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TITLE: In Vivo and Ex Vivo Models to Study Ischemia/Reperfusion Injury, Endothelial Cell Protection, and Limb Preservation in a Prolonged Field Care Scenario

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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 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 started the analysis of the collected tissue for comparison of the two models.
To be done: Additional in vivo perfusion experiments will be performed before the end of CY20 and analysis on the collected tissue and serum samples will be performed.

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?

Aim 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. ACURO permission obtained.
2. Performed extracorporeal perfusion using 6 pigs for both groups:1 hr ischemia and 9 hrs ischemia.
3. Started to perform in vivo reperfusion after COVID 19 lockdown; 2 pigs were used for the 1 hr ischemia and 1 for the 9 hrs ischemia.
4. Sample collection: plasma, serum and tissues.
5. Perfusion data has been collected and analyzed.
6. Tissue edema has been quantified and compared between the control and ischemic group.
7. Started immunofluorescent analysis of ischemic and control muscle tissue in ex vivo group.

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

In order to evaluate differences between the non-ischemic control (< 1 hr ischemia) and the ischemic limb (9 hrs ischemia) perfusion time was compared in both the extracorporeal (Figure 1A) and in vivo (Figure 1B) perfusion groups. As shown in figure 1 the reperfusion time in the ischemic group is shorter when compared to control, suggesting ischemia/reperfusion injury.

Statistical analysis between the two groups has not been performed yet, due to the low number of limbs analysed for the in vivo group. The in vivo experiments were on hold due to the COVID19 pandemic lockdown and have just started again. However, limitations on the number of possible surgeries are still ongoing.

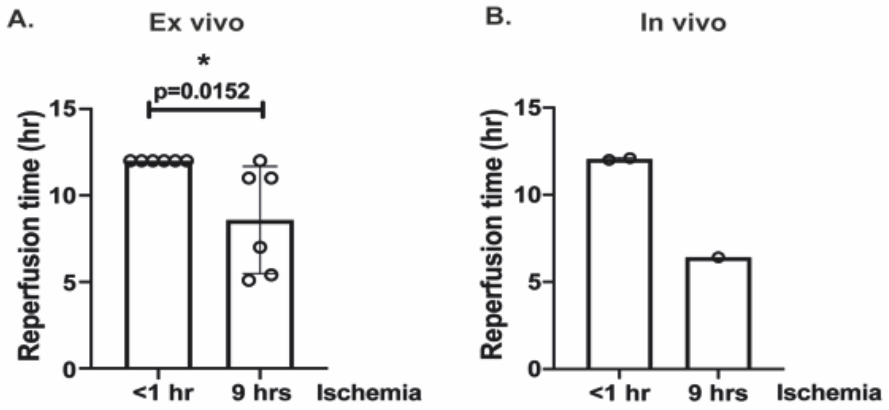

Figure 1. Limb perfusion time. (A) Extracorporeal reperfusion time in limbs which were exposed to < 1hr ischemia (control group) or 9 hrs ischemia (ischemic group). Mann-Whitney U-test (n= 6 limbs; * p=0.0152). **(B)** In vivo reperfusion time in limbs exposed to < 1hr ischemia (control group) or 9 hrs ischemia (ischemic group). No statistical analysis has been performed yet due to limited number of limbs (n= 2 for the control group and n=1 for the ischemic group).

Rhabdomyolysis (the rapid breakdown of skeletal muscle cells) may cause severe damage to the human body leading not only to heart and renal failure but also to disturbances of electrolyte balance. For this reason, electrolytes were measured every hour in our extracorporeal perfusion group (Figure 2) in both control and ischemic limbs. We observed increasing levels of potassium in the reperfused blood in the ischemic group (Figure 2 A), while no variation in potassium levels were observed in the control group

(Figure 2 A). Hyperkalemia is a common consequence of muscle damage. Reduction of calcium and chloride during the extracorporeal perfusion of ischemic limbs (Figure 2B and C) compared to control limbs was also detected. Swelling of the tissue may lead to destruction of muscle cells, leading to an initial release of calcium into the bloodstream. However, such calcium peak is immediately followed by a rapid reduction of calcium blood levels due to an intracellular accumulation of calcium. Interestingly, as shown in Figure 2B, extracorporeal perfusion of ischemic limbs shows such typical calcium profile, confirming damage of the muscle tissue. High lactate levels in blood of limbs exposed to 9 hrs of ischemia compared to controls also proves rhabdomyolysis (Figure 2D). Sodium imbalance, that can potentially lead to several physiological damage to the tissue, was not seen in either group of extracorporeal perfused limbs (Figure 2E).

To maintain the extracorporeal perfusion physiologically close to the in vivo counterpart and avoid additional tissue damage, we administered bicarbonate when necessary to maintain the pH at physiological level, and we stabilized blood glucose levels by adding glucose or insulin when necessary. CO_2 and O_2 were adapted to physiological levels when required. Limb temperature was also monitored during extracorporeal perfusion. No variations were observed between control and ischemic limbs.

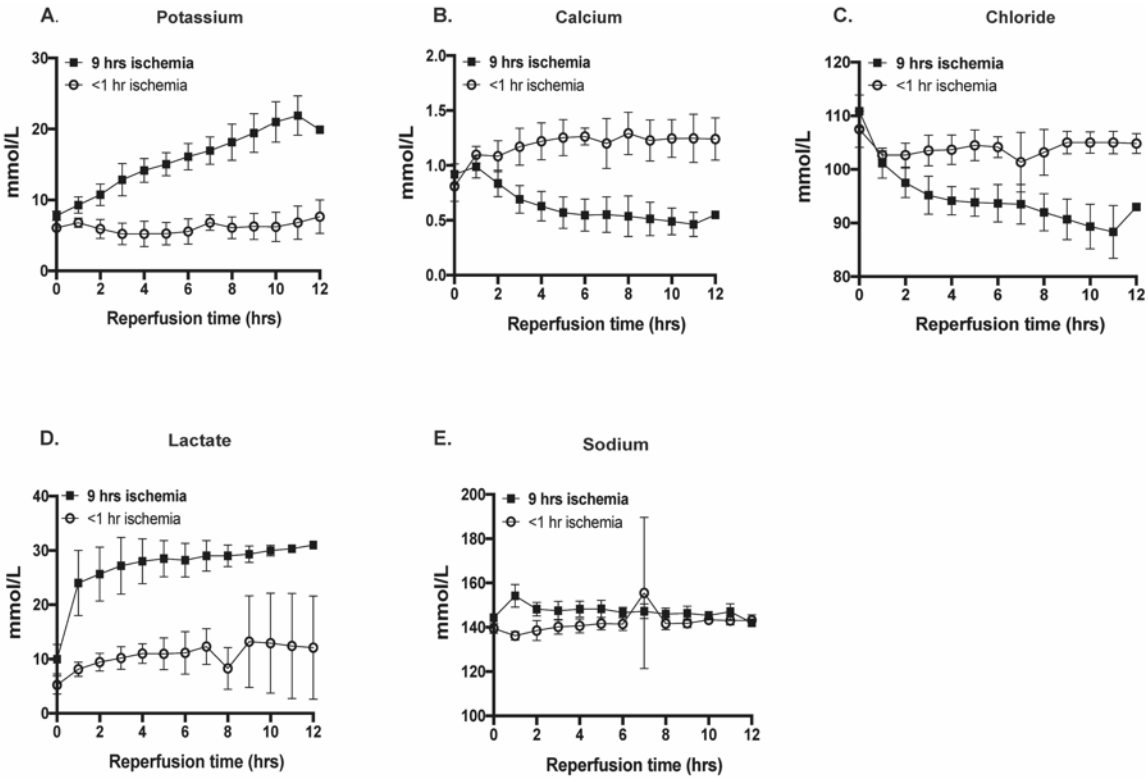

Figure 2. Blood levels of electrolytes and lactate during extracorporeal perfusion. Potassium **(A)**, Calcium **(B)**, Chloride **(C)**, Lactate **(D)** and Sodium **(E)** levels were monitored hourly during extracorporeal perfusion of limbs exposed to < 1hr ischemia (control group) or 9 hrs ischemia (ischemic group). n=6 limbs per group.

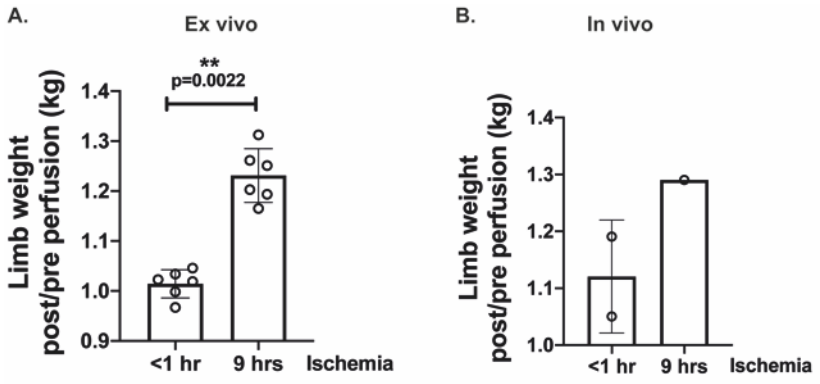

Figure 3. Limb weight ratio post- / pre-perfusion. The weight of control and 9 hrs ischemic limbs was measured before and after extracorporeal **(A)** and in vivo **(B)** perfusion. **(A)** Mann-Whitney U-test (n= 6 limbs; ** p= 0.0022). **(B)** No statistical analysis has been performed yet due to limited number of limbs (n= 2 for the control group and n=1 for the ischemic group).

Direct consequence of ischemia/reperfusion injury is severe edema in the tissue and increase of compartment pressure. Control and ischemic limbs from both extracorporeal and in vivo perfusion were weighted at baseline (immediately after amputation) and at endpoint. As shown in Figure 3A and B ischemia leads to a 1.2-fold increase in limb weight.

Edema in the tissue was further confirmed by analyzing the wet/dry ratio of the biopsies from limbs after perfusion.

Figure 4 clearly shows that ischemic limbs have a 2.4- **(A,** ex vivo**)** and 5-fold **(B,** in vivo**)** increase in the wet/dry ratio. The increase in wet/dry ratio was thus observed independently of the type of perfusion (**Figure 4**). Further in vivo reperfusion experiments are needed to confirm the respective data.

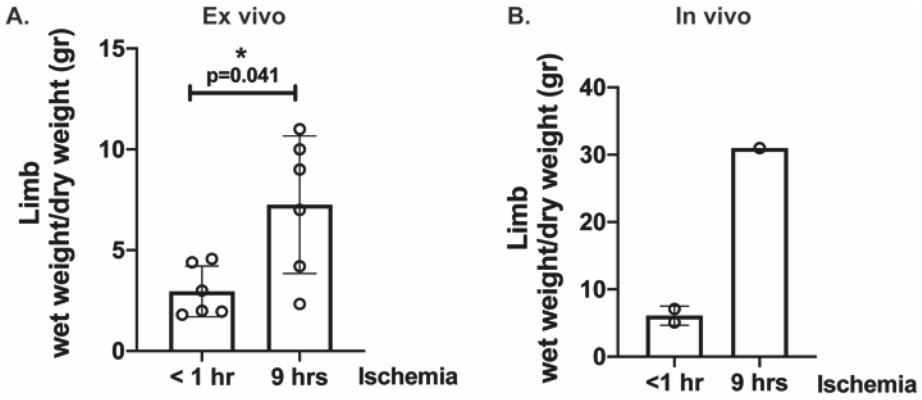

Figure 4. Limb wet/dry ratio after extracorporeal and in vivo reperfusion. Biopsies from both ischemic (9 hrs ischemia) and control limbs (<1 hr ischemia) were weighed immediately after extracorporeal **(A)** and in vivo **(B)** perfusion and after they were left to try dry for 40 hours at 80°C. **(A)** Mann-Whitney U test (n= 6 limbs; * p= 0.041). **(B)** No statistical analysis has been performed yet due to limited number of limbs (n= 2 for the control group and n=1 for the ischemic group).

Preliminary immunofluorescence stainings of muscle tissue samples from limbs which underwent extracorporeal perfusion suggest that 9 hrs ischemic limbs show major tissue damage upon ex vivo reperfusion. Figure 5 shows dystrophin (green) in muscle of extracorporeally perfused limbs. Two pigs were analyzed until now. The ischemic limb from pig 7 reached a perfusion time of 7 hrs, while the ischemic limb from pig 5 reached a perfusion time of 5.4 hrs.

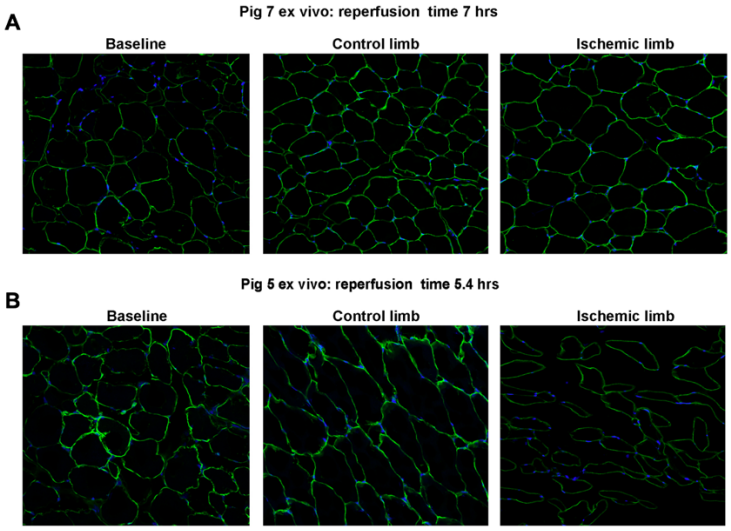

Figure 5. Dystrophin distribution in muscle of porcine limbs after extracorporeal reperfusion. Dystrophin (in green) distribution in limb tissue collected at baseline (before perfusion), from control limbs (< 1hr ischemia), and ischemic limbs (9 hrs of ischemia) after extracorporeal perfusion. Perfusion time lasted 11 hrs in pig 7 **(A)** and 5.4 hrs in pig 5 **(B)**.

Pig limbs from in vivo perfusion were also stained for dystrophin (Figure 6). The control limb (A) shows an intact dystrophin distribution while ischemic limb (B) shows a severely disrupted dystrophin pattern, suggesting muscle damage. Further stainings from all collected tissues will be performed and quantified.

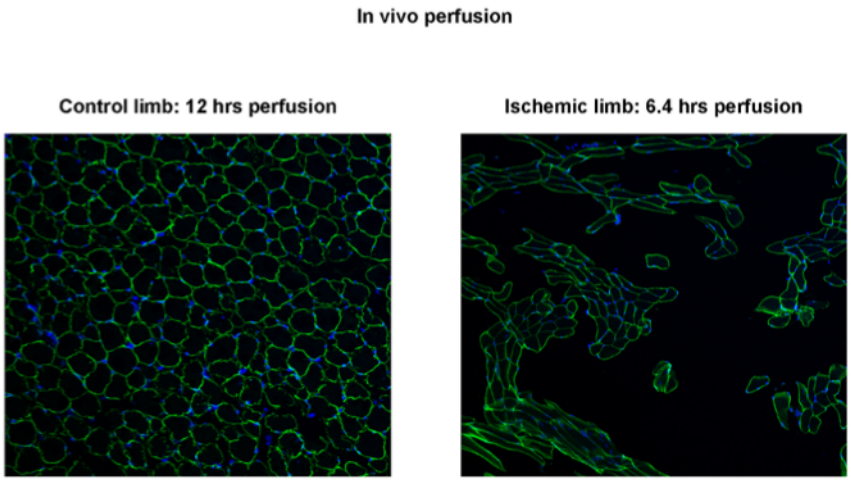

Figure 6. Dystrophin distribution in muscle of porcine limbs after in vivo reperfusion. Dystrophin (in green) distribution in control limb (**A;** < 1hr ischemia) and ischemic limb (**B;** 9 hrs of ischemia) after in vivo perfusion. Perfusion time lasted 12 hrs in the control limb and 5.4 hrs in the ischemic limb.

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 order to accomplish the goals of Milestone 2: Validation of ex vivo – in vivo models for the effect of EC protection on extremity I/R injury in a PFC scenario, we will perform additional in vivo perfusions of amputated and replanted pig experiments. Moreover, all collected muscle tissue will be analyzed for ischemia/reperfusion damage by immunofluorescence and histology. Endothelial cell activation complement, and coagulation will be evaluated in the tissue by immunofluorescence and in blood samples by ELISA. Data obtained from both extracorporeal and in vivo perfusion models will be then compared.

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

During the lockdown caused by the COVID19 pandemic (March – June 2020) no animal experiments could be performed. This has caused a delay in the animal experimentation of the in vivo perfusion group. However, the project is still on schedule because the setup of the experimental models was faster than expected and no technical difficulties occurred. In case the pandemic situation should again lead to a lockdown and reduction of animal experimentation, we would still be able to continue the project-related work by performing analyses of tissue and blood samples collected until now.

Our Experimental Surgery Facility closed for renovation in March 2020 and will only reopen in March 2021. Currently we are able to perform animal surgeries at the Veterinary Hospital (actually a bit under TRUCK / PLANE conditions, but that fits well with our project..), and we are planning to perform two more in vivo reperfusions by the end of the year.

Changes that had a significant impact on expenditures

Expenditure was lower than anticipated. This is due to the following reasons:

1) 2 months in which we did not pay a salary (Sept. 2019 – no postdoc recruited yet, June 2020 – postdoc Josip Mikulic left at the end of May and new senior research assistant Nicoletta Sorvillo only started beginning of July)
2) Less animal experiments could be performed because of the COVID-19 lockdown

As soon as the planned animal experiments can be performed the expenditures with rise again quickly.

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

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**

  Nothing to report

- **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?

1. Name:	Robert Rieben
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	ORCID: 0000-0003-4179-8891
Nearest person month worked:	1
Contribution to Project:	Prof. Robert Rieben is the PI advising on the design of the experiments, the development and acquisition of new methods as well as the evaluation of the obtained results. Furthermore, he offers support for the laboratory evaluations (antibodies and reagents, knowhow and access to technology).
2. Name:	Esther Vögelin
Project Role:	Co-PI
Researcher Identifier (e.g. ORCID ID):	ORCID: 0000-0003-4179-8891
Nearest person month worked:	1
Contribution to Project:	Prof. Esther Vögelin participates in the design and analysis of the experiments. As director of the Department of Plastic and Hand Surgery, Bern University Hospital, Esther Vögelin is responsible for all surgical aspects of the project.
3. Name:	Dr. Josip Mikulic (Oct 1, 2019 - May 31, 2020)
Project Role:	Postdoc
Researcher Identifier (e.g. ORCID ID):	not available
Nearest person month worked:	8
Contribution to Project:	Dr. Mikulic provided the support needed for the scientific and technical aspects of the project under the supervision of the PI. He was involved in planning and setup of the experiments, laboratory analyses, statistical evaluation and reporting.
4. Name:	Nicoletta Sorvillo (July 1, 2020 - present)
Project Role:	Senior Research Assistant
Researcher Identifier (e.g. ORCID ID):	not available
Nearest person month worked:	1
Contribution to Project:	Dr. Nicoletta Sorvillo provides the support needed for the scientific and technical aspects of the project under the supervision of the PI. She is involved in planning and setup of the experiments, laboratory analyses, statistical evaluation and reporting.

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