

THE EFFECTS OF EQUINE PLASMA TRANSFUSION ON MARKERS OF  
INFLAMMATION IN HEALTHY NEONATAL FOALS

BY

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THESIS

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## ABSTRACT

Very few studies in veterinary medicine have investigated the immunologic changes and inflammatory markers associated with transfusion of fresh frozen plasma in neonatal foals. Plasma transfusion is routinely used in the equine neonate for several reasons ranging from prophylaxis of disease to complementary treatment of ongoing conditions. Previous studies in hospitalized sick neonatal foals have reported a transfusion reaction rate close to 10% (Hardefeldt, 2010). In this prospective study, we examined the immunological impact of intravenous (IV) fresh frozen plasma administration in healthy newborn foals, using a three-fold approach. Systemic, plasma-invoked immune and inflammatory responses were assessed by measuring 1) peripheral blood leukocytes cell cytokine gene expression, 2) circulating cytokine concentration (interferon-gamma [IFN- $\gamma$ ], interleukin-1 $\beta$  [IL-1 $\beta$ ], tumor necrosis factor- $\alpha$  [TNF- $\alpha$ ], interleukin-6 [IL-6], interleukin-10 [IL-10] and interleukin-8 [IL-8], interleukin-4 [IL-4], interleukin-17 [IL-17]), and 3) changes in acute phase protein (fibrinogen [FIB] and serum amyloid A [SAA]) production. A total of 35 healthy, neonatal foals (mean  $\pm$  SD age 34  $\pm$  5 hours) were randomly allocated into one of two groups, and given either hyperimmune, fresh frozen plasma (treatment) or crystalloids (control) by IV administration through a preplaced jugular catheter. Blood samples were collected before (hour 0) treatment and at 2, 6 and 24 hours after transfusion. Serum cytokines concentrations were measured with either bead based multiplex technology (IFN- $\gamma$ , IL-4, IL-17, IL-10) or with commercial ELISA kits (IL-6, IL-8 and IL-1 $\beta$ , TNF- $\alpha$ , IL-10). Peripheral blood leukocyte gene expression was performed with qRT-PCR. Serum amyloid A [SAA] was measured with turbidometric immunoassay and fibrinogen concentration [FIB] was determined with the method of Clauss. Comparisons between groups were made using a Kruskal Wallis test, and changes overtime within groups were analyzed with

Friedman's test followed by Mann Whitney U. Parametric data was analyzed with repeated measures ANOVA with LSD correction. Plasma administration was associated with a significant decrease in TNF- $\alpha$  gene expression between 6 and 24 hours ( $p= 0.007$ ) and IL-1 $\beta$  gene expression at 24hrs. Crystalloid administration was associated with a significant decrease in IL-1 $\beta$  ( $p<0.005$ ) and IL-10 ( $p=0.003$ ) gene expression at 24hrs. In foals receiving plasma a significant increase from time 0 in serum [TNF-  $\alpha$ ] at 2, 6 and 24 hours ( $p<0.001$ ) and in [IL-8] at 2 hours ( $p=0.001$ ) was noticed. [IL-6] in plasma group was greater compared to the crystalloid group at 6 hours ( $p=0.051$ ) and 24 hours ( $p=0.044$ ). No significant overtime differences or between group variations were noticed for the other cytokines. A greater number of foals in the plasma group demonstrated an increase in [TNF-  $\alpha$ ], [IL-8] and [IL-6] compared to control group. On the contrary more foals in the control group demonstrated an increase over time of [IL-17] and [IL-4].

[FIB] was significantly different ( $p=0.048$ ) between plasma and crystalloid groups at 6 hours, while [SAA] was different at 24 hours between groups ( $p=0.0125$ ). In foals receiving plasma, [FIB] was significantly increased at 2, 6 and 24 hours ( $p<0.001$ ) compared to baseline. We concluded that plasma transfusion in healthy foals leads to quantifiable changes in concentrations of these cytokines and that commercially available plasma may influence foals [FIB] soon after transfusion. Inflammation was also found in the foals receiving crystalloids and an effect of catheterization and fluid shear force was hypothesized. In combination, these results indicate that IV crystalloid and plasma administration are associated with a significant, but clinically undetectable, systemic inflammatory response. The nature of this immunological response was qualitatively different and quantitatively greater in foals receiving IV plasma. Further studies are required to elucidate the mechanism and significance of this transfusion- associated systemic

immune response, and to determine how this might be different in sick or compromised individuals.

*To my family*

*For being with me even from far away*

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## CHAPTER 1

### INTRODUCTION

Fresh frozen plasma (FFP) is commonly administered in equine medicine and more frequently in neonates. Its use increased significantly over the past 15 years, in part due to its medical benefits, but also in line with commercial availability and affordability. Administration of plasma occurs commonly in both sick and healthy neonatal foals, for either therapeutic or prophylactic purposes. Healthy foals are commonly given plasma transfusion following the failure of acquisition of colostral immunoglobulins (failure of passive transfer or FPT) or as prophylaxis for the prevention of *Rhodococcus equi* infection (Giguere, 1997, 2002, 2011; Caston 2006; Madigan, 1991). The most frequent indication for use is based on the ability of plasma transfusion to increase circulating immunoglobulin concentrations in depleted recipients (Crump, 1992; Le Blanc, 1987; De Luca, 2001; McClure, 2001). However, sick foals also receive IV plasma to aid in the treatment of sepsis, FPT, hypoproteinemia, coagulation disorders or in order to provide additional colloidal oncotic support (De Luca, 2001; Wilkins 1994; Crump, 1992; Le Blanc 1987, 1985; Becht, 1985).

In people, the therapeutic benefits of various plasma fractions have been recognized since before the Second World War. These benefits include the addition of coagulation factors in patients with hemostatic disorders, the augmentation of circulating albumin levels to help maintain oncotic gradients, and the replacement or supplementation of immunoglobulin in inflammatory disorders or severe sepsis.

Despite the widespread application of IV plasma administration in horses, there are relatively few studies that have examined the potential side effects of this therapy in healthy or critically ill foals. In people, systemic inflammatory responses have been reported to occur following



transfusion of many types of blood components, with FFP being the most commonly implicated proinflammatory product (Shaz, 2011; Urner, 2012; Pandey, 2012). The most frequent mechanisms of immune reaction are transfusion related acute lung injury (TRALI), transfusion associated circulatory overload (TACO), and allergic transfusion reactions (ATR). Less common complications reported include red-cell alloimmunization or leukocyte-associated risks such as febrile non-hemolytic transfusion reactions (FNHTR), transfusion-associated graft versus host disease (TA-GVHD) and white blood cell (WBC) alloimmunization.

In a study performed on hospitalized neonatal foals, the incidence of clinically detectable transfusion reactions related to the administration of commercial plasma, was reported to be approximately 10% (Hardefeldt, 2010). This is not surprising considering that the neonatal foal immune system is fully functional, and able to mount a significant adaptive immune response to an appropriate stimulus, soon after birth (Perkins, 2015). While it is clear that FFP transfusion is not without risk, there are no published studies describing the nature and mechanism of plasma transfusion-mediated inflammatory responses in newborn foals. The inciting immunogen within the transfused plasma and the precise mechanism of these reactions are still unknown, and more studies are required to obtain a better definition in the equine species. The aim of this study was to investigate the inflammatory response induced by the IV administration of FFP in healthy neonatal foals. The FFP-associated inflammatory cascade was characterized using three approaches: changes in cytokine gene expression in peripheral blood leukocytes, alterations in serum cytokine, and acute phase protein concentrations. We hypothesized that IV FFP administration would induce a short-term, systemic inflammatory response characterized by increases in gene expression and concentration of proinflammatory cytokine (TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-17, IFN- $\gamma$ ), chemokines (CXCL8/IL-8), and anti-inflammatory cytokines (IL4, IL-10). An

accompanying increase in acute phase proteins such as FIB and SAA was also anticipated. The results of this, and similar studies, will be important in determining best practice and help clinicians understand the potential risks of FFP transfusions in young foals.

## CHAPTER 2

### LITERATURE REVIEW

#### PLASMA TRANSFUSION REACTIONS IN THE EQUINE, CANINE AND HUMAN SPECIES

Fresh frozen plasma (FFP) is a blood product obtained by separation from whole blood either by centrifugation or plasmapheresis. It is usually frozen within 8 hours of collection, and stored at -18—30 °C. It contains normal levels of all of the stable coagulation factors, along with anticoagulants, fibrinogen, fibronectin, albumin, globulins and alpha-macroglobulin. Both in human and veterinary medicine, FFP is commonly used in adults, neonates or pediatric patients for several purposes. Indications for plasma use are widely debated in both human and veterinary medicine. In human medicine, plasma is administered primarily to sick patients; whereas, in the veterinary field, especially in equine medicine, plasma transfusions are given for both therapeutic and preventative purposes. In human medicine, use of FFP is recommended in the sick patient with concomitant bleeding or coagulopathies (Holland, 2006; O’Shaughnessy, 2004; Yang, 2012, Parker, 2014; Karam, 2015, Goldenberg, 2006). Nonetheless, both human adults and neonates admitted to intensive care units receive FFP for reasons other than these primary indications, such as prophylactic administration before a planned surgical procedure (Baer, 2008; Puetz, 2012; Stanworth, 2004). In small animal medicine, FFP is primarily used in adult dogs and cats with coagulopathies, hypoproteinemia, sepsis or pancreatitis (Logan, 2001; Davidow, 2013) and in puppies affected by viral enteritis (Logan, 2001). Similarly, in equine medicine, FFP administration is routinely used for the treatment of failure of passive immunoglobulin transfer, sepsis, protein losing conditions, coagulation disorders or as additional colloidal support (Giguere, 1997, 2002, 2011; Caston 2006; Madigan, 1991, De Luca, 2001; Wilkins 1994;

Crump, 1992; Le Blanc 1987, 1985; Becht, 1985). Prophylactic use of hyperimmune *Rhodococcus equi* rich FFP is commonly performed, especially in areas where this pathogen is endemic (Giguere, 2002; Madigan, 1991; Caston, 2006). The administration of FFP is always associated with some level of risk, and adverse reactions have been reported in people and several animal species. In view of the potential clinical complications, several authors both in the human or veterinary medicine field are in favor of restricting the use of FFP transfusions to situations of strict necessity, thereby avoiding unnecessary risk in clinical cases when the potential benefit is questionable (Puetz, 2012; O'Shaughnessy, 2004; Beer, 2015). In people, reactions to transfused blood products are more frequent in young patients (< 21 years) than in adults with a reported rate of 6.2 versus 2.4 reactions per 1000 transfusions, respectively (Oakley, 2015). In these instances, platelets are the most frequently implicated component associated with adverse events, closely followed by RBCs and plasma. In human medicine, the reported incidence of adverse events to plasma administration is highly variable and depends on the process and consistency of event reporting. Reaction rates to plasma administration were reported to range between 1:1700 and 1:360 units transfused (Pandey, 2012). The incidence of plasma-related transfusion reactions is also not well documented in veterinary medicine but has been reported to range from 1% in dogs (Snow, 2010) to 10% in equine neonates (Hardefeldt, 2010). In both human and veterinary medicine, clinical signs associated with adverse reactions are first evident within 6 hours of FFP administration. However, delayed reactions occurring between 6 and 72 hours of plasma administration are also described (Thomovsky, 2014; Strobel, 2008).

A number of different mechanisms underlying plasma-associated transfusion reactions are reported in both human and veterinary medicine. These can be broadly classified as immunologic or non immunologic in origin (see table 1).

**Table 1: Mechanisms of plasma-related transfusion reactions in human and veterinary medicine**

<b>Categories of plasma-related transfusion reactions</b>	
<b>Immunologic</b>	<b>Non immunologic</b>
<b>Allergic</b>	<b>Sepsis</b>
<b>Transfusion-related acute lung injury (TRALI)</b>	<b>Transfusion-associated cardiac overload (TACO)</b>
<b>Transfusion related immuno modulation (TRIM)</b>	<b>Citrate toxicity (Hypocalcemia)</b>
<b>Febrile non hemolytic reactions (FNHTR)</b>	<b>Infectious disease transmission</b>
<b>Hemolytic reactions (HR)</b>	<b>Hyperammonemia</b>
	<b>Hypophosphatemia</b>
	<b>Hyperkalemia</b>
	<b>Hypothermia</b>

The most commonly reported plasma transfusion-associated reactions, in both human and veterinary medicine, are generated by immune-mediated mechanisms. They are invariably non-fatal and can be categorized as allergic transfusion reactions (ATR) or febrile non-hemolytic transfusion reactions (FNHTR) (Oakley, 2015). Fatalities are more commonly reported in association with transfusion-related acute lung injury (TRALI), transfusion-associated cardiac overload (TACO), hemolytic transfusion reactions (HTR) and anaphylactic reactions (Pandey,

2012; FDA reporting). In 2014, the food and drug administration (FDA) reported a human TRALI rate of 43% as the most implicated transfusion-related fatality, followed by TACO (17%), HTR (26%) and anaphylaxis (7%). The incidence of acute lung injury in dogs receiving transfusions has recently been reported to be 3.7% (Thomovsky, 2014).

A wide range of less common, non-immunological mechanisms of adverse responses to plasma administration have also been documented. These include plasma transfusion associated thrombotic events, especially in neonates (Puetz, 2012), metabolic derangements such as hyperkalemia (storage related), hyper and hypoglycemia, hypocalcemia (citrate toxicity), and hypothermia (Poterjoy, 2009).

### **Pathophysiology of plasma transfusion-associated inflammatory responses**

The pathophysiologic mechanisms underlying transfusion reactions in people have been more fully elucidated than those occurring in veterinary species. Several studies indicate that the inflammatory reactions that follow blood product transfusions are associated with two inter-related triggers. The first trigger is related to endothelial cell activation, elicited by surgery, infection, or tissue trauma. The second trigger occurs simultaneously, or shortly after the first, and can be characterized as a pro-inflammatory response associated with specific constituents of the transfused of blood product. This second trigger initiates or heightens the inflammatory response arising from the endothelial insult (Urner, 2012; Silliman, 2009; Shaz, 2011, Pandey, 2012).

Many studies have demonstrated the central role played by endothelium in vascular responses to inflammatory stimuli. Activated endothelial cells are potent sources of pro-inflammatory cytokines such as TNF- $\alpha$  and IL-1 $\beta$ , which recruit proinflammatory cells (leukocytes) to the site

of cellular damage. In non-diseased states, circulating leukocytes tether, roll, and adhere to the vascular endothelium by the action and interaction with cell surface glycoproteins, endothelial adhesion molecules, inflammatory mediators and chemokines (Petri, 2008). In a similar fashion, the expression of endothelial selectins (E and P-selectins) enables the adhesion and rolling of neutrophils along blood vessel walls (Shaz, 2011). In disease states, there is a site-specific up-regulation of soluble intercellular adhesion molecules (sICAM-1) that promotes leukocyte adhesion. The local production of cytokines, such as monocyte chemoattractant protein-1 (MCP-1), and chemokines, such as IL-8, stimulates these adhered leukocytes to accumulate in a specific area or to migrate from the vasculature (Carlow, 2009) into the injured area. The local production of vasoactive peptides and the concomitant increase in concentration of cytokines serve to amplify the local and systemic inflammatory response, as evidenced by accompanying signs such as fever, malaise, edema and urticaria.

### **Molecular pathways associated with plasma transfusion-associated inflammatory responses**

The intricate pathogenesis of plasma transfusion-invoked inflammatory responses can be attributed to the immunogenic complexity of the infused substance. Plasma contains protein (albumin, globulins, complement, coagulation factors, hormones) and non-protein (lipids, carbohydrates, anticoagulant, preservatives) immunogens. The immune system can be regarded as an important homeostatic regulator in terms of its purpose to recognize and respond to potentially noxious substances, or insults, in such a way that neutralizes or removes the danger and restores normality. The detection and elimination of potentially damaging, noxious insults involves the activation of numerous pathways that harmonically work together with the aim of restoring physiological health. The inflammatory cascade is the first immunological response of

the body to a noxious stimulus and is therefore a key element in restoring homeostasis.

The administration of incompatible blood products was found to increase the production of systemic proinflammatory cytokines (TNF, IL-8, IL-6, IL-1 $\beta$ ) and markers of endothelial activation in adults and preterm infants (Muylle, 1995). Interestingly, although concentrations of these inflammatory mediators increased significantly from baseline over time, they never reached the levels that have been reported in individuals afflicted with different inflammatory conditions (Keir, 2013). Several studies have shown that the quality of stored blood products can decline with time, and that this can be manifested by an increase in cytokine accumulation within the stored blood product itself, especially in non-leukocyte reduced whole blood or in platelet-rich plasma (Heddle, 1999; Silliman, 2003). In support of this, prestorage leukoreduction of whole blood product was reported to attenuate cytokine response (Baumgartner, 2009) and simultaneously reduce the rate of transfusion reactions (Lannan, 2013). The innate arm of the immune system has been described as the first line of defense against pathogens and cell damage. Danger from these two categories of noxious stimuli is detected through the presence of alarm signals on microbes (pathogen associated molecular patterns or PAMPs) or on damaged molecules or cells (damage associated molecular patterns, DAMP). These exogenous and endogenous danger signals are recognized by pattern recognition receptors (PRRs) that are present on the surface or in the cytoplasm of sentinel immune cells (macrophages, dendritic cells), strategically located at body surfaces, and found in key tissues, throughout the body (Takeuchi, 2010; Newton, 2012). The activation of PRRS, by the presence of PAMPs and DAMPS, triggers an intracellular signaling cascade that initiates the activity of nuclear factor (NF)- $\kappa$ B complex, which activate the gene expression and production of proinflammatory cytokines, chemokines and antimicrobial proteins (Takeuchi, 2010; Akira, 2006). TNF- $\alpha$  and IL-



1 $\beta$  are two early-phase cytokines, which evokes a cytokine production cascade, leading to the recruitment and activation of neutrophils to the damaged or infected tissue site. This influx of inflammatory cells is driven by increased endothelial and leukocyte expression of several families of transmembrane glycoproteins involved in intercellular (endothelial-leukocyte) adhesion, which is provoked by cytokines and complement fragments (Albrecht, 2004). Other substances such as vasoactive peptides, chemokines or lipid-based inflammatory mediators (prostaglandins, leukotriens) are also liberated in these early phases of the inflammatory response and recognized by other cellular receptors such as G protein-coupled receptor (GPCR). These proteins interact with ion channels stimulating generation of second messengers (IP3/DAG or Ca) and culminate in exocytosis and apoptosis of damaged cells (Newton, 2012; Liu, 2005). Activated neutrophils act as powerful effector cells, producing reactive oxygen species, lytic enzymes and antimicrobial peptides. Moreover, active cross talk between neutrophils and immune cells such as T, B cells or NK cells occurs, with the purpose of recruiting more effector cells for the inflammatory cascade.

### **Transfusion related acute lung injury- TRALI**

Transfusion related lung injury has been documented in both people and animals and is recognized as one of the most serious potential immune-mediated hazards of plasma and blood administration. In human medicine, the clinical syndrome has been termed TRALI, and similar criteria have been used to characterize its equivalent in veterinary medicine (veterinary acute lung injury; VetALI) (Wilkins, 2007). Clinically, TRALI is characterized by the presence of acute respiratory distress, hypoxemia and noncardiogenic pulmonary edema (Pandey, 2012; Skeate, 2007). Pathophysiologically, it is characterized by neutrophil accumulation and

activation within the respiratory tract, with subsequent pulmonary edema and alveolar damage. In many cases, the clinical presentation is similar to acute respiratory distress syndrome (ARDS). Data from both animal models and human studies suggests that TRALI is associated with both immune and non immune mechanisms (Triulzi, 2009; Shaz, 2011). Several studies have shown correlations between the presence of donor blood antibodies against human neutrophils antigens (HNA), human leukocyte antigens (HLA, MHC class I), granulocytes and monocytes (Nishimura, 2007; Shaz 2011; Goldmann, 2004) on the incidence of TRALI. Others have suggested that non immune factors such as the leukocyte derived factors (sCD40L) and lipids contained in the blood product (Shaz, 2011; Silliman, 1997 and 2003) contribute to the onset of TRALI. The presence of TRALI-associated antibodies in blood products is more common in pluriparous female donors (Reil, 2008; Triulzi, 2009) and in blood product contaminated with leukocytes or platelets. Interestingly FFP or plasma containing blood components are the most implicated in the pathogenesis of TRALI (Silliman, 2009; Pandey, 2012).

The two-trigger, or “two hit,” sequence of immunological activation described for plasma transfusion-mediated inflammation has also been implicated in TRALI. The first step involves a low-grade, provoking stimulus that causes endothelial activation and priming of circulating neutrophils. This is rapidly followed by a second inflammatory mediator within the transfused component that activates the primed neutrophils and triggers their degranulation. The resultant endothelial cell damage leads to vascular leakage and pulmonary edema, which causes tissue hypoxia and clinical signs of acute respiratory disease. In human patients several risk factors have been associated with the priming or pre-activation step of neutrophil stimulation. These include mechanical ventilation, pre-existing shock, smoke inhalation, liver surgery (transplant), end stage liver disease, increased levels of pre-transfusion IL-8, cardiac surgery, hematological

malignancy, sepsis, age, chronic alcohol abuse and pre-transfusion volume expansion (Pandey, 2012). Equivalent pre-inflammatory triggers have been suspected in animal VetALI/ARDS, with the observation that lung injury occurs in a wide variety of clinical settings including sepsis, systemic inflammatory response syndrome (SIRS), near-drowning, smoke inhalation, trauma, pancreatitis, aspiration of gastric contents, and in patients that have received multiple blood or plasma transfusions (Wilkins, 2007).

### **TACO- Transfusion associate circulatory overload (TACO)**

While TACO is reported as the second leading cause, after TRALI, of human ATR-related fatalities in the USA (source: FDA), it has only been anecdotally described in veterinary medicine. TACO is characterized by an increase in pulmonary capillary pressure, which causes a transcapillary transudation of fluid and pulmonary edema, leading to hypoxia and acute respiratory distress. A mild improvement in clinical signs has been observed with diuresis.

Differentiation between TRALI and TACO is difficult because of the similarities in the clinical signs of each disorder. However, some minor differences in laboratory and clinical parameters have been identified (Skeate, 2007). In general, TACO is correlated with large transfusion fluid volumes and rapid rates of infusion, although its occurrence has also been reported after administration of as little as one single unit of red blood cells (Li, 2011; Popovski, 1996). In people, risk factors such as age (old or young), left ventricular dysfunction, congestive heart failure or chronic renal failure are associated with the occurrence of TACO (Skeate, 2007; Li 2011; Murphy, 2013). In the same way, animals with renal or cardiac compromise may be at risk of TACO (Tocci, 2010).

### **Acute hemolytic reactions**

In human medicine, acute hemolytic reactions have been reported after transfusion of plasma containing high levels of iso-hemoagglutinins or high titers of antibodies directed against the recipient's RBC (Pandey, 2012). Patients experiencing a hemolytic transfusion reaction (HTR) can present with fever, chills, rigors, facial flushing, nausea and vomiting, hypotension, hemoglobinuria, oliguria or anuria (Poterjoy, 2009, Strobel, 2008; Tocci, 2010). Delayed post-transfusion hemolytic reactions have been reported in both human and veterinary medicine. In people, this delayed reaction has been correlated with the presence of alloantibodies against the minor groups of human RBC antigens, such as Jk, Fy, E, K (Strobel, 2008). In veterinary medicine, post-transfusion hemolysis has been recognized primarily in the small animal field and is more commonly associated with the transfusion of RBCs or whole blood and has been shown to be predominantly mediated by IgG in the dog and IgM in the cat (Tocci, 2010).

### **Febrile nonhemolytic transfusion reactions (FNHTR)**

Febrile, nonhemolytic transfusion reactions (FNHTRs) are commonly reported in both human and small animal medicine (Tocci, 2010; Hardefeldt, 2010; Pandey, 2012). They are often characterized by a rise in body temperature ( $>1^{\circ}\text{C}$  from baseline) and accompanied by symptoms of chills, cold, rigors, and discomfort (Poterjoy, 2009). Historically, FNHTRs to erythrocytes were believed to be caused by the release of endogenous pyrogens, secondary to the immune response of recipient anti-leukocyte antibody to leukocytes present in the transfused blood product. Hence, the use of leukoreduced whole blood was found to be beneficial in preventing these reactions (Paglino, 2004). FNHTRs to platelet products have also been reported and seem to be associated with various biologic response modifiers (BRM) such as cytokines, chemokines,

complement fragments, histamine, and lipids that often accumulate in platelet- and leukocyte-rich blood products during storage (Heddle, 1999). Since FFP is often considered to be non-cellular, FNHTRs are expected to be less common with plasma transfusions. However, in situations where the plasma is contaminated with red blood cells, leukocytes or platelets, adverse reactions can be significant. The absolute quantity of these cellular contaminants in a particular batch of plasma depends on the methods of separation and freeze-thaw employed during production. The residual quantity of white blood cell in plasma could range between  $0.02\text{--}8 \times 10^6$  WBCs per unit (Valbonesi, 2001; Hiruma, 2001). Generally, only a small number of leukocytes are considered to remain after the freeze-thaw process, but the thaw-associated destruction of these cells has been shown to release bioactive mediators that can mediate transfusion reactions (Willis, 1998).

### **Allergic transfusion reactions (ATR)**

Allergic reactions are another category of immune-mediated disorders related to the IV administration of blood products. In human medicine, the clinical presentation of ATR can be mild, characterized by edema, urticaria, pruritus, flushing, sickness behavior and anxiety (Savage, 2013; Pandey, 2012, Tocci, 2010) or severe, associated with anaphylaxis (hypotension, shock, bronchospasm, angioedema). Unfortunately, in some cases, ATR leads to death (Pandey, 2012). The pathophysiology of ATR is considered to be IgE-mediated, involving type I hypersensitivity-like reactions between soluble antigens in the donor plasma and preformed IgE in the recipient, leading to the activation and degranulation of basophils and mast cells and the subsequent release of histamine (Savage, 2014). The sickness behavior, fever and change in mentation are likely related to the action of peripherally released acute cytokines, such as TNF- $\alpha$ .

or IL-1, on the brain (Dantzer, 2001). Edema and urticaria are associated with vasodilation and extravasation of serum and constitute the local response to histamine, prostaglandins, leukotrienes, cytokines, and chemokines from activated mast cells (Hennino, 2006).

Post-transfusion anaphylactic shock has been associated with complement fraction C4, decreased Von Willebrand factor or factor IX (Hirayama, 2013), and with methylene blue-treated FFP (Nubret, 2011). Haptoglobin (hp) and human immunoglobulin A (IgA) in plasma have also been identified as initiators of allergic reactions in people (Homburger, 1981), but not in animals. While ATRs are observed frequently in canine and equine medicine, the pro-inflammatory or immunogenic elements in transfused blood product have not been clearly defined.

### **Treatment and prevention of adverse transfusion reactions**

In canine, equine and human medicine, blood product transfusion is generally performed in hospital settings. In human medicine, FFP is carefully selected based on donor match and intensive blood product screening for causative antibodies that may induce ATR. The hospital environment also allows close and careful patient monitoring during blood product administration; thereby, optimizing the process of identification and intervention in the event of a transfusion-related reaction or complication. In most hospital settings, ATR treatment is focused on supportive therapy. This may involve the immediate interruption or reduction of transfusion flow, gaining access to a large venous site, and the monitoring of vital signs and systemic and central blood pressure by central catheterization. In the event of blood pressure crises, aggressive fluid replacement therapy with crystalloid or colloid solutions is performed, until central pressures are optimized. Vasopressor agents, such as dopamine, to induce a vasodilation within the renal vascular bed may be considered. Depending on the degree of respiratory distress, the patient can be managed with oxygen administration (Capon, 1995). In severe cases, intubation

and mechanical ventilation are performed. In addition to the general systemic supportive therapies, specific inflammatory cascade blockade intervention has been proposed. Non-specific control of the inflammatory cascade, with immunosuppressive drugs such as corticosteroids, can be helpful in curtailing cytokine production. Furthermore, blockade of specific cytokines for the reduction of systemic inflammation and using anti-cytokine antibodies has been proposed in human medicine (Dinarello, 1993). Several case reports and isolated human studies on the use of anti- TNF or anti-IL-1 $\beta$  antibodies for the treatment of a variety of diseases are described in the literature (Gallelli, 2013; Waykole, 2009; Burns, 2008); however, its application in transfusion medicine is currently limited. In veterinary medicine, few studies involving animal patients are reported (Martuscelli, 2000); however, animal trials demonstrate therapeutic benefit (Dinarello, 1993; Kapoor, 2011).

In equine neonatal medicine, prophylactic plasma administration is often performed under ambulatory or field conditions. Under these circumstances, ATR detection, intervention, and the monitoring of therapeutic response, is based almost entirely upon clinical signs. In cases with suspected ATR, interruption or reduction of transfusion rate should be immediate and is often accompanied by the administration of anti-inflammatories (corticosteroids, non-steroidal anti-inflammatories, anti-histamine) and epinephrine if severe cardiac or circulatory effects are noted. Diuretics can be a useful adjunct if circulatory overload is suspected.

In view of the severity of clinical disease associated with many of the transfusion-reactions, there is an increasing interest in the use of preventative strategies. These include efforts to reduce the amount or ameliorate the effect of potential pro-inflammatory components found in the transfusate and therapeutics to minimize the magnitude of the immune reaction in the recipient. Although FFP is considered to be acellular, small numbers of residual leukocytes

present after separation from whole blood, may lead to leukocyte-associated transfusion reactions (Heddle, 1999). Processes to eliminate cellular contamination of plasma, such as leukoreduction, are routinely employed in human blood banks, together with more specific enumeration of residual cellular components (Masse, 2001). In veterinary medicine implementation of leukoreduction is becoming more common, but only for whole blood and platelet components that will undergo prolonged storage (McMichael, 2010). In veterinary medicine, the cellular component of plasma is frequently overlooked; however, more studies on the potential benefits of using leukoreduced plasma are warranted.



## CHAPTER 3

### MATERIALS AND METHODS

#### *Experimental Animals*

During the March to July 2014 foaling season, thirty-seven healthy, newborn foals (Standardbred n=36, Belgian draft n=1), aged between 24 and 36 hours old (mean ( $\pm$  SD) 34 ( $\pm$  5) hours), were enrolled in the study. Foals were defined as healthy using the following inclusion criteria:

- history of normal foaling
- ingestion of colostrum within 6 hours of birth
- no delayed placental passage of the dam
- normal attitude and activity of the foal in the 24 hours post-partum
- normal physical exam
- foals with mild flexural deformities (laxity) were included in the study

The foals were recruited from one of two local farms, one owned by the University of Illinois horse farm (Farm A, n=10) and the other a privately owned commercial equine breeding facility (Farm B, n=25). The two remaining foals were born from privately owned mares and delivery took place at the University of Illinois Veterinary Teaching Hospital (VTH). Informed consent was obtained from all of the owners prior to the foaling event.

Enrolled foals were allocated into one of two treatment groups (group 1 – plasma recipient; group 2 – control crystalloids recipient) using a randomly generated list (Microsoft Excel; Microsoft Office 2007). After physical examination, the foals were restrained by a trained handler, and a 14 gauge, 7 cm short term catheter jugular catheter was aseptically placed (DayCath- MILA International, INC., Erlanger, KY 41018) and secured with glue (3M Vetbond, Valleyvet, Marysville, KS. 66508). Group 1 foals (plasma recipient, n=18) each received 1 L of

commercially purchased FFP (Lake Immunogenics Inc., NY, USA) while group 2 (control crystalloid recipient, n= 19) received 1 L crystalloids (Normosol-R; Hospira Inc, Lakeforest, IL, USA). The entire 18 liters of plasma that were used for the study were obtained from a single donor by plasmapheresis, and the plasma mixed together prior to being aliquoted into individual units (personal communication with Lake Immunogenics). Prior to transfusion, the bags of plasma were slowly thawed by immersion in warm water, and both plasma and crystalloid fluids were warmed to body temperature. Administration of plasma was accomplished through a standard 1.4 m filtration device fitted with a 170-260 micron filter (Blood Component Recipient Set, Fenwal, Inc. Lake Zurich, IL). Crystalloids were administered through a sterile, 15 drops/ml standard infusion device (Abbott laboratories, IL, USA). Foals were closely monitored throughout the delivery of fluid infusion. Heart rate (HR), respiratory rate (RR) and rectal temperature were measured at the beginning of the transfusion and at five minutes intervals until the end of the transfusion. The administration of both fluids started slowly approximately 0.5 ml/kg for 10 minutes and then increased to 40 ml/kg/hr if no signs of abnormal reaction were observed. Administration of both fluids occurred over a 20- 30 minute period. Body weight of the foals was estimated at 50 kg. Foals were monitored for evidence of cardiovascular and/or respiratory compromise, piloerection and colic. Clinical parameters (HR, RR and rectal temperature) were measured at every time point (2, 6 and 24 hours) when blood was collected.

### ***Sample Collection***

A small aliquot of the plasma used in the study was requested from the manufacturer prior to shipment. The sample was collected into citrated tubes and shipped together with the bags of plasma. Additional samples were taken from randomly chosen plasma bags, at three other time

points in the study. These were used to measure concentrations of fibrinogen [FIB] and albumin [Alb] over the time course of the experiment.

Blood samples were collected from each manually restrained foal, at time 0 (pre fluid infusion), 2, 6 and 24 hours post-transfusion, by jugular venipuncture, using a 20 gauge 1 ½ inch sterile vacutainer needle (Vacutainer®, BD & Co, NJ, USA).

Blood was collected in sodium citrate-coated tubes (Vacutainer ®, BD & Co, NJ, USA) for measurement of [FIB] in serum tubes without anticoagulant (Vacutainer ®, BD & Co, NJ, USA) for measurement of [SAA], [TNF- $\alpha$ ], [IL-1 $\beta$ ], [IL-10], [IL-6] [IL-8], [IL4], [IL-17], and [INF- $\lambda$ ]. Whole blood was collected into specialized tubes for RNA stabilization (Tempus™ Blood RNA tube, Applied Biosystems, MA, USA) and stored at -80 °C until extraction was performed according to the manufacturer instructions. These samples were used for the measurement of cytokine gene expression (TNF- $\alpha$ , IL-1 $\beta$ , INF- $\lambda$ , IL-8, IL-6, and IL-10) (Brault, 2010).

Serum tube blood samples were allowed to clot, and centrifuged at 3800 rpm for 25 min at 20°C. The serum was removed, aliquoted and stored at -80 °C for batch analysis of [SAA], [TNF- $\alpha$ ], [IL-1 $\beta$ ], [IL-10], [IL-6], [IL-8], [IL4], [IL-17] and [INF- $\lambda$ ]. Citrated blood was centrifuged at 3800 rpm, for 15 minutes, at 4°C, and plasma was removed and stored at -20 °C. Samples were sent weekly to the internal clinical pathology laboratory at the University of Illinois Veterinary Teaching Hospital for the measurement of [FIB]. All samples were kept at -20 °C for a similar period of time to minimize the effects of potential degradation (Woodhams, 2001).

## ***Sample Assays***

### *IgG concentration measurement*

Serum [IgG] was measured for each foal at baseline and at 24 hours using a densimeter specific for equine IgG measurement (590B Foal IgG Analyzer Kit, Animal Reproduction, Systems, CA, USA) in accordance to manufacturer instructions.

### *RNA extraction, cDNA synthesis and real-time quantitative RT-PCR for cytokine expression*

RNA isolation and purification from RNA tubes (Tempus<sup>TM</sup> RNA tubes, Blood Applied Biosystems, MA, USA) was performed using the Tempus Spin RNA Isolation Kit (Applied Biosystems, WA, USA) in accordance with manufacturer instructions. Purified RNA was stored at -80 °C for batch analysis of all samples. The RNA concentration of each sample was measured by spectrophotometry at 260 nm (Nanodrop 2000, ThermoScientific, DE, USA). Synthesis of cDNA was performed using qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD, USA) and nuclease-free H<sub>2</sub>O according to manufacturer's instructions. The cDNA was diluted 1:4 with nuclease-free H<sub>2</sub>O and stored at 4°C pending further analysis. Real-time quantitative RT-PCR for TNF- $\alpha$ , IL-1 $\beta$ , INF- $\lambda$ , IL-8, IL-6, and IL-10 was performed using SYBR green (PerfeCTa® SYBR® Green SuperMix, Low ROX<sup>TM</sup>, Quanta Biosciences, Gaithersburg, MD, USA) and forward and reverse primers. Validated and published equine cytokine (TNF- $\alpha$ , IL-1 $\beta$ , INF- $\lambda$ , IL-8, IL-6, and IL-10), housekeeping ( $\beta$ -actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and beta 2 microglobulin (B2M)) gene primers were designed from the GenBank® database (Table 2). PCR amplification was performed in Thermocycler (ABI 7900, ThermoFisher Scientific, NY, USA). PCR cycling conditions were 95°C for 7 min

followed by 40 cycles of 95°C for 15 seconds, 60°C for 1 minute. The melting curve was composed of two cycles of 95°C for 15 seconds and of 65°C for 1 minute.

The RT-PCR conditions for each cytokine and housekeeping gene primers were validated and optimized using a pooled sample that contained aliquots of cDNA from each foal. All amplification reactions were performed in triplicate. A five- point standard curve was prepared using 5 µL of the pooled cDNA samples that was then serially diluted in nuclease-free H<sub>2</sub>O, as previously described (Whelan, 2003; Stordeur, 2002). Absolute quantification values were obtained against the standard curve generated using the pooled samples and were normalized by dividing by the geometric mean of the equine housekeeping genes. Efficiency of the PCR was calculated based on the slope of the standard curve for each plate run.

#### *Fibrinogen [FIB] and Serum Amyloid A [SAA] concentration measurement*

The [FIB] in each sample was measured using the Clauss clotting method (STA- Compact®, STAGO, Mount Olive, NJ, USA). The [SAA] in each sample was determined using a commercial turbidometric immunoassay at the University of Miami (Acute Phase Proteins Laboratory, University of Miami, Miami, FL, USA). This assay was developed for humans (LZ test SAA, Eiken Chemical©, Tokyo, Japan), and it was validated in horses (Jacobsen, 2006; Belgrave, 2013).

#### *Serum cytokines concentrations measurement*

Serum [TNFα], [IL-1β], [IL-6] and [IL-8] in each sample were measured using commercially available enzyme linked immune sorbent assay (ELISA) kits (TNFα, IL-1β, IL-6 and IL-10 by R&D System, Minneapolis, MN, USA; IL-8 by Genorise Scientific, Glen Mills, PA, USA). The

use of ELISA for the measurement of equine cytokines has been previously described (Wagner et al., 2006, 2009). Each of these assays had been validated by the manufacturer for use in equine cell culture supernatant samples, so each kit was validated for use with equine serum, by verifying recovery of sample and matrix interference, prior to their application in sample analysis. The assays were performed according to manufacturer instructions. All plates were analyzed using a spectrophotometric plate reader (Bio-tek®, EL800 model) at 450 nm, with a correction applied at 562 nm. Each sample was analyzed in duplicate for each cytokine. For analysis against the standard curve, most of the serum samples had to be diluted from between 1:2 to 1:10000. The samples did not require dilution for the [IL1-β] assay. There were significant technical difficulties and discrepancies in applying the [IL-10] assay (Burton et al, 2006), so the samples were submitted for analysis with a bead-based multiplex assay (Luminex, <http://www.luminexcorp.com/>), at Cornell University (Wagner, 2009). The results of both tests are reported.

### **Statistical Analysis**

The data for all of the assays were analyzed using commercially available statistical software (IBM®, SPSS®, 23<sup>rd</sup> version). Normality was confirmed with a Shapiro-Wilk test. Repeated measures ANOVA followed by a Least Significant Difference (LSD) post hoc test was performed for normally distributed data ([FIB] only). Non-normally distributed data was analyzed with the non-parametric Friedman's test for variables obtained at a given time point (time = 0, 2, 6 and 24 hours) followed by the Wilcoxon signed rank test. Because of the multiple comparisons that were made during the analysis, a Bonferroni correction was calculated by dividing the overall level of significance with the number of tests performed in each set (e.g.,

0.05/6). The p value used for statistical significance within each test was  $< 0.0083$ . The Kruskal-Wallis test was used to compare cytokine concentrations and gene expression values, between the two groups. Level of significance for this analysis, with Bonferroni correction, was  $P < 0.05/4 = 0.0125$ . Correlation between [SAA], [IgG], cytokine concentrations, and gene expression levels, was performed by Spearman rank correlation testing, and the results were considered significant at  $p < 0.05$ . Correlation of [IgG] and [SAA] between treatment groups, was performed using one-way ANOVA with Welch's test, and the difference was considered significant at  $p < 0.05$ . Categories of failure of passive immunity were defined using conventional cut-off values (Group 0, complete failure [IgG]  $< 400$  mg/dl; Group 1, partial failure [IgG]  $> 400$  and  $< 800$  mg/dl; Group 2, normal passive transfer [IgG]  $> 800$  mg/dl).

## CHAPTER 4

### RESULTS

Thirty-five (35) foals were enrolled in the study, 16 males and 18 females. In the plasma group (n=17), there were 9 males and 8 females. In the crystalloid group (n=18), there were 7 males and 11 females. Two enrolled foals were excluded from the study due to catheter failure and health problems (meconium impaction). None of the foals developed an increase in rectal temperature, tachycardia, tachypnea, piloerection or colic during or after transfusion. Three foals exhibited mild episodes of shivering during administration. For all foals, vital parameters were all within normal ranges throughout the period of study. No significant changes in temperature, heart rate or respiratory rate were noticed over time (Table 3).

#### **IgG Concentrations [IgG]**

As expected the mean [IgG] was significantly increased from baseline at 24 hours ( $\chi^2(1) = 4.000$ ,  $p = 0.046$ ) (Table 4). Crystalloids did not elicit a significant increase in [IgG] between baseline and 24 hours ( $p > 0.005$ ).

#### **Tumor necrosis factor alpha concentrations [TNF- $\alpha$ ]**

In the plasma group the mean [TNF- $\alpha$ ] at 2, 6 and 24 hours was significantly increased over baseline ( $p < 0.001$ ) (Figure 2, Table 6). [TNF- $\alpha$ ] did not statistically differ between treatment groups at any time point ( $p > 0.05$ ). A 6-fold increase in [TNF- $\alpha$ ], over baseline, was observed in 15/18 (88%) of the plasma group foals and in 6/18 (33%) of the crystalloid group foals. Donor plasma [TNF- $\alpha$ ] was 128.55 ng/ml.



### **TNF- $\alpha$ gene expression**

In the plasma group foals, TNF- $\alpha$  gene expression decreased significantly at 24 hours compared to 6 hours ( $Z = -2.698$ ;  $p = 0.007$ ) (Figure 3, Table 9). No significant correlation between [TNF- $\alpha$ ] and TNF- $\alpha$  gene expression was observed in any of the groups (plasma  $p = 0.901$ ; control  $p = 0.339$ ). No statistically significant differences in TNF- $\alpha$  gene expression were found between the plasma and crystalloid groups at any time point ( $p > 0.0125$ ).

### **Interleukin 1Beta concentration [IL-1 $\beta$ ]**

Serum IL-1 $\beta$  was not detected in any foals, from either group, at any time point. IL-1 $\beta$  was also not detected in the donor plasma.

### **IL-1 $\beta$ gene expression**

A time-dependent, statistically significant difference in IL-1 $\beta$  gene expression was found in both the plasma group ( $\chi^2(3) = 13.878$ ,  $p = 0.003$ ) and control group foals ( $\chi^2(3) = 27.057$ ,  $p < 0.001$ ) (Figure 1, Table 9). Compared to baseline, IL-1 $\beta$  gene expression was significantly lower at 24 hours in both plasma ( $Z = -3.064$ ;  $p = 0.002$ ) and control group ( $Z = -2.967$ ;  $p = 0.003$ ) foals and between 2 and 24 hours only in the control group ( $Z = -3.255$ ;  $p = 0.001$ ). There were no statistically significant differences in IL-1 $\beta$  gene expression between treatment groups at any time point ( $p > 0.0125$ ).

### **Interleukin-8 concentration [IL-8]**

A time-dependent, statistically significant difference in [IL-8] was found in plasma group foals ( $\chi^2(3) = 15.817$ ,  $p = 0.001$ ). Compared to baseline, [IL-8] was significantly higher at 2 hours in

the plasma group foals ( $Z = -3.195$ ;  $p = 0.001$ ) (Figure 4, Table 6). There were no statistically significant differences in [IL-8] between treatment groups at any time point ( $p > 0.0125$ ). No significant correlation between [IL-8] and IL-8 gene expression was observed in any treatment group, at any time point ( $p > 0.05$ ). [IL-8] was undetectable in 1/17 (6.0%) of plasma group and 3/18 (16.7%) of crystalloid group foals. A 1.3-fold (or greater) increase in [IL-8] was observed in 11/17 (64.7%) of plasma group foals. In 3/11 (17.6%) of these foals, there was a 4000-fold increase by 2 hours from plasma administration and an additional 8/17 (47.0%) at the following time points. A similar increase was noticed in the crystalloid group in only 5/18 (27.8%) foals (one animal presented with a 5000-fold increase and another over 1300-fold) at 2 hours and in 4/18 (22.2%) of foals at 6 and 24 hours.

### **IL-8 gene expression**

IL-8 gene expression was not statistically different over time, in any of the experimental groups (plasma:  $\chi^2(3) = 2.407$ ,  $p = 0.492$ ; controls:  $\chi^2(3) = 4.123$ ,  $p = 0.249$ ) (Figure 5, Table 9). [IL-8] were negatively correlated with [SAA] at 6 hours in the crystalloid group foals ( $r = -0.479$ ,  $p = 0.044$ ).

### **Interleukin-6 concentration [IL-6]**

No significant time-dependent changes in [IL-6] were found in either of the experimental groups (plasma:  $\chi^2(3) = 6.529$ ,  $p = 0.089$ ; control:  $\chi^2(3) = 3.933$ ,  $p = 0.269$ ) (Figure 6, Table 6). No statistically significant differences in [IL-6] were found between experimental groups at any time point ( $p > 0.0125$ ).

In the plasma group foals, [IL-6] was correlated with IgG concentrations at baseline ( $r=0.483$ ,  $p=0.049$ ). [IL-6] was not detectable in the commercial plasma samples. In the plasma group, a greater than two-fold increase in [IL-6] over baseline was observed in 7/17 (41%) of the foals at 2 hours, and in 8/17 (47.1%) of the foals at 6 and 24 hours. Of these foals, 4/7 (5.9%) had a greater than 100-fold increase (in [IL-6] over baseline, and 3/7 (17.6%) a 5000-fold increase in [IL-6] over baseline. In the control group, 1/18 (5.6%) foal demonstrated a 9-fold increase in [IL-6] over baseline, at 2 and 6 hours, and a 7-fold increase in [IL-6] over baseline at 24 hours.

### **IL-6 gene expression**

IL-6 gene expression was not detectable in any group and at any time point.

### **Interferon gamma concentration- [IFN- $\gamma$ ]**

[IFN- $\gamma$ ] was detectable in all foals at all time points (Figure 7, Table 7). No significant difference in [IFN- $\gamma$ ] was noticed between groups at any time point ( $p>0.0125$ ). No significant time dependent difference was noticed in any group at any time point (plasma=  $\chi^2(3) = 1.215$ ,  $p = 0.749$ ; control=  $\chi^2(3) = 4.786$ ,  $p = 0.188$ ). In the plasma group, a 1.1-fold increase in [IFN- $\gamma$ ] over baseline was observed in 3/17 (17.6%) foals at 2 hours, 7/17 (41.2%) foals at 6 hours and 8/17 (47.0%) foals at 24 hours. One foal in the plasma group demonstrated a 40-fold increase in [IFN- $\gamma$ ] over baseline, starting from 2 hours post-administration. In the crystalloid group, 4/18 (22.2%) foals demonstrated a 50-fold increase at 6 and 24 hours.

### **IFN- $\gamma$ gene expression**

No time-dependent change in IFN- $\gamma$  gene expression was found in either group (plasma=  $\chi^2$  (3) =2.170,  $p = 0.538$ ; control=  $\chi^2$  (3) =0.791,  $p = 0.852$ ) (Figure 8, Table 10). No statistically significant differences in IFN- $\gamma$  gene expression between groups were found between groups, at any time point ( $p>0.0125$ ).

### **Interleukin 10 concentrations [IL-10] (multiplex assay)**

Using the multiplex assay, [IL-10] was detectable in 14/35 (40.0%) of the foals and was under the detectable limits (15 pg/ml) in the remaining foals. The number of foals with detectable [IL-10] varied with time; t=0 hours (n=8/35, 23.0%); t=6 hours (n=4/35, 11.0%); and t=24 hours (n=1). No significant differences in [IL-10] between groups were found at any time point ( $p>0.0125$ ). No significant time-dependent differences in [IL-10] from baseline were found in any group, at any time points (plasma=  $\chi^2$  (3) =3.436,  $p = 0.329$ ; control=  $\chi^2$  (3) =4.818,  $p=0.186$ ) (Figure 9, Table 8). In the crystalloid group foals, [IL-10] demonstrated a positive correlation with IL-10 gene expression at 6 hours ( $r=0.581$ ,  $p=0.014$ ).

### **Interleukin 10 concentrations [IL-10] (ELISA)**

IL-10 concentrations were detectable in 34/36 foals by ELISA. IL-10 was undetectable in one foal from the plasma group (t=0 hours) and in one foal from the crystalloid group (t=6 hours). A time-dependent difference in [IL-10] was found in the plasma group ( $\chi^2$  (3) =16.775,  $p = 0.001$ ), but not in the crystalloid group foals ( $\chi^2$  (3) =13.306,  $p = 0.004$ ) (Figure 10). In the plasma group foals, a statistically significant difference in [IL-10] was noticed between 6 and 24 hours ( $p=0.005$ ) (Table 8 and Figure 9). No statistically significant difference in [IL-10] between

groups was noticed at any time point ( $p < 0.0125$ ). In the plasma group, a 1.3-fold increase in [IL-10] over baseline was noted in 10/17 (58.0%) of the foals at 2 hours, in 11/17 (65.0%) of the foals at 6 hours, and in 10/17 (58.0%) of the foals at 24 hours. A similar magnitude of increase in [IL-10] over baseline, was noticed in 1/18 (5.6%) of the control group foals at 6 hours.

### **IL-10 gene expression**

IL-10 gene expression was detectable in all foals (Figure 11, Table 10). A significant time-dependent change in IL-10 gene expression was observed in the crystalloid group, but not in the plasma group foals (plasma =  $\chi^2(3) = 2.032$ ,  $p = 0.566$ ; control =  $\chi^2(3) = 13.244$ ,  $p = 0.004$ ). In the crystalloid group foals, IL-10 gene expression at 24 hours was significantly reduced compared to baseline ( $t=0$ ) ( $Z = -3.015$ ,  $p = 0.003$ ). No statistically significant differences in IL-10 gene expression were found between groups at any time point ( $p > 0.0125$ ). There was a positive correlation between IL10 gene expression and the following:

- IL-8 gene expression at baseline ( $r = 0.435$ ,  $p = 0.009$ ), 6 hours ( $r = 0.523$ ,  $p = 0.001$ ), and 24 hours ( $r = 0.453$ ,  $p < 0.001$ )
- IFN- $\gamma$  gene expression at baseline ( $r = 0.718$ ,  $p < 0.001$ ), 2, 6 and 24 hours ( $r = 0.7$ ,  $p < 0.001$ )
- IL-1 $\beta$  gene expression ( $r = 0.421$ ,  $p = 0.012$ ) at 6 hours
- TNF gene expression at 2 ( $r = 0.391$ ,  $p = 0.020$ ) and 24 hours ( $r = 0.447$ ,  $p = 0.007$ )

### **Interleukin 4 concentration [IL-4]**

All foals had detectable [IL-4] and at all time points. No differences in [IL-4] were noticed between experimental groups at any time point ( $p > 0.0125$ ) (Figure 12, Table 7). No significant time-dependent changes [IL-4] were observed in any group, at any time point (plasma =  $\chi^2(3)$

=4.964,  $p = 0.174$ ; control= $\chi^2(3) = 6.318$ ,  $p = 0.957$ ). By visual examination crystalloid foals demonstrated a greater [IL-4] response compared to plasma foals:

Biologically significant differences observed between groups and over time are as listed:

- At baseline ( $t=0$ ), 5/18 (27.8%) crystalloid group and 7/17 (41.0%) plasma group foals had detectable [IL-4].
- Within the foals receiving crystalloids, 40 and 1800-fold increases in [IL-4] over baseline were noticed in 2/18 foals (11.1%) at 2 hours, in 3/18 foals (16.7%) at 6 hours, and in 2/18 (11.1%) at 24 hours.
- Within the foals receiving plasma, 200 and 1800-fold increases in [IL-4] over baseline were noticed in 2/17 (11.7%) of foals at 6 hours and 800-fold increase in [IL-4] over baseline in 1/17 (5.8%) foal at 24 hours.

### **Interleukin 17 concentration [IL-17]**

[IL-17] were detectable in all foals and at all time points (Table 7 and Figure 13). No difference in [IL-17] was noticed between groups at any time point ( $p > 0.0125$ ). No significant time-dependent differences in [IL-17] were noticed in any group, at any time point (plasma= $\chi^2(3) = 6.231$ ,  $p = 0.101$ ; control= $\chi^2(3) = 0.021$ ,  $p = 0.999$ ).

In the crystalloid group the number of foals, the magnitude of increase in [IL-17] over baseline varied with time as listed:

- $t = 2$  hours, 1/18 (5.6%) foals demonstrated a 1.2-fold increase
- $t = 6$  hours, 4/18 (22.2%) foals demonstrated a 5 to 440-fold increase
- $t = 24$  hours, 2/18 (11.1%) foals demonstrated a 12.5 to 25-fold increase

In the plasma group, 2/17 (11.7%) foals demonstrated a 5 to 10-fold increase in [IL-17] over baseline at 6 hours, with 1/17 (5.9%) demonstrating a 25-fold increase over baseline at 24 hours.

### **Fibrinogen concentration**

The fibrinogen concentration was normally distributed, and data are expressed as mean  $\pm$  SD (Table 11). There was a significant effect of time ( $F=17.633$ ,  $p<0.001$ ), and a treatment  $\times$  time interaction on [FIB] ( $F= 6.428$ ,  $p=0.010$ ). Fibrinogen concentration did not differ between groups at baseline ( $p>0.05$ ). A steady increase in [FIB] was noticed overtime in both experimental groups. In the plasma group foals, [FIB] was significantly increased at 2, 6 and 24 hours ( $p<0.001$ ) compared to baseline, and at 2 hours ( $p=0.002$ ) and 6 hours ( $p=0.004$ ) compared to 24 hours (Figure 15). In the crystalloid group foals, there was a statistically significant difference in [FIB] between 2 hours and 24 hours ( $p=0.004$ ). There was an increase in [FIB] between 0 and 24 hours in both the plasma (1.2 fold) and crystalloid (1.3 fold) group of foals. There was a statistically significant difference in [FIB] between the two experimental groups at 6 hours after fluid administration ( $p=0.048$ ).

### **Serum Amyloid A concentration**

At the beginning of the study, sixty percent (21/35) of the foals had [SAA] over normal adult ranges (20 mg/l), and measured values of [SAA] exceeded published normal values for similar aged foals (27.1 mg/l) (Stoneham, 2001) in 45.7% (16/35) of study foals. Serum amyloid A concentrations decreased overtime in both groups of foals, and a significant change in [SAA] was observed over time ( $\chi^2(3) = 31.036$ ,  $p<0.001$ ). In the crystalloid group foals, [SAA] was significantly decreased at 24 hours compared to 2 and 6 hours ( $Z= -3.010$ ,  $p=0.003$ ;  $Z= -3.408$ ,

p=0.001, figure 16, table 12). Serum amyloid A was significantly different between experimental groups at 24 hours (p=0.0125).

*Serum Amyloid A and passive transfer*

Using one-way ANOVA with Welch's test, there were statistically significant differences in [SAA] between foals identified as having complete failure of passive transfer of maternal immunoglobulins (Group 0, <400) and those categorized as reaching normal levels of passive immune transfer (Group 2, >800) (p=0.002).



## CHAPTER 5

### DISCUSSION

The aim of this study was to investigate the inflammatory effect of FFP administration in healthy, neonatal foals. This was achieved by measuring a range of systemic, molecular, inflammatory response markers in foals, at several time points over a 24 hour period following IV plasma transfusion, and comparing the response to foals receiving IV crystalloid fluids. The inflammatory markers included cytokine gene expression, circulating concentrations of cytokines, and acute phase proteins. Changes in systemic cytokine concentrations have been reported in human patients receiving blood products (Pandey, 2010; Keir, 2013; Muylle, 1995), but there is very limited information on the immunological impact of using this important category of therapeutics in animals. The results of our study reported that FFP administration was associated with a balanced, subclinical, acute, systemic inflammatory response in neonatal foals. The plasma-invoked response was characterized by increased circulating levels of TNF- $\alpha$ , IL-8 and IL-6, decreased IL-1 $\beta$ , and decreased TNF-  $\alpha$  gene expression in blood leukocytes.

These results contradict previous reports that did not demonstrate an up-regulation of cytokine gene expression (IFN- $\gamma$ , IL- 4, IL-8, IL-6, or IL-1  $\beta$ ) with plasma administration in neonatal foals (Gold, 2007) and that FFP could actually have an immune- protective effect on endothelial activation and systemic inflammation in critically ill human patients (Straat et al, 2015). These differences in results could arise from differences in study design and sampling frequencies. For instance, our study had a higher sampling frequency and shorter sampling interval than the studies performed by Gold et al, 2007, but our sampling was more delayed than Straat et al, 2015. The production of mRNA and accompanying protein synthesis can occur very rapidly, and gene expression can be extremely short-lived after stimulation.

In general, any inflammatory cascade involves the sequential activation and release of mediators (cytokines) early after recognition of tissue damage or the presence of pathogenic molecules. Tumor necrosis factor- $\alpha$  is one of the first cytokines produced during inflammation. Tumor necrosis factor- $\alpha$  gene expression occurs by activation of the NF- $\kappa$ B pathway, following interaction between immunostimulatory molecules and toll-like receptors (TLR) on CD4<sup>+</sup> Th1 cells, macrophages and neutrophils. The pro-inflammatory molecules that bind to cell surface TLRs include complement, cytokines, bacteria, viruses, LPS or immunoglobulins. The NF- $\kappa$ B pathway can also be activated by TLR-binding of molecules originating from damaged cells or tissues (DAMPs- damage associated molecular patterns) secondary to trauma or cytotoxicity (Mills, 2011). In our study, we saw a significant, and rapid, plasma-associated stimulation of TNF- $\alpha$  gene expression and protein synthesis. Interestingly, a similar, but smaller TNF- $\alpha$  response, was also observed in the crystalloid group foals. Activation of NF- $\kappa$ B, independent of TLR activation, has also been described in response to a variety of other stimuli including TNF- $\alpha$ , thrombin and fluid shear stress (Anrather, 1997). Thrombin has been shown to be a potent endothelial cell activator (Anrather, 1997), and temporary increases in circulating thrombin have been observed during IV catheter placement in healthy, adult horses (McGovern, 2012). Since there was significant increase in TNF- $\alpha$  production in both experimental groups, it seems likely that the pro-inflammatory stimulus was linked with IV catheter placement. Evaluation of coagulation parameters was not performed in this study, but the impact of IV catheter on thrombin production in neonatal foals receiving fluid therapy is warranted.

The importance of TNF- $\alpha$  in a variety of equine acute inflammatory conditions has been well documented (Wijnker, 2004, Kothari, 2013, Pusterla 2006). In healthy foals, TNF- $\alpha$  gene activation is present soon after birth, and undergoes age-related increases (Boyd, 2007). Gene

expression for TNF- $\alpha$  appears to be reduced in septic foals (Pusterla, 2006). In our study, the onset of TNF- $\alpha$  gene expression, and its associated protein production, were quite rapid and were observed within 2 hours of plasma administration. While TNF- $\alpha$  gene expression subsided over time, decreasing significantly by 24 hours, serum [TNF- $\alpha$ ] remained consistent for the duration of the study. Similar TNF- $\alpha$  production kinetics have been reported in human medicine (DeForge, 1991). The decrease in TNF- $\alpha$  gene expression was observed beginning at 6 hours in the plasma group foals. This significant decline could be explained by the presence of an anti-inflammatory stimulus or by the absence of pro-inflammatory stimuli. Tumor necrosis factor- $\alpha$  and IL-1- $\beta$  suppression have been previously attributed to increases in IL-6 concentrations (Schlinder, 1990). Interestingly, in this study [IL-6] reached peak serum levels 6 hours after the transfusion. An important piece of evidence supporting the pro-inflammatory impact of IV plasma administration was the significant difference in [TNF- $\alpha$ ] changes between the plasma group compared to the crystalloid group foals, both in terms of magnitude (5.4 fold increase over baseline within 2 hours post-transfusion) and with respect to the number of foals affected. Circulating [TNF- $\alpha$ ] is undetectable at birth in healthy, neonatal foals, but its concentration rises due to colostrum (not milk) consumption (Secor, 2012). While the initial upregulation of TNF- $\alpha$  gene expression was attributed to catheter-related endothelial activation, the early increase in circulating [TNF- $\alpha$ ] in our study was likely a combination of colostral TNF- $\alpha$  and an accompanying, substantial, plasma-dependent amplification of the initial endothelial-invoked inflammatory response. In other species, this two-step process has been described as the double-trigger inflammatory response (Shaz, 2011; Urner, 2012). Incompatible blood transfusion reactions have been reported to cause a dose-dependent increase in TNF- $\alpha$  production (Muyllé, 1995). While the specific role played by TNF- $\alpha$  in the sub-clinical inflammatory response

observed in this study is not certain, it is likely to be similar to that reported in other immune reactions. Tumor necrosis factor- $\alpha$  is a multi-functional cytokine, involved in a wide variety of processes. At the vascular endothelium level, this cytokine induces expression of adhesion molecules, stimulates innate immunity and recruitment of neutrophils, which is mediated by IL-8 (Kolaczowska, 2013). Specific measures of cell-surface adhesion molecule expression were not performed in this study, and there were no detectable changes in systemic neutrophil numbers. Additional studies are required to explore the relative importance and role of TNF- $\alpha$  in plasma-mediated transfusion reactions.

The chemokine IL-8 was another significant inflammatory mediator in our study. Measureable circulating levels of IL-8 were detected in foals from both experimental groups prior to the administration of any treatments. As reported in previous studies, IL-8 is expressed at birth, and circulating concentrations are detectable even prior to colostrum administration (Boyd, 2003; Mariella, 2014). Plasma administration appeared to produce a mild, and non-statistically significant, increase in IL-8 mRNA expression. In the absence of pro-inflammatory stimuli, IL-8 production is actively suppressed (Baggiolini, 1992), but following an immunological challenge, IL-8 is rapidly produced by monocytes, endothelial cells, or activated neutrophils and is a potent chemotactic molecule for promoting additional neutrophil recruitment (Baggiolini, 1992). Stimuli, such as IL-1 or TNF- $\alpha$  have been shown to up-regulate IL-8 through several signaling pathways, including activation of NF- $\kappa$ B. In these cases, the major sources of IL-8 production are usually endothelial cells. Significant amounts of IL-8 can usually be detected within thirty minutes of IL-1 stimulation (Hoffmann, 2002), and rapid changes in local concentrations can be attributed to the fact that this chemokine can be swiftly stored or released from pre-formed plasmalemmal vesicles within the endothelial cells (Rot, 1996). In the plasma group foals, [IL-8]

showed a 2-fold increase from baseline within 2 hours of plasma administration. The concomitant increase in [TNF- $\alpha$ ] and [IL-8] at this particular time point in the plasma group foals may indicate some kind of co-regulatory function for these two molecules. The relatively small changes in IL-8 gene expression observed in these early stages could be explained by the existence of a storage phase to the kinetics of this chemokine or the nature of the sampling schedule that precluded its detection because of its short half-life. Both experimental groups, after the initial 2 hour post-transfusion sampling point, exhibited a slow, but gradual, decrease in circulating [IL-8] over time. The reduced IL-8 clearance has been reported in human medicine (DeForge, 1991). Also of interest was the effect of IV crystalloid administration on [IL-8] production. Although variations were not statistically significant, [IL-8] increased over time, starting at 2 hours. These findings provide additional evidence for the impact of crystalloid fluid administration, catheter placement, and/or volume expansion on the systemic immune system.

The utility of adopting a multi-mechanistic approach in identifying the presence, and characterizing the nature of any transfusion-evoked inflammatory response, was illustrated by the observations regarding IL-1  $\beta$ . Examinations of IL-1  $\beta$  gene expression indicated that there was significant activation of this cytokine pathway in both groups of foals, and yet there were no measurable serum [IL-1  $\beta$ ] detected in our study. Elusive circulating [IL-1  $\beta$ ] in the face of active gene expression has previously been reported in blood transfusion reactions (Davenport, 1994). With this inflammatory cytokine, elevated systemic concentrations only occur if severe, acute inflammatory diseases are present (Kleiner, 2013; Mooradian, 1991; Hasdai 1996; Di Iorio, 2003). In horses, the presence of IL-1  $\beta$  gene activation, immediately after birth in foals, has been previously reported (Boyd, 2003), but there have been conflicting reports regarding the use of IL-1  $\beta$  gene expression as a marker of systemic inflammatory responses. Pusterla et al., 2006

and Gold et al., 2007 did not find any difference in IL-1  $\beta$  expression between septic or healthy foals; whereas Castagnetti et al., 2012 found that IL-1  $\beta$  gene expression was up-regulated during sepsis. Our results provide an additional perspective by demonstrating an immediate post-partum IL-1  $\beta$  gene activation in healthy foals that decreased over the time course of the study. The observed decline in IL-1 $\beta$  gene expression with time in both experimental groups could be attributed to the presence of anti-inflammatory cytokines such as IL-10 or IL-4 or to the influence of IL-6 as previously described (Schlinder, 1990). The anti-inflammatory functions of IL-10 are well documented (Saraiva, 2010; Gurung, 2015). The failure of our study to identify circulating [IL-1  $\beta$ ], despite the presence of active gene expression, may have been due to poor sensitivity of the assays. Alternatively, this finding could also reflect the unusual kinetics of this cytokine that have been described elsewhere. Interleukin-1 $\beta$  mRNA expression rises rapidly (15 minutes) after pro-inflammatory stimuli and has a short (4 hours) half-life (Dinarello, 2014). In addition, the ability for cytokines to swiftly associate with cell membranes or to bind to carrier proteins has been well-documented (Devonport, 1994; Pang, 1994). The sampling schedule used in our study may have impeded the detection of IL-1  $\beta$  if one of these rapid, local mechanisms occurred. The pro-inflammatory functions of IL-1 $\beta$  are somewhat similar to TNF- $\alpha$ , particularly in its capacity to stimulate the production of other pro-inflammatory cytokines such as IL-6 and IL-8 (Sironi, 1989; Rot, 1996). In our study, circulating [IL-6] was identified in foals from both experimental groups, but again there was no evidence of active gene expression at the time points examined. The dynamics of post-parturient IL-6 production has been described as low in neonatal foals at both the transcriptional and translational levels, but responsive to antigenic stimulation (Liu, 2009; Nerren, 2009). In view of fact that significant amounts of IL-6 are present in colostrum and that circulating [IL-6] is low in healthy foals prior to colostrum

ingestion (Burton, 2009; Robinson, 1993), it is reasonable to assume that the baseline [IL-6] detected in our study foals of study was maternally derived. This is supported by the observation of a strong correlation between [IL-6] and [IgG] in the plasma study group foals.

In our study, circulating [IL-6] was identified in foals from both experimental groups, but again there was no evidence of active gene expression at the time points examined. The dynamics of post-parturient IL-6 production have been described as low in neonatal foals at both the transcriptional and translational levels, but responsive to antigenic stimulation (Liu, 2009; Nerren, 2009). In view of the fact that significant amounts of IL-6 are present in colostrum and that circulating [IL-6] is low in healthy foals prior to colostrum ingestion (Burton, 2009; Robinson, 1993), it is reasonable to assume that the baseline [IL-6] detected in our study foals was maternally derived. This is supported by the observation of a strong correlation between [IL-6] and [IgG] in the plasma study group foals. Although no statistically significant difference was noticed in the [IgG] between the two groups, the plasma group contained a higher number of foals with [IgG] over 800 mg/dl and a lower number of foals with [IgG] below 800 mg/dl (table 5) than the crystalloid group. There was also an observable, but non-significant, trend towards higher median circulating [IgG] in the plasma group foals at both 6 and 24 hours. While the relationship between [IgG] and [IL-6] at these later time points is unclear, the kinetics of the changes in [IL-6] mirror that previously reported in human studies (DeForge, 1991; Chi, 2001). In addition, IL-6 has been shown to have a storage pathway associated with the formation of cellular vesicles, similar to that described for IL-8 (Stanley, 2010). The appearance of circulating [IL-6] in the absence of detectable gene expression at 6 hours could be explained by a plasma transfusion-associated release of preformed endothelial IL-6. Interestingly, in the present study the 6 hour increase in [IL-6] was correlated with a significant reduction in TNF- $\alpha$  gene. The

suppressive effect of IL-6 on transcription levels of other cytokines, such as TNF- $\alpha$  or IL-1 $\beta$  has been previously reported (Schlinder, 1990; Starkie, 2003).

The molecular markers used in this study were selected to encompass as wide a range of immunological pathways as possible. While some of the markers employed are associated with early innate processes in the inflammatory response, others are associated with activation of the adaptive immune system (e.g. IFN- $\gamma$ , IL-4, IL-17). Active IFN- $\gamma$  gene expression and protein production was demonstrated in all foals in this study, but no evidence of an impact by plasma or crystalloid fluid administration was observed. This is similar to other studies that showed that IFN- $\gamma$  gene expression is present at birth, but then undergoes an incremental, age-related change independent of colostrum consumption (Boyd, 2003; Breathnach, 2006). Generally IFN- $\gamma$  originates from Th<sub>1</sub> lymphocytes, natural killer cells and CD8<sup>+</sup> cytotoxic lymphocytes; these cell types are also major sources for IL-1  $\beta$  and TNF- $\alpha$ . There is some controversy in the literature about Th-cell responses in the neonatal and young foal. Some authors suggest that foals are IFN- $\gamma$  deficient (Breathnach, 2006; Forsthuber, 1996; Garcia, 2000) and that the neonatal immune system primarily exhibits a Th2-bias (Boyd, 2003). However, recent studies, showed that foals are capable of mounting a strong Th1 response (Wagner, 2010), but their macrophages may be hyporesponsive to IFN- $\gamma$  activation (Marodi, 2002a). Pandey, 2010 reported that allogenic blood product administration mediates a Th<sub>1</sub> to Th<sub>2</sub> cell switch and an IL-10-, IL-4- and IL-5-mediated reduction in IFN- $\gamma$  activation. Although the expected Th1-response to antigenic stimulation would be an increase in [IFN- $\gamma$ ] (Nerren, 2009), the absence of incremental IFN- $\gamma$  activity to the plasma group foals in our study could be attributed to the immunomodulatory action of plasma the healthy foal immune system, directing them towards a Th2 response.



The IL-17 family of cytokines is composed of 7 members (IL-17A - F), with important pro-inflammatory properties. These include the upregulation of chemokines (IL-8, IL-6, IL-4) activity and the accompanying recruitment of both neutrophils and eosinophils (Kolaczowska, 2013; Kawaguchi, 2004). The role of IL-17 in hypersensitivity reactions and airway responsiveness has been extensively studied in people and horses (Ainsworth, 2006; Kawaguchi, 2004). In our study, no overall difference in circulating [IL-17] was noticed between groups or over time. There was, however, significant individual variation in immune responsiveness with more crystalloid group foals than plasma group foals exhibiting marked increases in IL-17 at the later sample collection points. It was previously reported that IL-17 gene expression is reduced in foals at birth, and over the first week of life (Liu, 2011). The pro-inflammatory action of crystalloid administration could therefore be considered to be mild, and only detectable when the crystalloid group was analyzed in direct comparison to the plasma group. The immunomodulatory effect of plasma has been reported in human medicine (Subramanian, 2014). It is possible that plasma administration blunted the mild, IL-17-mediated inflammatory pathways associated with crystalloid infusion.

Interleukin-4 is broadly recognized as an anti-inflammatory cytokine. In this study circulating [IL-4] were not different overtime or between groups. The low numbers of foals with detectable [IL-4] levels (34%) was surprising in view of reports that indicated that colostral consumption has been associated with the passive transfer of IL-4 to newborn foals (Mariella, 2014). The fact that there was no correlation between circulating [IL-4] and [IgG], indicates that colostrum ingestion was not strongly associated with [IL-4] in our study population. The dynamics of [IL-4] change in this study were similar to that observed for [IL-17], with crystalloid administration being associated with a larger magnitude of [IL-4] change than plasma

administration. In foals, circulating [IL-4] has been shown to increase with age, reaching adult levels by three months of age. In the horse, in the immediate post-parturient period, IL-4 is produced by CD4<sup>-</sup> IgE<sup>+</sup> cells (basophils), followed later, as age increases, by CD4<sup>+</sup> T cells (Wagner, 2010). As with IL-17, it is possible that immunoglobulins from the plasma transfusion interfered with IL-4 expression and production. Since IL-4 plays an important role in the differentiation of naïve Th<sub>0</sub> cells into Th<sub>2</sub> cells and in the development of B cells into antibody producing plasma cells, plasma-transfusion associated IL-4 inhibition could down-regulate humoral immune responses in the young foal, and thus interfere with endogenous antibody production.

The anti-inflammatory cytokine IL-10 was also investigated in our study. Interleukin-10 is a cytokine produced by activated population of T (regulatory), B cells, monocytes-macrophages and keratinocytes (Moore, 1993). While this cytokine is not considered a normal component of colostrum (Burton, 2009), a recent study demonstrated that colostrum T cells retain the capacity to produce IL-10 after stimulation (Perkins, 2004). The major role of IL-10 is to modulate the inflammatory response through receptor dependent signals (Saraiva and O'Garra, 2010). The immunoregulatory activity of IL-10 occurs by its capacity to counteract immunoreactivity by stimulating the production of Th1 cytokines such as IL-1- $\alpha$ , IL-1 $\beta$ , IL-6, IL-8 and TNF- $\alpha$  (de Waal Malefyt, 1993, Pusterla, 2006). The use of IL-10 as a predictor for disease severity or injury has been proposed in both equine and human medicine (Pusterla, 2006; Neidhardt, 1997). Although IL-10 production from Treg cells is reduced in neonatal foals, compared to adults (Wagner, 2010), non-survivor foals seemed to have higher IL-10 gene expression than survivors (Pusterla, 2006). However, when measuring circulating [IL-10] in septic and healthy foals, the concentrations were not found to be significantly different (Burton, 2009). In this study, we used

a multiplex assay for IL-10 quantification. When compared to ELISA, this assay has a comparable sensitivity, but an improved specificity (Elshal, 2006). Using this technique, circulating [IL-10] was detectable in only 14/35 (40%) of the foals in our study and was considered below detectable limits (15 pg/ml) in the remaining foals. The absence of detectable [IL-10] could have been related to the excellent health status of the foals in this study or to their inability to mount a detectable and significant IL-10 response. There was also a great deal of foal-to-foal variation in [IL-10], indicating the need for a study population in future investigations. Despite the variability in circulating levels of this cytokine, significant IL-10 gene expression was detectable in many of the foals from both experimental groups. While the level of IL-10 gene expression remained relatively constant in the plasma group foals compared to time 0, there was a significant decline in gene expression by 24 hours in the crystalloid group foals. Clearly the crystalloid group foals can be considered as the normal controls in this study. It follows that the impact of plasma transfusion was a prolonged stimulation of IL-10 gene expression pathways. This supports the idea that IV plasma administration evokes a complex, time-dependent, balanced release of pro- and anti-inflammatory cytokines. This probable hypothesis provides a mechanism by which the immune system of the host can auto regulate the trajectory and magnitude of the systemic, plasma-induced inflammatory response.

Acute phase proteins (APP), such as FIB and SAA, are commonly used as markers of acute inflammation in equine medicine. Fibrinogen is considered to be a moderate APP compared to SAA (Hulten, 2002) and in the horse, undergoes a natural, physiologic increase from birth to about 5 months of age, when it reaches adult values (Barton, 1995; Harvey, 1984). In the present study, all foals underwent the expected physiological increase in [FIB], in both control and plasma groups. Moreover, none of the foals demonstrated increases in [FIB] outside

normal ranges. The highest [FIB] observed was 280 mg/dl at baseline (range  $200 \pm 70$  mg/dl). Plasma group foals demonstrated an earlier [FIB] increase compared to crystalline group foals. A recent study showed that the [FIB] in commercial plasma had little to no effect on [FIB] in sick patients [FIB] (Hollis, 2015). Our results deviate from those in that all foals except one, had increased [FIB] within 2 hours of plasma administration. The difference between studies can be explained by variation in study design, origin of transfused plasma and in the population of interest. For instance, the foals in the Hollis et al, 2015 study, all received an IV crystalloid bolus prior to plasma administration, and [FIB] were not measured immediately prior to transfusion. In our study the donor FFP fibrinogen concentration (mean 223.4 mg/dl) was higher than the baseline [FIB] measurements in all of the recipient foals. The majority of the plasma group foals demonstrated an increase in [FIB] at 2 hours post transfusion: 47% (8/17) of foals had a 10 mg/dl increase in [FIB] and 29% (5/17) had a 20 mg/dl increase in [FIB]. These observed increases in [FIB] may reflect an increased endogenous APP production or result from infusion of the plasma contents and volume-associated redistribution of vascular fluid in to the interstitial space. Fibrinogen production in hepatocytes is induced by IL-6 and glucocorticoid signaling pathways, (Mackiewicz, 1991) in response to inflammatory stimuli arising from inflammation, infection and tissue injury. In horses, a 2-10 fold increase in [FIB] is considered significant in the diagnosis of acute inflammation (Crisman, 2008). In horses, moderate increases in [FIB] can be detected within 24 hours of an inciting inflammatory insult, and peak levels are usually reached at 2–3 days (Gabay 1999, Schalm 1979; Hulten, 2002; Jacobsen et al., 2005; Pollack et al., 2005; Borges et al., 2007). Given the kinetics of FIB production, it is difficult to explain the early increases in [FIB] observed in the foals in our study, by *de novo* synthesis. It is possible,

however, that the gradual increase in [FIB] over 24 hours, was a result of a plasma-associated inflammatory stimulus.

There was an interesting, statistically significant, difference in [FIB] between the plasma and crystalline group foals at 6 hours after administration, largely due to a marked decline in [FIB] in the crystalline group. While this decline could be attributed to the hemodilutional effect of the crystalloid-associated increase in vascular volume, we would have expected this to occur more quickly (i.e. at 2 hours). Moreover, if the fluid volume changes were significant, we would have anticipated a similar trend with other serum proteins, but this was not observed. Increase in FIB degradation has been described after 4–6 hours of induced acidemia in pigs (Martini, 2007), but the crystalloid fluid employed in his study was Normosol-R, which is generally alkalinizing in effect, and therefore unlikely to affect the FIB in the samples. All samples were treated and stored, under similar conditions, making sample handling and laboratory error less likely causes. All foals were healthy throughout the study, and since no visible hematomas or excessive bleeding were identified, it is unlikely that coagulation cascade-associated degradation of FIB contributed to this decline in [FIB] in the crystalloid group foals. Another possible explanation for this delayed change in [FIB] could be a delayed hemodilution, arising from fluid redistribution from the interstitial to intravascular space. Equine neonates have a larger interstitial space compared to adults (Fielding, 2011) and a developing renal system, that impacts their ability to respond to, increased fluid loads (Palmer, 2004). These factors could lead to slightly unpredictable fluid dynamics early in life. Once again, if this were true, then changes in other serum protein concentrations would be expected.

Serum Amyloid A is also a commonly used marker for inflammation in equine medicine (Nunokawa, 1993; Hulten, 2002; Stoneham, 2001; Jacobsen, 2007). Several reports indicate that

SAA is a more sensitive marker of inflammation than [FIB], since rising circulating levels can be detected early in the inflammatory process and increases can expand several hundred-fold within 6 hours of the initial insult, with peak concentrations at 36-48 hours (Hulten, 2002). In the foal, circulating [SAA] can be increased due both endogenous production (Uhlar, 1999) or from an exogenous source, such as colostrum and early milk (Duggan, 2007, 2008). Precolostral [SAA] in newborn foals was previously reported to be relatively low, and a positive correlation between colostrum and foal [SAA] at 48 hours from birth has been described (Nunokawa, 1993; Duggan, 2007). In our study population, baseline [SAA] in both experimental groups were generally higher than previously reported (Nunokawa, 1993; Stoneham, 2001; Paltrinieri, 2008) with 60% of the foals demonstrating a mean baseline [SAA] 7-fold higher than published adult values and 5-fold higher than reported foals values. Physiological fetal or foal cortisolemia and spontaneous foaling have been reported as causes of elevated SAA production (Uhlar, 1999; Duggan, 2007). Pathophysiological stressors such as meconium impaction, sepsis or hypoxic insult may also induce an increase in [SAA] above and beyond that described for normal periparturient events (Duggan, 2007). Although SAA is primarily produced by the liver in response to inflammatory stimuli, the production of other SAA isoforms in extrahepatic tissues has been described (Uhlar, 1999). Serum amyloid A can be detected in colostrum and milk (Duggan, 2007; 2008), and specific milk and colostrum isoforms (AA3) have recently been determined in different species, including the horse (Berg, 2011; Chiba, 2009). The high baseline [SAA] was hypothesised to be due to passive acquisition from colostrum ingestion. All the foals in our study had consumed colostrum and the level of passive immune transfer was verified using IgG measurements. When assessing [SAA] in relationship with serum IgG groups, it was noticed that baseline [SAA] was significantly lower in foals with [IgG] concentration below 400 mg/dl, than in foals with IgG

concentration above 800 g/dl. This implies that successful acquisition of and absorption of colostrum may positively influence [SAA]. Normal foals demonstrate a mild age-related increase in [SAA] in the first 2-3 days of age, ranging 0–98.9 mg/l, most likely due to colostral and milk intake (Nunokawa, 1993; Paltrinieri, 2008; Duggan, 2008; Stoneham, 2001). As foals mature there is a physiologic decrease in [SAA]. Even though the foals in our study had greater baseline [SAA] than described in the literature, the gradual decline in [SAA] observed in our study was similar to that previously reported (Stoneham, 2011). The different measurement techniques and reagents used in the studies cited may have contributed to the discrepancy in results compared to the literature. Nunokawa and Paltrinieri used single radial immunodiffusion technique and ELISA respectively, whereas, the immunoturbidimetric assay was used by the Stoneham study as well as in this study. In our study, the increase in [SAA] at 24 hours after plasma administration may be related with the pro-inflammatory effects of plasma and be linked with the observed increase in pro-inflammatory cytokines, IL-6, IL-1 $\beta$  and TNF- $\alpha$ . Additional studies with prolonged sampling intervals are necessary to improve our understanding of SAA kinetics.

#### *Individual foals concentration assessments*

Variation in the individual peripheral blood cytokines gene expression and concentrations can be observed in Figure 14. Interestingly 4 foals allocated in the control group and 2 allocated in the plasma group presented with a similar behavior for IL-4, IL-17, IL-10 and IFN- $\gamma$ . Foal 13 and 33, both enrolled in the study with adequate passive transfer (IgG >2000 mg/dl) had very high cytokines concentrations at baseline followed by a significant decrease starting at 2 hours, reaching close to undetectable levels by 6 hours post crystalloid administration. Moreover, foal 13 demonstrated a very high TNF- $\alpha$  and IL-10 gene expression at baseline compared to its

cohort. Although it is known that TNF- $\alpha$  is colostrum derived at birth, the very high TNF- $\alpha$  expression at baseline could be indicative of endogenous production. It could be speculated that this foal was potentially in the process of fighting septicemia and that the fluids administered were able to mitigate the systemic effects. Interestingly, foals 14 and 34, both belonging to the crystalloid group, had total failure of passive transfer and demonstrated lower baseline [IL-10], [IL-17], [IL-4] and [IFN- $\gamma$ ]. [IgG] in these foals were 479 and 334.7 mg/dl respectively. Both foals responded to crystalloid administration with a substantial increase in those cytokines at 6 hours after administration. Individual variation in the ability to recognize and respond to pathogen colonization in several species was previously reported and attributed to MHC polymorphism (Sommer, 2005; Shad, 2004; Simkova, 2006; Antunes, 1998; Batchelor, 1990). While the real cause for this biological behavior cannot be entirely explained in our study, it could be speculated that foals 14 and 34 immune systems were in a more reactive status. Colostrum-deprived foals are more likely to be septicemic (Baldwin, 1993), and increased IL-10 expression was reported in non-surviving foals (Pusterla, 2006). Therefore, it could be hypothesized that the innate immune system of these foals was already primed, which could have potentially stimulated a more dramatic response to catheter placement and fluid administration. Individual variations were noticed also in the plasma group with foals 2, 9, 26, 35 and 36 demonstrating these to be more reactive.

Several trends for common cytokines were noticed and can be summarized as follows:

- Foal 2 demonstrated an increase in [IL-17], [IL-4] and [IFN- $\gamma$ ] at 24 hours
- Foal 9 increased in [IL-17] and [IFN- $\gamma$ ] at 24 hours
- Foal 35 demonstrated an exuberant decrease in [IL-10] starting at 2 hours after plasma administration



- Foal 26 and 36 demonstrated a uniformly increased [IL-10], [IL-17], [IL-4] and [IFN- $\gamma$ ] starting at 6 hours after plasma transfusion

Of the foals listed, only foal 26 demonstrated failure of passive transfer ([IgG] 138.9 mg/dl). While the clinical presentation remained unremarkable for all foals enrolled, it is possible that their immune system was involved in a more marked immunomodulation than the rest of the cohort. As stated previously, one of the limitations of this study was the lack of individual complete blood counts for the population of foals. Correlating immune cells concentration with cytokines behavior would have been extremely important in the clarification of otherwise unexplained interleukin trends, especially for the outliers in the population of study. In order to better define the pathway of interest in the immune behavior noticed in our study, immune cells receptors expression and activation of specific expression pathways could also be included in a future study design. Moreover, due to the highly variable and exquisitely individual behavior of the cytokines of interest, future studies should take into account to enroll a larger population of study.

### *Study Limitations*

An important limitation of the study was that the sampling duration only extended over 24 hours after administration of plasma. Further data collection points, at 48 or 72 hours after administration could have provided a more complete picture of the extent of the inflammatory condition induced by plasma. This is particularly important in regard to the acute phase proteins, because the kinetics of production and metabolism extend to days rather than hours. Another limitation of the current study was the absence of hematological data in our experimental foals. All foals appeared clinically healthy throughout the period of study. However, a baseline

leukogram examination would have provided additional information regarding their systemic health status prior to administration of IV plasma or crystalloids. Moreover, evaluation of the dynamics of the leukocyte populations over time would have been beneficial in determining the cytological associations with fluxes in cytokine production. Both IL-1 $\beta$  and TNF- $\alpha$  induce activation of endothelial adhesion molecules, which are essential for the attachment of leukocytes to the endothelial surface, prior to migration into the tissues. The pathogenesis of immune-mediated transfusion reactions involves a “two hit” mechanism initiated by endothelial cell activation elicited by surgery, infection, or tissue trauma and followed by a second triggering action induced by pro-inflammatory components of blood products (Urner, 2012; Silliman, 2009; Shaz, 2011, Pandey, 2012). Since inflammatory cells (leukocytes) are recruited by activation of the endothelium, monitoring hematological changes would have provided more information regarding the nature and extent of the inflammatory response.

## CHAPTER 6

### CONCLUSIONS

The present study demonstrated that FFP administration in neonatal foals has an immunomodulatory effect as revealed by the significant increase in TNF- $\alpha$ , IL-8 and IL-6. Similar cytokine dynamics have been described with acute hemolytic transfusion reactions in people (Devonport, 1995). This profile of rapid cytokine activation, associated with plasma administration could be associated with an acute inflammatory response by cells of the innate immune system or by an endothelial-based inflammatory cascade induced by TNF- $\alpha$  and IL-1 $\beta$  production. Both IL-1 $\beta$  and TNF- $\alpha$  are able to stimulate IL-8 and IL-6 production by endothelial cells. Although circulating [IL-1 $\beta$ ] was not detected, the presence of active IL-1 $\beta$  gene expression indicates a role for this cytokine in the plasma-invoked inflammatory response. The presence of active IL-1 $\beta$  and TNF- $\alpha$  gene expression, together with increased circulating TNF- $\alpha$ , IL-6 and IL-8 concentrations in response to FFP administration, supports the hypothesis that plasma is capable of inducing an immunomodulatory response in young foals. Interestingly, an inflammatory response was also detected in foals receiving crystalloid fluids, indicating that catheter placement and/or shear stress due to fluid administration alone are capable of inducing a mild, but detectable inflammatory response. Based on previous models of adverse reactions to blood component transfusions, the results of this study support the concept of a two hit model for the induction of inflammation (Urner, 2012). We speculate that catheter placement and fluid shear stress prime or sensitize endothelial or innate immune cells for immune reactivity. The pro-inflammatory constituents of plasma then provide the “second hit” or trigger capable of inducing an acute systemic inflammatory response, as demonstrated by the increase in proinflammatory cytokines and subsequent generation of acute phase protein generation. In our study SAA

demonstrated pattern of flux over 24 hours in the plasma group foals, consistent with its inflammatory kinetics. Circulating [FIB] also seems to be influenced by plasma administration and can be detected starting at the second hour after administration. Clinically, the increase in [FIB] in foals shortly after a blood transfusion can therefore be interpreted as an expected physiological response, but circulating [SAA] may be a more reliable marker for gauging the duration and magnitude of and an acute inflammatory response.

In contrast to the findings of other studies, plasma did not generate a profound anti-inflammatory response in our foals (Pandey, 2010). Active IL-10 gene expression, [IL-10] and [IL-4] were only detectable in some foals, and plasma administration did not seem to induce an increase in these cytokines. However, the great variation in standard deviation within the cytokines of interest indicates that individual variation in response to fluid therapy may play a role in detecting significance. Studies with larger populations are warranted.

## CHAPTER 7

### FUTURE PERSPECTIVES

Although blood products are commonly used in human medicine for the treatment and prevention of a variety of diseases, inappropriate or unjustified transfusion may expose patients to unnecessary risk. Fresh frozen plasma transfusion guidelines have been proposed in human medicine in order to limit the inappropriate prophylactic or inadequate use of FFP (Shaz, 2011; Puetz, 2012). Similar recommendations have recently been reported in canine medicine (Beer, 2015). In the equine neonate, incidence of transfusion reactions is reported to be higher than adults but limited recommendations on the use of FFP are reported (Hardefeldt, 2010). Based on the results of our study, the concomitant presence of an activated endothelium and the administration of a blood product can be sufficient stimuli for the induction of a subclinical acute inflammatory response, even in healthy neonatal foals. Studies involving a population of sick foals are required to investigate the extent of the inflammatory response in individuals whose immune response may be already active due to the presence of an underlying disease. The administration of FFP to sick neonatal foals may result in a greater inflammatory response possibly contributing to increased morbidity and mortality of hospitalized intensive care patients. The results of this study may provide useful indications and recommendation for the administration of FFP in the neonatal foal.

CHAPTER 8

FIGURES AND TABLES

<b><i>Gene</i></b>	<b><i>Primer F</i></b>	<b><i>Primer R</i></b>	<b><i>Source</i></b>
<b>TNF<math>\alpha</math></b>	CGGGATCCCAGCTGGACTTGAGCCCCT	CCCAAGCTTGCCGATCACCCCAAAGTG	<i>Boyd et al., 2007</i>
<b>IL1<math>\beta</math></b>	TGTACCTGTCTTGTGGGATGAAA	TTCTGCTTGAGAGGTGCTGA	<i>Sanchez-Matamoros et al., 2013</i>
<b>IFN<math>\gamma</math></b>	GATCTGAAGGTCCAGCGCAA	TCCGGCCTCGAAATGGATTC	<i>Vendrig et al., 2014</i>
<b>IL8</b>	TACTGCGTGGCACAATGAAA	TGCCAATGAAACTTCAAGCA	<i>Cappelli et al., 2007</i>
<b>IL10</b>	GAGAACCACGGCGCAGACATCAAG	GACAGCGCCGCACCCTCACT	<i>Vendrig et al., 2014</i>
<b>IL6</b>	TGGCTGAAGAACACAACAACCT	GAATGCCCATGAACTACAACA	<i>Boyd et al., 2007, Vendrig et al., 2014; Giguere and Prescott, 1999; Allen et al., 2007</i>
<b><i>Housekeeping genes</i></b>			
<b>B2M</b>	CGGGCTACTCTCCCTGACT	GCGTGACGTGAGTAAACCTGAAC	<i>Mienaltowsky et al., 2008</i>
<b>B Actin</b>	CGTGGGCCGCCCTAGGCACCA	TTGGCCTTAGGGTTCAGGGGGG	<i>Mette et al., 2010</i>
<b>GAPDH</b>	GGGTGGAGCCAAAAGGGTCATCAT	AGCTTTCTCCAGGCGGCAGGTCAG	<i>Mette et al., 2010</i>

**Table 2** Primer of the genes analyzed and literature source

	<b>0 hours</b>	<b>2 hours</b>	<b>6 hours</b>	<b>24 hours</b>
<b>Heart rate (bpm)</b>				
<b>Plasma</b>	100.0 ± 14.2	100.0 ± 17.8	96.0 ± 8.1	100.0 ± 12.8
<b>Control</b>	100.0 ± 20.7	90.0 ± 20.3	100.0 ± 15.9	94.0 ± 13.4
<b>Temperature (°F)</b>				
<b>Plasma</b>	101.0 ± 0.59	101.2 ± 0.65	101.3 ± 0.40	101.4 ± 0.50
<b>Control</b>	101.2 ± 0.44	100.9 ± 0.60	101.1 ± 0.56	101.2 ± 0.51
<b>Respiratory rate (rpm)</b>				
<b>Plasma</b>	48.0 ± 17.1	40.0 ± 10.6	36.0 ± 14.1	48.0 ± 18.0
<b>Control</b>	40.0 ± 11.3	50.0 ± 15.1	28.0 ± 15.3	32.0 ± 12.7

**Table 3** Clinical parameters (mean and standard deviation) at the time of blood sampling of the foals receiving blood transfusion or crystalloid administration.

Foal Group	IgG		Foal Group	IgG	
	<i>Baseline</i>	<i>24 Hours</i>		<i>Baseline</i>	<i>24 hours</i>
<b>Plasma (n=17)</b>			<b>Control (n=18)</b>		
<b>Min</b>	138.9	498.1	<b>Min</b>	220.6	202.6
<b>25th</b>	651.3	1025.5	<b>25th</b>	470.4	392.8
<b>Median</b>	1529.7 <sup>a</sup>	1930.7 <sup>b</sup>	<b>Median</b>	852.2	851.6
<b>75th</b>	1933.6	2000.0	<b>75th</b>	2000.0	2000.0
<b>Max</b>	2000.0	2000.0	<b>Max</b>	2000.0	2000.0
<b>Mean</b>	1314.6	1562.0	<b>Mean</b>	1188.0	1166.0
<b>SD</b>	658.8	548.8	<b>SD</b>	746.1	771.2

**Table 4** [IgG] Concentration (Min, 25<sup>th</sup>, Median, 75<sup>th</sup>, Max, Mean and SD) at baseline and 24 hours in foals receiving a plasma transfusion or infusion of crystalloids. Different lower case letters define statistical significance. A significant increase in [IgG] was noticed in plasma foals after transfusion of FFP (p=0.046).



<b>IgG concentration (mg/dl)</b>	<b>Number of foals</b>	
	<b>Baseline</b>	<b>24 Hours</b>
<b><u>GROUP 1</u></b>		
<b>IgG &lt;400</b>		
<b>Control</b>	2	2
<b>Plasma</b>	1	0
<b>Total</b>	3	2
<b><u>GROUP 2</u></b>		
<b>IgG 400-800</b>		
<b>Control</b>	6	6
<b>Plasma</b>	5	2
<b>Total</b>	11	8
<b><u>GROUP 3</u></b>		
<b>IgG &gt;800</b>		
<b>Control</b>	10	10
<b>Plasma</b>	11	15
<b>TOTAL</b>	21	15

**Table 5** Table reporting the total number of foals in the study with failure of passive transfer ([IgG] <400 mg/dl), partial failure of passive transfer ([IgG]<400 and >800 mg/dl) and with positive passive transfer ([IgG]>800 mg/dl) for the two treatment groups.

Variable (time)	Group	N	Min	25 <sup>th</sup>	Median	75 <sup>th</sup>	Max	Mean	Std. Dev	P
<b>TNF-<math>\alpha</math> (baseline)</b> <b>(ng/ml)</b>	<b>Plasma<sup>a</sup></b>	17	0.2	38.5	201.9	204.4	205.8	133.4	89.4	0.947
	<b>Control</b>	18	0.3	25.8	166.0	204.9	6100.0	476.5	1451.6	
<b>TNF-<math>\alpha</math> (2 hours)</b>	<b>Plasma<sup>b</sup></b>	17	375.1	515.2	1101.8	1934.6	1962.8	1138.8	644.2	0.099
	<b>Control</b>	18	0.3	21.6	253.4	1795.2	2059.8	734.1	844.3	
<b>TNF-<math>\alpha</math> (6 hours)</b>	<b>Plasma<sup>b</sup></b>	17	283.7	450.2	1251.8	1800.6	1984.2	1112.2	645.9	0.121
	<b>Control</b>	18	0.3	28.5	274.5	1633.4	2060.2	712.7	811.3	
<b>TNF-<math>\alpha</math> (24 hours)</b>	<b>Plasma<sup>b</sup></b>	17	406.8	489.5	1237.4	1867.1	2121.8	1177.6	674.6	0.075
	<b>Control</b>	18	0.3	19.1	293.2	1838.8	2091.0	760.6	877.4	
<b>IL-8 (baseline)</b> <b>(ng/ml)</b>	<b>Plasma<sup>a</sup></b>	17	-	-727.2	3546.7	6538.9	86677.8	7675.1	20787.8	0.338
	<b>Control</b>	18	-	-830.8	982.1	6987.4	31326.1	5630.7	9278.9	
<b>IL-8 (2 hours)</b>	<b>Plasma<sup>b</sup></b>	17	-526.7	826.1	7112.5	15256.8	83677.8	13594.5	20747.1	0.619
	<b>Control</b>	18	-	6.9	3245.0	7107.5	35891.3	6270.2	10645.3	
<b>IL-8 (6 hours)</b>	<b>Plasma</b>	17	-676.7	1247.5	5817.8	11231.4	72677.8	10212.9	17243.2	0.921
	<b>Control</b>	18	-926.7	166.7	2101.7	7428.3	31478.3	5887.9	8843.6	
<b>IL-8 (24 hours)</b>	<b>Plasma</b>	17	-676.7	1037.9	4784.4	11700.8	78455.6	10947.4	18827.2	0.753
	<b>Control</b>	18	-976.7	280.1	1165.4	9461.7	32826.1	6450.8	9814.0	
<b>IL-6 (baseline)</b> <b>(ng/ml)</b>	<b>Plasma</b>	17	-	98.5	2955.0	3234.0	12639.0	3390.7	4610.3	0.248
	<b>Control</b>	18	-334.0	642.0	3496.0	3978.3	13013.0	3241.4	3271.2	
<b>IL-6 (2 hours)</b>	<b>Plasma</b>	17	2811.0	3089.5	3253.0	8060.0	12329.0	5872.0	3553.6	0.075
	<b>Control</b>	18	-282.0	611.3	3305.5	3907.0	12736.0	3246.7	3194.9	
<b>IL-6 (6 hours)</b>	<b>Plasma</b>	17	-676.7	1247.5	5817.8	11231.4	72677.8	10212.9	17243.2	*0.051
	<b>Control</b>	18	-287.0	371.8	3097.5	3827.3	13046.0	3188.3	3276.2	
<b>IL-6 (24 hours)</b>	<b>Plasma</b>	17	2377.0	3019.5	4610.0	9204.5	12671.0	6128.8	3789.5	*0.044
	<b>Control</b>	18	-284.0	544.3	3316.0	3789.3	13094.0	3251.5	3248.0	

**Table 6** Data regarding cytokine concentration (median, mean, minimum, maximum, 25-75th percentiles and standard deviation) of healthy foals at baseline, 2 hours, 6 hours and 24 hours after a plasma transfusion or infusion of crystalloids. Different lowercase letters indicate statistical significance within group. \* Indicates statistically significant differences between groups. [TNF- $\alpha$ ] was significantly different at 2, 6 and 24 hours compared to baseline ( $p < 0.001$ ) in plasma group; [IL-8] was significantly different at 2 hours compared to baseline ( $p = 0.001$ ) in plasma group. [IL-6] was significantly different at 6 hours ( $p = 0.051$ ) and 24 hours ( $p = 0.044$ ) in the plasma group compared to crystalloid group.

<b>Variable (time)</b>	<b>Group</b>	<b>N</b>	<b>Min</b>	<b>25<sup>th</sup></b>	<b>Median</b>	<b>75<sup>th</sup></b>	<b>Max</b>	<b>Mean</b>	<b>Std. Dev</b>	<b>P</b>
<b>IL-4 (baseline)</b> <b>(pg/ml)</b>	<b>Plasma</b>	17	0.0	0.0	0.0	161.0	5390.0	557.0	1387.2	0.612
	<b>Control</b>	18	0.0	0.0	0.0	116.3	32089.0	2151.2	7550.6	
<b>IL-4 (2 hours)</b>	<b>Plasma</b>	17	0.0	0.0	0.0	167.0	2780.0	370.1	813.8	0.764
	<b>Control</b>	18	0.0	0.0	0.0	111.8	26916.0	1848.5	6371.6	
<b>IL-4 (6 hours)</b>	<b>Plasma</b>	17	0.0	0.2	0.0	42.0	1886.0	196.1	513.8	0.764
	<b>Control</b>	18	0.0	0.0	0.0	9.5	18256.0	1338.9	4402.7	
<b>IL-4 (24 hours)</b>	<b>Plasma</b>	17	0.0	0.0	0.0	28.0	1537.0	153.8	408.7	0.535
	<b>Control</b>	18	0.0	0.0	0.0	121.8	16450.0	1200.8	3919.9	
<b>IFN-<math>\gamma</math> (baseline)</b> <b>(U/ml)</b>	<b>Plasma</b>	17	3	8.5	19.0	117.0	310.0	75.6	101.3	0.960
	<b>Control</b>	18	3	8.5	16.5	149.0	1320.0	144.6	317.2	
<b>IFN-<math>\gamma</math> (2 hours)</b>	<b>Plasma</b>	17	2	9.5	21.0	112.0	297.0	71.1	93.0	0.817
	<b>Control</b>	18	3	6.3	17.5	139.5	1218.0	134.7	298.0	
<b>IFN-<math>\gamma</math> (6 hours)</b>	<b>Plasma</b>	17	3	6.5	19.0	39.0	177.0	36.5	49.1	0.498
	<b>Control</b>	18	2	6.5	25.5	185.8	1198.0	164.4	304.6	
<b>IFN-<math>\gamma</math> (24 hours)</b>	<b>Plasma</b>	17	3	11.5	27.0	82.5	154.0	49.9	50.2	0.429
	<b>Control</b>	18	2	6	14.0	170.0	1152.0	135.3	279.2	
<b>IL-17 (baseline)</b> <b>(U/ml)</b>	<b>Plasma</b>	17	1	2.5	6.0	43.0	220.0	39.2	66.3	0.435
	<b>Control</b>	18	1	2	4.5	15.8	2181.0	143.4	512.3	
<b>IL-17 (2 hours)</b>	<b>Plasma</b>	17	1	2	3.0	24.5	112.0	20.6	34.1	0.907
	<b>Control</b>	18	1	2	4.5	14.3	1835.0	126.3	433.1	
<b>IL-17 (6 hours)</b>	<b>Plasma</b>	17	1	2	4.0	14.5	152.0	18.6	38.0	0.920
	<b>Control</b>	18	2	2	3.5	28.3	2218	174.6	529.1	
<b>IL-17 (24 hours)</b>	<b>Plasma</b>	17	2	2	3.0	6.5	150.0	19.4	43.9	0.350
	<b>Control</b>	18	1	2	3.5	77.8	1955.0	156.6	461.8	

**Table 7** Data regarding cytokine concentration (median, mean, minimum, maximum, 25-75th percentiles and standard deviation) of healthy foals at baseline, 2 hours, 6 hours and 24 hours after a plasma transfusion or infusion of crystalloids.

Variable (time)	Group	N	Min	25 <sup>th</sup>	Median	75 <sup>th</sup>	Max	Mean	Std. dev	P
<b>Bead based multiplex assay</b>										
<b>IL-10 (baseline)</b> (pg/ml)	<b>Plasma</b>	17	0.0	0.0	0.0	0.0	2226.0	168.8	541.5	0.560
	<b>Control</b>	18	0.0	0.0	0.0	83.8	5274.0	346.2	1235.6	
<b>IL-10 (2 hours)</b>	<b>Plasma</b>	17	0.0	0.0	0.0	0.0	1698.0	99.9	411.8	0.193
	<b>Control</b>	18	0.0	0.0	0.0	26.0	4895.0	308.8	1148.4	
<b>IL-10 (6 hours)</b>	<b>Plasma</b>	17	0.0	0.0	0.0	0.0	1348.0	91.6	325.7	0.394
	<b>Control</b>	18	0.0	0.0	0.0	140.5	4524.0	297.7	1059.0	
<b>IL-10 (24hours)</b>	<b>Plasma</b>	17	0.0	0.0	0.0	21.5	1195.0	84.8	288.4	0.699
	<b>Control</b>	18	0.0	0.0	0.0	7.0	4188.0	245.4	984.7	
<b>ELISA</b>										
<b>IL-10 (baseline)</b> (ng/ml)	<b>Plasma</b>	17	-0.3	82.8	970.0	6038.0	34988.0	4714.9	8661.4	0.974
	<b>Control</b>	18	0.3	39.6	268.6	4333.8	26068.5	3786.0	7394.7	
<b>IL-10 (2 hours)</b>	<b>Plasma</b>	17	367.6	500.1	1492.6	5943.3	33918.3	4683.5	8306.3	0.969
	<b>Control</b>	18	-0.3	59.7	415.8	3456.8	21261.0	2748.2	5363.9	
<b>IL-10 (6 hours)</b>	<b>Plasma</b>	17	484.6	702.6	1611.6	4460.0	47173.3	5521.8	11386.0	*0.056
	<b>Control</b>	18	0.5	54.4	296.8	4313.5	20506.0	3300.5	5733.3	
<b>IL-10 (24hours)</b>	<b>Plasma</b>	17	359.6	465.1	1389.6	3617.5	61085.0	5433.5	14497.1	0.086
	<b>Control</b>	18	0.7	77.9	332.1	5175.5	23448.0	3668.2	6480.3	

**Table 8** Data regarding [IL-10] concentration (median, mean, minimum, maximum, 25-75% percentiles and standard deviation) of healthy foals in plasma and crystalloid group at baseline, 2 hours, 6 hours and 24 hours after a plasma transfusion or crystalloid infusion. Above values reported are obtained with Multiplex. Below values are obtained with ELISA test. \* Indicates statistically significant differences between groups. [IL-10] measured with ELISA were significantly different between groups at 6 hours (p=0.056).

Gene expression	Group	Min	25 <sup>th</sup>	Median	75 <sup>th</sup>	Max	Mean	SD
TNF- $\alpha$ (baseline)	Plasma	0.6	0.8	0.9	1.0	2.0	1.0	0.3
	Control	0.5	0.9	1.1	1.3	3.8	1.2	0.7
TNF- $\alpha$ (2 Hrs)	Plasma	0.5	0.9	1.0	1.2	3.8	1.3	1.2
	Control	0.8	1.0	1.2	1.4	1.6	1.2	0.3
TNF- $\alpha$ (6 Hrs)	Plasma <sup>a</sup>	0.7	0.9	1.0	1.3	2.5	1.2	0.5
	Control	0.7	0.9	1.2	1.2	1.5	1.1	0.2
TNF- $\alpha$ (24 Hrs)	Plasma <sup>b</sup>	0.5	0.7	0.8	1.1	1.4	0.9	0.2
	Control	0.7	0.9	1.0	1.2	1.3	1.0	0.2
IL-8 (baseline)	Plasma	0.4	0.7	1.15	2.1	3.3	1.7	0.9
	Control	0.9	1.0	1.6	2.1	4.4	1.5	0.9
IL-8 (2 Hrs)	Plasma	0.5	1.0	1.3	1.5	3.3	1.7	0.8
	Control	1.0	1.1	1.4	2.1	3.5	1.6	0.7
IL-8 (6 Hrs)	Plasma	0.2	0.9	1.3	1.9	2.7	1.4	0.6
	Control	0.0	0.9	1.2	2.0	2.8	1.6	0.8
IL-8 (24 Hrs)	Plasma	0.5	0.9	1.1	2.0	5.1	1.5	1.2
	Control	0.6	1.0	1.4	1.7	3.1	1.4	0.6
IL-1 $\beta$ (baseline)	Plasma <sup>*</sup>	0.0	0.5	0.7	1.1	4.7	1.0	1.1
	Control <sup>**</sup>	0.0	0.5	0.8	1.1	7.1	1.2	1.6
IL-1 $\beta$ (2 Hrs)	Plasma	0.0	0.4	0.5	0.8	3.9	0.8	1.0
	Control <sup>#</sup>	0.0	0.4	0.5	0.8	1.3	0.6	0.3
IL-1 $\beta$ (6 Hrs)	Plasma	0.0	0.3	0.4	0.8	2.3	0.6	0.7
	Control	0.0	0.3	0.4	0.8	1.3	0.5	0.3
IL-1 $\beta$ (24 Hrs)	Plasma <sup>*</sup>	0.0	0.2	0.3	0.6	0.9	0.4	0.3
	Control <sup>**#</sup>	0.0	0.2	0.3	0.6	0.8	0.4	0.2

**Table 9** Data regarding gene expression of TNF- $\alpha$ , IL-8, IL-1 $\beta$  in peripheral blood samples collected from healthy foals at baseline, 2 hours, 6 hours and 24 hours after a plasma transfusion or infusion of crystalloids. Expression is reported as relative quantification obtained by normalizing the absolute quantification against the geometric mean of 3 house keeping genes. Similar lowercase symbols indicate statistical significance within group. IL-1 $\beta$  gene expression was statistically significantly decreased at 24 hours compared to baseline in both groups (plasma, p=0.001; crystalloids, p=0.003) and between 24 hours compared to 2 hours in the crystalloid group (p=0.002).

Gene expression	Group	Min	25 <sup>th</sup>	Median	75 <sup>th</sup>	Max	Mean	SD
<b>IFN-<math>\gamma</math> (baseline)</b>	<b>Plasma</b>	0.3	0.6	0.9	1.1	2.3	1.0	0.5
	<b>Control</b>	0.3	0.6	0.8	1.5	2.3	1.0	0.6
<b>IFN-<math>\gamma</math> (2 Hrs)</b>	<b>Plasma</b>	0.2	0.6	1.2	1.7	6.5	1.4	1.4
	<b>Control</b>	0.3	0.5	0.8	1.5	2.7	1.0	0.7
<b>IFN-<math>\gamma</math> (6 Hrs)</b>	<b>Plasma</b>	0.0	0.8	1.2	1.8	2.5	1.3	0.7
	<b>Control</b>	0.3	0.6	0.6	1.5	2.4	0.9	0.6
<b>IFN-<math>\gamma</math> (24 Hrs)</b>	<b>Plasma</b>	0.3	0.7	0.9	1.4	3.0	1.1	0.7
	<b>Control</b>	0.2	0.4	0.8	1.0	2.1	0.8	0.5
<b>IL-10 (baseline)</b>	<b>Plasma</b>	0.6	0.7	0.9	1.1	2.1	1.0	0.4
	<b>Control<sup>***</sup></b>	0.5	0.7	1.0	1.2	2	1.0	0.4
<b>IL-10 (2 Hrs)</b>	<b>Plasma</b>	0.2	0.7	0.8	1.1	3.3	1.0	0.7
	<b>Control</b>	0.3	0.6	0.9	1.0	1.6	0.8	0.3
<b>IL-10 (6 Hrs)</b>	<b>Plasma</b>	0.0	0.8	0.9	1.3	1.6	0.9	0.4
	<b>Control</b>	0.4	0.6	0.9	1.0	1.4	0.9	0.3
<b>IL-10 (24 Hrs)</b>	<b>Plasma</b>	0.4	0.6	0.9	1.2	1.6	0.9	0.3
	<b>Control<sup>***</sup></b>	0.4	0.6	0.8	1.0	1.3	0.8	0.3

**Table 10** Data regarding gene expression of IFN-  $\gamma$ , IL-10 in peripheral blood samples collected from healthy foals at baseline, 2 hours, 6 hours and 24 hours after a plasma transfusion or infusion of crystalloids. Expression is reported as relative quantification obtained by normalizing the absolute quantification against the geometric mean of 3 house keeping genes. Similar lowercase symbols indicate statistical significance within group. IL-10 gene expression was significantly decreased at 24 hours compared to baseline in the crystalloid group ( $p=0.003$ ).

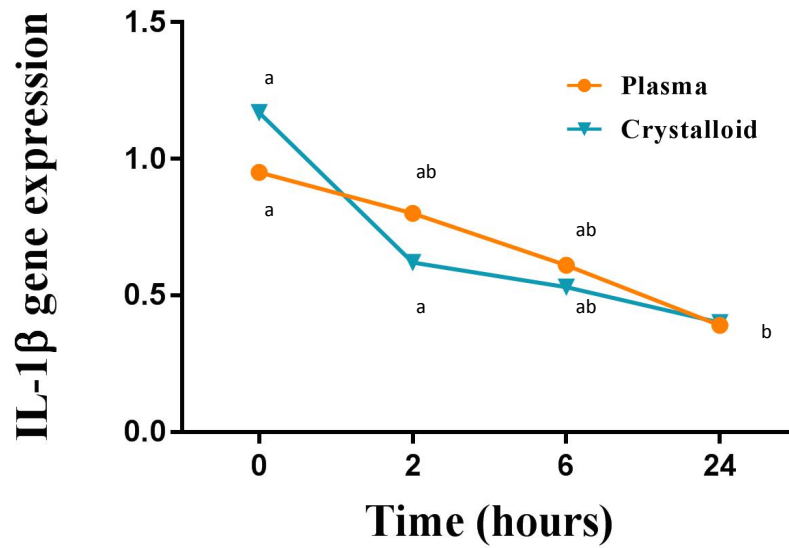
Variable	Group	N	Mean (mg/dl)	Std. Dev	P
FIB (baseline)	Control	18	175.6	44.4	
	Plasma <sup>a</sup>	17	180.5	33.4	
FIB (2 hours)	Control <sup>z</sup>	18	180.9	30.9	
	Plasma <sup>b</sup>	17	197.4	28.8	
FIB (6 hours)	Control	16	179.3	39.7	*0.048
	Plasma <sup>b</sup>	17	203.9	28.4	
FIB (24 hours)	Control <sup>y</sup>	17	198.2	36.5	
	Plasma <sup>c</sup>	17	218.3	30.9	

**Table 11** Fibrinogen concentration (mg/dl) at baseline and at 2, 6 and 24 hours in healthy foals after receiving a plasma transfusion or infusion of crystalloids. Different lowercase symbols indicate significant difference overtime within group; \* indicates significance between groups. Mean [FIB] was significantly increased at 2, 6 and 24 hours compared to baseline ( $p < 0.001$ ) and between 2 and 24 ( $p = 0.002$ ) and 6 and 24 hours ( $p = 0.004$ ) in the plasma group. [FIB] at 6 hours was significantly different between the two groups ( $p = 0.048$ ). Mean [FIB] was different between 2 and 24 hours ( $p = 0.004$ ) in the control group.

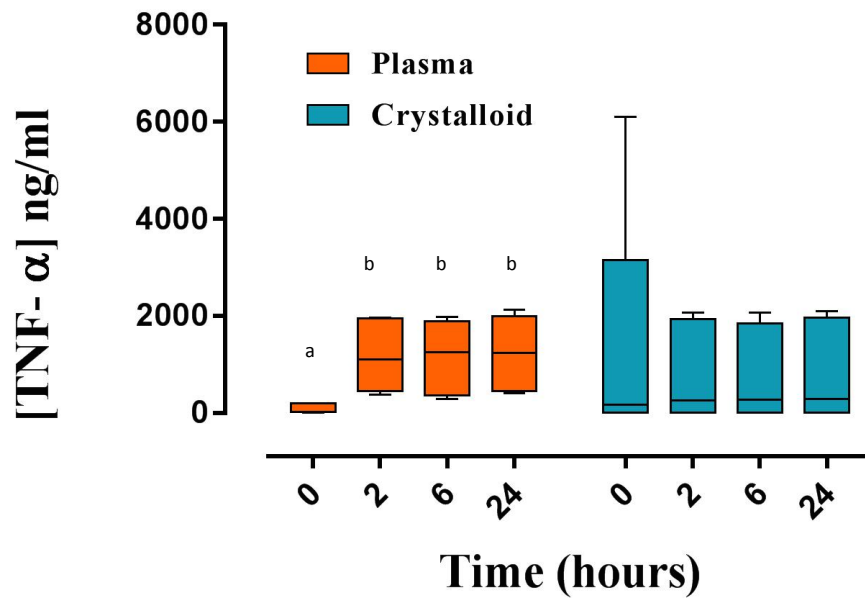
Variable (time)	Group	N	Min	25 <sup>th</sup>	Median	75 <sup>th</sup>	Max	Mean	SD	P value
SAA (baseline)	Plasma	17	2.0	39.7	193.7	233.8	915.5	227.3	348.4	
	Control	18	0.0	13.3	43.5	247.5	951.8	145.1	139.9	
SAA (2 hours)	Plasma	17	4.4	44.4	143.4	179.8	707.1	191.7	290.6	
	Control <sup>b</sup>	18	0.0	10.6	36.5	246.4	917.3	149.1	129.4	
SAA (6 hours)	Plasma	17	0.0	10.6	59.2	144.1	440.1	123.7	232.4	
	Control <sup>b</sup>	18	0.0	5.5	30.6	246.6	930.0	142.5	118.3	
SAA (24 hours)	Plasma	17	0.0	10.0	100.0	173.7	456.8	171.8	345.7	*0.0125
	Control <sup>a</sup>	18	0.0	0.0	11.8	237.5	913.7	113.8	96.3	

**Table 12** SAA concentration (mg/l) at baseline and at 2, 6 and 24 hours in healthy foals after receiving a plasma transfusion or infusion of crystalloids. Different lowercase letters indicate significant difference overtime within group; \* indicates significance between groups. Significant difference was found at 24 hours between groups (p= 0.0125). SAA was significantly decreased at 24 hours compared to 2 hours (p=0.003) and at 24 hours compared to 6 hours in the control group (p=0.001).

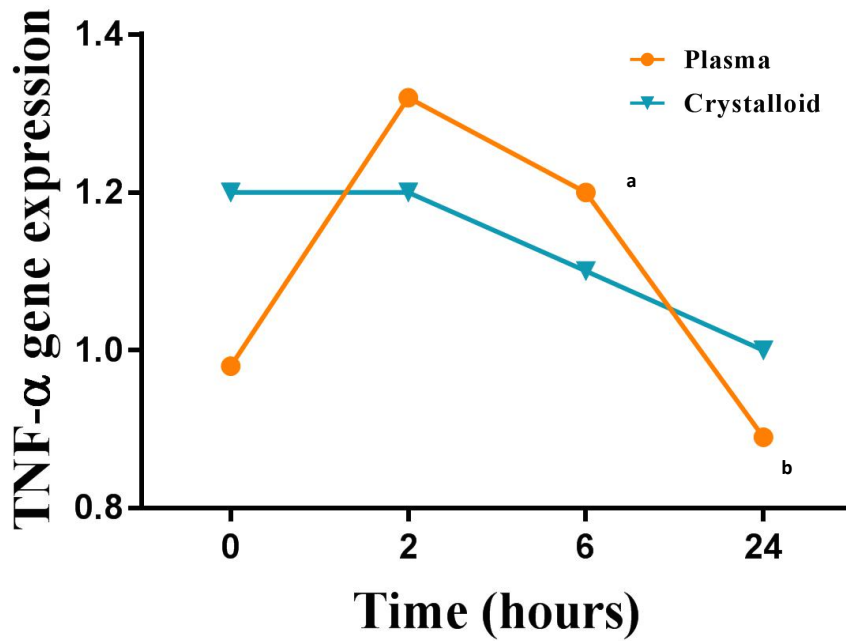




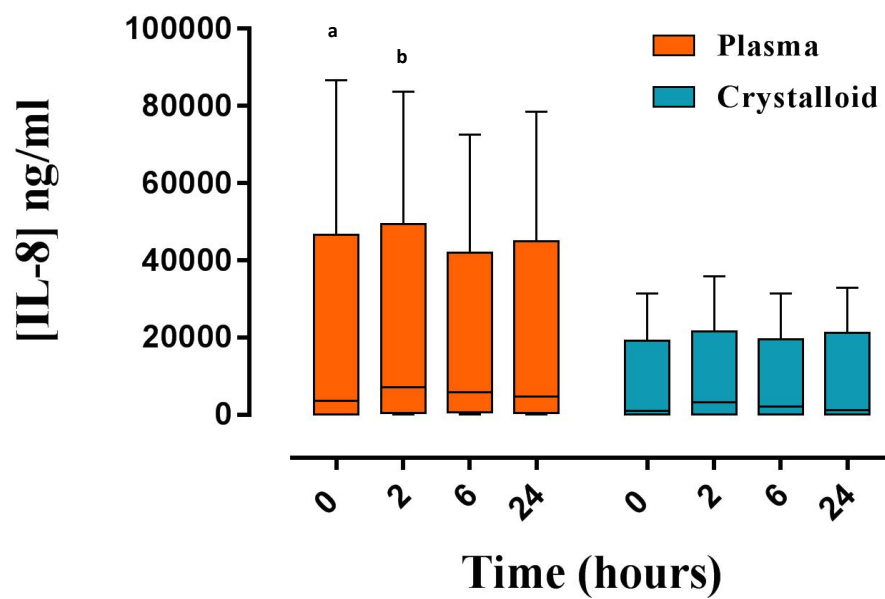
**Figure 1** IL-1 $\beta$  gene expression (mean copy numbers) in peripheral blood collected from healthy foals at baseline, 2, 6 and 24 hours after a plasma transfusion (circles) or infusion of crystalloids (triangles). Both treatment groups demonstrated a significant decrease in gene expression at 24 hours compared to baseline (crystalloid group  $p=0.003$ ; plasma group  $p=0.002$ ;) and in the crystalloid group between 2 and 24 hours ( $p=0.001$ ). Different lowercase letters indicate significant differences over time. Error bars have been omitted for clarity.



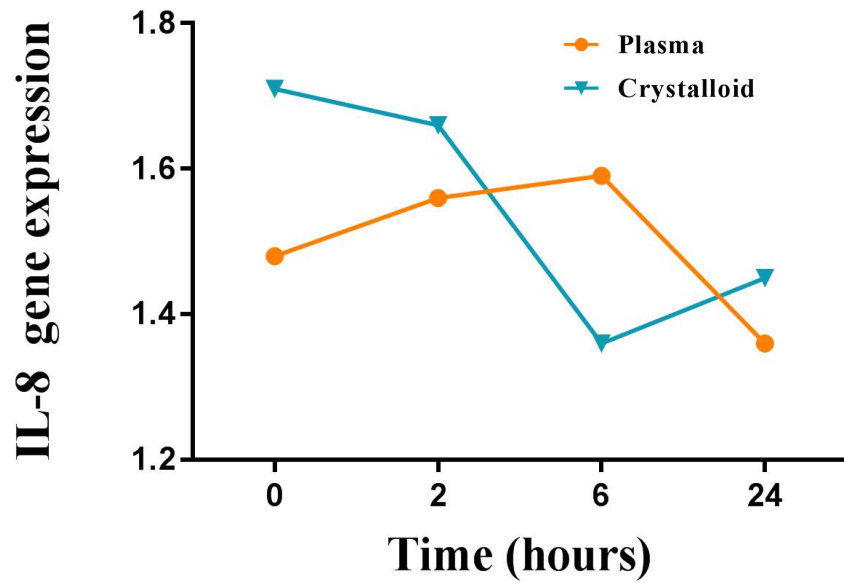
**Figure 2** Box and whiskers plot (from bottom to top: min, 25<sup>th</sup> percentile, median, 75<sup>th</sup> percentile, max) of serum [TNF- $\alpha$ ] (ng/ml) in healthy foals at baseline, 2, 6 and 24 hours after plasma transfusion (left, orange) or crystalloid infusion (right, blue). Median concentrations at 2, 6 and 24 hours are statistically significant compared to baseline in the plasma group ( $p < 0.001$ ). Different lowercase letters indicate significant differences over time.



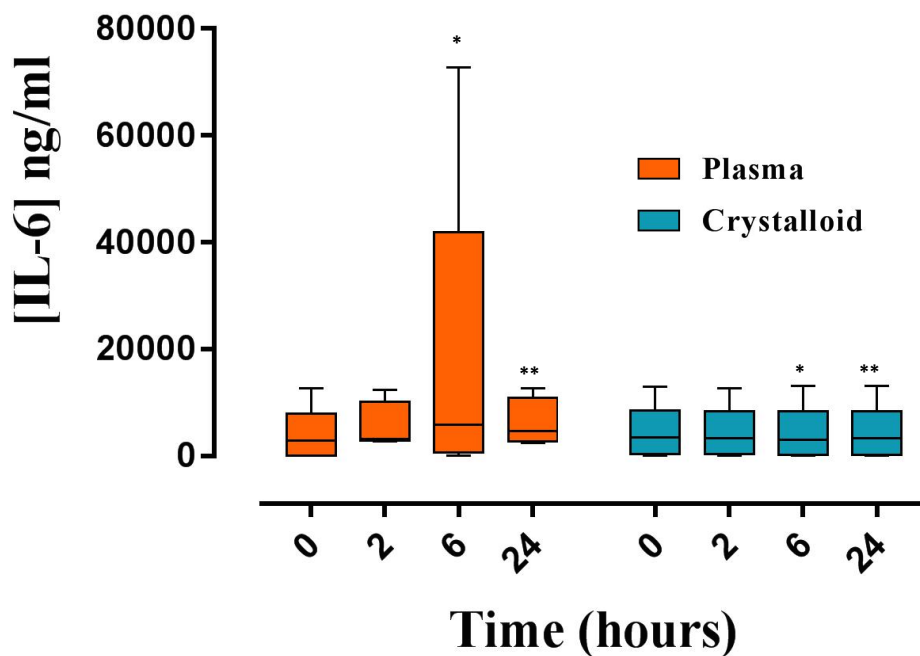
**Figure 3** TNF- $\alpha$  gene expression (mean copy numbers) in peripheral blood collected from healthy foals at baseline, 2, 6 and 24 hours after a plasma transfusion (circles) or infusion of crystalloids (triangles). Gene expression is different between 6 and 24 hours in the plasma group ( $p=0.007$ ). Different lowercase letters indicate significant differences over time. Error bars have been omitted for clarity.



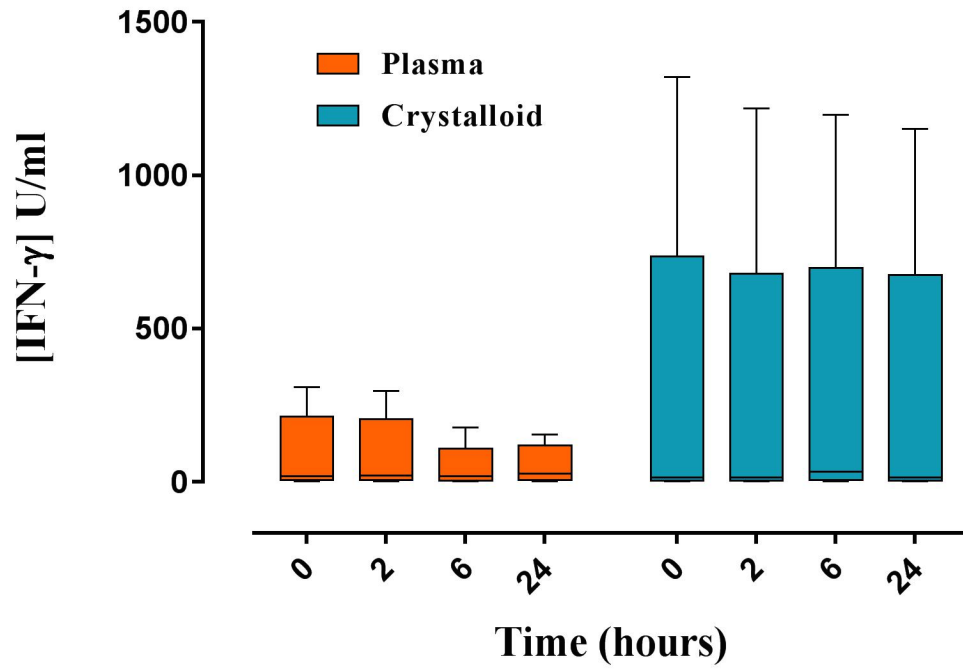
**Figure 4** Box and whiskers plot (from bottom to top: min, 25<sup>th</sup> percentile, median, 75<sup>th</sup> percentile, max) of serum [IL-8] (ng/ml) in healthy foals at baseline, 2, 6 and 24 hours after plasma transfusion (left, orange) or crystalloid infusion (right, blue). Median [IL-8] is different at 2 hours compared to baseline in the plasma group (p=0.001). Different lowercase letters indicate significant differences over time.



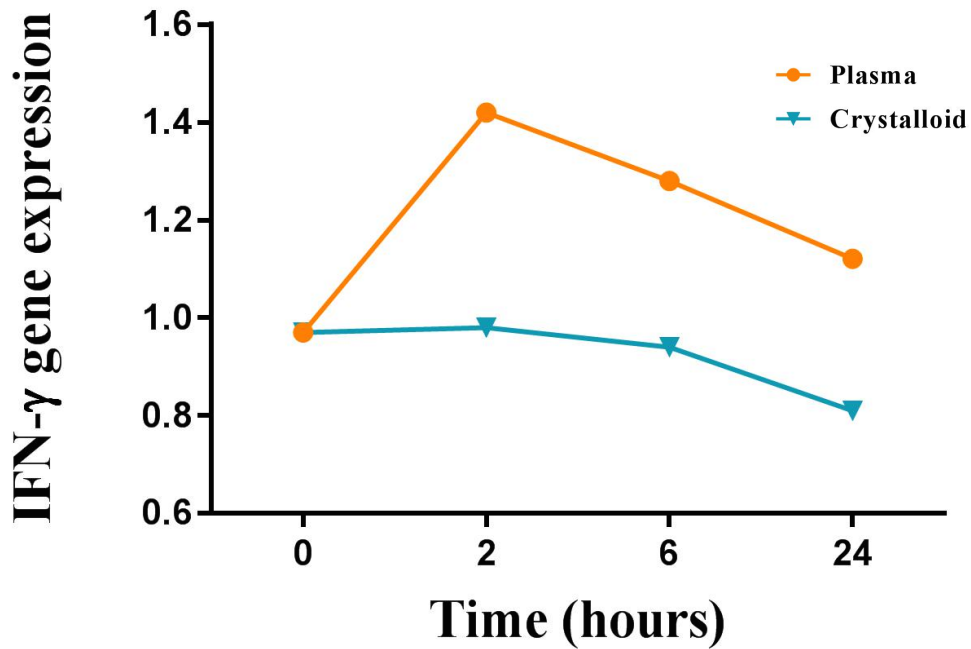
**Figure 5** IL-8 gene expression (mean copy numbers) in peripheral blood collected from healthy foals at baseline, 2, 6 and 24 hours after a plasma transfusion (circles) or infusion of crystalloids (triangles). No statistically significant differences were found overtime or between groups. Error bars have been omitted for clarity.



**Figure 6** Box and whiskers plot (from bottom to top: min, 25<sup>th</sup> percentile, median, 75<sup>th</sup> percentile, max) of serum [IL-6] (ng/ml) in healthy foals at baseline, 2, 6 and 24 hours after plasma transfusion (left, orange) or crystalloid infusion (right, blue). Median [IL-6] is different between groups at 6 (p=0.051) and 24 hours (p=0.044) prior to Bonferroni correction. Median [IL-6] is different at 2 hours compared to baseline in the plasma group (p=0.001). Similar lowercase symbols indicate significant differences between plasma and crystalloids.

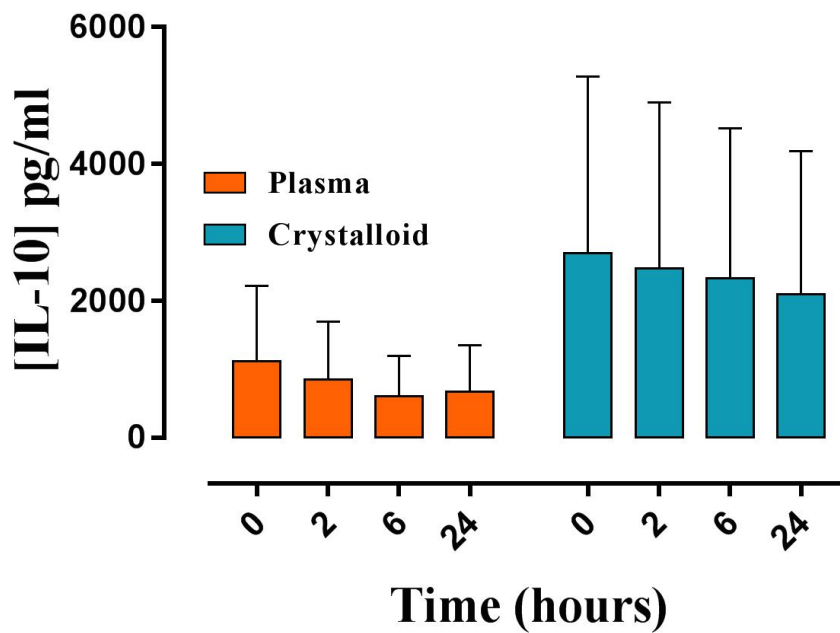


**Figure 7** Box and whiskers plot (from bottom to top: min, 25<sup>th</sup> percentile, median, 75<sup>th</sup> percentile, max) of serum [IFN-  $\gamma$ ] (ng/ml) in healthy foals at baseline, 2, 6 and 24 hours after plasma transfusion (left, orange) or crystalloid infusion (right, blue). Median [IFN-  $\gamma$ ] were not different at any time point or between groups.

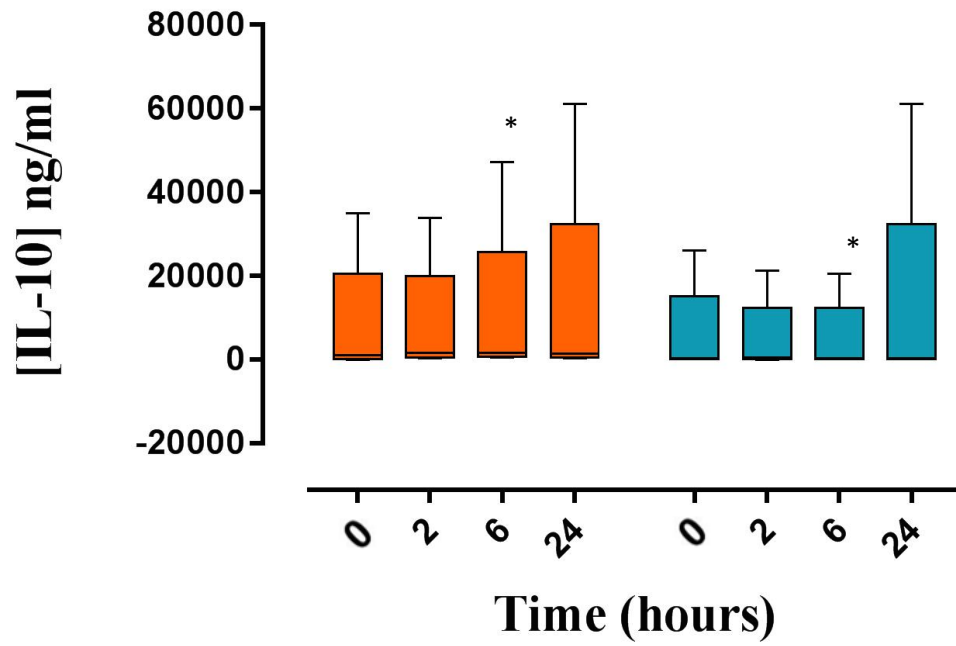


**Figure 8** IFN-  $\gamma$  gene expression (mean copy numbers) in peripheral blood collected from healthy foals at baseline, 2, 6 and 24 hours after a plasma transfusion (circles) or infusion of crystalloids (triangles). No statistically significant differences were found overtime or between groups. Error bars have been omitted for clarity.

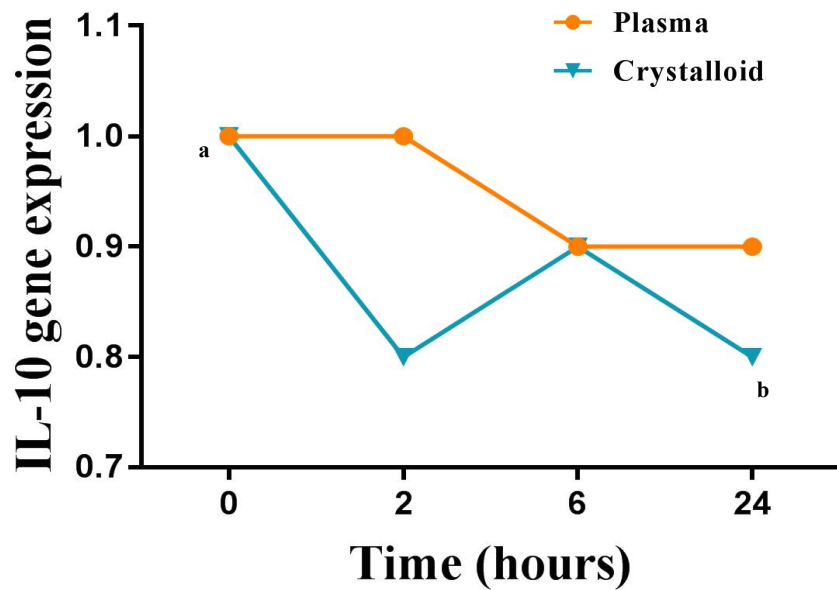




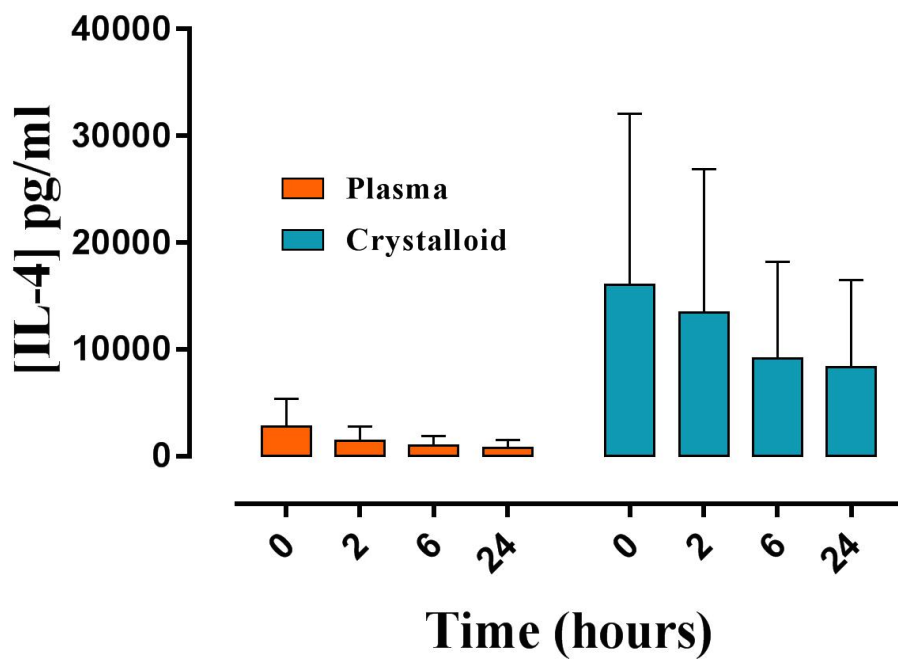
**Figure 9** Box and whiskers plot (from bottom to top: min, 25<sup>th</sup> percentile, median, 75<sup>th</sup> percentile, max) of serum [IL-10] measured with Multiplex technology (pg/ml) in healthy foals at baseline, 2, 6 and 24 hours after plasma transfusion (left, orange) or crystalloid infusion (right, blue). Median [IL-10] did not differ at any time point or in any group.



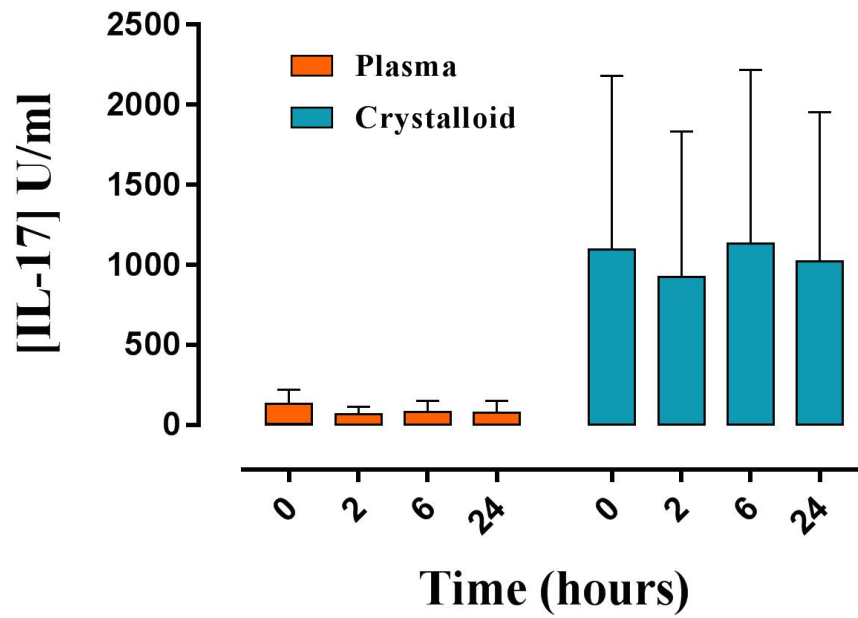
**Figure 10** Box and whiskers plot (from bottom to top: min, 25<sup>th</sup> percentile, median, 75<sup>th</sup> percentile, max) of serum [IL-10] (ng/ml) measured with ELISA in healthy foals at baseline, 2, 6 and 24 hours after plasma transfusion (left, orange) or crystalloid infusion (right, blue). Median [IL-10] differed at 6 hours between groups (p=0.056).  
 \* Indicates significant differences between plasma and crystalloids.



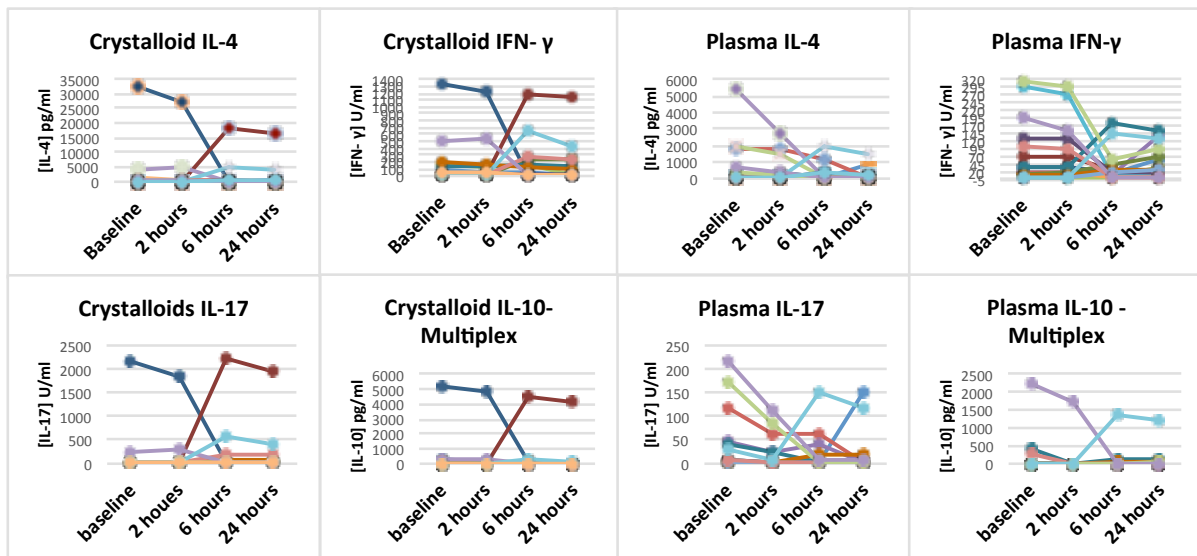
**Figure 11** IL-10 gene expression (mean copy numbers) in peripheral blood collected from healthy foals at baseline, 2, 6 and 24 hours after a plasma transfusion (circles) or infusion of crystalloids (triangles). in peripheral blood collected from healthy foals at baseline, 2, 6 and 24 hours after a plasma transfusion or infusion of crystalloids. Gene expression was significantly decreased at 24 hours compared to baseline in the control group ( $p=0.003$ ). Different lowercase letters indicate significant differences over time. Error bars have been omitted for clarity.



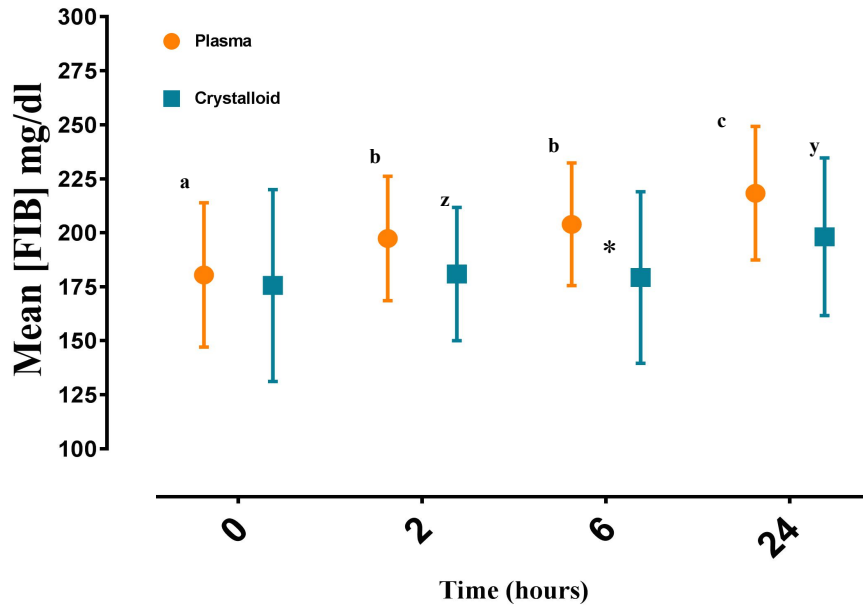
**Figure 12** Box and whiskers plot (from bottom to top: min, 25<sup>th</sup> percentile, median, 75<sup>th</sup> percentile, max) of serum [IL-4] (ng/ml) measured in healthy foals at baseline, 2, 6 and 24 hours after plasma transfusion (left, orange) or crystalloid infusion (right, blue). Median [IL-4] did not differ at any time point or in any group.



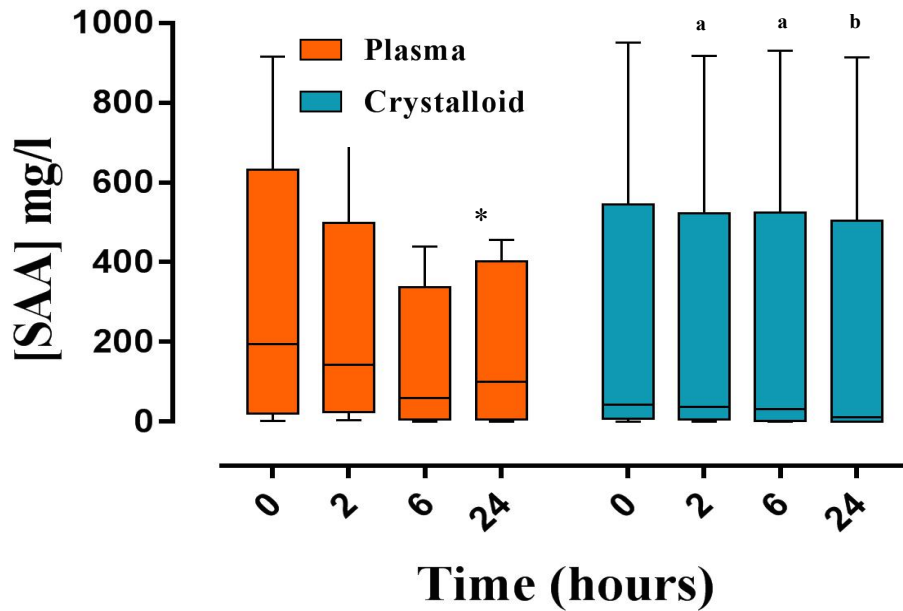
**Figure 13** Box and whiskers plot (from bottom to top: min, 25<sup>th</sup> percentile, median, 75<sup>th</sup> percentile, max) of serum [IL-17] (U/ml) measured in healthy foals at baseline, 2, 6 and 24 hours after plasma transfusion (left, orange) or crystalloid infusion (right, blue). Median [IL-17] did not differ at any time point or in any group.



**Figure 14** Individual variation in cytokine concentration for the cytokines analyzed with Multiplex technology (IL-4, IL-17, IL-10 and IFN- $\gamma$ ) for plasma and crystalloid group. Within the crystalloid group foal 13 (dark blue), foal 33 (purple), foal 14 (maroon) and foal 34 (light blue) have similar cytokine behavior. In the plasma group foal 35 (purple) and 36 (light blue) showed similar cytokine behavior for all cytokines of interest. Foal 13 demonstrated a very high TNF- $\alpha$  and IL-10 gene expression at baseline compared to its cohort. Foal 14 and 34 had partial and total failure of passive transfer ([IgG] were 479 and 334.7 mg/dl respectively). Both foals responded to crystalloid administration with a substantial increase in those cytokines at 6 hours after administration. Plasma group foal 2, 9, 26 also had interesting cytokine concentration variations. While foal 2 demonstrated an increase in [IL-17], [IL-4] and [IFN- $\gamma$ ] at 24 hours, foal 9 in [IL-17] and [IFN- $\gamma$ ] at 24 hours, and foal 26 demonstrated a uniformly increased [IL-10], [IL-17], [IL-4] and [IFN- $\gamma$ ] starting at 6 hours after plasma transfusion. Of the foals listed only foal 26 demonstrated failure of passive transfer ([IgG] 138.9 mg/dl).



**Figure 15** Fibrinogen concentration (mg/dl), expressed as mean and standard deviation in healthy foals at baseline and at 2, 6 and 24 hours after a plasma transfusion (circles) or crystalloid infusion (squares). Mean concentration was significantly increased at 2, 6 and 24 hours compared to baseline ( $p < 0.001$ ) and between 2 and 24 ( $p = 0.002$ ) and 6 and 24 hours ( $p = 0.004$ ) in the plasma group. Mean concentration was different between 2 and 24 hours in the crystalloid group ( $p = 0.004$ ). [FIB] at 6 hours was significantly different between the two groups ( $p = 0.048$ ). Different lowercase letters indicate significant differences overtime and within group. \* Indicates significant difference between plasma and crystalloids.



**Figure 16** Box and whiskers plot (from bottom to top: min, 25<sup>th</sup> percentile, median, 75<sup>th</sup> percentile, max) of serum [SAA] mg/l measured in healthy foals at baseline, 2, 6 and 24 hours after plasma transfusion (left, orange) or crystalloid infusion (right, blue). [SAA] is significantly different at 24 hours between groups ( $p=0.0125$ ), between 2 and 24 hours ( $p=0.003$ ) and 6 and 24 hours in the control group ( $p=0.001$ ). Different lowercase letters indicate significant differences overtime within group. \* Indicates significant differences between plasma and crystalloids.



## CHAPTER 9

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