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14. ABSTRACT

In this project, endothelial cell protection and extracorporeal machine perfusion will be combined to achieve a massive prolongation of the time between traumatic amputation and replantation. In the first year, Milestone 1: 'Establishment of ex vivo – in vivo models for extremity I/R injury in a PFC scenario' was reached and work on Milestone 2: 'Validation of ex vivo – in vivo models for the effect of EC protection on extremity I/R injury in a PFC scenario' was started. In particular, a massively increased I/R injury was observed in pig limbs perfused after 9 h ischemia as compared to less than 1 h, both in extracorporeal and in vivo limb perfusion. Currently available analyses include limb weight gain, wet/dry ratio of muscle tissue, perfusion blood electrolytes and lactate, as well as immunofluorescence analysis of dystrophin patterns for muscel tissue integrity. Despite a 4-month lockdown of animal experimentation due to the COVID-19 pandemic the project did not incur a major delay until today.

15. SUBJECT TERMS

Ischemia/reperfusion injury, complement, endothelial activation, muscle damage, perfusion time, glycocalyx

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1. INTRODUCTION

The study consists in the development of *in vivo* and *ex vivo* models to assess strategies for the prevention of ischemia reperfusion (I/R) injury in a Prolonged Field Care (PFC) scenario. There is a limited intervention capacity at the point of injury that may force the use of battlefield tourniquet to prevent fatal blood loos. Moreover, several hours or even days prior to revascularization or replantation may lead to severe I/R injury. Mainly extensive muscle tissue damage will be the local consequence in the affected extremity, but severe I/R injury may also lead to systemic inflammatory response syndrome and even multiorgan failure. The models to be developed will therefore be used to provide guidance in the management of I/R injury and to test technical feasibility and clinical efficacy of promising therapeutic interventions in the RUCK-TRUCK-HOUSE-PLANE operational context. Our project aims at reducing I/R injury after surgical revascularization or replantation of traumatically devascularized or amputated extremities in a PFC scenario in order to reduce limb loss and prevent systemic consequences of I/R injury. The goal of the project is to prove that a combination of pharmacological endothelial protection via simple perfusion of the devascularized or amputated extremity at RUCK or TRUCK level, followed by machine perfusion of the extremity at HOUSE / PLANE level, will allow prevention of I/R injury and successful surgical revascularization or replantation even if performed 24 or more hours after the injury was incurred.

2. KEYWORDS

Ischemia/reperfusion injury, complement, endothelial activation, muscle damage, perfusion time, glycocalyx shedding

3. ACCOMPLISHMENTS

What were the major goals of the project?

Our specific aims are:

1. Develop an ex vivo – in vivo large-animal model to study the consequences of I/R injury in extremity vascular injury as occurring in a PFC scenario.

Milestone 1: Establishment of ex vivo – in vivo models for extremity I/R injury in a PFC scenario
Target date: CY19/20-Models for extremity I/R injury in a PFC scenario
Completion: Both -*ex vivo* and -*in vivo* models have been established.

2. Validate these models for the screening of pharmacological interventions that can be applied at RUCK/TRUCK level to promote EC protection and reduce/control I/R injury.

Milestone 2: Validation of ex vivo – in vivo models for the effect of EC protection on extremity I/R injury in a PFC scenario
Target date: End CY20 and CY21
Completion: Partially completed. We have completed groups 3 (6/6 pigs) and 4 (7/7 pigs), and established and performed ex vivo re-perfusion with C1-INH intervention using 10 Limbs (5/6 limbs for group 5 and 5/6 limbs for group 6). Moreover, we have started writing the paper on the comparison of the two models.

3. Combine and test the full PFC scenario comprising pharmacological EC protection and prolonged machine perfusion in the surgical replantation setting.

Milestone 3: Experimental assessment of the whole PFC scenario for massive prolongation of the time window to replantation / revascularization as compared to the situation today – 33 hours vs. 6 hours.
Target date: CY22

What was accomplished under these goals?

Overview of accomplished work packages according to time line

Work Package	Groups	Year 1	Year 2 Sep. 2020-Jan. 2021	Year 2 Feb.-Aug. 2021	Status at end of Year 2
WP 1	Group 1	6 / 6 limbs			completed
	Group 2	6 / 6 limbs			completed
	Group 3	1/ 6 pigs	1 / 6 pigs	4 / 6 pigs	completed
	Group 4	3 / 7 pigs	1 / 7 pigs	3 / 7 pigs	completed
WP 2	Group 5	0	0	5 / 6 limbs	5 / 6 limbs completed
	Group 6	0	0	5 / 6 limbs	5 / 6 limbs completed
	Group 7	0		0	blind study 3 / 12 pigs planned
	Group 8	0		0	

WP 1. Develop an ex vivo – in vivo large-animal model to study the consequences of I/R injury in extremity vascular injury as occurring in a PFC scenario.

Major activities and specific objectives

1. Completed groups 3 (6/6 pigs) and 4 (7/7 pigs) by performing in vivo re-perfusion with 5 pigs were used for the 9h ischemia, 4 pigs were used for 1h ischemia.
2. Sample collection: plasma, serum and tissues from muscle, lung, liver, kidney, arterial and vein
3. Perfusion data from both in vivo and ex vivo models was collected and analyzed: plasma/serum and muscle tissue samples (muscle damage, complement activation, pro-inflammatory cytokines, growth factors).

WP 2. Validation of the models for testing the efficacy of pharmacological interventions aimed at preventing endothelial cell (EC) activation at RUCK/TRUCK stage

Major activities and specific objectives

1. Prepared sterile limb flushing solutions, C1-inhibitor (C1-INH) and vehicle control, pairwise blinded.
2. Established and performed ex vivo re-perfusion with C1-INH intervention using 10 Limbs (5/6 for group 5 and 5/6 for group 6), double blinded study until data analysis.
3. Sample collection: plasma, serum and muscle tissues.
4. Limb weight post/pre- re-perfusion and wet/dry ratio was analyzed.
5. Perfusion data was collected and analyzed: muscle tissue samples (muscle damage, endothelial cell activation, deposition of complement activation products C1q and C3b/c).

Significant results and/or key outcomes, including major findings, developments and/or conclusions

1. Limb perfusion time

In order to evaluate differences between the control group and the group with 9-hour ischemia, the perfusion time was compared in both the extracorporeal and in vivo perfusion groups (groups 1, 2, 3, 4), and in ex vivo perfusion with C1-INH intervention (groups 5 and 6). Un-paired one-way ANOVA was used to analyze the difference between groups. It was shown that there was no significant difference between the ischemia group and controls except for the groups with ex vivo perfusion (Figure 1). This might be due to the additional pro-inflammatory and pro-coagulant effect of the ex vivo perfusion setting itself.

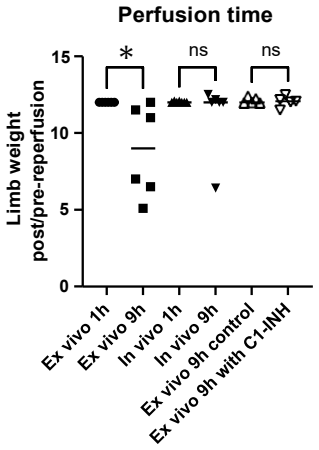

Figure 1. Limb perfusion time. Comparison between the control groups and the groups with 9h ischemia, from extracorporeal re-perfusion time and in vivo re-perfusion time, and the ex vivo perfusion time with C1-INH intervention. Extracorporeal re-perfusion time in limbs which were exposed < 1h ischemia (control group) or 9h ischemia (ischemic group), n= 6 limbs. In vivo re-perfusion time in limbs undergone < 1h ischemia (control group, n=7) or 9h ischemia (ischemic group, n=6). The ex vivo perfusion time undergone 9h ischemia rinsed with vehicle buffer, or rinsed with C1-INH (n=5). Un-paired one way ANOVA, *p＜0.05.

2. Comparison of ex vivo and in vivo perfusion models

Ischemia re-perfusion (I/R) injury occurs upon re-perfusion of vascularized tissue after an extended period of ischemia. It is a common source of illness and death in a wide variety of conditions, including myocardial infarction and stroke. I/R injury is also an unavoidable event in organ transplantation and has a major effect on short- and long-term graft survival. The pathogenesis of I/R injury is complex, and multiple factors (i.e., complement activation, the coagulation system, leukocytes, cytokines, chemokines, and adhesion molecules) are all thought to contribute to its development.

2.1 Tissue damage from porcine limbs exposed to extracorporeal and in vivo re-perfusion

The measurement of plasma enzyme activity is important in the diagnosis of muscle disease, and while the activities of several enzymes may be elevated, creatine kinase (CK) is the most sensitive indicator of muscle damage. Very high levels of CK are found in skeletal muscle, primarily the isoform pattern in muscle MM form. We showed that CKMM levels were increased in ischemic limbs at end point (EP) when compared to base line (BL), but there was no difference between ischemic and control limbs, exposed to extracorporeal re-perfusion (Figure 2A). There was no difference either between EP and BL or ischemic and control limbs exposed to in vivo re-perfusion (Figure 2B).

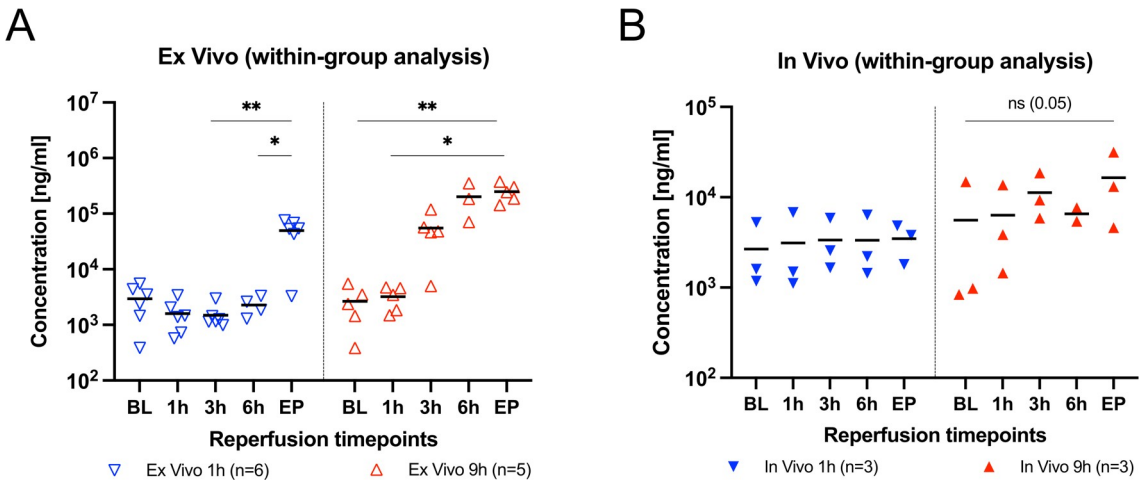

Figure 2. Plasma concentration of CKMM from the limbs after extracorporeal and in vivo re-perfusion. (A) Comparison of plasma concentration of CKMM across different timepoints of extracorporeal re-perfusion between ischemic (9h of ischemia) and control limbs (< 1h ischemia). (B) Comparison of plasma concentration of CKMM across different timepoints of in vivo re-perfusion between ischemic (9h of ischemia) and control limbs (< 1h ischemia). Un-paired one-way ANOVA (*p< 0.05; **p< 0.01).

2.2 Growth factors from porcine limbs exposed to extracorporeal and in vivo re-perfusion

Vascular endothelial growth factor (VEGF), a major angiogenic factor, mediates a variety of disease conditions through promotion of angiogenesis. It also plays a critical role as a potent proinflammatory cytokine in a variety of physiologic and pathologic immune responses. We showed that VEGF levels were increased in ischemic limbs at EP exposed to extracorporeal and in vivo re-perfusion, when compared to BL (Figure 3A&B).

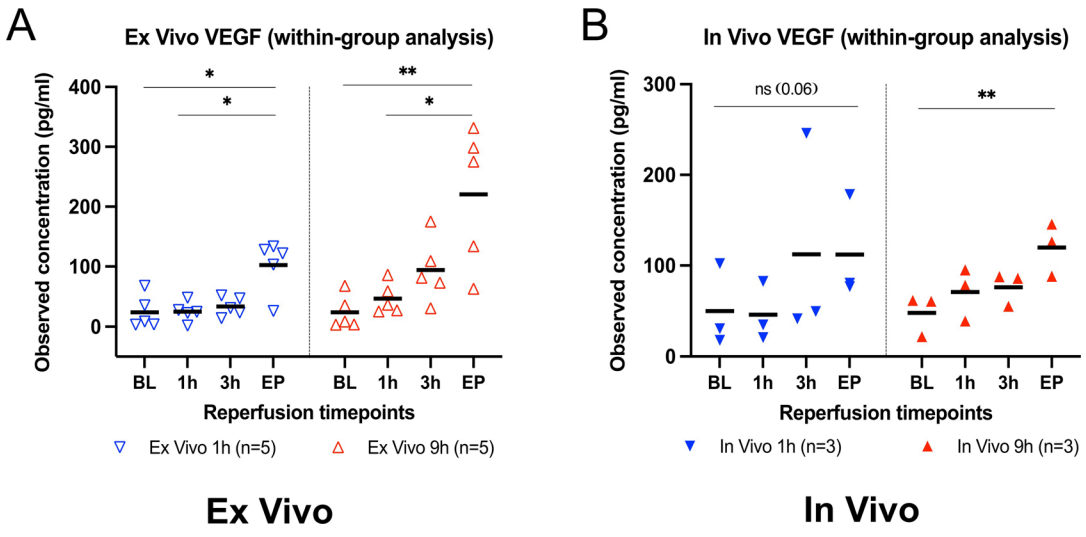

Figure 3. Plasma concentration of VEGF from the limbs after extracorporeal and in vivo re-perfusion. (A) Comparison of plasma concentration of VEGF across different timepoints of extracorporeal re-perfusion between ischemic (9h of ischemia) and control limbs (< 1h ischemia). (B) Comparison of plasma concentration of VEGF across different timepoints of in vivo re-perfusion between ischemic (9h of ischemia) and control limbs (< 1h ischemia). Un-paired one-way ANOVA (*p< 0.05; **p< 0.01).

Basic fibroblast growth factor (bFGF) is a potent angiogenic and mitogenic polypeptide produced mainly by mast cells. Several studies have shown that bFGF delivered during re-perfusion protects against ischemia re-perfusion injury and has therapeutic potential in ischemic injury (Zhao et al., 2022). We found no significant difference in bFGF expression levels between either EP and BL, or ischemic and control limbs exposed to extracorporeal perfusion (Figure 4A), however, its level was significantly higher in ischemic limbs at EP exposed to vivo perfusion, when compared to BL (Figure 4B).

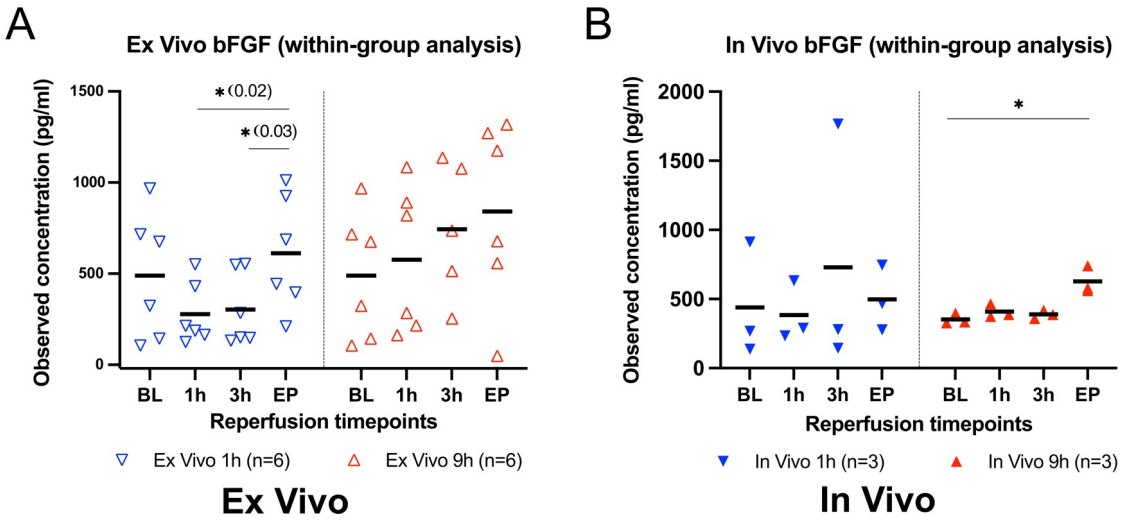

Figure 4. Plasma concentration of bFGF from the limbs after extracorporeal and in vivo re-perfusion. (A) Comparison of plasma concentration of bFGF across different timepoints of extracorporeal re-perfusion between ischemic (9h of ischemia) and control limbs

(< 1h ischemia). (B) Comparison of plasma concentration of bFGF across different timepoints of in vivo re-perfusion between ischemic (9h of ischemia) and control limbs (< 1h ischemia). Un-paired one-way ANOVA (*p< 0.05).

Platelet-derived growth factors (PDGF) have an ambiguous role in microvascular dysfunction caused by ischemia re-perfusion injury. On the one hand, PDGF may exert vascular stabilizing and antiapoptotic actions, on the other, PDGF signaling mediates neointimal formation and exacerbates chronic rejection in allografts. The balance between these potentially harmful and beneficial actions determines the final outcome of the allografts. We showed that PDGF levels were increased in ischemic limbs at EP when compared to BL, but there was no difference between ischemic and control limbs, exposed to extracorporeal re-perfusion (Figure 5A). There was no difference either between EP and BL or ischemic and control limbs exposed to in vivo re-perfusion (Figure 5B).

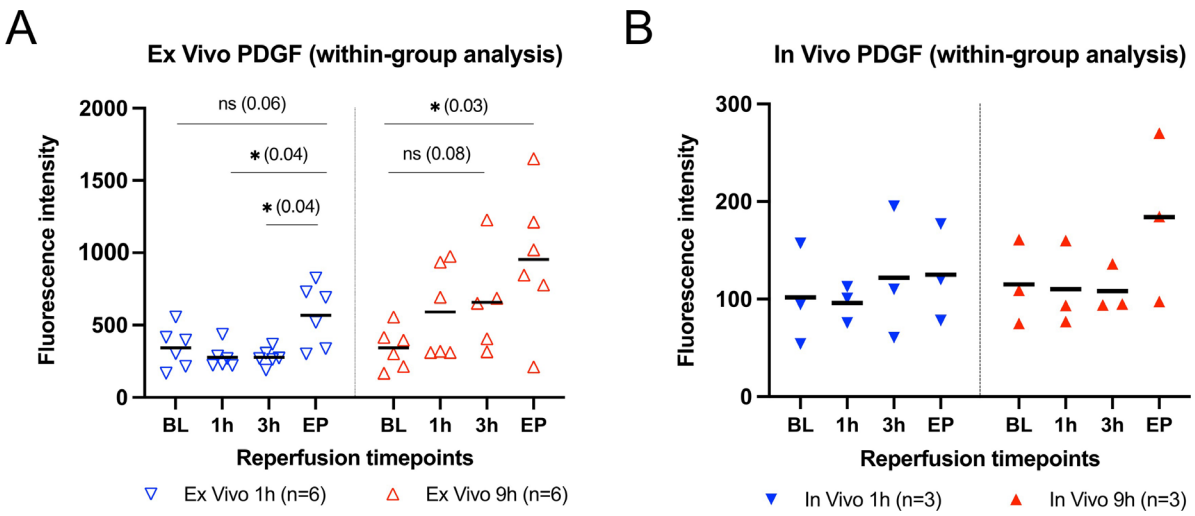

Figure 5. Plasma concentration of PDGF from the limbs after extracorporeal and in vivo re-perfusion. (A) Comparison of plasma concentration of PDGF across different timepoints of extracorporeal re-perfusion between ischemic (9h of ischemia) and control limbs (< 1h ischemia). (B) Comparison of plasma concentration of PDGF across different timepoints of in vivo re-perfusion between ischemic (9h of ischemia) and control limbs (< 1h ischemia). Un-paired one-way ANOVA (*p< 0.05).

2.3 Pro-inflammatory cytokines from porcine limbs exposed to extracorporeal and in vivo re-perfusion

Interruption of blood flow by occlusion of afferent blood vessels and subsequent re-perfusion initiates an inflammatory response in the jeopardized tissue. Within minutes of re-perfusion, reactive oxygen species are generated, stimulating the release of cytokines and expression of adhesion molecules on damaged cells in re-perfused tissues. Several hours after the onset of re-perfusion, neutrophils and other inflammatory cells are activated and adhere to damaged cell membranes, further enhancing the inflammatory response. This inflammatory response ultimately leads to cell damage. We showed that plasma level of pro-inflammatory Interleukin-6 (IL-6) was significantly higher in both control and ischemic limbs exposed to extracorporeal re-perfusion, when compared to BL (Figure 6A). There was no difference either between EP and BL or ischemic and control limbs exposed to in vivo re-perfusion (Figure 6B).

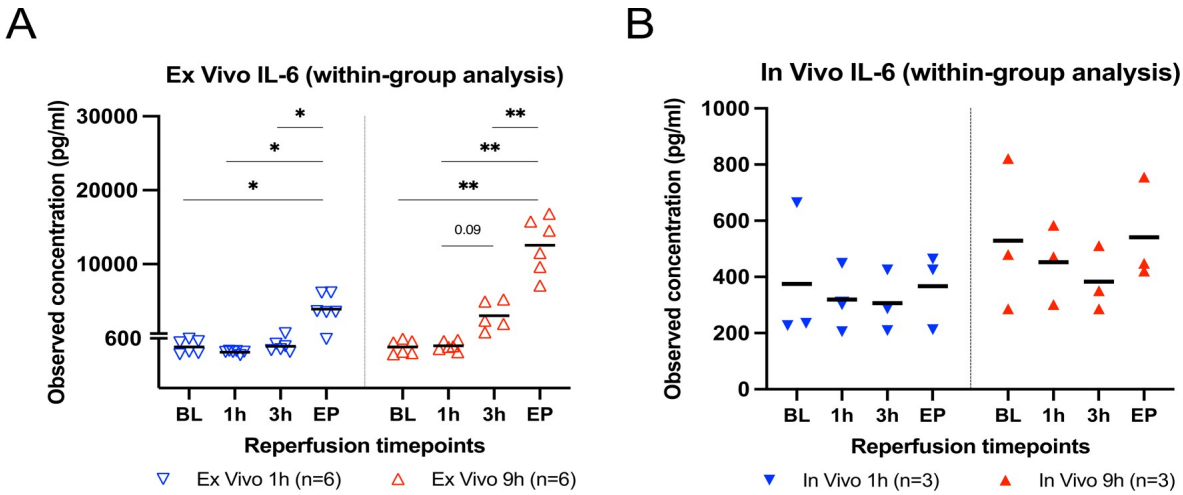

Figure 6. Plasma concentration of IL-6 from the limbs after extracorporeal and in vivo re-perfusion. (A) Comparison of plasma concentration of IL-6 across different timepoints of extracorporeal re-perfusion between ischemic (9h of ischemia) and control limbs (< 1h ischemia). (B) Comparison of plasma concentration of IL-6 across different timepoints of in vivo re-perfusion between ischemic (9h of ischemia) and control limbs (< 1h ischemia). Un-paired one-way ANOVA (*p< 0.05; **p< 0.01).

Tumor Necrosis Factor alpha (TNF-alpha), is an inflammatory cytokine produced by macrophages/monocytes during acute inflammation and is responsible for a diverse range of signaling events within cells, leading to necrosis or apoptosis. We showed that there was no difference either between EP and BL or ischemic and control limbs exposed to both extracorporeal and in vivo re-perfusion (Figure 7A&B).

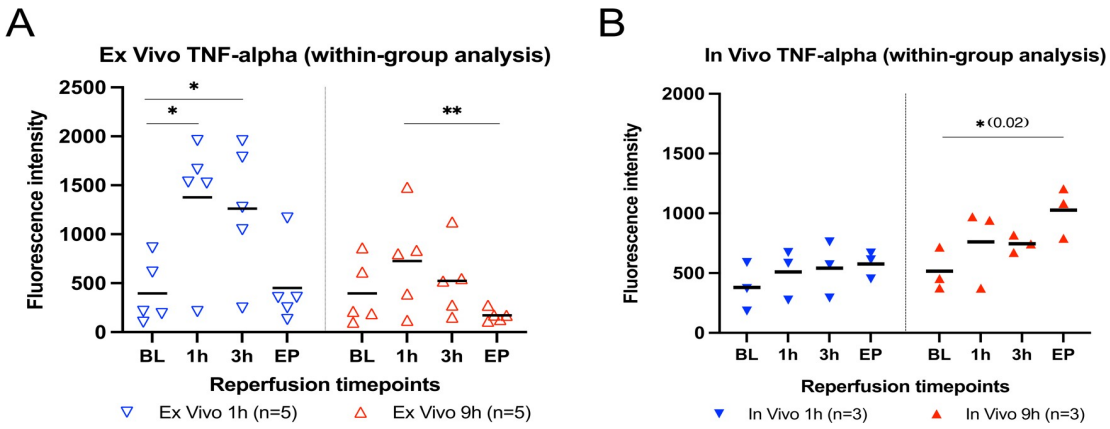

Figure 7. Plasma concentration of TNF-alpha from the limbs after extracorporeal and in vivo re-perfusion. (A) Comparison of plasma concentration of TNF-alpha across different timepoints of extracorporeal re-perfusion between ischemic (9h of ischemia) and control limbs (< 1h ischemia). (B) Comparison of plasma concentration of TNF-alpha across different timepoints of in vivo re-perfusion between ischemic (9h of ischemia) and control limbs (< 1h ischemia). Un-paired one-way ANOVA (*p< 0.05).

2.4 Macrophage related cytokines from porcine limbs exposed to extracorporeal and in vivo re-perfusion

In addition to macrophages, IL-8 is also released by epithelial cells, airway smooth muscle cells, and endothelial cells. We showed that the plasma level of IL-8 expression was significantly higher in ischemic limbs compared to control limbs at EP exposed to both extracorporeal and in vivo perfusion, when compared to BL (Figure 8A&B).

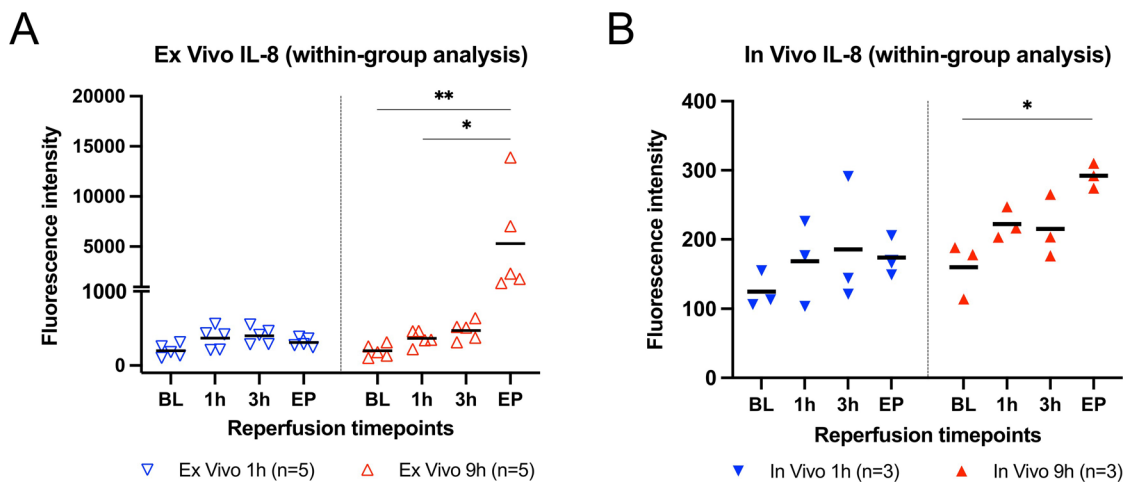

Figure 8. Plasma concentration of IL-8 from the limbs after extracorporeal and in vivo re-perfusion. (A) Comparison of plasma concentration of IL-8 across different timepoints of extracorporeal re-perfusion between ischemic (9h of ischemia) and control limbs (< 1h ischemia). (B) Comparison of plasma concentration of IL-8 across different timepoints of in vivo re-perfusion between ischemic (9h of ischemia) and control limbs (< 1h ischemia). Un-paired one-way ANOVA (*p< 0.05; **p< 0.01).

Monocyte chemoattractant protein-1 (MCP-1/CCL2) is one of the key chemokines that regulate migration and infiltration of monocytes/macrophages. We showed that the plasma level of MCP-1 expression was significantly higher in ischemic limbs compared to control limbs at EP exposed to both extracorporeal and in vivo perfusion, when compared to BL (Figure 9A&B).

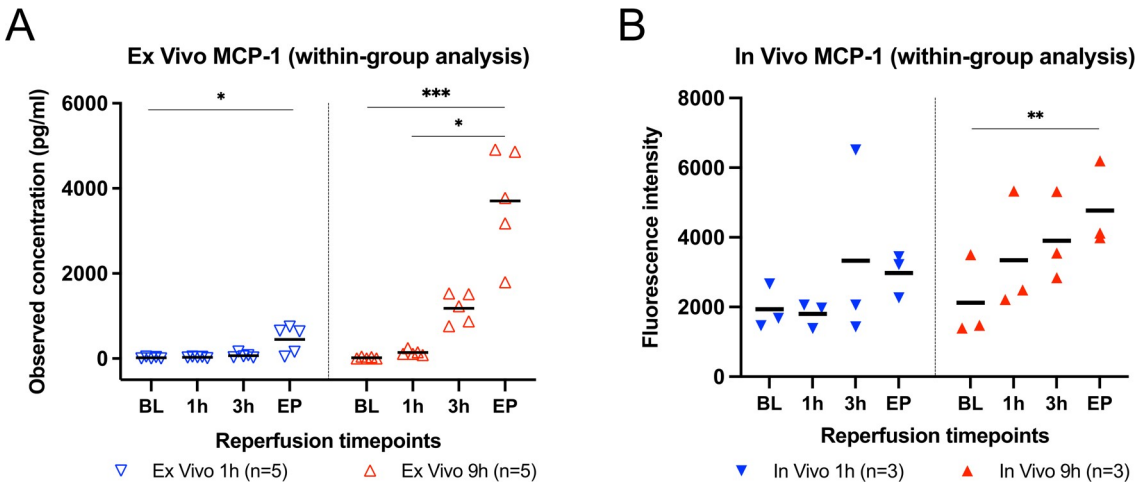

Figure 9. Plasma concentration of MCP-1 from the limbs after extracorporeal and in vivo re-perfusion. (A) Comparison of plasma concentration of MCP-1 across different timepoints of extracorporeal re-perfusion between ischemic (9h of ischemia) and control limbs (< 1h ischemia). (B) Comparison of plasma concentration of MCP-1 across different timepoints of in vivo re-perfusion between ischemic (9h of ischemia) and control limbs (< 1h ischemia). Un-paired one-way ANOVA (*p< 0.05; **p< 0.01).

2.5 Complement activation from porcine limbs exposed to extracorporeal and in vivo re-perfusion

The complement system consists of a set of distinct plasma proteins that react in a cascade manner upon triggering by various stimuli, such as infection and tissue injury. Previous studies of ours and others have demonstrated that complement activation contributes to the pathogenesis of I/R injury (Abdelhafez et al., 2017, Zhou et al., 2000). Several studies have also suggested that the formation of MAC (C5b-9) in epithelial cells is a critical effector mechanism through which complement mediates I/R injury (Zhou et al., 2000). We showed that the plasma levels of sC5b_9 kept increasing over the re-perfusion time course in both ischemic and control limbs at 1h, 3h and EP exposed to extracorporeal re-perfusion, when compared

to BL (Figure 10A). The plasma levels of sC5b_9 was significantly higher in both ischemic limbs at EP exposed to in vivo re-perfusion, when compared to BL (Figure 10B).

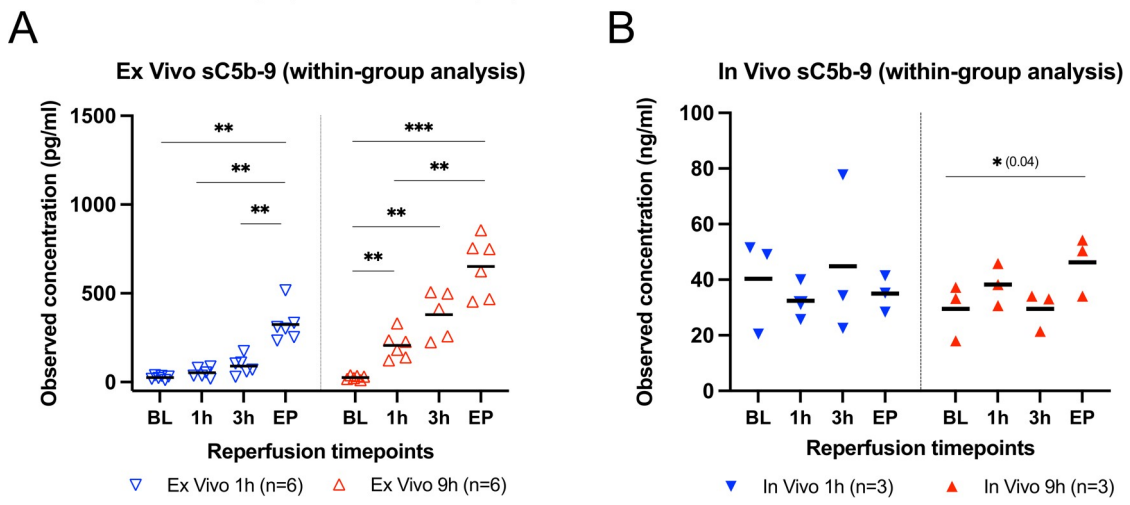

Figure 10. Plasma concentration of sC5b_9 from the limbs after extracorporeal and in vivo re-perfusion. (A) Comparison of plasma concentration of sC5b_9 across different timepoints of extracorporeal re-perfusion between ischemic (9h of ischemia) and control limbs (< 1h ischemia). (B) Comparison of plasma concentration of sC5b_9 across different timepoints of in vivo re-perfusion between ischemic (9h of ischemia) and control limbs (< 1h ischemia). Un-paired one-way ANOVA (*p< 0.05; **p< 0.01).

C3a and C5a, which are released during complement activation, play an important role in the initiation and regulation of inflammatory responses. The plasma levels of C3a were decreased at 1h re-perfusion then increased over the re-perfusion time course in ischemic limbs exposed to extracorporeal re-perfusion (Figure 11A). The plasma levels of C3a were significantly higher in ischemic limbs at EP exposed to in vivo re-perfusion, when compared to BL (Figure 11B).

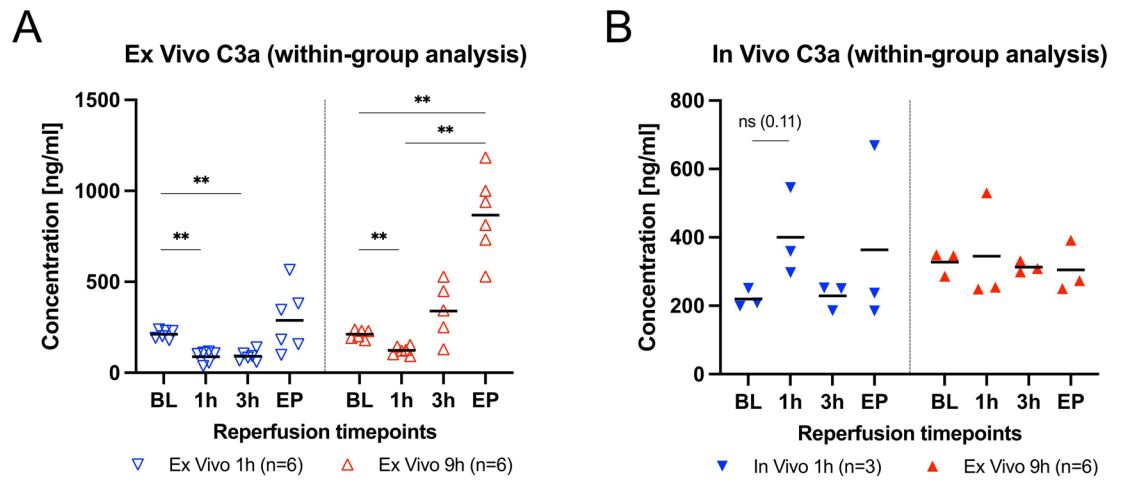

Figure 11. Plasma concentration of C3a from the limbs after extracorporeal and in vivo re-perfusion. (A) Comparison of plasma concentration of C3a across different timepoints of extracorporeal re-perfusion between ischemic (9h of ischemia) and control limbs (< 1h ischemia). (B) Comparison of plasma concentration of C3a across different timepoints of in vivo re-perfusion between ischemic (9h of ischemia) and control limbs (< 1h ischemia). Un-paired one-way ANOVA (**p< 0.01).

The plasma levels of C5a were increased at 1h re-perfusion then decreased over the re-perfusion time course in ischemic limbs exposed to extracorporeal re-perfusion (Figure 12A). However, there was no difference in the plasma levels of C5a between ischemic limbs at EP exposed to in vivo re-perfusion, when compared to BL (Figure 12B).

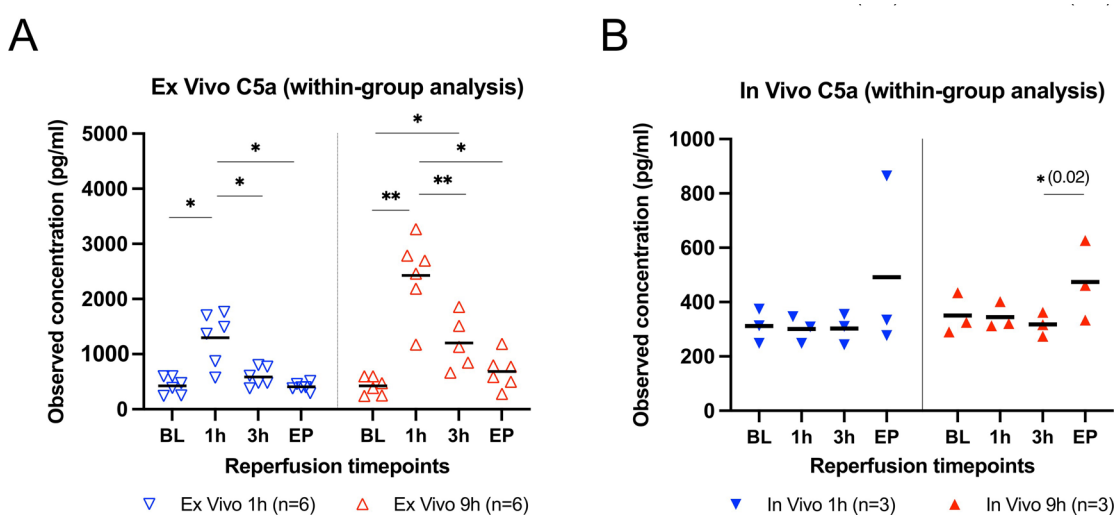

Figure 12. Plasma concentration of C5a from the limbs after extracorporeal and in vivo re-perfusion. (A) Comparison of plasma concentration of C5a across different timepoints of extracorporeal re-perfusion between ischemic (9h of ischemia) and control limbs (< 1h ischemia). (B) Comparison of plasma concentration of C5a across different timepoints of in vivo re-perfusion between ischemic (9h of ischemia) and control limbs (< 1h ischemia). Un-paired one-way ANOVA (*p< 0.05; **p< 0.01).

2.6 Thrombotic – Fibrinolytic systems from porcine limbs exposed to extracorporeal and in vivo re-perfusion

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. Tissue factor (TF), an important activator of the extrinsic pathway, can be released from both activated vascular endothelium as well as neutrophils that are recruited at the site of I/R injury. Inhibition of TF has been shown to reduce renal injury in a model of kidney I/R injury (Sevastos et al., 2006). We also showed that the plasma levels of TF were lower in ischemic limbs exposed to extracorporeal re-perfusion (Figure 13A). However, there was no difference in the plasma levels of TF between ischemic limbs at EP exposed to in vivo re-perfusion, when compared to BL (Figure 13B).

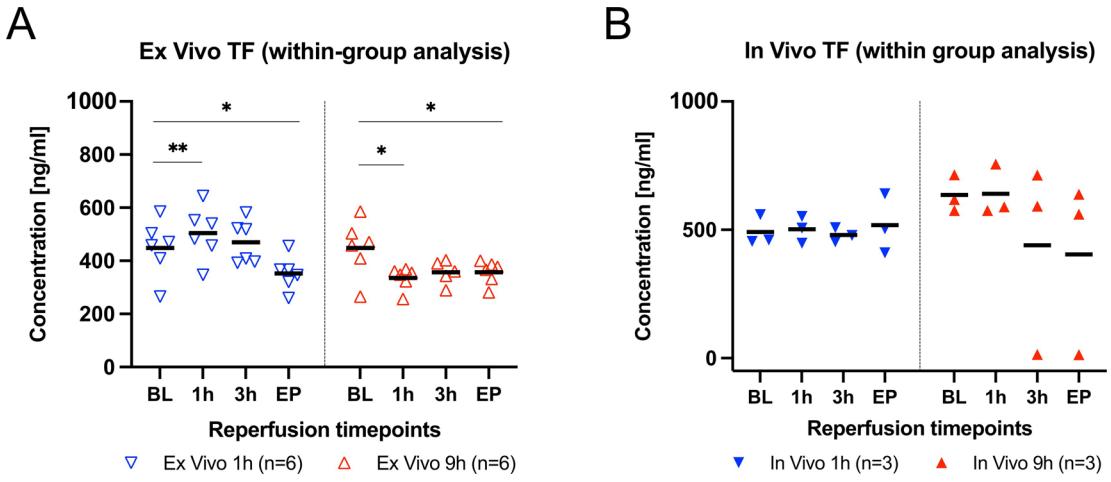

Figure 13. Plasma concentration of TF from the limbs after extracorporeal and in vivo re-perfusion. (A) Comparison of plasma concentration of TF across different timepoints of extracorporeal re-perfusion between ischemic (9h of ischemia) and control limbs (< 1h ischemia). (B) Comparison of plasma concentration of TF across different timepoints of in vivo re-perfusion between ischemic (9h of ischemia) and control limbs (< 1h ischemia). Un-paired one-way ANOVA (*p< 0.05; **p< 0.01).

Fibrinolysis is a fundamental biological process that enables the maintenance of tissue perfusion by preventing clot formation in blood vessels. Plasmin is the principal effector protease in the fibrinolytic

system, mediating the dissolution of fibrin polymers. Its zymogen form, plasminogen, is activated by tissue-type plasminogen activator (tPA) and, to a much lesser degree, by urokinase-type plasminogen activator. Beyond their established role in the fibrinolytic system, these serine proteases have previously been documented to contribute to different biological processes, including the regulation of distinct steps in leukocyte recruitment to postischemic tissue (Praetner et al., 2018). Studies have demonstrated that PAI-1 plays a pivotal role during the initiation of the postischemic inflammatory response by directing neutrophils to the site of injury (Praetner et al., 2018). We also showed that the plasma levels of PAI-1/tPA were increased over the re-perfusion time course in both ischemic and control limbs at 1h, 3h and EP exposed to extracorporeal and in vivo re-perfusion, when compared to BL (Figure 14A&B).

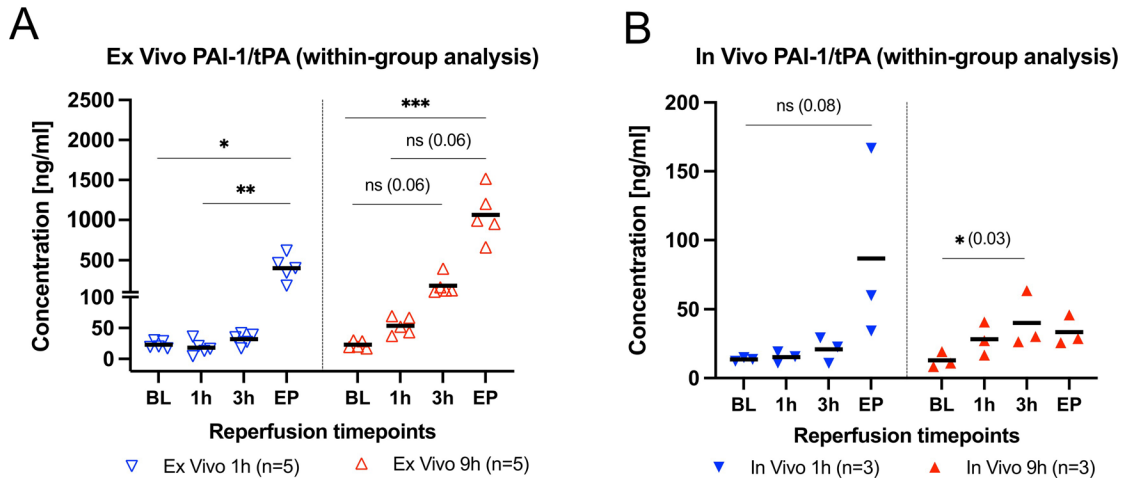

Figure 14. Plasma concentration of PAI-1/tPA from the limbs after extracorporeal and in vivo re-perfusion. (A) Comparison of plasma concentration of PAI-1/tPA across different timepoints of extracorporeal re-perfusion between ischemic (9h of ischemia) and control limbs (< 1h ischemia). (B) Comparison of plasma concentration of PAI-1/tPA across different timepoints of in vivo re-perfusion between ischemic (9h of ischemia) and control limbs (< 1h ischemia). Un-paired one-way ANOVA (*p< 0.05; **p<0.01).

3. Effect of C1-inhibitor (C1-INH) from porcine limbs exposed to extracorporeal re-perfusion

3.1 Tissue damage from porcine limbs exposed to extracorporeal re-perfusion with or without C1-INH intervention

Immunofluorescence staining of muscle tissue samples from limbs that underwent extracorporeal perfusion (n=5 limbs) suggested that 9h ischemic limbs showed major tissue damage upon ex vivo re-perfusion. There was an improvement in the muscle damage from the limbs that had undergone extracorporeal perfusion with C1-INH intervention. Figure 15 shows dystrophin (green) in the muscle of extracorporeally perfused limbs with or without C1-INH intervention.

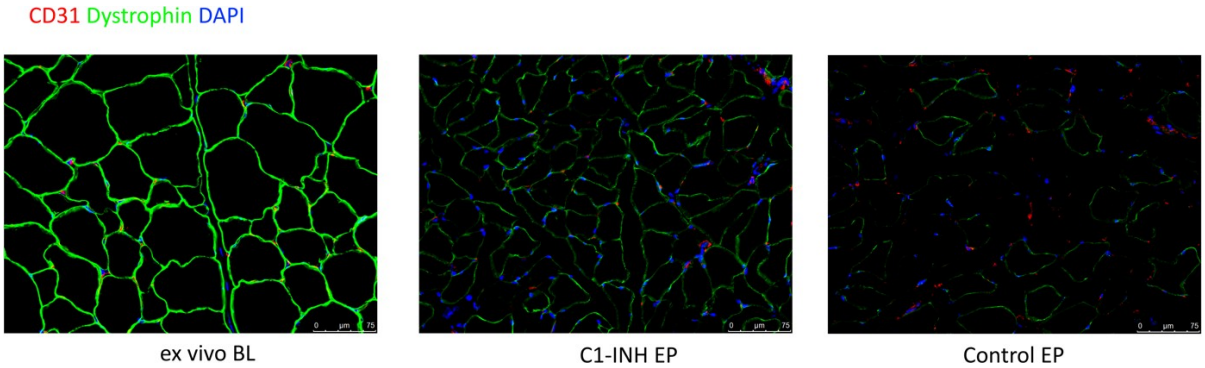

Figure 15. Dystrophin distribution in muscle of porcine limbs after extracorporeal re-perfusion with or without C1-INH intervention. Dystrophin (in green) distribution in limb tissue collected at baseline (before perfusion) (left), from ischemic limbs (9h of ischemia) after extracorporeal perfusion with C1-INH intervention (middle), and without C1-INH intervention (right).

3.2 Endothelial cell activation from porcine limbs exposed to extracorporeal re-perfusion with or without C1-INH intervention

Vascular endothelial cells in the skeletal muscle are the first cells that come in contact with the blood during re-perfusion. Healthy endothelial cells provide protection against inflammation and spontaneous activation of the plasma cascades. E-selectin, also known as CD62 antigen-like family member E (CD62E), is a selectin cell adhesion molecule expressed only on endothelial cells activated by cytokines. As shown in Figure 16, both CD31 and E-selectin immunostaining showed a less expression in ischemic limbs both with and without C1-INH intervention, the quantification is in process.

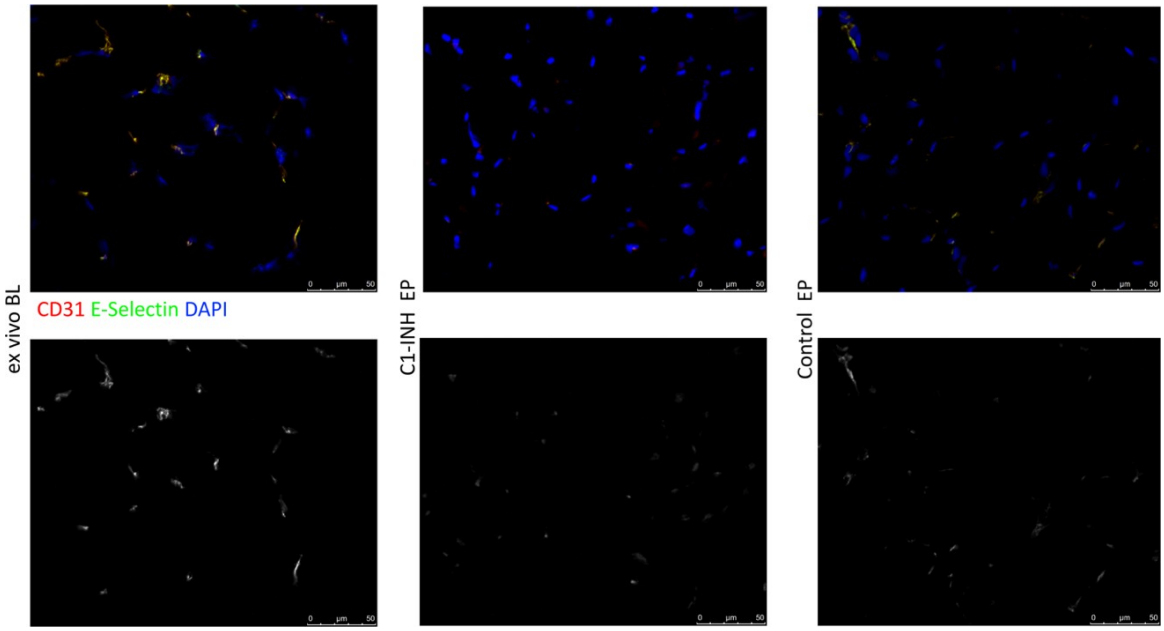

Figure 16. E-selectin in muscle of porcine limbs after extracorporeal re-perfusion with or without C1-INH intervention. E-selectin (in green, co-localization with CD31 in yellow) distribution in limb tissue collected at baseline (before perfusion) (left), from ischemic limbs (9h of ischemia) after extracorporeal perfusion with C1-INH intervention (middle), and without C1-INH intervention (right). E-selectin (in grey) distribution in limb tissue collected at baseline (before perfusion) (left), from ischemic limbs (9h of ischemia) after extracorporeal perfusion with C1-INH intervention (middle), and without C1-INH intervention (right).

3.3 Complement deposition from porcine limbs exposed to extracorporeal re-perfusion with or without C1-INH intervention

A role of complement activation in I/R injury was first demonstrated by Hill and Ward in the early 70s and thereafter a key role of the complement system in the mediation of tissue injury was further established in different animal models (D'Ambrosio et al., 2001). Convincing evidence for such a role is provided by studies in which I/R injury is attenuated or prevented using knock out models or intervention with complement inhibitors (Abdelhafez et al., 2017). Our previous studies showed a significant deposition of complements in 4-6 hour ischemic limbs and the use of inhibitor C1-INH led to a significant reduction of complement deposition in the tissue at the end of re-perfusion, suggesting a key role for complements in I/R injury (Abdelhafez et al., 2017). As shown in Figure 17 and 18, both C1q and C3b/c immunostaining showed less expression in ischemic limbs with C1-INH intervention, when compared to those without C1-INH intervention. The quantification is in process.

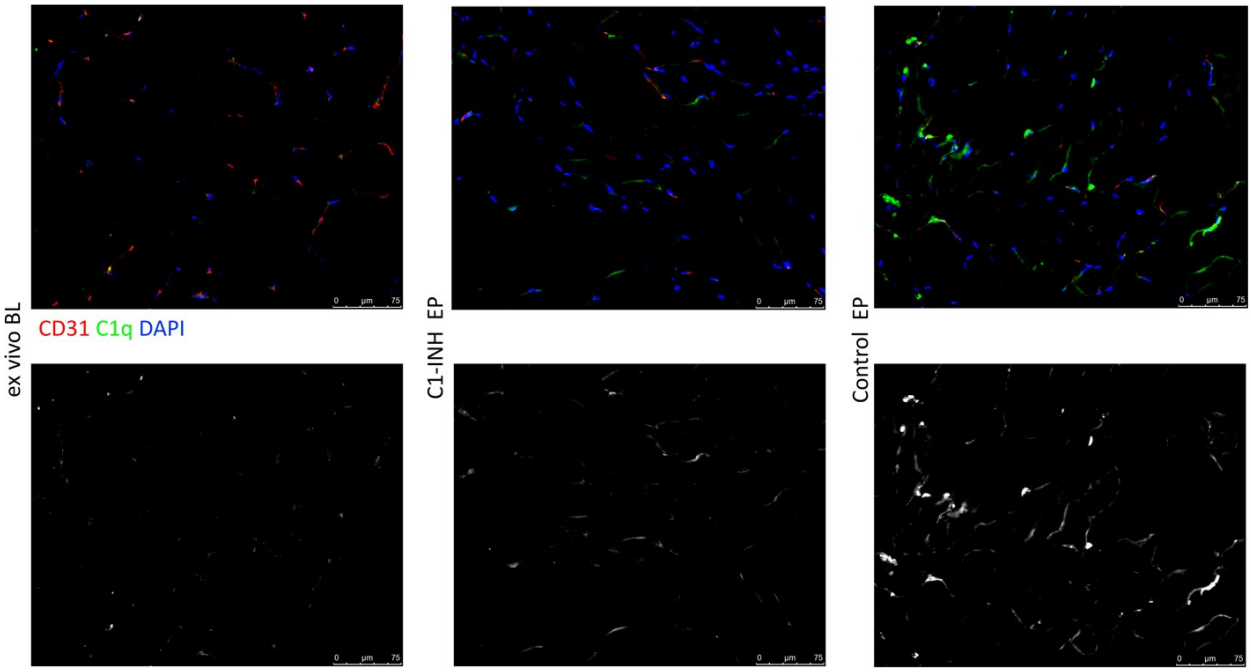

Figure 17. C1q deposition in muscle of porcine limbs after extracorporeal re-perfusion with or without C1-INH intervention. C1q (in green) deposition in limb tissue collected at baseline (before perfusion) (left), from ischemic limbs (9h of ischemia) after extracorporeal perfusion with C1-INH intervention (middle), and without C1-INH intervention (left). C1q (in grey) deposition in limb tissue collected at baseline (before perfusion) (left), from ischemic limbs (9h of ischemia) after extracorporeal perfusion with C1-INH intervention (middle), and without C1-INH intervention (right).

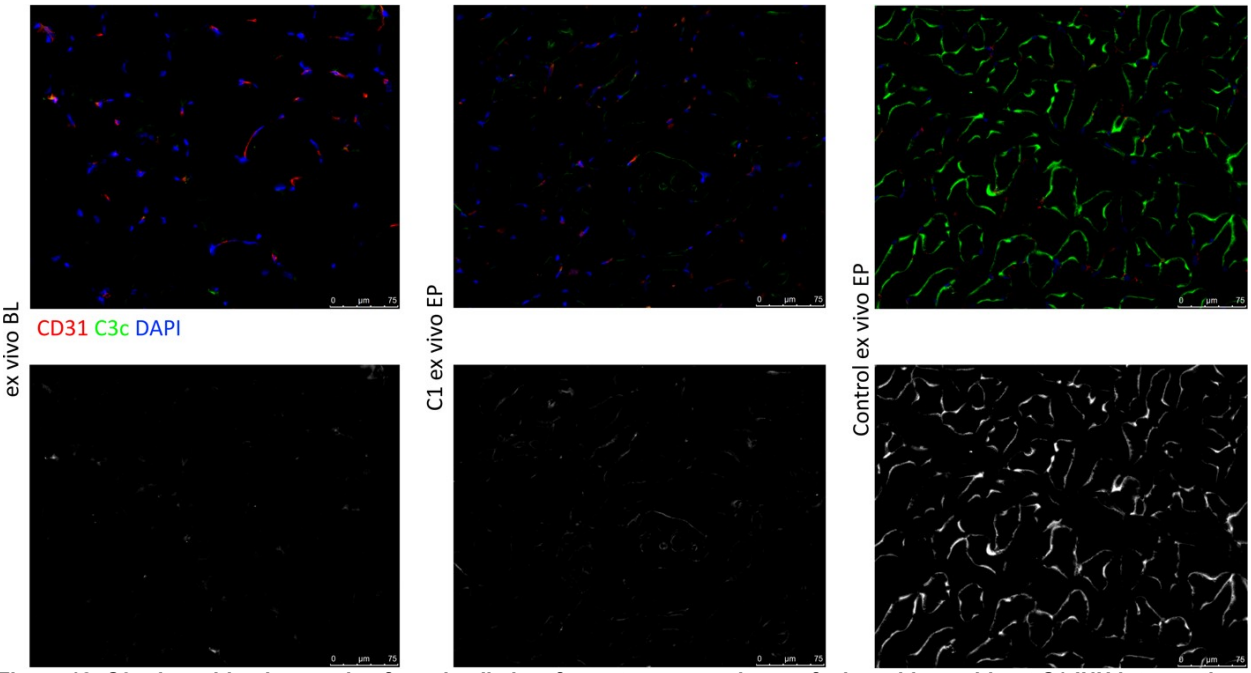

Figure 18. C3c deposition in muscle of porcine limbs after extracorporeal re-perfusion with or without C1-INH intervention. C3c (in green) deposition in limb tissue collected at baseline (before perfusion) (left), from ischemic limbs (9h of ischemia) after extracorporeal perfusion with C1-INH intervention (middle), and without C1-INH intervention (right). C3c (in grey) deposition in limb tissue collected at baseline (before perfusion) (left), from ischemic limbs (9h of ischemia) after extracorporeal perfusion with C1-INH intervention (middle), and without C1-INH intervention (right).

What opportunities for training and professional development has the project provided?

Nothing to report

How were the results disseminated to communities of interest?

Nothing to report.

What do you plan to do during the next reporting period to accomplish the goals?

In the next quarter, we will perform the last two limbs to complete groups 5 and 6, analyze all the samples obtained from ex-vivo perfusion with C1-INH intervention from groups 5 and 6 including endothelial cell activation, antibody deposition in the tissue, immune cell infiltration, pro-inflammatory and anti-inflammatory cytokines, and growth factors. In addition, we will start groups 7 and 8 from WP2, with 3 more in vivo experiments with C1-INH intervention, collect the samples from in vivo perfusions (groups 7 and 8) including blood, muscle, liver, kidney, and lung.

4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?

Nothing to report.

What was the impact on other disciplines?

Nothing to report.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS

Changes in approach and reasons for change

Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them

Problem description: The Animal Facility of the Department for BioMedical Research was closed for six months for renovation until March 2021. And due to the COVID-19 pandemic, research activities at the

University of Bern were restricted. We informed our Science Officer and Contract Officer and will be requesting a cost-neutral extension in 2022.

Solutions: From February to August 2021, to catch up with some of the experiments, we had an intensive period of performance and completed the groups 3 and 4 and the whole of WP1, and analyzed all the samples obtained from ex-vivo and in-vivo perfusion from groups 1, 2, 3, 4 including endothelial cell activation, complement deposition in the tissue, immune cell infiltration, pro-inflammatory and anti-inflammatory cytokines, and growth factors. Moreover, we performed 10 limbs from groups 5 and 6, analyzed the samples obtained from ex-vivo perfusion with C1-INH intervention from groups 5 and 6 including endothelial cell activation, and complement deposition in the tissue. In addition, we have planned to complete groups 5 and 6, and to start groups 7 and 8 from WP2 before the end of calendar year 2021.

Changes that had a significant impact on expenditures

Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report.

Significant changes in use or care of human subjects

Nothing to report.

Significant changes in use or care of vertebrate animals.

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS

- **Publications, conference papers, and presentations**

  ### Journal publications

  Nothing to report.

  ### Books or other non-periodical, one-time publications

  Nothing to report.

- **Other publications, conference papers, and presentations**

1. Emara AF, Brahim BB, Hoyos IA, Wang J, Zollet V et al., Ex vivo machine perfusion access ischemia re-perfusion injury of amputated porcine limbs. ESOT 2021, Milan, 29 Aug. - 1 Sep. 2021 (Poster)
2. Brahim BB, Emara AF, Hoyos IA, Wang J, Zollet V et al., The role of complement in ischemia/re-perfusion injury of amputated limbs from ex vivo and in vivo re-perfusion. ICW 2021, Berlin, 6-10 Dec. 2021
3. Hoyos IA, Hirsiger S, Zollet V, Fuest L, Wang J, et al., Ischemia re-perfusion injury: extracorporeal or in vivo perfusion? 57th Swiss Plastic surgery congress 2021, Lucerne, 22-23 Oct. 2021

- **Website(s) or other Internet site(s)**

  Nothing to report.

- **Technologies or techniques**

  Nothing to report.

- **Inventions, patent applications, and/or licenses**

  Nothing to report.

- **Other Products**

  Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS
What individuals have worked on the project?

Name: **Junhua Wang** (from February 2021)
Project Role: Scientific Associate
Researcher Identifiers: ORCID 0000-0002-6110-3711
Nearest person month worked: 4
Contribution to Project: Prepare the experiments, perform and lead the experiments, perform the lab measurements, collect the samples, archive and record the project results, contribute to the reports.

Name: **Robert Rieben**
Project Role: PI
Researcher Identifiers: ORCID 0000-0003-4179-8891
Nearest person month worked: 2
Contribution to Project: Global supervision of the project.

Name: **Esther Vögelin**
Project Role: Co-PI
Researcher Identifier: ORCID: 0000-0003-4179-8891
Nearest person month worked: 1
Contribution to Project: Responsible for all surgical aspects of the project.

Name: **Nicoletta Sorvillo**
Project Role: Senior Research Assistant
Researcher Identifiers: none
Nearest person month worked: 5
Contribution to Project: Analyze the data obtained from both ex vivo and in vivo re-perfusion.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report.

What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

An updated Quad Chart has been submitted.

9. APPENDICES