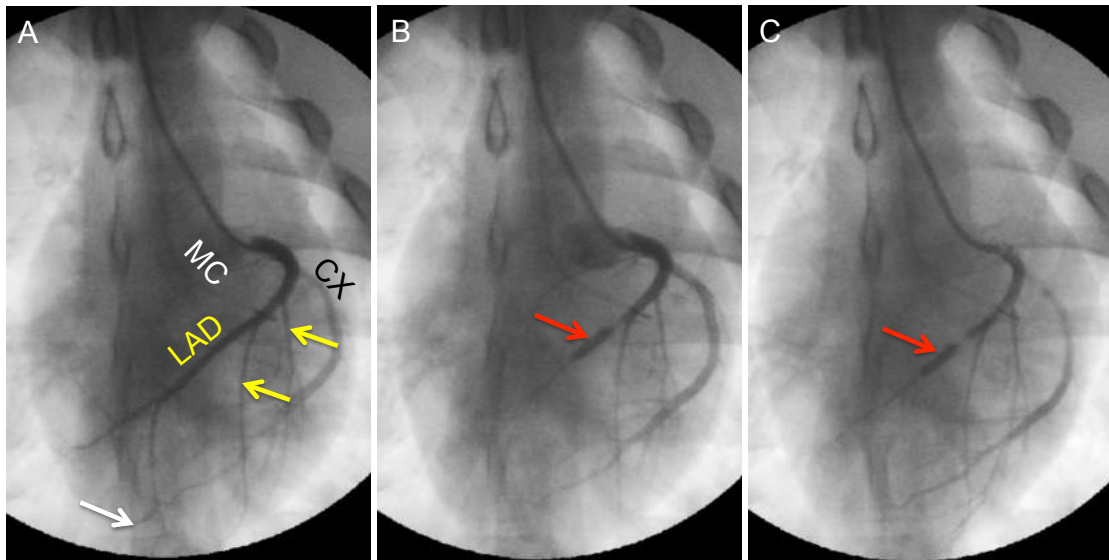


Figure 4

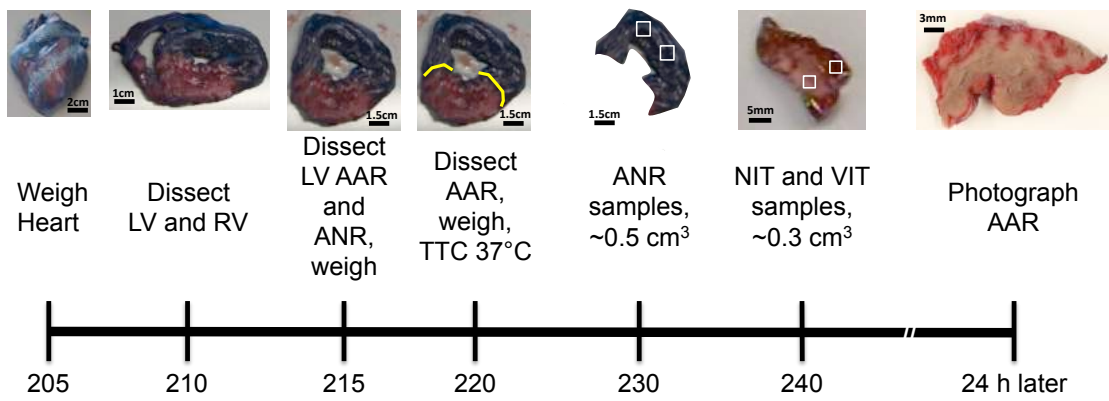


Figure 5



Figure 6

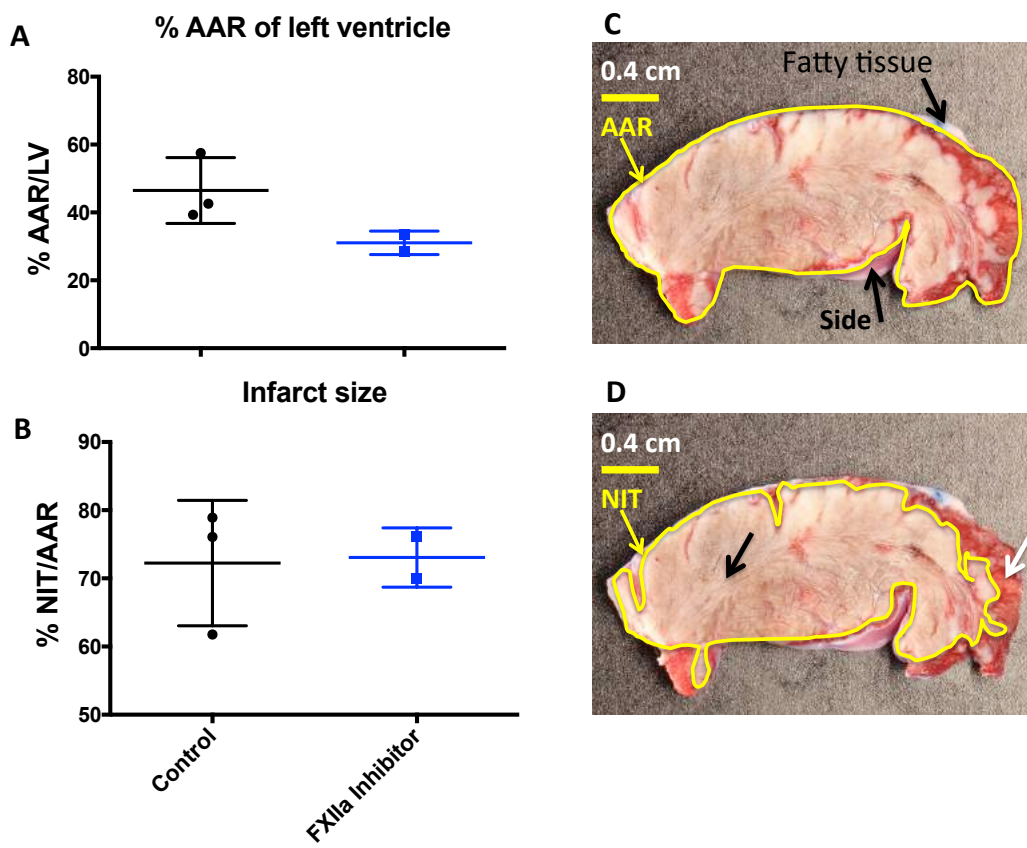
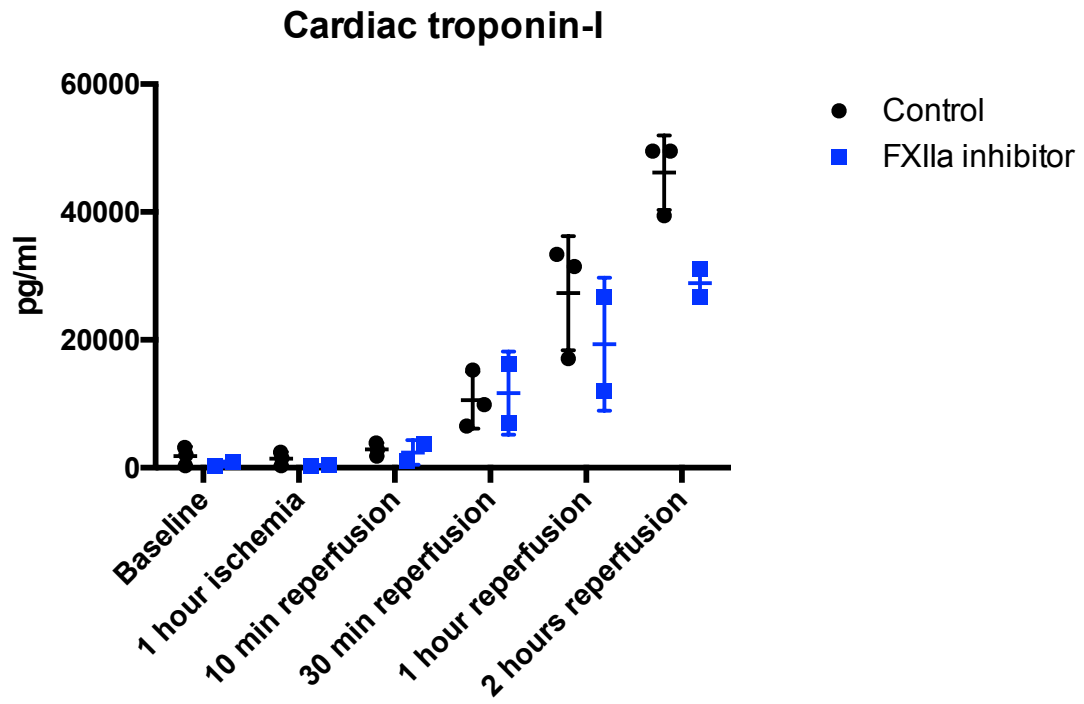


Figure 7



Paper V: The architecture of the IgG anti-carbohydrate repertoire in primary antibody deficiencies (PADs)

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†These authors contributed equally to this publication

Status: In preparation for submission to Science Translational Medicine

Contribution: Suspension array experiments were made by Mai M. Abdelhafez

Background: Common variable immunodeficiency is the most common form of the primary immunodeficiency diseases. It is an inherited disease characterized by low levels of immunoglobulins (Ig). The characterization of the Ig deficiency quality is still unknown, although it is an important factor for determination of the pathophysiology of the disease

Aim: To access the human IgG-mediated carbohydrate repertoire of in the common variable immunodeficiency patients

Conclusion: The degree of disease severity in patients suffered from primary antibody deficiency does not depend only on the quantitative value for the antibodies but also on the type of the deficient antibody. This open a new scope for the diagnosis of the primary antibody deficiency

The architecture of the IgG anti-carbohydrate repertoire in primary antibody deficiencies (PADs)

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KEY WORDS: Primary immunodeficiency diseases (PID), primary antibody deficiencies (PAD), common variable immunodeficiency disorders (CVID), glycan array, xenotransplantation

ABSTRACT

Immune system failure in primary antibody deficiencies (PADs) has been linked to recurrent infections, autoimmunity and cancer, yet clinical judgment is often based on the reactivity to a restricted panel of antigens. Previously, we demonstrated that the human repertoire of carbohydrate-specific IgG exhibits a universal structure and modular organization. The current study compares the glycan-specific IgG repertoires among different PAD entities. Despite their heterogeneity, similar repertoire architecture, with extensive glycan-recognition defects characterized by the dominant loss of α -Gal- and GalNAc-reactivity, was observed, in addition to disease-specific recognition of microbial, self-antigens and tumor-associated carbohydrate antigens. Our findings demonstrate the power of antibody repertoire analysis to better understand the dimension and clinical implications of the immune system failure in individual patients.

INTRODUCTION

The immune system, organized as a highly coordinated cellular and humoral network (1), executes manifold functions beyond the defence against foreign invaders. As a consequence, primary antibody deficiencies (PADs), the most common forms of immunodeficiencies in humans, are associated with a plethora of clinical sequelae including severe and recurrent infections, microbial dysbiosis, autoimmunity, granulomatous disease and malignancy (2-4). The complexity of the immune system network is reflected in the plethora of monogenetic, polygenetic and still unexplained defects, which are not limited to antibody producing cells, but affect other immune system components relevant to antibody generation, class switch recombination (CSR) or somatic hypermaturation (SHM) (4).

Inadequate humoral responses to carbohydrate-structures are a frequent feature of PADs. Patients with specific antibody deficiency (SAD) exhibit poor responses to structural or capsular polysaccharides of bacteria (e.g. *S. pneumoniae*, *H. influenzae*), despite the presence of normal serum concentrations of IgG, IgM and IgA (5). Insufficient responses to glycan-based vaccines or low titers of isohemagglutinins, naturally occurring antibodies to polysaccharide blood group antigens, are typical features of common variable immunodeficiency (CVID), the most frequent symptomatic antibody deficiency diagnosed in adulthood (6, 7). Antibodies from different IgG subclasses are known to contain different specificities for glycan-structures (8, 9), which may explain the predisposition of certain patients with IgG subclass deficiency to infections with encapsulated bacteria (5). However, the clinical assessment of carbohydrate-recognition defects by diagnostic vaccination (e.g. pneumococcal vaccines) or measurement of pre-existing antibody titers (e.g. isohemagglutinins) currently relies on a restricted number of glycan epitopes, thus providing only a narrow perspective of the actual dimensions of the immunodeficiency. While the interpretation of such data is challenging and controversially discussed, they have important implications in terms of diagnostic delay, disease classification and treatment decisions, including life-long IgG replacement therapy (6, 7, 10, 11).

Glycan array technology allows the high-throughput analysis of specific antibody responses to carbohydrate antigens (12-14). In a previous systems immunology study using glycan array version 5.1 of The Consortium for Functional Glycomics (CFG) we found that in healthy individuals the IgG repertoire is characterized by a universal architecture with modular organization that depends on structural features of glycans that determine their immunogenicity (8). In the present study, we employed this technology to investigate the IgG antibody repertoire of PAD patients in terms of clinically relevant carbohydrate epitopes, including microbial glycans, self- or xenoantigens, and tumor-associated carbohydrate antigens (TACAs).

RESULTS

Broad carbohydrate reactivity defects (CRD) in diverse PAD entities

Sera from PAD patients or healthy donors were screened on CFG glycan array version 5.1 to analyze the IgG binding reactivity to 610 distinct glycans. Cohorts included patients with symptomatic hypogammaglobulinemia (HGG), SAD or IgGSD, CVID, CVID with low pneumococcal polysaccharide vaccine (PPV) response (CVID PPV^{low}). In addition, sera from CVID patients were screened to investigate their glycan-reactivity profiles beyond pneumococcal carbohydrate antigens. Raw data are presented in table S1.

Using this platform, we observed broad IgG reactivity to printed glycans in pooled sera from healthy donors, which is consistent with previously published data (8, 9, 14-17), (Fig. 1A and 1B). In contrast, marked reduction of overall glycan binding intensities was observed for all investigated PAD patient subsets, with lowest averaged relative fluorescence units (RFU) in CVID, particularly in CVID PPV^{low} patients (Fig. 1A). Next, IgG was purified from CVID, HGG and healthy donor sera by affinity chromatography, and then screened on glycan array CFG version 5.1 at 180µg/ml. In accordance with previous reports (8, 9), this assay concentration was determined to be optimal, resulting in reproducible IgG glycan-binding patterns with minimal background. As with whole sera, anti-glycan reactivity of equimolar, purified IgG from immunocompromised patients presented lower RFU values on average compared to healthy controls (Fig. S2).

In an effort to compare the carbohydrate-specific IgG repertoires between PAD subsets and healthy individuals, hierarchical clustering analysis was performed. Figure 1B illustrates the computed reactivity matrix ordered by a dendrogram clustering algorithm (18). Rows represent the antibody reactivity profiles (reactivity of each specific glycan for the different sera samples), and the columns reflect the immune profiles for each patient subgroup. The dendrogrammed reactivity matrix illustrates the broader spectrum of glycan reactivity in healthy donor sera compared to all screened PAD patient subgroups. At the next level of the hierarchical tree, the glycan-binding profile of IgGSD sera was computed distinct to the other subgroups. A close relationship of the

immunoprofiles between SAD and HHG patients was revealed. The glycan-binding profiles of CVID sera displayed a narrower spectrum of glycan reactivity, which was most impaired in the CVID PPV^{low} subgroup.

To better understand the degree of relationship among the immunoprofiles between diseased and healthy donors the correlation matrix was computed by Spearman's rank correlation analysis (Fig. 1C). Corroborating our previous observation, the correlation of glycan-binding profiles compared to healthy individuals ranged from low to intermediate ($r_s = 0.39 - 0.68$) reflecting the widely impaired antibody reactivity to glycans in all PAD patient groups. The most impaired anti-glycan antibody repertoire was found in the CVID PPV^{low} subgroup ($r_s = 0.39$), which was considerably more aberrant compared to CVID ($r_s = 0.57$). The IgG glycan reactivity profile of IgGSD patients was different compared to those of healthy donors ($r_s = 0.60$) and other PAD subgroups ($r_s = 0.42-0.61$), which supports the notion that IgG subclasses harbor unique spectra of antigen specificity (5). Notably, the immunoprofile from SAD patients exhibited a more extensive defect than expected ($r_s = 0.67$). The SAD reactivity profile presented with higher similarity with the repertoires of symptomatic HGG ($r_s = 0.81$) and CVID ($r_s = 0.72$) patients compared to healthy individuals ($r_s = 0.67$), suggesting a similar repertoire architecture defect, despite the normal serum immunoglobulin concentrations in these patients (5).

Recognition of bacterial and blood group antigens in PAD

PADs are characterized by recurrent, severe or unusual bacterial infections, especially by bacteria protected by a polysaccharide capsule. Thus, we decided to analyze the IgG reactivity profiles to known bacterial carbohydrate. On glycan array CFG version 5.1, we previously identified 121 bacterial carbohydrate antigens consulting the Bacterial Carbohydrate Structure Data Base (BCSDB) (8), providing a platform for high-throughput analysis for antibody reactivity to carbohydrate epitopes of multiple bacterial species. The printed glycans include capsular and structural oligosaccharides, as well as exopolysaccharides of commensal and pathogenic bacterial species (8). Figure 2A illustrates the computed reactivity matrix to bacterial antigens for the different PAD subsets.

The anti-bacterial reactivity profile of SAD and HGG was related, but different to healthy donors and the other PAD entities. The most profound loss of anti-bacterial reactivity was found for CVID PPV^{low} patients (Fig. 2A, B). Spearman's rank correlation analysis revealed a reactivity correlation of 0.34 CVID PPV^{low} patients and 0.57 for CVID in comparison to healthy controls (Fig. 2C). Moreover, hierarchical clustering and Spearman's rank correlation analysis revealed a unique immunoprofile for IgGSD also for bacterial glycan epitopes, while the IgG signatures for HGG and SAD were more closely related among each other ($r_s = 0.79$), and exhibited less aberrant recognition of bacterial antigens ($r_s = 0.68$ and 0.69 , respectively) (Fig. 2A, C).

The glycan array technology combined with the consultation of the BCSDB databank allowed to screen in parallel several distinct epitopes that were identified as glycan constituents of specific bacteria. Figure 2D shows the IgG reactivity to glycans found in *S. pneumonia* (n=5), *H. influenzae* (n=5), *N. meningitidis* (n=15), *E. coli* (n=36), *H. pylori* (n=47), and *Salmonella* (n=10) species. Serum antibodies from CVID and CVID PPV^{low} cohorts bound glycan epitopes of these bacterial species with reduced intensity, with the exception of *H. pylori* epitopes, which were readily recognized by IgG from all cohorts (Fig. 2D). Differential binding activity to bacterial antigens was found between PAD cohorts, including lost or lowered reactivity of IgGSD and SAD IgG to *N. meningitidis* and *Salmonella* epitopes, and low recognition of *S. pneumonia* glycans by IgGSD IgG.

Fig. 2E depicts IgG reactivity patterns of the PAD cohorts to different blood group antigens. While low isohemagglutinins are frequently observed in CVID patients (7), we found reduced IgG reactivity to blood group A and B antigens also in the HGG, SAD and IgGSD cohorts.

Recognition of TACA and Siglec ligands in PAD

One life-threatening complication of PAD, in particular of CVID, is the occurrence of malignancies (4, 19-22). Since altered surface glycosylation is a hallmark of cancer and influences different aspects of tumor progression and anti-tumor immunity (23-25), we set out to explore IgG reactivity with tumor-associated

carbohydrate antigens. Figure 2F demonstrates the IgG reactivity profiles to twenty-two glycan epitopes on CFG glycan array version 5.1 that constitute well-established TACAs. In accordance with our previous data (8), healthy donors were shown to produce natural antibodies against TACAs on the array. High reactivity was found against Lewis antigen-related TACAs, gangliosides, Thomsen-Friedenreich and globo-series-associated antigens. Loss of anti-TACA IgG reactivity was found in all examined PAD patient subgroups. The reduced binding activity by IgG was most dominant in CVID PPV^{low} donors, followed by CVID patients, as computed based on ABR profiles and represented as circular dendrogram (Fig. 2G).

As sialoglycans recognized by immunoregulatory Siglecs have been proposed to act as self-associated molecular patterns and to be relevant for the escape of malignant or infected cells (24-26), IgG binding to Siglec ligands was investigated. While the recognition of most Siglec ligands was similar or reduced, antibody responses to two ligands of CD22/Siglec-2 on B cells was high (# 377) or even increased (# 268) in PAD cohorts (Fig. 2C).

Failure to raise a-Gal- and GalNAc-directed IgG antibodies in PAD

The human IgG anti-carbohydrate repertoire in healthy individuals is shaped by structural features of glycans that determine their immunogenicity (8). This raised the question about the effect of the relationship between immune system failure (ISF) in PAD subgroups and the structure-related immunogenicity on the architecture of the glycan-specific IgG repertoire. A binary deviation matrix was computed considering statistical deviation ($P < 0.05$) of IgG binding (RFU values) for each PAD cohort and each glycan compared to healthy donor data and reordered by a dendrogram clustering algorithm (Fig. 3A). The rows in this matrix indicate the binary antibody reactivity profiles, and the columns represent the deviation immune profiles for each PAD subgroup. By hierarchical clustering analysis 4 major subgroups were identified: predominant were clique 4 representing glycans with deviation in all PAD cohorts (n = 293; 48%) and clique 2 containing glycans without deviation in all PAD cohorts (n = 245; 40%); of lower magnitude were clique 1 (n = 5; 1%) and clique 3 (n = 67; 11%)

encompassing glycans with heterogeneous IgG binding activities between subgroups.

Given the association between immunogenicity and terminal carbohydrate moiety of glycans (8, 27), the clique distribution of glycans was investigated based on their structure. The most dominant deviation was found for Gala- and GalNAc-terminated glycans (Fig. 3B). 47 (85.5%) versus only 1 (1.8%) Gala-structure(s), and 37 (68.5%) versus 12 (22.2%) GalNAc-terminated glycans were represented in clique 4 or clique 2, respectively. In accordance, the IgG antibody-binding levels (RFU values) of Gala- and GalNAc-terminated glycans were lower in all cohorts (Fig. 3C, D). In depth analysis computing a deviation matrix based on *P* values and hierarchical clustering analysis revealed large glycan clusters that were concomitantly either non-aberrant (clique B) or highly aberrant (clique E) in all disease entities (Fig. 3E), whereas Gala- (70.9%) and GalNAc- (62.9%) terminated glycans were most prevalent in latter (Fig. 3F), indicating failed antibody responses to these specific structures.

As a consequence of the inactivation of the *GGTA1* gene encoding for α 1,3galactosyltransferase (GalT), humans, apes and Old World monkeys do not express the Galili epitope Gal α 1-3Gal β 1-4GlcNAc β (28). The high levels of natural antibodies to this xenoantigen in most humans, also generated in response to the microbiota of the host, is a key factor in the rejection of xenografts (29), and necessitated the development of GalT knockout (GalTKO) animals (30, 31). Using a suspension array, we examined individual sera from patients (HGG, *n* = 37; CVID, *n* = 15; IgGSD, *n* = 8) patients and healthy donors (*n* = 18) for IgG reactivity to the Galili epitope and other Gala-terminated and GalNAc-terminated structures including, aLN (Gal α 1-4GlcNAc β), Aa3GN (Gal α 1-3GlcNAc β), or A and B blood group antigens (Fig. 4A and S3). The IgG reactivity to these Gala- and GalNAc-terminated epitopes was consistently lower in most PAD patients, whereby the reduction was most significant for the Galili epitope, as evidenced by statistical analysis.

Given the significant loss of anti-Galili reactivity in PAD patients, functional implications were tested in a xenoreactivity assay, analyzing antibody-

dependent cell cytotoxicity (ADCC) of primary human natural killer (NK) cells directed against the porcine kidney cell line PK15. On this cell line the Galili epitope is highly expressed on the surface and lost following enzymatic digestion by α -galactosidase as assessed by flow cytometry (Fig. 4B). Sera from healthy donors induced substantial NK cell-mediated ADCC activity against the Galili-positive PK15 cells, which was abolished following α -galactosidase treatment of the porcine target cells (Fig. 4C), illustrating the dependence of the xenogeneic activity on Gal-a epitopes, including Galili. In contrast, CVID sera failed to promote NK cell-mediated ADCC against the porcine Galili-positive target cells at equimolar IgG concentrations (1mg/mL), indicating a repertoire defect for the recognition of xenogeneic antigens. Moreover, the loss of the xenogeneic potential of CVID sera was demonstrated in a modified version of an established model for antibody-mediated skin damage (32, 33), involving cryosections of porcine skin incubated with patients' sera and leukocytes from healthy donors and the assessment of dermal-epidermal separation. Porcine skin damage was significantly lower in the presence of CVID sera compared to human control sera at equimolar IgG concentrations (Fig. 4D). Together, these data provide evidence for humoral immune system failure beyond quantitative antibody deficiency, which involves repertoire defects in PAD.

DISCUSSION

The emerging picture is that the human IgG anti-carbohydrate repertoires in health (8), and in primary antibody deficiencies, exhibit a modular organization, yet with a different architecture in PAD. Translated to Cohen's concept of immune computation (34), our data imply that primary antibody deficiency is not solely characterized by aberrant antibody production, but features disturbed immune-system response states (the output) due to an altered algorithm to compute immunogenic states (the input) that is dictated by impaired cellular and molecular networks of innate and adaptive immunity (1, 35). Despite the heterogeneous genetic basis across PAD entities (4), deviation mapping revealed significant repertoire similarities pointing toward a common disease-modifying algorithm that drives humoral immune system failure. Dominant was the reduced or lost specificity for Gala- or GalNAc-terminated glycan epitopes for which healthy individuals express high levels of naturally occurring antibodies, eventually in response to immune stimulation by carbohydrate antigens of their microbiota (8, 28).

Our broad analysis demonstrates the extent of the skewed IgG repertoire with impaired reactivity to biologically relevant glycan epitopes, including those linked to PAD-associated clinical manifestations, such as infection, malignancy and autoimmunity (4, 6). The loss of IgG reactivity to tumor antigens was found to be most evident in the CVID cohort, a disorder in which malignancy is common (6), supporting the concept of aberrant immune surveillance in PID (36). Our systems immunology approach highlights the power of high-throughput assessment of humoral immune system failure by microarray technology, which has important ramifications for the diagnosis, classification and therapy of PAD patients.

MATERIALS AND METHODS

Study design

This non-randomized study was designed to investigate the human IgG anti-carbohydrate repertoire, in healthy and disease conditions, using glycan array technology combined with a computational system level approach. The total sera from the individuals included in the study and respectively purified IgG, as well as, IgG control mix were screened on CFG glycan array version 5.1, whereby glycan binding was assessed at least six times for each sample.

Patient samples

Human blood was collected from healthy individuals or patients upon informed and written consent in accordance with the Declaration of Helsinki. All experimental protocols were approved by the local institutional and/or licensing committees. Patients admitted to the University Hospital of Bern from January 2005 to December 2011, were retrospectively identified. CVID was defined in accordance with the criteria of the Pan-American Group for Immunodeficiency and the European Society for Immunodeficiency (37). Inclusion criteria for the IgGSD cohort were selective or combined IgG subclass deficiency (38) with recurrent episodes of infection. SAD was diagnosed in patients with normal total immunoglobulin and IgG subclass concentrations but impaired PPV response, as detected 4 to 6 weeks after vaccination. The characteristics of the different groups are summarized in Table S3. The sera from the different patients were pooled within the indicated groups. IgG purification was performed by affinity chromatography in Ab SpinTrap columns (GE Healthcare). Pooled sera from patients and healthy donors were directly applied to the columns and the purification procedures performed according to manufacturer instructions. The quality of the isolated antibodies was checked by SDS-PAGE under reducing and non-reducing conditions. The control IgG preparation (IgG control mix) was prepared by mixing two monoclonal human myeloma proteins, IgG1l (67%) and IgG2k (33%), purchased from Sigma-Aldrich. This process resulted in a k/l ratio of 0.5, which is well within the range found in normal serum (0.26 to 1.65).

Glycan array analysis

The glycan microarrays from the CFG (<http://www.functionalglycomics.org/static/consortium/resources/resourcecece-reh8.shtml>) were prepared from amine functionalized glycan structures covalently coupled in microarrays to N-hydroxysuccinimide-derivatized microscope slides as previously described (39). The mixed human sera, purified IgG and IgG control mix were screened at 180 μ g/ml for binding to glycans on CFG glycan array version 5.1 (610 different glycans) using a biotinylated anti-human IgG mAb at 5 μ g/ml followed by Alexa633-coupled streptavidin. To determine the specific binding to selected glycans, the antibody binding ratio (ABR) was calculated. The computed ABR represents the quotient of the respective sample RFU and the corresponding IgG control mix RFU. Data are expressed as the mean of RFU or ABR values from six repeated experiments, if not indicated otherwise.

Database search

The identity or characteristics of glycans was investigated by consulting the databases of the Consortium of Functional Glycomics (<http://www.functionalglycomics.org/fg/>) or PubMed (<http://www.ncbi.nlm.nih.gov/pubmed/guide/>). The online Bacterial Carbohydrate Structure Data Base (BCSDB) was consulted to identify the bacterial origin of the glycans (<http://csdb.glycoscience.ru/bacterial/>).

Suspension array

The Bio-Plex glycan suspension array was performed as previously described (40, 41). Briefly, end-biotinylated glycopolymers (Laboratory of Carbohydrate Chemistry, Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow, Russian Federation) were coupled to fluorescent carboxylated beads with a distinct ratio of red and infrared fluorescent dye (Bio-Rad Laboratories Inc., Hercules, CA, USA). Antibody diluent (PBS-1%BSA, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) incorporating 2000 beads of each region/well (50 μ L/well) was added to a 96-well Multiscreen HTS filter plate

(Millipore Corp., Billerica, MA, USA) previously soaked in 100µl of antibody diluent for 5 min. The plate was washed twice with 100µl washing buffer (PBS-0.02% Tween 20) using a vacuum manifold (Bio-Rad). Human serum samples were added to wells (in antibody diluent 1:4 (50µl/well)) and incubated on a shaker for 1h at RT in the dark. After incubation, the plate was washed three times with washing buffer. Secondary antibodies (R-PE conjugated goat anti-human IgG H+L; Southern Biotechnology Associates Inc., Birmingham, AL, USA, 25ng/well) were added and incubated on a shaker for 1h at RT in the dark. The plate was washed three times with washing buffer and beads were resuspended and shaken vigorously for 30s in 100µl of antibody diluent. The plate was analyzed on the Bio-Plex array reader, with which data were acquired in real time, analyzing 50 beads by their median fluorescence intensity (MFI) using Bio-Plex Manager 6.1 software (Bio-Rad).

Cell culture

Total peripheral blood leukocytes were isolated by dextran sedimentation using 3% dextranT500 (Amersham Pharmacia Biotech). Erythrocytes were subsequently lysed with increasing concentrations of sodium chloride. Leukocytes were washed with PBS and resuspended in DMEM (DMEM plus GlutaMAX™, Gibco®, Life Technologies). Cells were counted by a Sysmex cell counter (Sysmex Digitana AG, KX-21) and adjusted to a concentration of 30×10^6 cells/mL in DMEM. Mononuclear cells were obtained by density centrifugation using Pancoll solution (PAN-Biotech). For antibody-dependent cell-mediated cytotoxicity (ADCC) experiments, NK cells were isolated using the EasySep™ Human NK Cell Enrichment Kit (StemCell Technologies), according to manufacturers' instructions. Purified human NK cells were cultured in RPMI medium (plus GlutaMAX™, Gibco®, Life Technologies) supplemented with 10% FCS (Life Technologies), 1% Penicillin/Streptomycin (Life Technologies) and pre-stimulated with rhIL-2 (100 U/ml; Proleukin, Roche Diagnostics) at the indicated concentration. The pig kidney cell line, PK15, was maintained in DMEM supplemented with 10% FCS (Life Technologies), 1% Penicillin/Streptomycin (Life Technologies) and 0.1% of Endothelial Cell Growth Medium 2 (PromoCell).

Cytotoxicity assay

The antibody-dependent cell-mediated cytotoxicity assay against the pig kidney cell line PK15 was assessed in a 4 hours LDH release assay (Roche). To determine the effects of induced ADCC, dilutions of the human sera were adjusted to 1 mg/mL of IgG and added to the mixture of primary NK cells and PK15 cells. The PK15 cells were pre-treated with 0.4 units of α -Galactosidase from green coffee beans (Sigma) for 45min at 37°C (42) when indicated. The detection of released LDH was performed according to the manufacturer instructions.

Dermal-epidermal separation assay

Skin was obtained from healthy pigs kindly provided by the facility of Experimental Surgery of the Department of Clinical Research, University of Bern, Switzerland. The pigs were used in animal experiments related to surgical device testing and approved by the animal experimentation committee of the Canton of Bern, Switzerland. Pig skin was washed, depilated, prepared and embedded in optimum cutting temperature compound (Tissue-Tek® O.C.T.™ compound, Sakura Finetek). Cryosections of 6 μ m thicknesses were cut and placed on adhesive microscope slides (Starfrost®, Knittel Glass). Per slide six cryosections were placed. Common variable immune deficiency (CVID) patient sera, IgGSD patient sera or healthy human sera was added on the pig skin cryosections and incubated for 2 hours at 37°C according to a previously described protocol (32). Briefly, the sera were diluted 1:2 with PBS. After washing of the slides with PBS, human leukocytes were added and incubation proceeded for 3 hours at 37°C. Slides were fixed with formalin 3.7% and stained with hematoxylin and eosin. Dermal-epidermal separation (DES) was evaluated by light microscopy. Both the length of the dermal-epidermal junction (DEJ) and the DES along this DEJ were calculated. This allowed us to determine DES in percentages. To correct for the different total IgG levels of the sera, DES per mg of IgG was calculated and expressed as arbitrary units.

Statistical analysis

Correlation matrixes, heatmaps and hierarchical clustering were performed using “R” (The R Foundation for Statistical Computing, Version 3.0.2), statistical analysis and other illustrations were performed using Microsoft Excel (Microsoft Corporation, 2011, Version 14.0.0) and GraphPad PRISM (Graphpad Software, Inc., Version 6.0c). For clique distribution analysis, only groups with common terminal carbohydrate moiety that are represented at least with 12 glycans (2% of total) on CFG glycan array version 5.1 were taken into consideration. For statistical analysis, Kruskal-Wallis, paired Student’s *t*-test, Spearman correlation and Two-way ANOVA tests were used.

SUPPLEMENTARY MATERIALS

Fig. S1. Recognition of carbohydrate structures on CFG glycan array version 5.1 by purified IgG from patients and healthy donors.

Fig. S2. Blood group antigens recognition by individual sera from patients and healthy individuals.

Table S1. List of glycans bound for antibodies contained in the sera of healthy donors and patients.

Table S2. List of differentially recognized glycans by healthy donor and patients in cliques 1 and 3.

Table S3. Characteristics of patients with Primary Antibody Deficiency disorders included in the study.

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Author contributions: S.V.G. and P.J. designed the study. K.F.B., P.J. and S.V.G. wrote the manuscript. K.F.B. and S.V.G. analyzed the data. Glycan array experiments at the CFG were conducted under supervision of D.F.S. and R.D.C. Database searches and computational analysis of the data set was performed by K.F.B. Patient sample collection, classification and preparation were done by B. G., C.J. and P.J. Experimental work was done by K.F.B., E.D.G., M.A., A.D. under supervision by D.S., H.U.S., R.R. and S.V.G. Glycan synthesis was done by N. B. All authors had full access to the data, helped draft the report or critically revised the draft, contributed to data interpretation, reviewed and approved the final version of the report. Competing interests: The authors declare no conflict of interest.

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FIGURE LEGENDS

Figure 1. Antibody repertoire profiling on CFG glycan array version 5.1 reveals broad carbohydrate recognition defects in symptomatic primary antibody deficiencies (PADs). (A) Binding reactivities to 610 glycans expressed as relative fluorescence units (RFU) of serum-derived IgG at 180 mg/ml, pooled from symptomatic patients with PAD (n=76), specific antibody deficiency (SAD; n=5), common variable immunodeficiency (CVID; n=25), IgG subclass deficiency (IgGSD; n=8), or healthy donors (HD; n=43). (B) Glycan-binding reactivity matrices computed by the dendrogram clustering algorithm as outlined in Materials and Methods. The color key and distribution histogram are depicted. (C-D) Spearman's rank correlation matrix for healthy donors and immunodeficient patients in color code representation (C) and selected scatter diagrams (D). Significant values are reported, Kruskal–Wallis test.

Figure 2. Recognition of bacterial carbohydrate epitopes, blood group antigens, tumor-associated carbohydrates (TACAs) and Siglec ligands in PADs. (A) Dendrogrammed glycan reactivity matrix for PAD cohorts and healthy individuals towards bacterial antigens as identified by BCSDB analysis. (B) IgG binding reactivities to bacterial glycan epitopes expressed as relative fluorescence units (RFU). (C) Spearman's rank correlation matrix for recognition of the bacterial carbohydrate structures. (D) Recognition of glycan epitopes from *S. pneumoniae* (n=5), *H. influenzae* 357 (n=5), *N. meningitidis* (n=15), *E. coli* (n=36), *H. pylori* (n=47), and *Salmonella* (n=10) species. (E) Heatmap presentation showing blood group binding, expressed as antibody-binding (ABR) ratio. (F) Dendrogrammed glycan reactivity matrix for TACAs based on ABR. (G) Radial dendrogram for recognition of TACAs based on RFU. (H) Heatmap presentation illustrating reactivity to Siglec ligands based on ABR values. ABR values were computed based on isotype controls, as outlined in Materials and Methods. The color keys and distribution histograms are depicted. Significant values are reported, Kruskal–Wallis test.

Figure 3. Deviant recognition of distinct terminal carbohydrate moieties and specific loss of Gala reactivity in symptomatic PAD. (A) Binary deviation map ($P < 0.05$) of IgG immunoprofiles compared to healthy controls based on two-way ANOVA and hierarchical clustering analysis with identification of cliques 1-4. (B) Terminal carbohydrate moieties of epitopes in reactivity cliques 2 (non-significant deviation) and 4 (significant deviation). Numeric occurrence is indicated in parenthesis. (C, D) Recognition of Gala- (C) or GalNAc- (D) epitopes within clique 4 (significant deviation). (E) Deviation map based on degree of significance with identification of cliques A-E. (F) Bubble chart displaying terminal glycan structure distribution across cliques A-E. The size key and percentage numbers indicate frequencies.

Figure 4. Diminished recognition of Gala-terminated glycan epitopes and reduced Gala-dependent xenogeneic anti-porcine reactivity of CVID and symptomatic IgGSD sera. (A) IgG antibody reactivity to Galili, aLN and Aa3GN glycans as assessed by suspension array (multiplex immunoassay). Individual sera from healthy donors ($n=18$), HGG patients ($n=37$), CVID ($n=15$) and IgGSD ($n=8$) patients were analysed. Box-and-whisker diagrams. Significant values are reported, Kruskal–Wallis test. (B) Reduced surface staining of anti-Galili mAb reactivity following α -galactosidase treatment of porcine PK15 cells, as assessed by flow cytometry. Representative histogram (left panel) and summary (right panel). (C) Antibody dependent cellular cytotoxicity (ADCC) activity of primary human NK cells against PK15 cells at E/T ratio of 10:1, in absence or presence of α -galactosidase. (D) Histopathologic analysis of pig skin damage in cryosections incubated with healthy and patient's sera in presence of total leukocytes. Representative examples for each analyzed disease and control (PBS) are shown (left panel). The dashed lines indicate the dermal-epidermal junction. Pictures were taken with 40X magnification, scale bars 75 μ m. The damage induced was calculated as the dermal-epidermal separation normalized to the IgG concentration in the sera (right panel) (at least $n=7$). Results are representative of at least three (C) or seven (D) experiments. Significant values are reported (Kruskal–Wallis test). Paired Student's *t*-test.

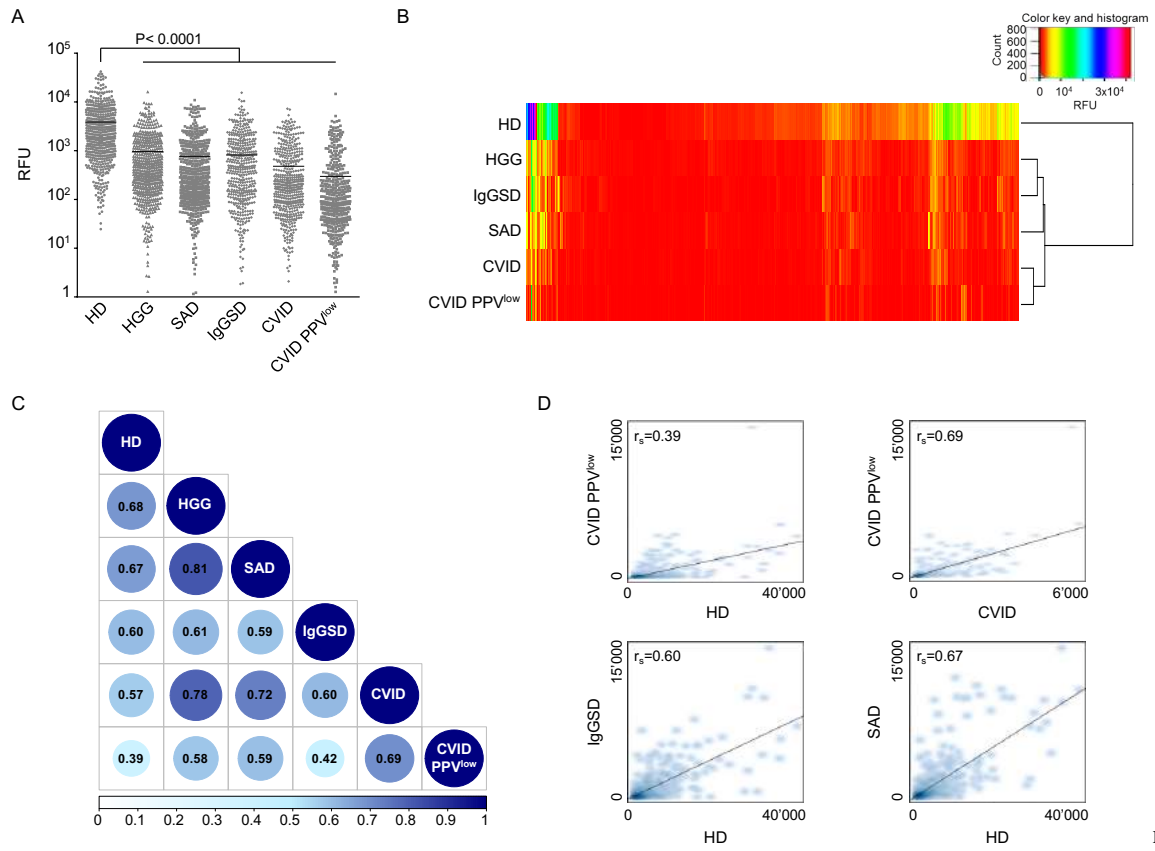


Fig.1

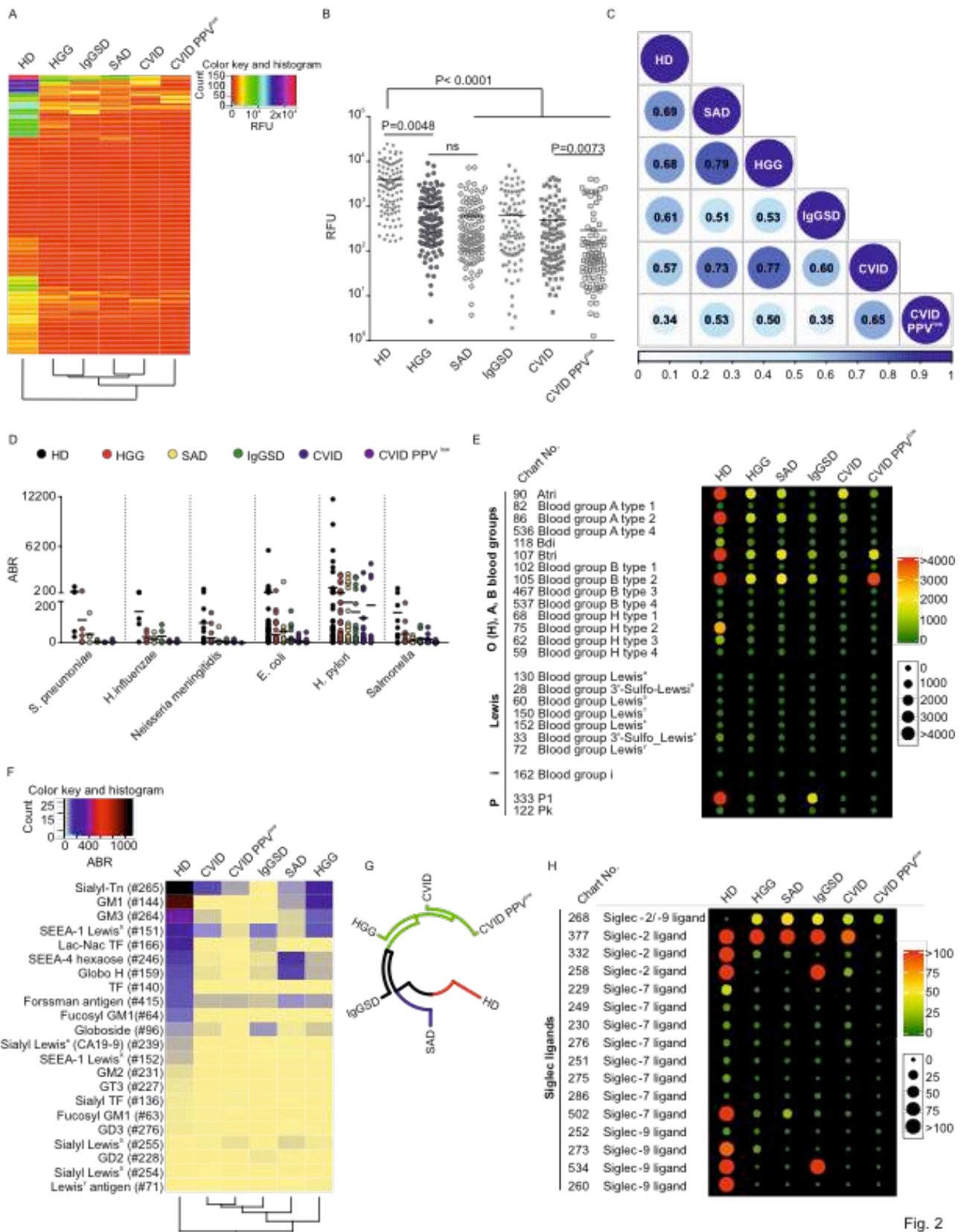


Fig. 2

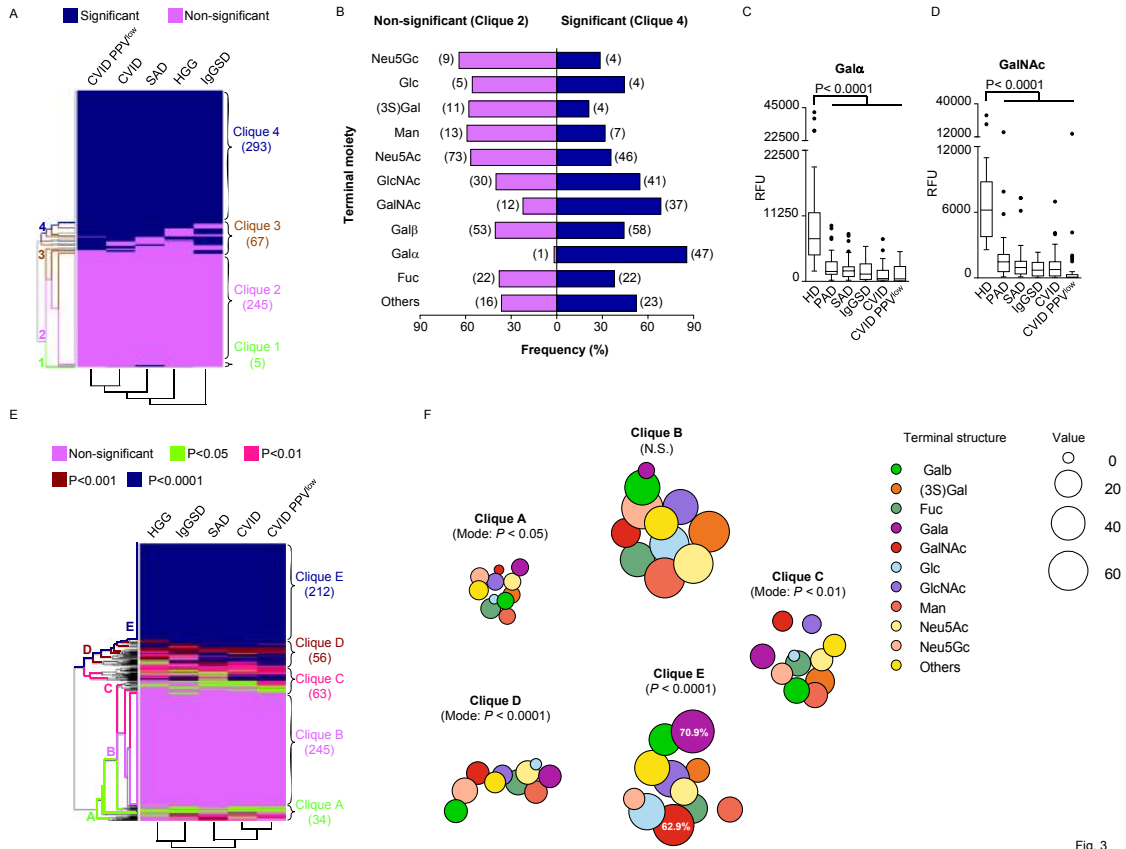


Fig. 3

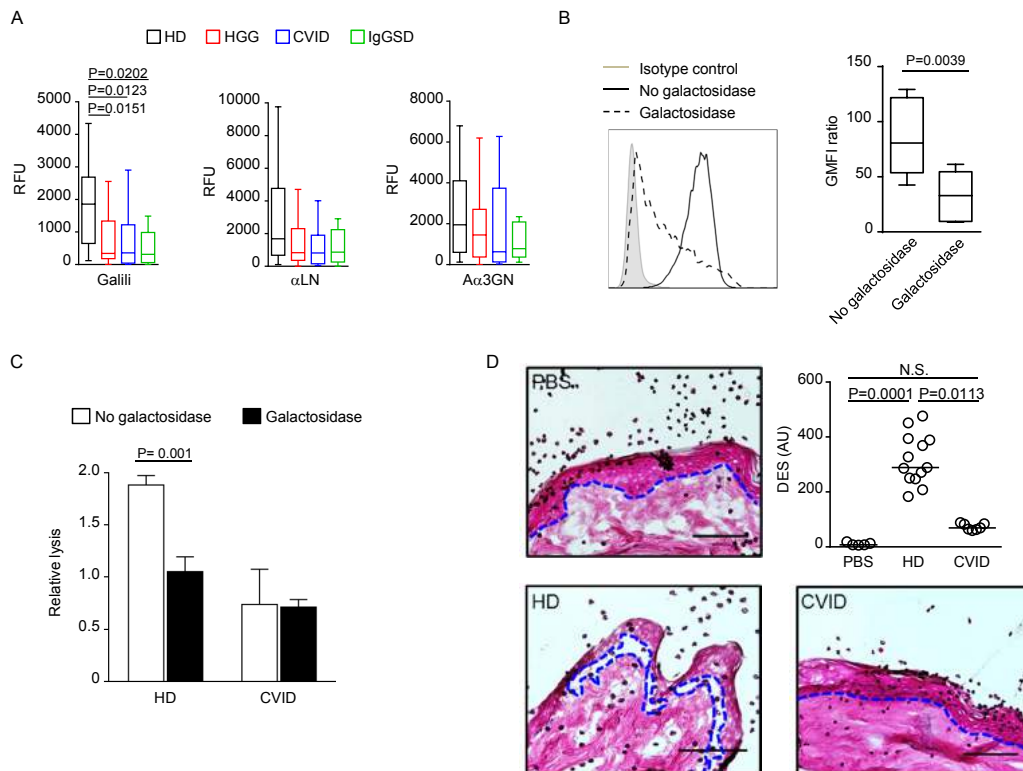


Fig. 4

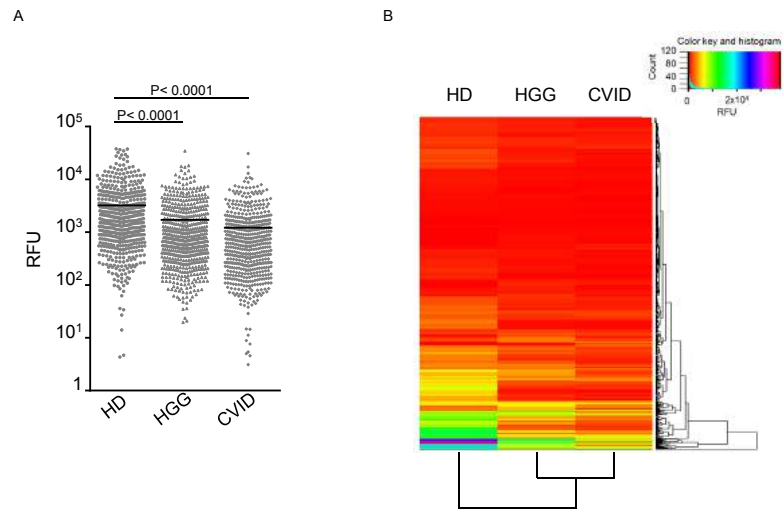


Fig. S1

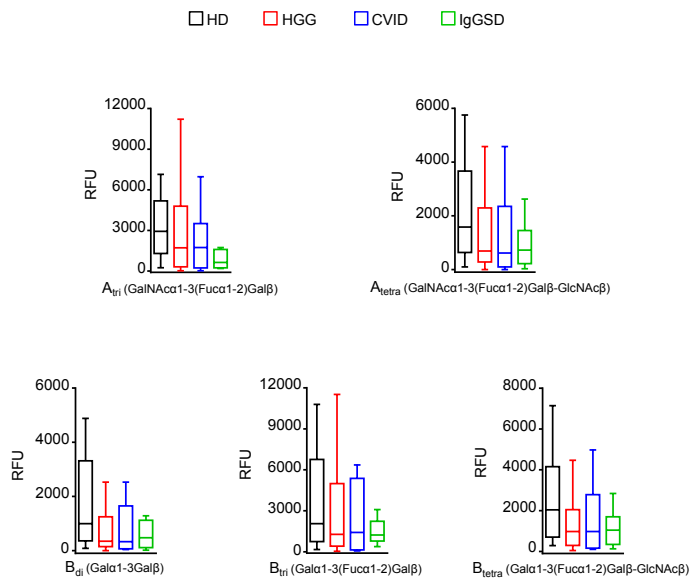


Fig. S2

Table S3. Characteristics of patients with Primary Antibody Deficiency disorders included in the study

Variable	HGG	SAD	CVID	PPV ^{low}	IgGSD
No. of patients	76	5	25	5	8
Age, years (range 11-83)*	44.55±15.63	39.8±13.82	39.08±13.98	48.4±13.78	47.8±14.79
Gender					
Male	30	2	13	3	3
Female	46	3	12	2	5
Immunoglobulin level (g/L) [¶]					
IgG (8.54-12.6)	6.85	11.99	5.23	4.62	8.91
IgG ₁ (4.9-11.4)					4.82
IgG ₂ (1.5-6.4)					4.50
IgG ₃ (0.2-1.11)					0.4
IgG ₄ (0.08-1.4)					0.68
IgA (0.98-2.03)	1.30	1.73	0.50	0.38	1.55
IgM (0.76-1.48)	0.99	0.60	0.66	0.45	1.14

* Representation of the mean age ± the standard deviation

¶ The values represent the mean immunoglobulin levels, reference values of serum immunoglobulins are defined in parentheses

Overall conclusion

Although ischemia reperfusion injury affects a wide variety of clinical conditions, there is no approved treatment available in clinical practice. Here, we focused on two types of I/R injury: firstly skeletal muscle I/R injury in a porcine model simulating the clinical situation of limb amputation (e.g. accidents) and replantation, and secondly a myocardial I/R injury model simulating acute myocardial infarction and its treatment in the clinical settings.

Based on the extensive experiments that we conducted in the current thesis, I can conclude that I/R injury either in the skeletal muscle or in the myocardium involves not only the activation of all plasma cascades but also the activation of the endothelial cells. This leads to a vicious circle that amplifies the damage and results in cell death and organ failure.

The ischemic endothelial cells express neo-epitopes and become a target for natural antibody deposition. The latter ends with complement activation through the classical and lectin pathways. In the current skeletal muscle and myocardial I/R injury porcine model, the lectin pathway showed an important role in the complement cascade activation. Moreover, the amplification loop of the alternative pathway increases the complement activation in the myocardial I/R model.

The ischemic endothelial cells also shed their protective glycocalyx layer as well as being activated during the reperfusion phase leading to upregulation of several proteins, among which FGL-2. This protein has a direct prothrombinase effect that leads to activation of thrombin generation and fibrin deposition on the surrounding tissue (both skeletal muscle and myocardium). Thrombin generation is also involved in more complement activation via its known direct cleavage of C5, generating C5b as well as C5a.

Pathophysiologically, I/R injury not only comprises the complement and coagulation systems, but also upregulation of bradykinin receptors. The kinin system also plays another important role in feeding the vicious circle of the I/R injury.

The known effects of C1-INH on different plasma cascade levels encouraged us to test its effect on skeletal muscle I/R injury in a porcine model of limb amputation (9 hours ischemia) and ex-vivo reperfusion (12 hours). The

use of C1-INH in the model indeed protected the endothelial cells from being activated and reduced the deposition of natural antibodies as well as expression of FGL-2. Furthermore, it reduced the glycocalyx shedding and provided more protection for the endothelial cells against skeletal muscle I/R injury. Overall, the use of C1-INH in this model reduced endothelial cell, complement and kinin activation as well as fibrin deposition. Based on these results, I would like to propose the use of C1-INH in clinical trials on I/R injury, as we were able to prove its efficacy in a clinically relevant large animal model.

The expression of hCD46 on porcine cells reduced coagulation activation; namely fibrin deposition as well as endothelial expression of FGL-2 and TF. Moreover, the significant increase of endothelial cell expression of tPA provides more protection against coagulation in the porcine model of limb amputation and ex-vivo reperfusion. In the myocardial I/R model, the use of pigs transgenic for human membrane cofactor protein (hCD46), with/without human thrombomodulin (hTM), leads to protection against reperfusion injury in the myocardial infarction model resulting in a significantly lower infarct size in the transgenic pigs compared to wild type. The expression of hCD46 inhibits the activation of lectin, alternative and classical pathways resulting in less endothelial cell activation and less glycocalyx shedding. This led to less activation of the coagulation system and reduced bradykinin receptor expression.

In summary, targeting the plasma cascades (complement, coagulation and kinin systems) is a very promising approach to reducing or preventing I/R injury in both skeletal and myocardial muscle in a pre-clinical setting.

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Dr. Kayluz Frias and Dr. Stephan von Gunten from ²Institute of Pharmacology, University of Bern

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Dr. Daniel Mettler, Ms. Olgica Beslac, and Mr. Kay Nettelbeck as well as their former colleague Mr. Daniel Zalokar from the Experimental Surgery Unit, Department for Biomedical Research, University of Bern.

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Curriculum Vitae

Personal details

Name: Mai Moustafa Ahmed Abdelhafez
Date of Birth 02nd December, 1982
Nationality Egyptian

Languages: Arabic (mother tongue), English and German

Educational background

2013-2017 PhD in immunology, University of Bern
2006-2010 Master in Clinical Pharmacy, Ain- Shams University
2000-2005 Bsc Pharmaceutical Sciences, Ain- Shams University

Education and training

2017 European meeting on Complement (Copenhagen, Denmark)
Annual Meeting of the Society of Thrombosis and
Swiss Society for pharmacology and toxicology (Bern,
Switzerland)
Hemostasis Research (Basel, Switzerland)
Cardiovascular and metabolic meeting (Fribourg, Switzerland)
Scientific writing (University of Bern)

2016 Scientific communication (University of Bern)
Continuing training for performance in animal experiments
(SGV meeting, Basel, Switzerland)
Introduction to Systematic Literature Searching:
Biomedical, Molecular and Chemical Databases (University
of Bern)

2015 Biostatistics for Non-Statisticians: Good Practices, Misuse
and Pitfalls (University of Bern)
Book Club- cellular and Molecular Immunology (University
of Bern)

2014 ABS Animal Science Day 2015 (Bern, Switzerland)
Practical Course in Fluorescent Staining, Confocal
Microscopy and Image Analysis (University of Bern)
LTK1- Introduction course in laboratory animal sciences
(University of Zurich)
Flow Cytometry (FACS Course, University of Bern)
Swiss Experimental Surgery Symposium (Bern, Switzerland)

Awards

09.2017 Outstanding abstract achievement award in
European Meeting of Complement in Human
Disease

List of publications, abstracts and presentation

Publication in peer-reviewed journals

Mai M Abdelhafez, Jane Shaw, Damian Sutter, Jonas Schnider, Yara Banz, Hansjörg Jenni, Esther Voegelin, Mihai A. Constantinescu, and Robert Rieben. Effect of C1-INH on ischemia/reperfusion injury in a porcine limb ex vivo perfusion model. **Molecular Immunology**. 2017 June 20; **88:116-124**

Manuscripts in preparation/submitted

Mai M. Abdelhafez, Jane Shaw, Alain Despont, and Robert Rieben. Refinement of a closed chest porcine myocardial infarction model and standardizing sampling procedure for subsequent molecular and cellular analysis. Submitted to Journal of Visualized Experiments

Mai M Abdelhafez, Anjan K. Bongoni, Alain Despont, David Kiermeir, Jonas Schnider, Hansjörg Jenni, Pavan Garimella, Andrea Bähr DVM, Nikolai Klymiuk, Eckhard Wolf, David Ayares, Esther Voegelin, Mihai A. Constantinescu, Jörg D Seebach, and Robert Rieben. Investigation of the protective role of human CD46 expression on plasma cascade and endothelial cell activation in the context of autologous ex-vivo perfusion of pig limbs. Manuscript in preparation

Mai M. Abdelhafez, Jane Shaw, Alain Despont, Yara Banz, Sarah Longnus, Andrea Bähr, Nikolai Klymiuk, Eckhard Wolf, Robert Rieben. Reduction of myocardial ischemia reperfusion injury in pigs by overexpression of human membrane co-factor protein. Manuscript in preparation

Peter Jandus, Kayluz Frias Boligan, Richard D. Cummings, Elisabeth de Graauw, Camilla Jandus, **Mai M. Abdelhafez**, Alain Despont, Robert Rieben, Dagmar Simon, Hans-Uwe Simon, Stephan von Gunten. Patients with Primary Antibody Deficiency exhibit a qualitative defect in carbohydrates recognition by circulating immunoglobulins. Manuscript in preparation

Oral and poster presentations at national and international conferences

Reduced susceptibility of pig hearts overexpressing human membrane cofactor protein to ischemia/reperfusion injury **presented as poster** at the 14th International Xenotransplantation Association. Baltimore, MD, USA. September 2017.

Reduction of myocardial ischemia reperfusion injury in pigs by overexpression of human membrane co-factor protein **orally presented** at the 16th European Meeting on Complement in Human Diseases. Copenhagen, Denmark. September 2017.

Reduction of ischemia reperfusion injury by C1-esterase inhibitor in a porcine limb amputation and an ex-vivo reperfusion model **presented as poster** at the Spring Meeting of the Swiss Society of Pharmacology and Toxicology. Bern, Switzerland. April 2017.

The cross talk between coagulation cascade and endothelial cells in a porcine limb ischemia reperfusion injury model and the protective role of C1-INH **orally presented** at the 61st Annual Meeting of the Society of Thrombosis and Hemostasis Research. Basel, Switzerland. February 2017.

Reduction of myocardial ischemia reperfusion injury in pigs by (over)expression of endothelial cell protective human genes **orally presented** at 6th Cardiovascular and Metabolic Research meeting. Fribourg, Switzerland. January 2017.

The protective role of C1-INH on ischemia reperfusion injury in a porcine limb amputation and reperfusion model **presented as poster** at the 15th European Meeting on Complement in Human Diseases. Kanazawa, Japan. September 2016.

Declaration of Originality

Last Name, First Name: **Abdelhafez Mai**

Matriculation Number: **12-137-501**

I hereby declare that this thesis represents my original work and that I have used no other sources except as noted by citation.

All data, tables, figures and text citations, which have been reproduced from any other source, including the internet, have been explicitly acknowledged as such.

I am aware that in case of non-compliance, the Senate is entitled to withdraw the doctorate degree awarded to me on the basis of the present thesis, in accordance with the "Statut der Universität Bern (Universitätsstatut, UniSt)" Art. 69, of 7 June 2011.

Place, date

Bern, 21.08.2017

Signature

A handwritten signature in blue ink that reads "Mai Abdelhafez". The signature is written in a cursive style with a long horizontal stroke at the end.