INVESTIGATION OF HAEMATOLOGIC, INFLAMMATORY, AND HAEMOSTATIC CHANGES IN HORSES EXPERIMENTALLY INFECTED WITH AFRICAN HORSE SICKNESS VIRUS

By

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TABLE OF CONTENTS

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LIST OF ABBREVIATIONS

%	percent
α-angle	speed of fibrin accumulation
AHS	African Horse Sickness
AHSV	African Horse Sickness Virus
APP	acute phase proteins
APR	acute phase reactants
aPTT	activated partial thromboplastin time
ARRIVE	Animal Research: Reporting of In Vivo Experiments
AT	antithrombin
BTV	Bluetongue Virus
°C	degrees Celsius
CBC	complete blood count
CHCM	mean haemoglobin concentration per cell
CL30	blood clot lysis index at 30 minutes
CL60	blood clot lysis index at 60 minutes
DAMP	danger-associated molecular patterns
DIC	disseminated intravascular coagulation
ds	double-stranded
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
F	factor
FDP	fibrin degradation products
fL	femtolitre
G	clot strength

h	hours
HCT	haematocrit
H&E	haematoxylin and eosin
HGB	haemoglobin
IFN	interferon
IL	interleukin
JAK	Janus kinase
K	time for clot formation
kg	kilogram
L	litre
LY30	lysis at 30 minutes
LY60	lysis at 60 minutes
LLOD	lowest level of detection
MA	maximum amplitude
MCH	mean cell haemoglobin
MCHC	mean cell haemoglobin concentration
MCP	monocyte chemoattractant protein
MCV	mean cell volume
mg	milligram
min	minutes
mL	millilitre
mM	millimolar
MPC	mean platelet concentration
MPM	mean platelet mass
MPV	mean platelet volume

MPXI	myeloperoxidase index
MRL	maximum rate of lysis
MRTG	maximum rate of thrombus generation
NF	nuclear factor
NK	natural killer cell
nm	nanometre
nM	nanomolar
NS	non-structural protein
PAMP	pathogen associated molecular patterns
PCR	polymerase chain reaction
PCT	plateletcrit
PDW	platelet distribution width
PLT	platelet concentration
PML-NB	promyelocytic leukaemia nuclear body
PRR	pathogen recognition receptors
PT	prolonged prothrombin time
R	time to start of clot formation
RBC	red blood cell concentration
RNA	ribonucleic acid
RT-PCR	real time polymerase chain reaction
SAA	serum amyloid A
sec	seconds
ss	single-stranded
STAT	signal transducers and activators of transcription
TAFI	thrombin- activated fibrinolysis inhibitor

TAT	thrombin antithrombin complexes
TEG	thromboelastography
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TG	thrombus generation
TGF	transforming growth factor
TICAM1/TRIF	Toll-IL-1 receptor domain-containing adaptor inducing interferon-β/ Toll-IL-1 domain-containing adaptor molecule 1
TLR	Toll-like receptors
TMRL	time to maximum rate of lysis
TMRTG	time to maximum rate of thrombus generation
TNF	tumour necrosis factor
tPA	tissue plasminogen activator
TT	thrombin time
uPa	urokinase-type plasminogen activator
µL	microliter
VP	viral protein
vWf	von Willebrand factor
WBC	white blood cell concentration

LIST OF FIGURES

LIST OF TABLES

SUMMARY

INVESTIGATION OF HAEMATOLOGIC, INFLAMMATORY, AND HAEMOSTATIC CHANGES IN HORSES EXPERIMENTALLY INFECTED WITH AFRICAN HORSE SICKNESS VIRUS

By

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Department: Companion Animal Clinical Studies
Degree: PhD

African horse sickness (AHS) is an infectious disease affecting equids. It is caused by the African horse sickness virus (AHSV), a double-stranded RNA *Orbivirus* with 9 different serotypes which is transmitted by insect vectors, particularly Culicoides midges and is endemic in sub-Saharan Africa. Infection with AHSV results in one of four disease forms, the pulmonary (“dunkop”), cardiac (“dikkop”), mixed, and fever form and morbidity and mortality ranges of up to 100% are described in naïve horses. Clinical signs such as dyspnoea, fever, haemorrhages, and pulmonary oedema are thought to be reflective of inflammation and endothelial damage due to viral replication in the vascular endothelial cells. To date, the understanding of the underlying pathology is marginal, and no therapy has been recognized as effective. The enzootic virus has important implications on animal welfare, the equine industry and the local economy of rural communities which depends on working equids.

Given the limited knowledge of the inflammatory response to infection with AHSV and the resulting haematological changes and alterations in haemostasis, the broad objectives of this study were to 1) evaluate the haematological changes and changes in acute phase reactants; 2) describe the changes in selected cytokines; and 3) characterize the haemostatic changes occurring in horses experimentally infected with AHSV.

The study was designed as a prospective, longitudinal, experimental study which included four healthy AHS-susceptible Boerperd cross horses that had tested negative for AHSV group-specific antibodies using a commercial competitive enzyme-linked immunosorbent assay (ELISA) against all nine AHSV serotypes. These horses were infected intravenously with low passage mouse brain suspension (5 mL) that contained at least 10^5 mouse infective doses/mL of virulent AHSV serotypes. Each horse was inoculated with a different AHS serovar: horse 1, AHSV-2 (horse origin); horse 2, AHSV-4 (horse origin); horse 3, AHSV-6 (horse origin) and horse 4, AHSV-6 (dog origin). All horses developed severe clinical signs typical of AHS post infection and were humanely euthanized.

All horses developed significant haemoconcentration in the late stages of the disease. Significant thrombocytopenia with increased markers of platelet activation developed; however, changes in leukocytes and acute phase reactants serum amyloid A (SAA) and serum iron were significant but not considered clinically relevant. This suggested possible derangements in the host's immune response which contribute to the observed dampened immune response in reaction to the inflammatory stimuli triggered by the virus.

To further elucidate the immune response to infection with AHSV, selected plasma cytokines interleukin (IL)-1α, IL-2, IL-6, IL-8, IL-10, IL-12, IL-17, interferon (IFN)-γ, tumour necrosis factor (TNF)-α, and monocyte chemoattractant protein (MCP)-1, which represent mediators of both innate and adaptive immunity pathways, were evaluated throughout the course of the disease. Unexpectedly, an almost complete absence of proinflammatory cytokines in blood was observed, as only TNF-α increased in the final stages of the disease while an increase in IL-10, considered an anti-inflammatory cytokine, was predominant. This correlates with the previous findings of a mild acute phase response and mild haematological changes as these responses are mediated by cytokines. The lack of a significant cytokine response could indicate viral immune evasion mechanisms. In *Orbiviruses*, *in vivo* studies have documented inhibition of the immune response by the virus – specifically of IFN and Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathways and this is a likely cause of the lack of a proinflammatory response.

Horse infected with AHSV develop haemorrhages; this finding is thought to be mainly due to the endothelial damage caused directly by viral replication in endothelial cells. It is now understood that inflammation and haemostasis are closely integrated, and inflammatory cytokines activate haemostatic pathways by increasing tissue factor expression on circulating endothelial cells, monocytes and macrophages. Specifically, horses developed overt disseminated intravascular coagulation (DIC), a consumptive coagulopathy, and clinical signs of bleeding, and procoagulant activation, inhibition of anticoagulants and fibrinolysis was detected on both traditional coagulation tests and viscoelastic tests. Given the lack of proinflammatory cytokines, inflammatory activation

of the haemostatic pathways is likely secondary while endothelial damage is the probable primary trigger for activation of haemostasis.

The findings of this study further elucidate the pathogenesis of the AHSV. The results suggest that AHSV is capable of interfering with the innate immune response, possibly via interference with the Janus kinase/signal transducers and activators of transcription (JAK/STAT) signalling pathways or promyelocytic leukaemia nuclear bodies (PML-NBs), while simultaneously initiating haemostatic pathways, most likely via endothelial damage, and causing overt DIC. Early identification of haemostatic derangements allows for earlier intervention which may improve outcome. Recognition of the virus's capability to interfere with the innate immune system may be used to develop new treatment strategies, including direct cytokine or antibody therapy to improve the development of more effective vaccines.

1 CHAPTER 1: BACKGROUND

1.1 African Horse Sickness

African horse sickness (AHS) is an infectious disease with a mortality rate of up to 100% in naive populations. The causative pathogen, the African horse sickness virus (AHSV), is a double-stranded (ds) ribonucleic acid (RNA)-*Orbivirus* from the *Sedoreoviridae* family (Matthijnssens et al., 2022) with nine different serotypes (Calisher and Mertens, 1998; Howell, 1962; Verwoerd et al., 1979). The virus is not directly transmissible between animals and is transmitted by insect vectors. The midge species *Culicoides imicola,* and to a lesser extent *C. bolitinos,* have been implicated as the main vectors (Boorman et al., 1975; Mellor et al., 1975); however, other arthropod vectors have also been shown to be capable of transmitting AHSV (Carpenter et al., 2017).

AHSV is endemic in the tropical and subtropical regions of sub-Saharan Africa, and all equids are susceptible to all nine serotypes. Zebras have natural immunity and are considered the virus reservoir. Horses have no innate immunity against AHSV, and African donkeys are partially immune. The virus can also infect dogs (*Canis familiaris*); with transmission taking place via *Culicoides* midges or the ingestion of infected meat (Hanekom et al., 2023; M'Fadyean, 1910; McIntosh, 1955; Salama et al., 1981). In rare cases, sheep and goats can be infected with AHSV; however, they rarely develop clinical signs. Antibodies against AHSV have been demonstrated in wild carnivores like hyena (*Crocuta crocuta*), jackals (different *Canis spp*.), African wild dogs (*Lycaon pictus*), cheetahs (*Acinonyx jubutus*), lions (*Panthera leo*) and Southern Cape genets

(*Genetta maculata*), and ingestion of infected zebra meat is the likely cause (Binepal et al., 1992). Antibodies to AHSV have also been found in camels (*Camelus bactrianus, C. dromedarius*), black and white rhinoceros (*Diceros bicornis, Ceratotherium simum*), and African elephants (*Loxodonta africana*) but none of these species are considered of epidemiological significance (Erasmus et al., 1978; Fischer-Tenhagen et al., 2000; Lubroth, 1992; Miller et al., 2011; Wilson et al., 2009). Experimental infections revealed that the virus does not replicate in African elephants (Barnard et al., 1995) and seroconversion is not observed in hyenas. Neither viral replication nor seroconversion were observed in experimentally infected mink (*Mustela vison*) (Sahu and Dardiri, 1979), while virus could be isolated from experimentally infected ferrets (*Mustela putorius furo*) (McIntosh, 1955).

1.1.1 History of African Horse Sickness

The first known description of disease assumed to be AHS dates to the early 14th century. In an Arabic document “Kitâb el-Akouâl el Kafiah wa el Foucoûl ef Charfiah” (Mornet, 1968; Moulé, 1896) a scene is described in Yemen: “a purchaser and a salesman are discussing the price of a horse when, suddenly, the horse falls on the floor and dies” (Zientara S., 2012).

It is generally believed that the AHSV originated in Africa, where it was first described in 1569 by Father Monclaro (Theal, 1899). As explorers and colonial powers imported horses into southern Africa, the first large disease outbreak was observed in 1729 where more than 1700 horses died. At least 10 more outbreaks were observed over the next 200 years. During the biggest recorded outbreak from the spring to fall of

1845/55 more than 70,000 horses perished, equivalent to approximately 40% of the entire horse population in the Cape of Good Hope (Bayley, 1856). Outbreaks have decreased both in frequency and severity over the last century, likely due to a decreasing number in equines but also as a result of vaccine development (Mellor and Hamblin, 2004b). While the virus is endemic in sub-Saharan Africa, incursions of the AHSV into other regions occur, resulting in devastating outbreaks in naïve horse populations. In 1943/44, outbreaks were reported in Egypt, Syria, Jordan, Lebanon, and Palestine, and between 1959 and 1960 more than 300,000 horses died from AHS in the middle east and Southwest Asia (Cyprus, Turkey, Lebanon, Iran, Iraq, Syria, Jordan, Palestine, Pakistan, India). In 1965/66, the virus also circulated in Northern Africa (Morocco, Algeria, Tunisia) before it was introduced into Spain in 1966 (Lubroth, 1992). Between 1987 and 1990 several outbreaks occurred in Spain, Portugal, and Morocco (Portas et al., 1999; Rodriguez et al., 1992). In 2020, AHSV serotype 1 was introduced into Thailand with the import of a clinically healthy zebra that was an AHSV carrier. This resulted in at least 15 outbreaks and the death of more than 500 horses (Castillo-Olivares, 2021; King et al., 2020) and additional incursion into the neighbouring country of Malaysia (WOAH, https://www.woah.org/en/disease/african-horse-sickness/, accessed 25.7.2022).

1.1.2 Description of African Horse Sickness Virus

The virus was first described scientifically by Sir Arnold Theiler. Theiler described “a disease of equines caused by an ultravisible virus, probably transmitted by a blood-sucking insect” (Theiler, 1921). The virus has since been classified as an *Orbivirus* in the family of *Sedoreoviridae* (Calisher and Mertens, 1998; Matthijnssens et al., 2022). The virion is a non-enveloped spherical particle with a diameter of ± 80 nm

(Oellermann et al., 1970). The virus has three concentric protein layers (Bremer, 1976; Bremer et al., 1990; Grubman and Lewis, 1992) with the AHSV genome and its 10 linear segments of dsRNA in the centre. The genome encodes seven structural (four major and three minor) and five non-structural (NS) proteins (Roy et al., 1994). The outer capsid layer consists of the major structural proteins, viral protein (VP)2 and VP5; the virus core particle is made up of the two major structural proteins VP3 and VP7 and the NS proteins VP1, VP4, and VP6. Much of our knowledge of AHSV relies on research on the closely related Bluetongue Virus (BTV) which is often used as a model for related large, non-enveloped dsRNA viruses (Roy, 2017) as studies on AHSV are less specific.

The AHSV inner core is made up by 120 VP3 proteins with 60 asymmetric VP3 dimers that organize into twelve decamers with five VP3 dimers each. The outer core and the protein layers are scaffolded around this inner shell (Figure 1-1). The minor structural proteins VP1 (polymerase), VP4 (capping enzyme), and VP6 (helicase) attach directly to the inside of the VP3 layer and form transcription complexes, while VP7 attaches to the outside of the VP3 layer and forms the outer core. The VP2 and VP5 trimers form the outer capsid on the outside (Grimes et al., 1998; Manole et al., 2012; Nason et al., 2004). Furthermore, a cell infected by AHSV will also synthesizes the five non-structural proteins, NS1, NS2, NS3 and NS3a, and NS4. These proteins play important roles in the replication, assembly, and the transport of the virus from infected cells (Roy et al., 1994). The NS1 protein is important for upregulation of viral protein expression (Boyce et al., 2012), NS2 forms inclusion bodies for viral replication (Patel and Roy, 2014; van Staden et al., 1991), NS3 and NS3a mediate release of virus from

the cell (Boyce et al., 2012; Quan et al., 2008), and NS4 has been shown to interfere with the host's innate immune system (Ratinier et al., 2016; Zwart et al., 2015).

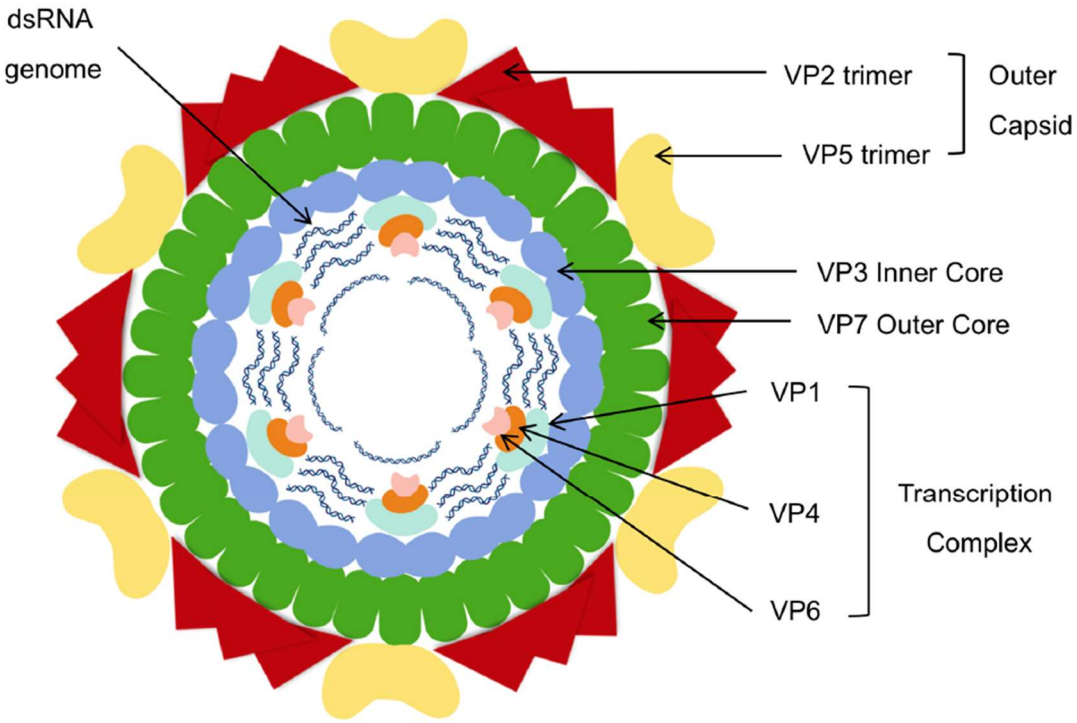

Figure 1-1:Schematic representation of the AHSV virion.
Image reprinted with permission from Dennis et al. (2019)

1.1.3 Infection and Replication

While the exact mechanism of infection and replication have not been elucidated in AHSV, BTV is used as a model for these events. The infection of the host cell with AHSV is thought to be initiated by binding of the outer capsid proteins VP2 and VP5 to host cell surface receptors (Forzan et al., 2007). Following removal of the outer capsid proteins, the viral core is released into the host cell where it becomes transcriptionally active. The VP1 enzymatic protein produces single-stranded (ss)RNAs (Boyce et al., 2004) that are modified by VP4 enzyme (Ramadevi et al., 1998) and only then released into the cytoplasm. The host cell's ribosomes are then

used to synthesize viral protein and dsRNA genes. Assembly of the virus is then modulated by NS2 (Kar et al., 2007), and enzymatic proteins VP1, VP4, and VP6 and VP3, an inner core protein, encapsidate the new sub-core particles (Patel and Roy, 2014). The NS3 protein then facilitates an exocytosis pathway, leading to acquisition of VP5 and VP2 and egression of virus from the cell (Beaton et al., 2002; Wirblich et al., 2006).

1.1.4 Clinical signs of African Horse Sickness

The clinical signs in horses with AHSV were first described in detail in 1921. Sir Arnold Theiler classified the disease into three forms: the pulmonary ("dunkop"), cardiac ("dikkop") and fever forms (Theiler, 1921). A fourth mixed form with clinical signs occurring in the heart and the lungs has since been described (Coetzer and Guthrie, 2004). To date it is not clear if these different forms are different diseases or rather manifestations in different tissues, or related to the time-course of the disease, or caused by infections with viruses with different organ tropism in the heterogenous virus population (Burrage and Laegreid, 1994).

The pulmonary form begins with peracute fever of up to 42°C, depression, and anorexia. Acute dyspnoea develops, often followed by frothy nasal discharge. The mortality of the pulmonary form is commonly up to 100%. The main findings on necropsy include lung oedema with severe pleural effusion.

The cardiac form is more protracted and commonly subacute. Infected horses develop fever (39-41°C) and characteristic oedema of the supraorbital fossa, the conjunctiva and the intermandibular region, hence the name "dikkop", translated as "thick head".

The cardiac form is fatal in 50-70% of cases, and infected horses usually die within 4-8 days after developing clinical signs. Necropsy findings include hydropericardium, epi- and endocardial petechial and ecchymotic haemorrhages, and oedema in the subcutis, the fascia and the muscles of the neck and head. The myocardial lesions are not uniform, and increases in creatinine kinase and lactate dehydrogenase, markers of muscle damage, are not seen in most cases.

The fever form is usually subacute and mild. The main clinical sign is mild fever, and no characteristic lesions are observed on necropsy. The mixed form is usually confirmed during necropsy where lesions of both the pulmonary and the cardiac forms are noted: severe oedema in the lung and the subcutis, pleural and pericardial effusion and myocardial haemorrhages (Erasmus, 1972; Henning, 1956; Maurer and McCully, 1963; Newsholme, 1983).

1.1.5 Diagnosis of African Horse Sickness

In endemic regions, the diagnosis is usually made based on clinical signs. The definitive diagnosis is made by demonstration of the virus in cell cultures or via intracerebral inoculation of new-born mice or in chicken eggs (Howell, 1962). Indirect identification is possible using serologic antigen tests (enzyme-linked immunosorbent assay, ELISA) using antibodies that target VP7. (Maree and Paweska, 2005) The current test of choice is real-time polymerase chain reaction (RT-PCR) that allows for identification, classification and typing of the virus within hours (Aguero et al., 2008; Bachanek-Bankowska et al., 2014; Guthrie et al., 2013; Quan et al., 2010; Rodriguez-Sanchez et al., 2008; Sailleau et al., 2000; Weyer et al., 2015).

1.1.6 Pathogenesis of African Horse Sickness

To date, details of the pathogenesis of AHS are unknown. The virus is transmitted during the biting act of the vector, a *Culicoides* midge. Initial viral replication occurs in the regional lymph nodes, followed by a primary viraemia. Within three days post infection, the virus is found in the endothelial cells of its target organs. The AHSV is organotropic and the virus can be found in the lung and lymphoid tissues such as the spleen, lymph nodes, the caecum, and the pharynx (Coetzer and Guthrie, 2004). A secondary viraemia of usually 4-9 (up to 21) days ensues. Disease severity is determined by the viral serotype and the host's immune status (Mellor and Hamblin, 2004a).

Studies have demonstrated that the AHSV targets endothelial cells as well as monocytes and macrophages (Laegreid et al., 1992a; Wohlsein et al., 1997b). In both experimental and natural infections, tropism of the endothelium of the heart and lungs is observed (Clift and Penrith, 2010); however, the underlying mechanism is not understood. Virus can be found in the lymph nodes, spleen, liver, and gastrointestinal tract during early infection (Brown et al., 1994; Clift and Penrith, 2010). Virus replication has been observed in endothelial cells, pulmonary intravascular macrophages, interstitial macrophages, and fibroblasts. It is likely that both the direct endothelial cell damage and the activation of the pulmonary intravascular macrophage population result in the observed vascular changes (Carrasco et al., 1999; Gomez-Villamandos et al., 1999). Endothelial cell damage and the resulting loss of function result in oedema formation, effusion, and haemorrhages observed in the affected tissues on necropsy and histopathology (Gomez-Villamandos et al., 1999; Laegreid et al., 1992a; Newsholme, 1983).

It is still not understood if the different clinical manifestations are caused by different virus variants or represent different stages of infection or organotropism. It is now standard to serotype the AHSV found during an outbreak and a database of different virus isolates can be found in the AHS genome sequencing Bioproject (http://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA271179) where each unique virus strain is identified by a unique name. During outbreaks in South Africa in 2011 (Weyer et al., 2016), and 2016 (Grewar et al., 2019) and Thailand in 2020, AHSV1 was identified as the causative serotype. All different clinical forms were observed in the affected horses. These results suggest that the clinical course of the disease is not dependent on the serotype but rather the virulence of the variant present.

1.1.7 Prevention of African Horse Sickness

To date, vaccination is the most effective prevention against AHS. The only licensed vaccine in South Africa is a polyvalent, attenuated live virus vaccine (OBP, Onderstepoort, South Africa). The vaccine contains two different vaccine vials with two mixtures of different serotype combinations. To date, this vaccine combination is considered the best prevention against AHS. However, many limitations exist regarding its efficacy. The immune response to the different serotypes varies widely and it may take up to 6 years with annual boosters to develop protective immunity (Mirchamsy and Taslimi, 1968; Molini et al., 2015; von Teichman et al., 2010; Weyer et al., 2017). Immunological studies also showed that not all horses develop adequate immunity against all serotypes (Crafford et al., 2013). These findings explain why younger horses whose immune system has not been stimulated repeatedly by booster vaccination are more susceptible to infection than older horses. The transfer of

maternal antibodies in the colostrum depends on the dam‘s immune status and antibody titres. It has also been shown that the duration of the passive protection via maternal antibodies is influenced by the AHSV serotype; maternal antibodies against serotypes 2, 5, 7, 8, and 9 only afford protection for 2-3 months postpartum (Crafford et al., 2013). Furthermore, there is a risk for outbreaks caused by reversion to virulence in the attenuated live virus as well as through reassortment of genomic sequences and the formation of new variants. Reversion to virulence of AHSV 1 and reassortment with genetic sequences from serotypes 1, 3, and 4 led to outbreaks in the Western Cape in 2004 and 2014 (Weyer et al., 2016). Finally, the vaccine does not allow for differentiation between vaccinated and naturally infected animals, this distinction is important for surveillance purposes. Due to these concerns, there is ongoing research to develop improved AHS vaccines.

1.2 Host immune response to infection

To protect the body from invasion by pathogens or damage, the immune system has evolved a network of biological processes. The immune system recognizes inflammation-inducing molecules by detecting highly conserved structures that are common on pathogens, termed pathogen-associated molecular patterns (PAMPs) for extracellular pathogens or danger-associated molecular patterns (DAMPs) for proteins of intracellular origin (Gong et al., 2020; Kumar et al., 2011). These structures bind to pattern recognition receptors (PRRs) on innate immune cells. Three major classes of PRRs exist: Toll-like receptors (TLR), nucleotide-binding domain leucine-rich repeat containing proteins, and retinoic acid-inducible gene-1 like receptors (Kumar et al., 2011). Toll-like receptors 3 and 7 are especially important for the recognition of invading viruses (Kumar et al., 2011). Once activated, PRRs transmit signals to the

nucleus and initiate a cellular response that leads to the transcription, production, and release of cytokines (Figure 1-2) (Kumar et al., 2011).

Specifically, binding of DAMPs or PAMPs to a PRR activates nuclear factor (NF)-κB and activator protein-1 via proteins such as myeloid differentiation primary response 88 or Toll-IL-1 receptor domain-containing adaptor inducing interferon-β/TIR domain-containing adaptor molecule 1 (TICAM1/TRIF). Once activated, NF-κB and activator protein-1 enter the nucleus where they stimulate gene transcription for numerous molecules. These include coagulation factors, inducible nitric oxide synthase, acute phase proteins (APP), and proinflammatory cytokines, such as tumour necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, IL-8, and IL-12 (Lewis et al., 2012). Activation of the TICAM1/TRIF pathway by PAMPs and DAMPs also leads to phosphorylation of interferon regulatory factor-3 and -7. These also enter the nucleus and activate genes transcribing for interferon (IFN)-α, IFN-β, and other type I IFNs (Lewis et al., 2012). Similarly, IFN-γ is produced by T-helper cells and many antigen presenting cells (Schroder et al., 2004).

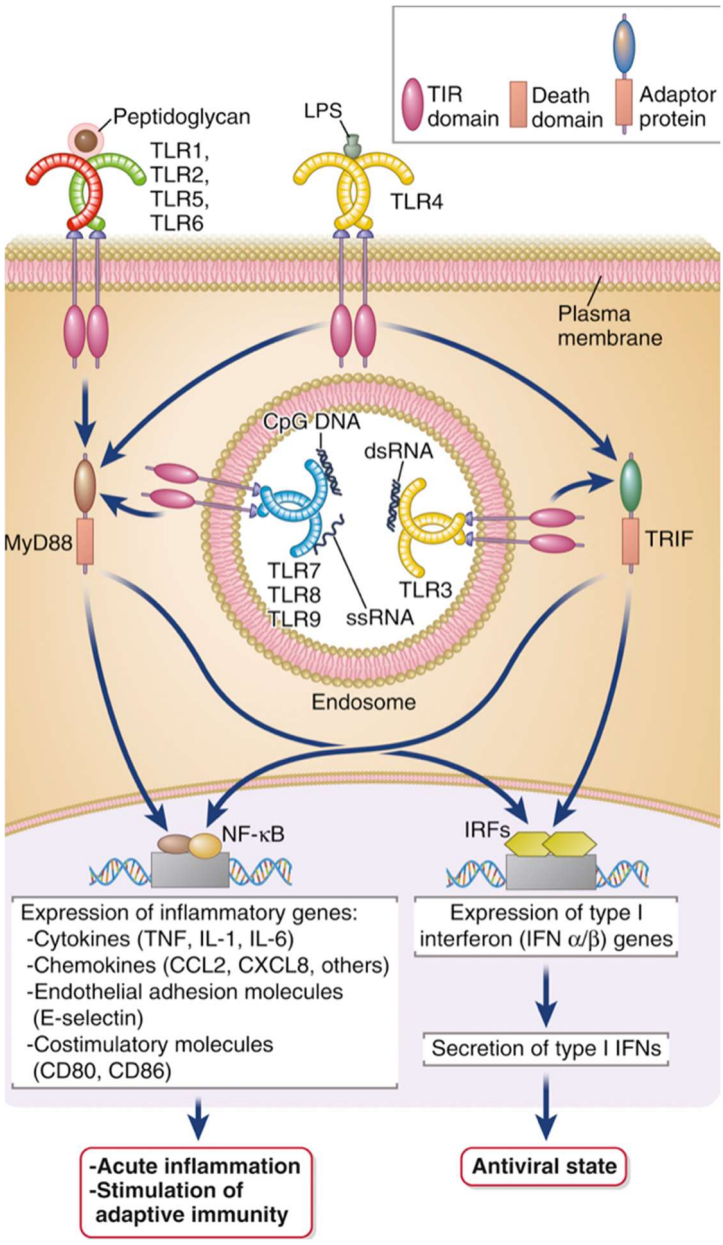

Figure 1-2: Signalling pathways and functions of Toll-like receptors.
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Cytokines bind to and activate receptors on target cells. Receptor expression varies highly between different tissues and individual cell types, controlling the cell's response to cytokines (Lokau and Garbers, 2020). Expression of cytokine receptors has been described for macrophages, neutrophils, lymphocytes, and other effector cells of the immune system and for epithelial and endothelial cells and myocytes

(Chao, 2009; Rakoff-Nahoum et al., 2004). Many of the cell-surface cytokine receptors, including receptors for IFNs, use non-receptor tyrosine kinases called Janus kinases (JAKs) and transcription factors called signal transducers and activators of transcription (STATs) in their signal transduction pathways. To date, four JAKs (JAKs 1 to 3 and tyrosine kinase (TYK2)) and seven STATs (STATs 1 to 4, 5a, 5b, and 6) have been described (Schindler et al., 2007). Binding of an appropriate cytokine to one of these receptors activates the receptor associated JAKs and phosphorylates tyrosine residues in the cytoplasmic portions of the clustered receptors. This facilitates binding of monomeric cytosolic STAT proteins to the cytoplasmic part of the cytokine receptor. The proximity of the STAT proteins to the JAKs allows for their phosphorylation by these receptor-associated kinases resulting in the formation of STAT dimers. These STAT dimers then migrate to the nucleus, bind to specific DNA sequences in the promoter regions of cytokine-responsive genes and activate gene transcription (Figure 1-3) (Abbas AK, 2022). The Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway is specifically important for IFN production, thus critical to the function of the immune response against viruses (Lokau and Garbers, 2020).

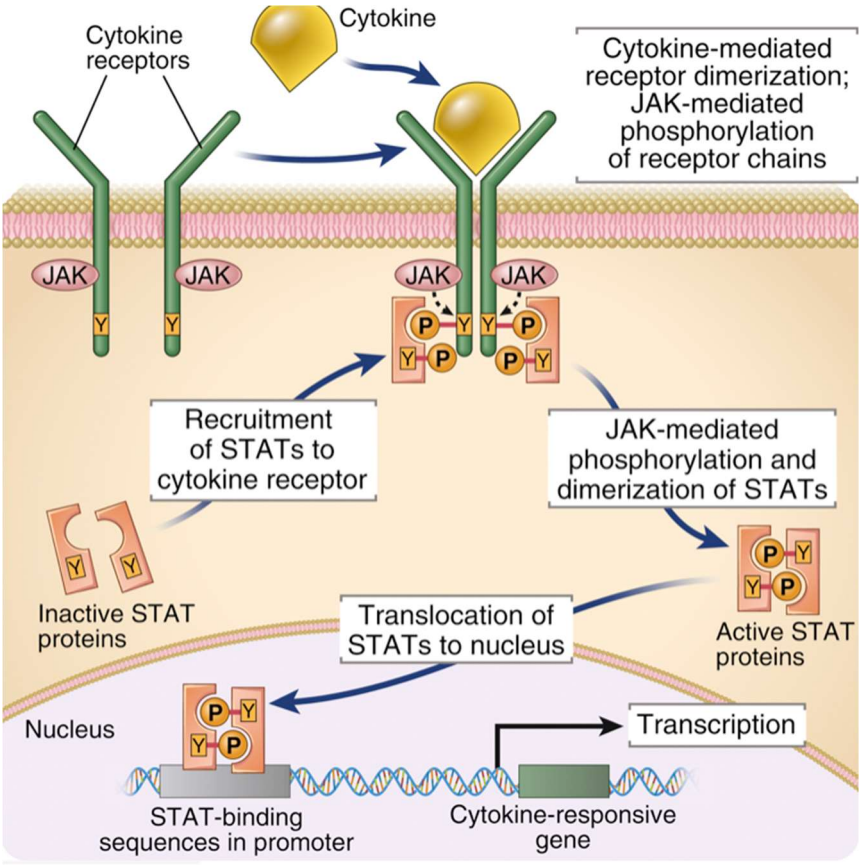

Figure 1-3: JAK/STAT signalling induction by cytokines.
Image reprinted with permission from Abbas AK (2022)

1.2.1 Cytokines

Cytokines are low-molecular-weight proteins which are synthesized by various immune cells, including T lymphocytes, neutrophils, and macrophages. They function to promote, regulate, and orchestrate the immune response by acting on other cells and also as effector molecules (Figure 1-4). Cytokines can act in an autocrine, paracrine, and endocrine fashion and stimulate the effector cells towards sites of inflammation, infection, or trauma, act as primary lymphocyte growth factors and on signalling molecules and cells (Horohov, 2003; L. Ferreira et al., 2019).

Cytokines are commonly classified into different groups, these include chemokines, IFNs, ILs, lymphokines, and TNF.

Once cytokines have been produced, they act on their many different cellular targets and have important functions in the regulation of the maturation, proliferation, and function of immune cells. Cytokines can be secreted by different cells and have effects on different cells with often different biological responses, while at the same time cells can be stimulated by different cytokines. While cytokines are often categorized as either pro- or anti-inflammatory, they can have both properties, depending on the context, such as the secreting cell and the activated cell (Table 1-1). Cytokines are highly regulated and abnormal or dysregulated cytokine production can result in organ failure and death. It is therefore helpful to determine cytokine concentrations in the face of disease as cytokine quantification allows a better understanding of the immune status of the animal and allows for possible intervention.

Table 1-1: Overview of selected cytokines

Cytokine	Biological Activity	Source
TNF	Activation of endothelial cells, activation of neutrophils, synthesis of acute phase proteins (APP) in liver, fever, catabolic effect on muscle and fat, apoptosis of target cells	Macrophages, T lymphocytes
IL-1	Activation of endothelial cells, fever, synthesis of APP in liver, differentiation of T-helper (Th)17 cells	Macrophages, endothelial cells
IL-2	Stimulation of growth and proliferation of T lymphocytes	Th 1 cells, natural killer (NK) cells
IL-6	Synthesis of APP in liver, proliferation of B lymphocytes into antibody-producing cells, differentiation of Th 17 cells	Macrophages, endothelial cells, T lymphocytes
IL-8	Neutrophil chemotaxis	Macrophages
IL-10	Anti-inflammatory actions: inhibition of the expression of IL-12, costimulators and class II major histocompatibility complex molecules	Macrophages, T lymphocytes
IL-12	Stimulator of Th 1 differentiation, stimulates NK and cytotoxic T lymphocyte function	Dendritic cells, macrophages
IL-17	Neutrophil recruitment	Th 17 cells
MCP-1	Regulation of the migration of monocytes, memory T lymphocytes, and natural killer cells	endothelial, epithelial, smooth muscle, fibroblasts, mesangial, and microglial cells
IFN-γ	Antiviral, immunomodulatory, and anti-proliferative actions	Th 1 cells

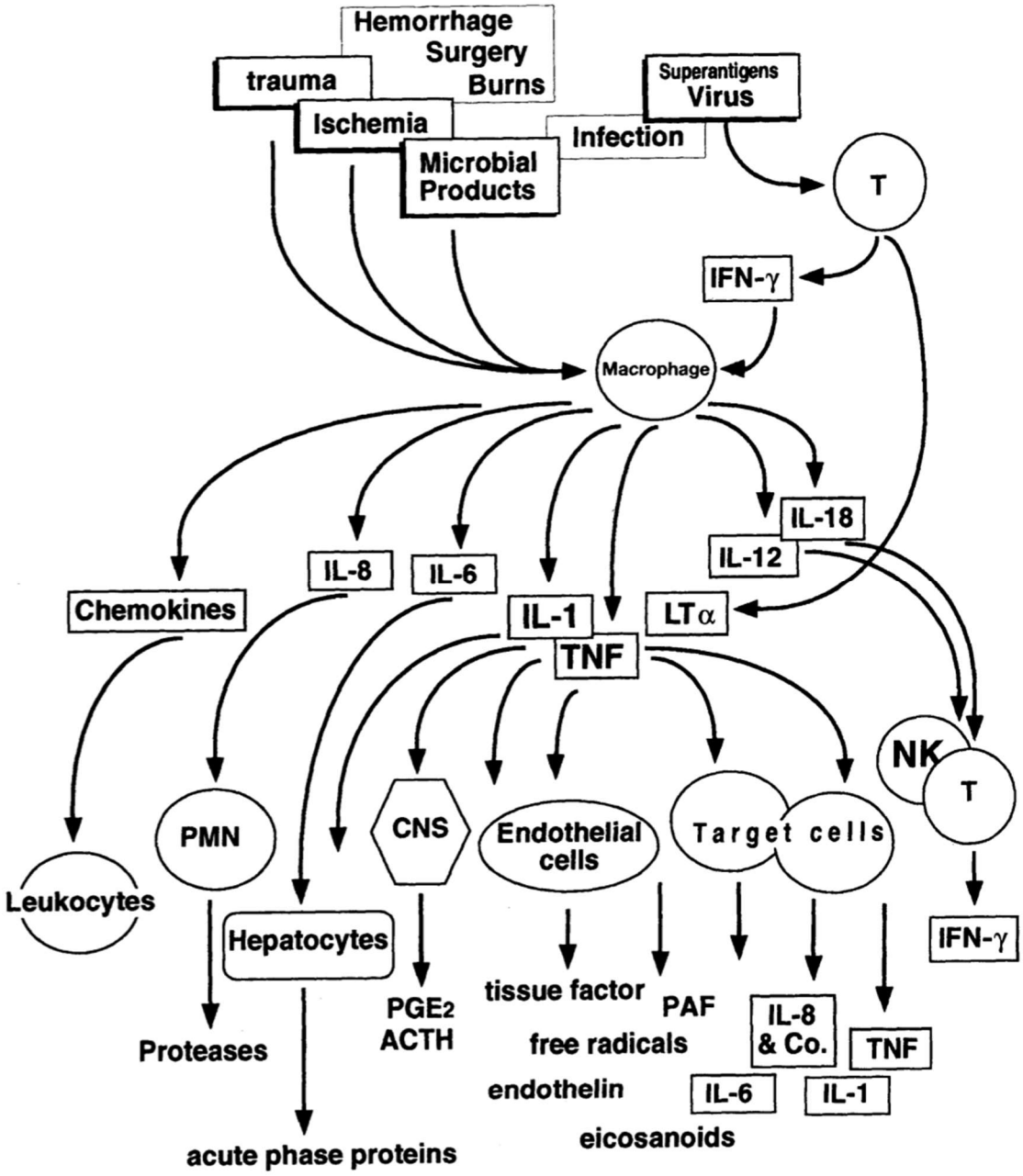

Figure 1-4: Basic representation of the proinflammatory cytokine cascade.
Image reprinted with permission from Cavaillon and Adib-Conquy (2002)

1.2.1.1 Cytokine Kinetics in Horses

As stated earlier, a detailed understanding of cytokine secretion patterns in disease may help elucidate disease pathology and possibly allow for improved therapeutical intervention. To date, cytokine studies in diseased horses are sparse. One challenging

aspect is the fact that blood cytokine profiles differ widely between different populations and age groups and thus cytokines are currently not used for diagnostic or prognostic purposes (Taylor, 2015). Research in horses with endotoxin-induced sepsis reported an increase in TNF-α which induces IL-1 (Cudmore et al., 2013; Holcombe et al., 2016; Morris et al., 2018), as well as IL-6 (Tadros and Frank, 2012). In foals with sepsis, increased IL-1β expression has been observed (Castagnetti et al., 2012) and increases in IL-6 (Ratinier et al., 2011) and IL-10 (Pusterla et al., 2006) have been associated with a poor prognosis, while IFN-γ was not increased (Gold et al., 2007). However, another study did not find increases in either IL-6 or IL-10 in foals with sepsis but suggested the use of an IL-6:IL-10 ratio for prognostic purposes (Burton et al., 2009).

1.2.1.2 Cytokine Kinetics in Orbivirus infections and Bluetongue Virus immune evasion strategies

While the dynamics of cytokine expression have not yet been investigated in AHS, the closely related BTV has been shown to induce IFN and other cytokines both in vitro (Chauveau et al., 2012; Coen et al., 1991; DeMaula et al., 2002; Drew et al., 2010; Huismans, 1969; Rinaldo et al., 1975; Rojas et al., 2017; Russell et al., 1996; Sanchez-Cordon et al., 2015; Umeshappa et al., 2012) and in vivo (Channappanavar et al., 2012; Foster et al., 1991; Jameson et al., 1978; MacLachlan and Thompson, 1985; Ruscanu et al., 2012). These studies report conflicting findings on the cytokine kinetics during experimental BTV infection in vitro and in vivo. During experimental BTV infection, upregulation of IL-1, IL-6, and IL-8 has been observed in microvascular endothelial cells in vitro (DeMaula et al., 2002), while in vitro increases in TNF-α, IL-1β, and IL-8 were identified in pulmonary artery endothelial cells and macrophages

(Drew et al., 2010). In stimulated peripheral blood mononuclear cells from vaccinated sheep, upregulation of IL-1β, IL-6, IL-12 as well as IFN-γ was detected in vitro (Umeshappa et al., 2012). In experimentally infected sheep, increases in IL-12, IFN-γ and TNF-α in lymph nodes, spleen and peripheral blood mononuclear cells were recorded in one in vitro study (Channappanavar et al., 2012), while an in vivo study reported increases in serum IL-1β, TNF-α, IL-4 and IL-10 but absence of IFN-γ and IL-12 (Sanchez-Cordon et al., 2015). In another, more recent in vivo study, repeated experimental infections with BTV resulted in increased concentrations of peripheral blood mononuclear cells expression of IL-6, IL-12, and IFN-γ -induced protein 10, while IL-1β only increased after the third infection (Rojas et al., 2017). While an increase in pro- and anti-inflammatory cytokines was observed in these studies, the reported discrepancies are likely due to the different methods used in the different studies as well as different BTV serotypes and different host species. These results also highlight the immensely intricate balance of the cytokine response and the challenge of testing and interpreting changes in cytokine secretion.

Over time BTV has developed different strategies to interfere with or evade the host immune response (Rojas et al., 2021). Certain genetic changes in the BTV-NS3 protein have been identified as a factor for virus virulence (Janowicz et al., 2015) and may play a role in decreased host cellular protein production (Avia et al., 2019; Stewart et al., 2015). The BTV-NS3 protein has also been found to be an antagonist for IFN through various mechanisms (Avia et al., 2019; Chauveau et al., 2013; Doceul et al., 2014; Li et al., 2021; Pourcelot et al., 2016). The BTV-NS4 protein also plays a role in decreasing the IFN response via different mechanisms such as decreased IFN production, as well as IFN-I and IFN-II signalling (Avia et al., 2019; Li et al., 2021;

Ratinier et al., 2016). The NS4 protein also interacts with the NS3 protein to potentiate its effect on transcription activity (Li et al., 2021). Other BTV proteins that interfere with IFN production are VP3 and VP4 (Chauveau et al., 2013; Pourcelot et al., 2021) and NS1 and NS2 (Stewart and Roy, 2010). The NS5 protein has also been postulated to inhibit cellular gene expression (Stewart et al., 2015). It is clear that BTV employs multiple proteins to target the IFN response.

1.2.2 Changes in acute phase reactants during inflammation

The inflammatory response with proinflammatory cytokine secretion, especially IL-1, IL-6, and TNF-α, generally also induces changes in the concentration of acute phase reactants (APR), such as serum iron, and APP like serum amyloid A (SAA) and fibrinogen (Eckersall and Bell, 2010). The APP are proteins mainly synthesized in the liver during the innate immune system's acute phase response. Commonly, APP are classified as either positive or negative based on an increase or decrease in serum or plasma levels during the acute phase response that occurs after injury or infection (Long and Nolen-Walston, 2020). Typical positive APR include SAA, haptoglobin, and fibrinogen, while albumin and serum iron are negative APR (Jacobsen, 2007). The extent of the change in concentration is dependent on the species and the extent of the trauma but also differs between various APP. During the acute phase response, serum or plasma concentrations of APP change by a minimum of 25%. The positive APP are again subcategorized as major, moderate or minor APP. While minor or moderate APP such as fibrinogen or haptoglobin are present in healthy individuals and their concentrations increase up to tenfold during the acute phase response, the major APP SAA is often undetectable in healthy animals and increases more than 100-fold during inflammation (Jacobsen, 2007). Serum amyloid A has become the

APP of choice for detection of inflammation in equines over the last decade (Nolen-Walston, 2015). In contrast, serum iron is a negative APR, and its concentration decreases during systemic inflammation in order to limit iron availability for pathogen metabolism (Borges et al., 2007; Brosnahan et al., 2012; Ratledge and Dover, 2000). The decrease in serum iron concentration is induced by hepcidin, a positive APP which is derived from the liver. Hepcidin inhibits export of iron from the enterocytes and macrophages into the circulation and hepcidin synthesis is upregulated during inflammation (Oliveira-Filho et al., 2012). In horses, decrease in serum iron concentration as a useful diagnostic and prognostic indicator has been demonstrated during diseases causing systemic inflammation (Borges et al., 2007; Corradini et al., 2014) and acute equine herpesvirus type 1 infection (Brosnahan et al., 2012) but serum iron concentration has been reported to not be a useful inflammatory marker for systemic inflammatory response syndrome in neonatal foals (Sanmarti et al., 2020).

1.3 Haematological changes during inflammation

The inflammatory response caused by infection with a pathogen such as a virus or a bacterium typically results in alterations in the haematopoietic system with resulting changes in either the quality or the quantity of erythrocytes, leukocytes, and platelets (McKenzie and Laudicina, 1998). Inflammation with the secretion of cytokines results in vasodilation with increased vascular permeability, as well as the activation of immune cells, and also has effects on cellular adhesion and coagulation with the goal of localising and controlling the infection (McKenzie and Laudicina, 1998). Thus, inflammation results in typical haematological changes dependent on the severity of

the insult, and evaluation of the leukogram, erythron and changes in platelet concentration and size is commonly performed to evaluate a patient's disease status.

Leukocytes are the main immune cells targeting infectious agents and increases in leukocyte numbers are commonly observed in response to an infectious process. In horses, the predominant type of leukocytes are neutrophils (Carrick and Begg, 2008). During inflammation, proinflammatory cytokines trigger the release of increased numbers of neutrophils from the bone marrow into the blood with an increase in immature stages reflecting increased severity of the disease, while leukopenia is observed when tissue demands exceed the bone marrow's neutrophil production (Carrick and Begg, 2008). Similarly, neutrophil morphologic changes (called "toxic changes") such as cytoplasmic basophilia, Döhle bodies, cytoplasmic vacuolation, nuclear immaturity, and toxic degranulation, indicate immaturity and accelerated release of neutrophils caused by inflammation (Freeman et al., 2022). Leukocytes are a vital part of the immune response as they help eliminate the pathogen by promoting phagocytosis, ingestion, and destruction of the pathogen.

Erythrocytes are not actively involved in the immune processes; however, erythrocyte concentrations can be influenced by systemic disease resulting in blood loss, haemolysis, or production disorders.

Finally, platelets are an important component of haemostasis and the maintenance of vascular integrity. Inflammation results in platelet activation, which results in morphologic changes that lead to improved function. Following injury to the vascular endothelium, adhesion to the subendothelial matrix is initiated by binding of the platelet

glycoprotein (GP) receptor GP1b/V/IX complex to von Willebrand factor (vWf) as well as the platelet surface receptors GPVI and GPIa/IIa binding to the exposed subendothelial collagen (Yun et al., 2016). This process activates the platelets, they change shape and release their granules. These granules are alpha, dense, and lysosomal granules. The dense granules store adenine nucleotides, serotonin, calcium, and inorganic phosphates as well as proteins with important functions for cell signalling, molecular chaperones, the cytoskeleton, glycolysis, and platelet function. The alpha granules contain the proteins beta-thromboglobulin and platelet factor 4, von Willebrand factor, multimerin, and factor V (FV), fibrinogen, thrombospondin, and fibronectin. Finally, lysosomal granules contain hydrolases which include glycosidases, proteases, and lipases (Boudreaux and Christopherson, 2022).

Platelet activation is usually triggered by exposed collagen. Glycoprotein receptors are then bound by either collagen directly or by vWf bound to the collagen (Yun et al., 2016). Platelet activation is further stimulated by local prothrombotic factors including tissue factor (TF) (Yun et al., 2016). Once platelets become activated by either exposure to collagen or as a result of thrombin generation during inflammation, the granules' content is released, and receptors are expressed on the platelet surface allowing for platelet aggregation (Yun et al., 2016). An important structural change occurs during activation to generate a procoagulant surface layer. In the nonactivated platelet, the outer layer is composed of neutral phospholipids such as phosphatidylcholine and sphingomyelin, while phosphatidylserine and phosphatidylethanolamine are confined to the inner layer. With platelet activation, the ATP-dependent protein scramblase is activated and translocase is inhibited, resulting in exposure of phosphatidylserine and phosphatidylethanolamine on the outer

membrane layer (Bevers et al., 1996; Schroit and Zwaal, 1991; Wolfs et al., 2005). Phosphatidylserine expression on the platelet's outer membrane layer serves as a procoagulant surface which is required to assemble other coagulation factors (Leventis and Grinstein, 2010).

Markers of activation include the presence of certain receptors, including P-selectin. P-selectin is important for the interaction between platelets, leukocytes, and endothelial cells as it cross-links platelets and leukocytes and mediates the formation of platelet-leukocyte aggregates, as well as CD40L, which interacts with dendritic cells, B and T lymphocytes (Yun et al., 2016). The resulting changes in shape and size are reflected in changes in the platelet indices which can be measured by modern automated haematology analysers. These include variables representing the mean platelet volume (MPV), the variation in platelet size (platelet volume distribution width, PDW), the percentage of blood consisting of platelets (plateletcrit, PCT), the platelet density (mean platelet component concentration, MPC), the variation in platelet shape (platelet component distribution width, PCDW), the mean platelet mass (MPM), the platelet distribution width (PMDW), and the number of large platelets (Giacomini et al., 2001).

1.3.1 Haematological changes in horses with AHS

Haematological changes previously reported in horses with AHS include leukopenia, thrombocytopenia, and increased erythrocyte and haemoglobin (HBG) concentrations (haemoconcentration) (Skowronek et al., 1995). Variables reflecting platelet indices recognized as surrogate markers of platelet activation such as MPV, MPC, and MPM, have not been evaluated in AHS. In veterinary medicine, these variables have been

demonstrated to be useful markers of platelet activation in dogs infected with canine parvovirus (Engelbrecht et al., 2021) and *Babesia rossi* (Goddard et al., 2015). The resulting changes in platelet indices are used as indicators for thrombopoiesis and platelet activation and have also been described in human diseases caused by endotheliotropic viruses such as Hantavirus (Laine et al., 2016) Denguevirus (Mukker and Kiran, 2018), Coronavirus (Liu et al., 2020), or Crimean-Congo fever (Yilmaz et al., 2016). In these diseases, these indices have been reported as useful tools for diagnostic and prognostic purposes and are now considered important as part of the evaluation of the pathophysiology of many diseases (Yun et al., 2016) and might also be of use in AHS.

1.4 Haemostasis

Precise control of haemostasis is vital to protect the body from injury and allow for repair of vascular lesions and prevent blood loss while simultaneously inhibiting blood from coagulating within the vasculature. It has now been established that activation of the haemostatic systems can also be triggered by inflammation and “crosstalk” between these systems is vital to coordinate tissue healing with activation of an immune response during injury and infection. Inflammation aims to restore tissue integrity following injury or infection, while haemostasis stops bleeding due to a damaged blood vessel. Imbalances in this communication may result in inappropriate activation of haemostasis commonly observed in severe inflammation.

1.4.1 The cell-based model of haemostasis

To understand the interaction between haemostasis and inflammation, it is important to recognize the physiologic pathways involved in haemostasis. Haemostasis has

been described as an interaction of several cells and proteins and the cell-based model of haemostasis suggests that haemostasis happens in three overlapping phases and involves two cell types: the cell that expresses TF and platelets (Figure 1-5).

Phase 1: Initiation

The most important step for the initiation of haemostasis is the expression of TF. Tissue factor is normally only expressed extravascularly, thus preventing haemostasis under normal circumstances. Following injury to the endothelium, exposure of subendothelial cells bearing TF to the bloodstream ensues. The exposed TF is bound by circulating FVII, resulting in activation to FVIIa. The TF-FVIIa complex catalyses the activation of more FVII to FVIIa, further propagating TF-FVIIa complex activity and also activating small amounts of FIX and FX. FV is activated by FXa to form the prothrombinase complex, which then cleaves prothrombin to form a small amount of thrombin (FIIa) (Hoffman, 2003).

Phase 2: Amplification

The thrombin dissociates from the TF-presenting cell and binds to the protease-activated receptors on the platelet. Platelet activation results in the upregulation of integrin adhesion receptors, particularly activating the GPIIb/IIIa receptor. Activation of this receptor leads to fibrin or vWF cross-linking between the receptors and results in adhesion of further platelets and platelet aggregation (Yun et al., 2016). As previously described, activation of platelets also results in externalization of phosphatidylserine which is important for the assembly of coagulation complexes in the upcoming propagation phase and the release of microparticles enriched with

phosphatidylserine (Weisel and Litvinov, 2019). Activated platelets also release procoagulant factors such as FV, fibrinogen, and vWF that promote further platelet activation and thrombin formation (DeNotta and Brooks, 2020). Furthermore, endothelial injury also allows circulating vWF to bind to the subendothelial matrix (Roscher, 2019). Van Willebrand factor then binds platelets via the GP1b receptor, thus allowing for platelet adhesion (Sang et al., 2021).

Phase 3: Propagation

Activation of platelets and release of granules results in the recruitment of more platelets. The expression of phosphatidylserine on the outer platelet membrane further results in binding of FIX and FVIII to form the tenase complex, which can also activate FX, and V and X, which form the prothombinase complex that converts prothrombin to thrombin (DeNotta and Brooks, 2020; Wang et al., 2022). In this final stage, a large-scale burst of thrombin is generated, which suffices to form an insoluble, cross-linked fibrin clot (DeNotta and Brooks, 2020).

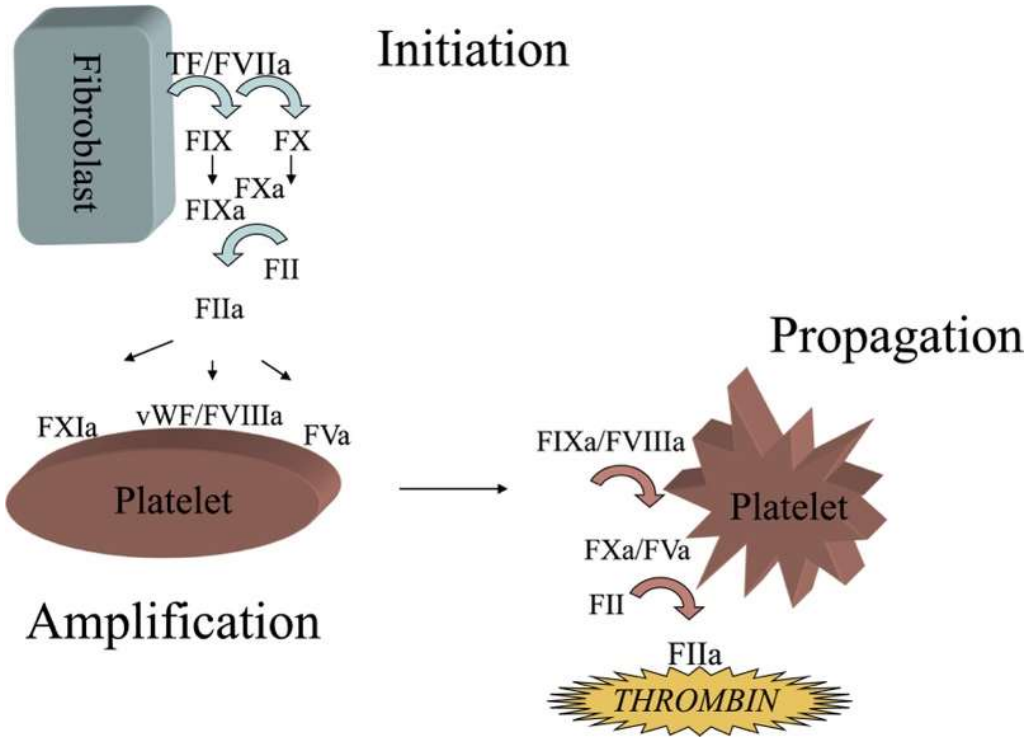

Figure 1-5: Diagram depicting the cell-based model of blood coagulation.
Image reprinted with permission from DeNotta and Brooks (2020)

To prevent excessive haemostasis, the body has a balanced system to prevent generalized haemostasis and to localize haemostasis. Fine control of anti- and procoagulant activity is paramount to prevent excessive haemostasis that could occlude blood vessels. The three main natural anticoagulants in plasma are the plasma serine inhibitors tissue factor pathway inhibitor (TFPI), antithrombin (AT) and thrombomodulin via protein C and its co-factor protein S (Verhamme and Hoylaerts, 2009). Antithrombin is the most important anticoagulant, affecting several aspects of haemostasis (Abildgaard, 1984; Bick, 1982) It is an α2-globulin that is synthesized in the liver (Rezaie and Giri, 2020) and circulates in the plasma but can also be found on endothelial cells and platelets (Opal et al., 2002). Antithrombin specifically inhibits thrombin and FXa but also, to a lesser extent, factors IXa, XIa, and XIIa, modulating

the formation of fibrin (Opal et al., 2002). While horses have a higher AT activity than other domestic animals, this does not seem to have clinical implications (Morris, 1988). Free thrombin is bound by AT in a 1:1 ratio (Iba and Saitoh, 2014), forming thrombin-antithrombin complexes (TAT). During excess thrombin production, more free thrombin is available and an increase in TAT is observed, this is considered a useful marker of a prothrombotic state (Rimpo et al., 2018), and has also been reported in horses (Topper et al., 1996). Furthermore, the formation of the AT-protease complex can be increased up to 1000-fold by heparin, which increases the affinity of AT to the protease and promotes TAT complex formation (Iba and Saitoh, 2014).

Tissue factor pathway inhibitor is synthesized by the endothelium of the microvasculature and is found either circulating, intracellularly in platelets, or bound to the endothelium (Mast, 2016). Tissue factor pathway inhibitor blocks the activity of both the TF-FVIIa (Girard et al., 1989) and prothrombinase complexes (Wood et al., 2013) and thus inhibits the procoagulant response in the initial phases.

The third important anticoagulant is the protein C/protein S complex. Thrombin is bound by the endothelial receptor thrombomodulin to form a complex which has an anticoagulant effect. The thrombin-thrombomodulin complex further activates protein C which, in the presence of protein S, inhibits factors Va and VIIIa in plasma-bound and free form and thus prevents excessive thrombin formation (Ikezoe, 2015).

1.4.2 Fibrinolysis

When haemostasis is activated, fibrinolysis is initiated simultaneously to prevent excessive clot formation which could result in thrombosis. Tissue plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) activate plasminogen to plasmin, plasmin then cleaves fibrin at lysin residues (Longstaff and Kolev, 2015). Fibrin-degradation products and D-dimer are end-products of fibrinolysis (May et al., 2021).

To maintain the fine balance between haemostasis and to prevent excessive fibrinolysis, inhibitors of fibrinolysis including serpins, such as plasminogen activator inhibitor (PAI)-1 and α_2-antiplasmin (AP), as well as thrombin-activatable fibrinolysis inhibitor (TAFI), a nonserpin inhibitor (May et al., 2021) are in place. Plasminogen activator inhibitor-1 inhibits both tPA and uPA and is released by endothelial cells, monocytes, adipocytes and hepatocytes (Simpson et al., 1991); while α_2-AP is produced by hepatocytes and inactivates plasmin directly unless plasmin is bound to fibrin (Schneider and Nesheim, 2004). Thrombomodulin-associated thrombin activates TAFI, which then cleaves lysin from the clotted fibrin (Mosnier and Bouma, 2006). All of these mechanisms decelerate the generation of plasmin, impair fibrinolysis and stabilize the clot.

1.4.3 Haemostasis and inflammation

During inflammation, TF expression on intravascular cells, particularly monocytes, is rapidly upregulated as a consequence of secretion of proinflammatory cytokines such as IL-1, IL-6 and TNF (Grignani and Maiolo, 2000) or generation of oxygen free

radicals (Golino et al., 1996). The exposed TF then interacts with circulating factor VIIa, which then activates factors IX and X and triggers the conversion of prothrombin into thrombin. (Foley and Conway, 2016b). With a sustained inflammatory insult, the generated amount of thrombin can exceed negative regulation and promote further platelet activation, the formation of fibrin clots, and other proinflammatory cascades (Danckwardt et al., 2013) which include the activation of monocytes, neutrophils, and platelets, as well as complement, and the release of proinflammatory cytokines (Demetz et al., 2010). This promotes a further increase in TF expression on monocytes and can induce a positive feedback loop, which results in sustained TF expression, propagated by cytokines and complement factors and promoted by activated leukocytes, platelets and endothelial cells (Foley and Conway, 2016b). Thus, inflammation can cause haemostatic derangement by promoting prothrombotic activity.

1.4.4 Disseminated intravascular coagulation

Disseminated intravascular coagulation (DIC) has been defined as a pathological activation of haemostasis mechanisms which may lead to a systemic hypercoagulable state. This results in both micro- and macrovascular thrombosis with perfusion derangements and ultimately in multiple organ dysfunction (Costello and Nehring, 2022; Morris, 1988). Disseminated intravascular coagulation is not a primary pathology but the consequence of an underlying disease and may vary in severity from subclinical to fulminant (Hamilton et al., 1978). Given the described “crosstalk” between haemostasis and inflammation, it is now accepted that DIC can occur due to an exaggerated inflammatory response and be a complication of severe infections,

resulting in multiorgan failure and death (Costello and Nehring, 2022). Due to the wide clinical spectrum of presentations, DIC has also been named “consumptive thrombohaemorrhagic disorder” (Marder et al., 2006). More recently, the scientific committee of the International Society of Thrombosis and Haemostasis (ISTH) has defined DIC as a multicausal acquired syndrome in which unlocalized intravascular coagulation is present. This condition can originate from the microvasculature whilst damaging it at the same time and result in organ dysfunction in severe cases (Wada et al., 2013).

The key factor in the initiation of DIC is the activation of haemostasis followed by excess thrombin generation. This may be caused by two events: The first is the production of excess procoagulant agents with TF, phospholipids, platelet activating factors, or tumour products directly activating coagulation factors. The second event is blood coming into contact with abnormal surfaces as the endothelium becomes disrupted and collagen is exposed. This can lead to the activation of factor XII or activate platelets. Disseminated intravascular coagulation often involves both mechanisms (Colman et al., 1979). As previously described, inflammation can also promote the expression of TF on monocytes and other cells and propagate the generation of thrombin (Foley and Conway, 2016b), resulting in the conversion of fibrinogen to produce fibrin. As thrombin has multiple positive feedback mechanisms, it perpetuates its own procoagulant effects. This then overwhelms the anticoagulant capacities of TFPI, AT and protein C, resulting in systemic thrombosis (de Gopegui et al., 1995; Levi and van der Poll, 2014). Eventually, this leads to hypercoagulability with simultaneous haemorrhagic tendencies due to platelet depletion, consumption of coagulation factors, and acceleration of plasmin formation. Both hyper- and

hypocoagulative states can occur simultaneously (de Gopegui et al., 1995; Rodgers, 2013). It is recommended to diagnose DIC based not only on clinical signs but also on diagnostic tests. In human medicine, criteria have been established to categorize DIC as non-overt or overt. In non-overt DIC, the haemostatic system is considered stressed but not yet decompensated, while in overt DIC decompensation has occurred and clinical signs of haemostatic derangements, like diffuse haemorrhage, are present (Taylor et al., 2001). Categorization criteria include test results indicating activated procoagulant pathways (such as prolonged prothrombin time (PT) or activated partial thromboplastin time (aPTT), hypofibrinogenaemia, or thrombocytopenia) or test results indicating consumption of coagulation inhibitors (like decreased AT or protein C activity) or test results indicating fibrinolysis (such as increased D-dimer concentration or FDP) (Goggs et al., 2018). These tests results, together with clinical signs, are then used in a scoring system to differentiate between overt and non-overt DIC. Relevant test results include decreases in platelet concentration, AT-, and protein C activities with increases in PT, TAT, fibrinogen or FDP concentration (Di Nisio et al., 2012; Levi et al., 2009; Taylor et al., 2001; Wada et al., 2010a). Additional diagnostic modalities were later included in people and dogs, specifically a thromboelastogram indicating hypercoagulation, and increases in D-dimer and plasminogen concentrations (Egi et al., 2009; Hayakawa et al., 2007; Taylor et al., 2001; Toh and Downey, 2005; Toh et al., 2007; Wada et al., 2010b; Wiinberg et al., 2008). The scoring system differentiates overt from non-overt DIC by using negative scores to reflect compensated stages in non-overt DIC (Lee and Song, 2010; Taylor et al., 2001). An association between the diagnosis of overt DIC and mortality using similar scoring criteria has been described in dogs (Goggs et al., 2018; Wiinberg et al., 2010). In veterinary medicine, additional tests including a thromboelastogram indicating

hypocoagulation (Wiinberg et al., 2005) and increases in D-dimer concentrations (Stokol, 2003) have been evaluated to improve the early detection of DIC in dogs. No scoring system has been established for use in horses. Early identification of DIC allows for improved intervention and likely a better outcome as the presence of non-overt DIC has a better prognosis compared to overt DIC (Goggs et al., 2018; Wiinberg et al., 2008).

In the last decade, thromboelastography (TEG) has become more widely available as a diagnostic modality to assess haemostasis. Thromboelastography allows for the in vitro assessment of haemostasis by evaluating the cellular and soluble aspects of the haemostasis process. It is thus assessing all steps of haemostasis, which include initiation, amplification, propagation, and fibrinolysis and also evaluates platelet contribution to haemostasis.

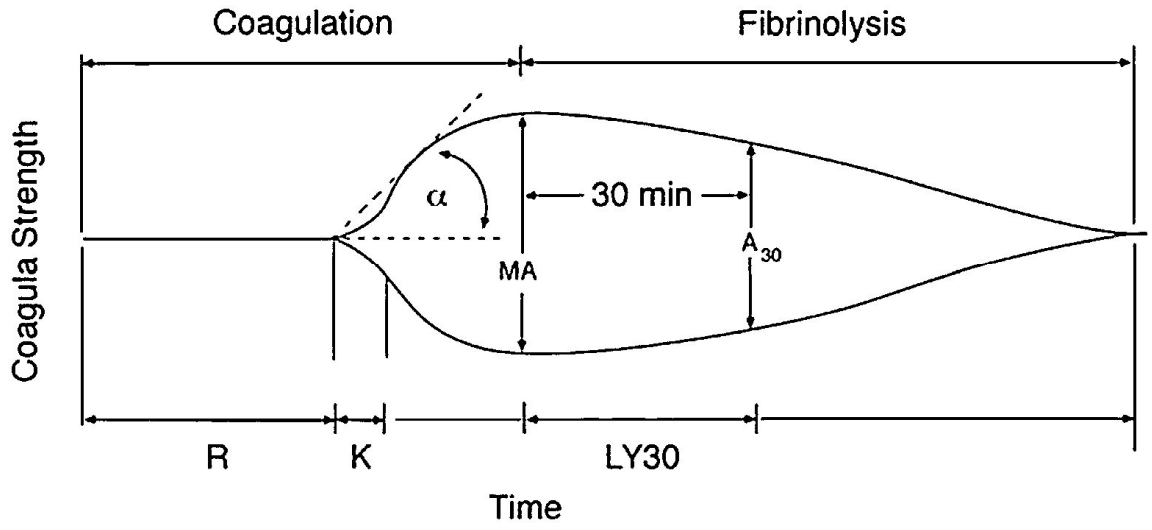

Figure 1-6: Schematic diagram of a thromboelastogram tracing.

Image reprinted with permission from Saeveraas et al. (2019)

The variables typically evaluated in a thromboelastogram include (Figure 1-6) (Thakur et al., 2012):

- Reaction time, R: The time in minutes from initiation of the clot until production of the first fibrin strand. It is the time measured from the start of the test until the amplitude reaches 2mm. The reaction time is affected by availability of coagulation factors and inhibitor activity (Burton and Jandrey, 2020).
- Clotting time, K: Represents the speed of clot formation. It is the time from the end of R until the amplitude reaches 20 mm. The clotting time is affected by platelet concentration and function, factor II, factor VIII, fibrinogen concentration, and HCT (Burton and Jandrey, 2020).
- Angle, α: It represents the kinetics or acceleration of the formation crosslinking of fibrin and is the angle (in degrees) to the tangent to the curve drawn from K. The angle is affected by platelet concentration and function, factor II, factor VIII, fibrinogen concentration, and HCT (Burton and Jandrey, 2020).
- Maximal clot strength, MA: It reflects maximum or final clot strength and is the maximum amplitude. The maximum amplitude is influenced by fibrinogen concentration, platelet concentration and function, thrombin concentration factor XIII, and HCT (Burton and Jandrey, 2020).
- Global clot strength, G: It is the modification of MA to physical units and a measure of the overall coagulant state. It is calculated: $G = 5000 \times MA / (100 – MA)$ dyn/cm^2. The global clot strength is influenced by fibrinogen concentration, platelet concentration and function, thrombin concentration, factor XIII, and HCT (Burton and Jandrey, 2020).
- Lysis, LY 30/60: These represent the percent of clot lysis that is recorded 30 or 60 minutes after MA is reached.

- Whole blood clot lysis index, CL30/60: These represent the measurements of fibrinolysis at 30 and 60 minutes after maximum clot strength has been reached.
- Ly30/60 and CL30/60 are both variables indicating clot stability and influenced by the concentration of clot inhibitors (Burton and Jandrey, 2020).

Generally, hypocoagulation is depicted by prolonged R and K times and decreases in the α-angle and MA values compared to reference values (Figure 1-7). Hypercoagulation on the other hand is characterized by a decrease in R and K times with an increase in α-angle and MA values (Mendez-Angulo et al., 2012).

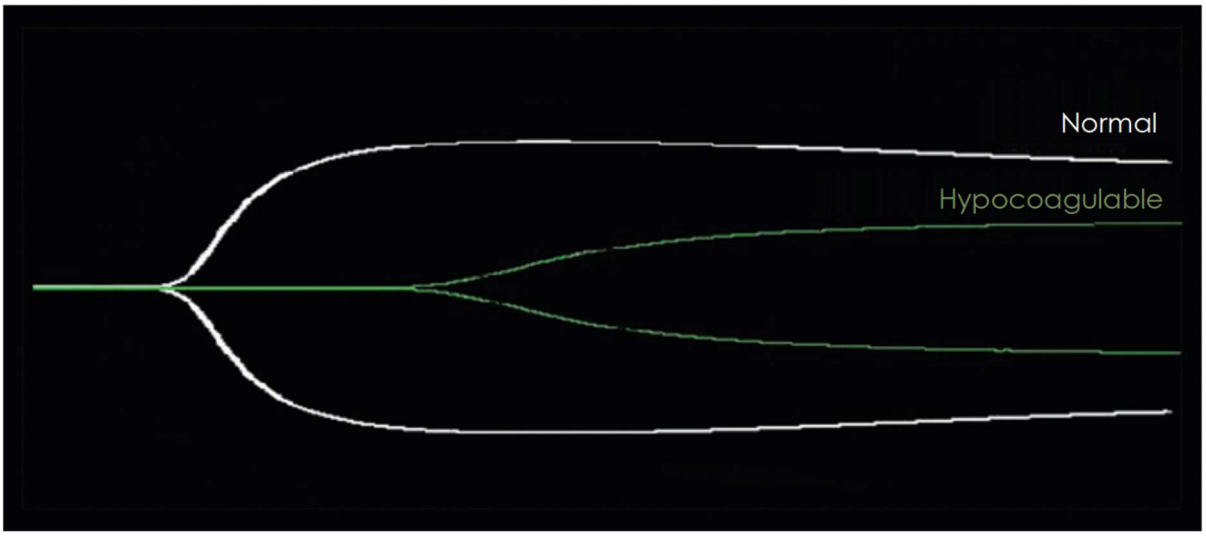

Figure 1-7: Superimposed thromboelastography tracings from a healthy and a hypocoagulable horse.
Image reprinted with permission from Mendez-Angulo et al. (2012)

Clot-velocity curve

A correlation between thromboelastographic coagulation kinetics and in vivo thrombin generation has been shown and a clot-velocity curve (V-curve) has been established (Sorensen et al., 2003). The V-curve allows assessment of the global integrity of coagulation by measuring thrombin generation, thus assessing the rate and strength

of thrombus formation (Rivard et al., 2005). The V-curve is calculated from the TEG values from the mathematical first derivative of the TEG tracing and reflects thrombus formation velocity (plotted above the x-axis) and the dissolution of the thrombus (plotted below the x-axis) (Figure 1-8) (Gonzalez et al., 2010; Pommerening et al., 2014).

The values typically evaluated in a V-curve include (Pommerening et al., 2014):

- Maximum rate of thrombus generation, MRTG: the time in minutes to the peak of the curve, represents the maximum rate of thrombus generation. It is considered more sensitive than TEG angle α as a marker of clot formation, and allows more precise evaluation of clot kinetics (Ellis et al., 2007).
- Time to maximum rate of thrombus generation, TMRTG: represents the time from the start of the curve to its peak. It is influenced by the availability of clotting enzymes (Gonzalez et al., 2010)
- Thrombus generation, TG: calculated from the area under the curve, indirect measurement of clot strength and thus affected by fibrinogen concentration, platelet concentration and function, thrombin concentration, factor XIII, and HCT (Burton and Jandrey, 2020).
- Maximum rate of lysis, MRL: indicated by the negative peak of the curve, represents the maximum rate of lysis. It is influenced by the concentration of clot inhibitors.
- Time to maximum rate of lysis, TMRL: the distance from the start of the fibrinolysis curve to its negative peak and influenced by clot inhibitor concentration.

- Total lysis, L: indirectly measures the total amount of lysis, reflected by total reduction in clot amplitude (mm).
- The values reflecting lysis are influenced by the concentration of clot inhibitors (Burton and Jandrey, 2020).

Evaluations in human medicine compared the MRTG to the TEG parameter α and observed a less marked increase for α while MRTG increases exponentially, therefore allowing for more precise assessment of clot kinetics (Ellis et al., 2007):

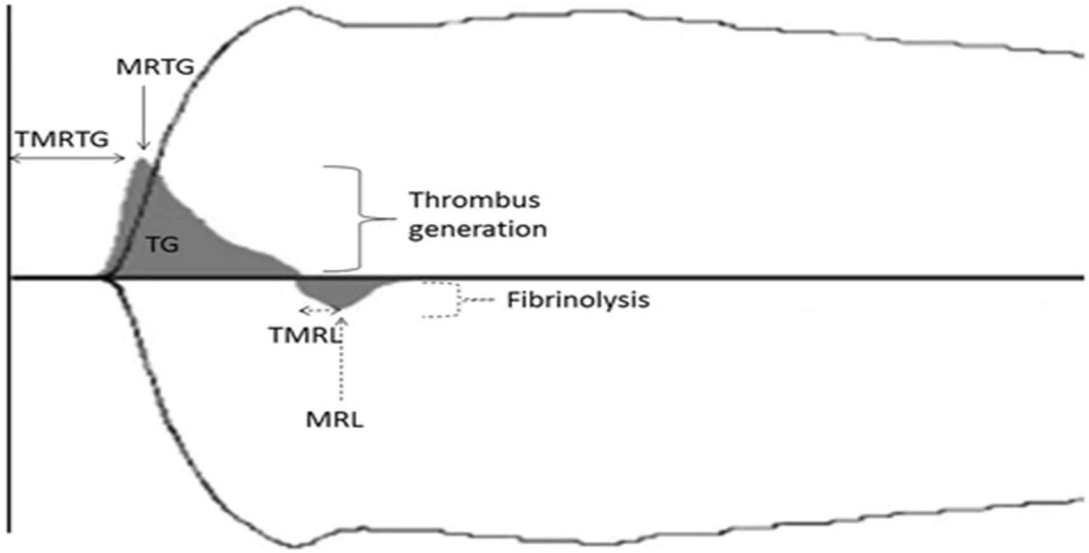

Figure 1-8: Thrombus velocity curve.
Image reprinted with permission Engelen et al. (2017)

Thromboelastography has been validated in horses (Paltrinieri et al., 2008) and used to assess haemostatic derangements occurring in different diseases in adult horses such as gastrointestinal diseases (Dunkel et al., 2010; Epstein et al., 2011; Mendez-Angulo et al., 2010), exercise induced pulmonary haemorrhage (Giordano et al., 2010), and asthma (Leclere et al., 2015) as well as in healthy, sick non-septic, and

septic neonates (Mendez-Angulo et al., 2011). While the degree of correlation between the traditional coagulation tests and TEG differs between different studies (Alexander et al., 2010; Park et al., 2009; Wagg et al., 2009), TEG has been shown to improve early recognition of bleeding in dogs (Bucknoff et al., 2014; Wiinberg et al., 2009) and may be superior to the use of traditional coagulation tests in clinical patients.

Disseminated intravascular coagulation has been recognized as a complication of various pathologies, and in horses these include sepsis in the neonate, inflammatory and ischemic gastrointestinal tract disease (Armengou et al., 2008; Dallap Schaer and Epstein, 2009; Monreal et al., 2000; Welch et al., 1992). Endotoxemia, a common cause of severe systemic inflammation in these pathologies, has been demonstrated to induce the expression of TF on monocytes (Ouellette et al., 2004), making this a likely factor for the haemostatic disturbances.

1.4.5 African Horse Sickness and haemostasis

Very little has been reported on the haemostatic changes observed in horses with AHS. Clinically, petechiae and prolonged bleeding after venepuncture are often observed in infected horses (Coetzer and Guthrie, 2004) and one study reported the presence of thrombocytopenia with prolonged PT, aPTT, and TT (thrombin time) as well as an increase in FDP (Skowronek et al., 1995). Investigations into the changes in other laboratory variables used to evaluate haemostasis, such as TEG and activities of individual coagulation factors, have not been described to date. Generally, while coagulation factor deficiency is uncommon in horses, in humans, cytokines and autoantibodies acting as acquired factor inhibitors and resulting in disturbance of haemostasis have been described in viral infections (Bennett et al., 2021; Chuang et

al., 2013). This may exacerbate the haemostasis abnormalities observed due to inflammation and it is unknown if AHSV may act in this fashion.

Viruses have been implicated to cause DIC via the formation of circulating antigen-antibody complexes that activate the endothelium and the contact system (Yazdanparast Tafti et al., 2022). African horse sickness virus is an endotheliotropic virus and has been shown to replicate in endothelial cells (Clift and Penrith, 2010; Laegreid et al., 1992a; Wohlsein et al., 1997a). It is thus possible, that resultant damage to the endothelium leads to exposure of TF bearing cells and thus activation of haemostasis.

1.5 Conclusion

As highlighted, there is a scarcity of knowledge on the inflammatory and haemostatic changes that infection with AHSV causes in naïve horses. Clinical signs including fever, bleeding tendencies, and pulmonary oedema, especially in horses developing the pulmonary form, suggest severe inflammation, possibly caused by a cytokine storm resulting in endothelial damage and coagulation disorders and ultimately death. To date, therapy attempts in infected horses are limited to symptomatic treatment and subjectively have very little impact on the outcome. A better understanding of the underlying pathophysiology of the disease and the inflammatory changes occurring after infection with AHSV may inform treatment strategies. Furthermore, additional knowledge on the inflammatory response to infection with AHSV may also be valuable to improve vaccine development and lead to the development of a more effective vaccine. More effective treatment for, and vaccination against AHSV, would improve

equine welfare and in extension aid in supporting the South African equine industry as well as rural economies that often depend on working horses.

2 CHAPTER 2: RESEARCH HYPOTHESES

The aims of this research were to investigate the haematologic, inflammatory, and haemostatic changes in horses experimentally infected with AHS.

The objectives of this research were to:

1. Evaluate the haematological changes occurring in horses experimentally infected with AHSV.
2. Investigate platelet activation in horses experimentally infected with AHSV.
3. Appraise the acute phase response represented by acute phase reactants SAA and serum iron concentration in horses experimentally infected with AHSV.
4. Describe the changes in selected cytokines during experimental infection with AHSV.
5. Characterize the haemostatic changes occurring in horses experimentally infected with AHSV.

The research hypotheses of the project were:

1. Horses experimentally infected with AHSV develop leukopenia with pronounced neutropenia as well as thrombocytopenia as result of the host immune response.
2. Platelet activation, indicated by changes in platelet indices and platelet activation markers, is present in horses experimentally infected with AHSV.

3. Horses with experimental infection with AHSV mount a severe inflammatory response resulting in marked increases in the acute phase reactant SAA following infection, while serum iron concentration decreases.
4. Experimental infection with AHSV initiates an immune response with a pronounced proinflammatory cytokine response in alignment with the severe clinical signs such as pyrexia and vasculitis observed during AHS.
5. Horses experimentally infected with AHSV develop overt DIC which can be demonstrated both clinically and using laboratory haemostasis tests.

3 CHAPTER 3: MATERIALS AND METHODS

3.1 Ethical aspects regarding the use of animals for experimental purposes

This thesis consists of three studies with the evaluation of one data set derived from one group of experimental animals included in a vaccine trial. Approval of the vaccine trial including the virus challenge was obtained from the Deltamune Ethics Committee before commencement of the trial (PD 17-23). Research and animal ethics approval for our research was granted by the Research Ethics Committee of the Faculty of Veterinary Science and Animal Ethics Committee of the University of Pretoria (REC 195-19). As this study involved the use of experimental animals, the study design and reporting of results was carried out in line with Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines (see appendix 11.1).

3.2 Study population

The horses included in this research project were participants in an independent AHSV virulence study required for a vaccine trial conducted by a pharmaceutical company (Deltamune (Pty) Ltd, Pretoria, South Africa). Using molecular techniques, Deltamune Ltd. has developed attenuated AHS viruses and to evaluate the efficacy of potential vaccine candidates, it is necessary to conduct animal experiments, since no in vitro model is available.

Infection with passaged AHSV subtypes commonly only results in mild clinical signs due to loss of virulence because of repeated passaging during the tissue culture process. Therefore, it is necessary to establish the serovars' virulence prior to using

them in a clinical vaccine trial. The four horses used in our study were required to test the virulence of four strains of virulent field AHSV isolates (one horse per serovar) which were available at the time. With the scarcity of knowledge on the detailed pathophysiological changes caused by AHSV and the importance of AHS in South Africa, blood samples were obtained from these experimentally infected horses to reduce the number of animals sacrificed for future research purposes and maximize information gain from this sacrifice. All samples obtained are stored in a biobank to allow for inclusion in further research.

3.2.1 Animal selection

Unvaccinated AHS-susceptible horses were sourced from a farm in an area with a very low prevalence of natural AHS outbreaks in the eastern Free State province, South Africa. Prior to inclusion, horses were tested for AHSV group-specific antibodies with a competitive ELISA as well as the presence of neutralizing antibodies against all nine AHSV serotypes (Ingezim AHSV compac plus, Eurofins Technologies, Madrid, Spain). The completely susceptible horses were transported to a BSL-2+ vector-free facility and housed in individual stalls 14 days before the start of the trial to allow environmental adaptation. Peripheral blood smears were examined by the principal investigator, the veterinarian on site, and an experienced clinical pathology technologist for the presence of blood-borne parasites, specifically piroplasms.

Additionally, prior to inclusion, a complete blood count (CBC) with manual evaluation of blood smears by an experienced technologist for the presence of blood-borne parasites, specifically piroplasms, was performed, as well as a panel of serum biochemistry tests, including total protein, albumin, creatine kinase, aspartate

transaminase, urea, creatinine, alkaline phosphatase, gamma-glutamyl transferase, bilirubin, and glutamate dehydrogenase, was performed to further ensure general good health of the included horses.

3.2.2 Animal housing and management

The horses were individually housed in stables in the vector-free facility with a minimum of 8.5 m^2 floor space per horse. Windows and gates allowed for visual contact between horses. Wood shavings were supplied as bedding, faeces and wet shavings were removed once a day and clean dry wood shavings were added daily. Temperature of the incoming air was regulated between 16 – 26˚C by electrical elements and an evaporative cooling system in the ventilation ducting. Air flow was maintained. A photoperiod of 12 hours light: 12 hours darkness was maintained by electrical lights and automatic timers throughout the study. Natural lighting occurred via panels in the roof. Relative humidity was not controlled or measured. Good quality *Eragrostis curvula* hay, which had been fumigated using phosphine gas, was fed *ad libitum* and commercial horse-pellets were supplied twice a day. Fresh, potable water was available *ad libitum*. Horses were evaluated visually throughout the day, physical examinations including rectal temperatures were recorded twice daily from two days before infection until euthanasia.

3.3 Study design

3.3.1 Experimental infection

Horses were infected intravenously with 5 mL of low passage mouse brain suspension, containing at least 10^5 mouse infective doses/mL of virulent AHSV

serotypes. As different serovars were assessed for virulence during the vaccine trial, each horse was inoculated with a different AHS serovar: horse 1, AHSV-2 (horse origin); horse 2, AHSV-4 (horse origin); horse 3, AHSV-6 (horse origin) and horse 4, AHSV-6 (dog origin).

3.3.2 Animal monitoring and welfare considerations

Every challenged horse was physically evaluated twice a day as previously described and checked according to a welfare score (Table 3-1). Horses that developed severe discomfort, including severe dyspnoea (respiratory rate >40 breaths per minute, severely increased respiratory effort), colic, dehydration, or any other condition, related or unrelated to the challenge (welfare score 3 and 4), were humanely euthanized. Humane euthanasia was performed by intravenous administration of sodium pentobarbital at 200 mg/kg.

All euthanized animals were subjected to necropsy and histopathological examination at the Section of Pathology, Department Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, Onderstepoort.

Table 3-1: Animal Welfare Score.

ANIMAL WELFARE SCORE		
Score	**Severity of discomfort**	**Clinical criteria on which the score is based**
0	Normal	No local or systemic reaction to challenge
1+	Mild discomfort	Mild systemic reaction to challenge (temperature rise of less than 1˚C
2+	Moderate discomfort	Moderate systemic reaction to challenge (temperature rise of 1-2˚C, accompanied by signs of discomfort such as decreased appetite, shifting stance etc.)
3+	Severe discomfort	Severe, but not life-threatening reaction (significant fever reaction for prolonged period, accompanied by intramuscular oedema – typical dikkop AHS)
4+	Unbearable discomfort	Severe, life-threatening reaction – usually seen as significant fever reaction accompanied by pulmonary oedema and/or hydrothorax (laboured breathing/tachypnoea/flared nostrils/stretched neck) and/or severe colic and/or cardiac failure (weak peripheral pulse/tachycardia)

3.4 Sampling procedures

Horses were restrained in stocks for sampling and at each sampling time point blood was collected from each horse in the same order. Blood was obtained prior to infection, then every 12 hours post-infection until the horses tested positive for AHS using polymerase chain reaction (PCR, see chapter 3.5 for details). A PCR for AHSV was performed daily, then twice daily once horses became febrile until viraemia was confirmed using a method previously described (van Rijn et al., 2018a). Once a horse tested positive on PCR, samples were obtained from that horse every 2 hours for 12 hours, followed by every 4 hours for another 12 hours. After this, 12-hour sampling intervals were reinstituted until the horse was humanely euthanized (Figure 3-1).

Sample acquisition was performed by venepuncture from the jugular vein using 20-gauge needles directly into vacutainer tubes, through vacuum assistance. Veins were alternated between sampling time points. Blood was collected in specific sequence into a serum tube (4 mL), a 3.2% sodium citrate tube (4 mL), a heparin tube (4 mL), and an EDTA tube (4 mL).

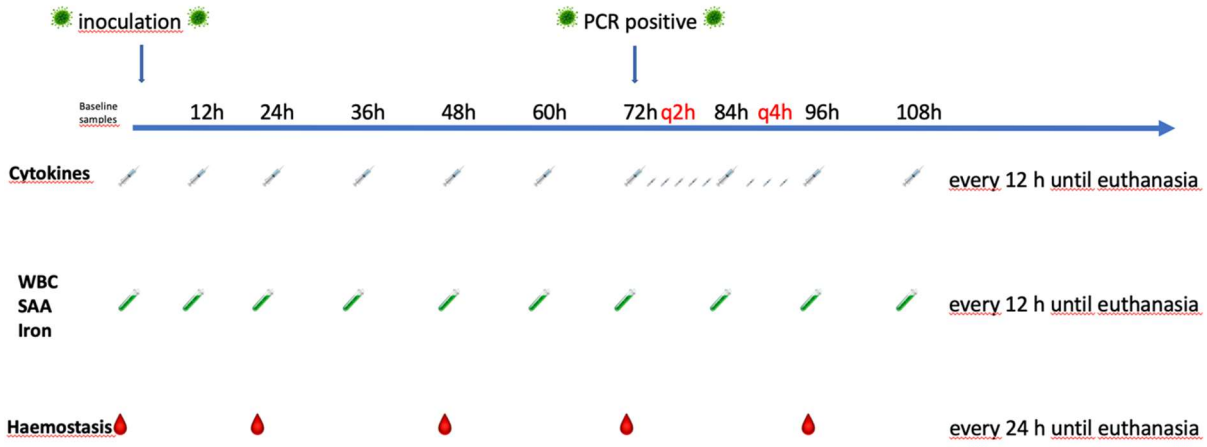

Figure 3-1:Timeline for the sample collection.

The EDTA blood was used to perform a CBC, and cytokine concentrations were measured on the EDTA plasma. Serum was used to measure the APR concentrations, SAA and iron, as well as a panel of biochemistry tests (total protein, albumin, creatine kinase, aspartate transaminase, urea, creatinine, alkaline phosphatase, gamma-glutamyl transferase, bilirubin, and glutamate dehydrogenase). The sodium citrate whole blood sample was used to perform the TEG assay and the citrated plasma to perform various haemostasis assays. The EDTA plasma, serum, and citrated plasma samples were stored for the assays to be run in batches to minimize inter-assay variability. The EDTA plasma, citrated plasma, and serum samples were separated from the red blood cells within 90 minutes of collection and stored in cryovials at -80°C.

3.5 Polymerase Chain Reaction

Real-time-polymerase chain reaction for AHSV was performed using a previously described method (van Rijn et al., 2018b), pan AHSV RT-PCR (a PCR detecting all AHSV nine serotypes) daily for each horse, then twice daily once the horses became febrile until viraemia was confirmed. Briefly, the primers (F-pan-S4: TTAGGATGGAACCTTACGC and R-pan-S4: ATTCTGCCCCTCTCTAACCA) and probe (P-pan-S4: FAM-CTTTGAGTAGGTATTCGATCTCCTGCG-BBQ) used for testing were synthesized by Eurogentec (Seraing, Belgium) and TIBMOLBIOL (Berlin, Germany), respectively. RT-PCR assays (20 µL final volume) consisted of 800 nM primers each, 300 nM of probe, 2 mM MnO_2, 7.5 µL RT-PCR mix (Roche LC480 RNA Master HybProbes, Roche, Basel, Switzerland) and 5 µL of purified RNA. RT-PCR was performed in the LightCycler Nano (Roche): 98°C for 20 sec (dsRNA denaturation), 55°C for 20 sec and 61°C for 10 min (reverse transcription), 95°C for 30 sec, and 40 cycles of 95°C for 10 sec, 55°C for 10 sec and 61°C for 30 sec (pre-denaturation, reverse transcription, target amplification). Purified genomic dsRNA (extracted from cells infected with AHSV 32/62 (the Office International des Epizooties reference strain of AHSV4) was used as a positive control. Quantification cycle values of 37-39 were considered suspect, quantification cycle values lower than 37 were positive.

3.6 Complete blood count

An EDTA blood sample was utilized to perform a CBC to determine the haematocrit (HCT), platelet concentration (PLT) and platelet indices, as well as a total (WBC) and differential leukocyte count. The CBC was performed on the Advia 2120i (Siemens, Germany), an automated haematology analyser. Internal quality control procedures

for the ADVIA were performed daily using three levels of manufacturer-supplied quality control material, and results were within target ranges. A blood smear was prepared to perform a manual 100-cell leukocyte differential count by an experienced technologist. Blood smears were further evaluated for platelet aggregation and the presence of blood-borne parasites.

Erythrocyte variables included:

- Haemoglobin concentration (HGB, g/L)
- Haematocrit (HCT, L/L)
- Red blood cell (RBC, $x10^9$ cells/L) concentration
- RBC distribution width (RDW)
- Mean corpuscular volume (MCV, fL)
- Mean corpuscular haemoglobin (MCH, pg)
- Mean corpuscular haemoglobin concentration (MCHC, g/L)
- Mean haemoglobin concentration per cell (CHCM, g/L)

Leukocyte variables included:

- WBC ($x10^9$ cells/L) concentrations
- absolute mature neutrophil- ($x10^9$ cells/L) concentrations
- band neutrophil- ($x10^9$ cells/L) concentrations
- lymphocyte- ($x10^9$ cells/L) concentrations
- monocyte- ($x10^9$ cells/L) concentrations
- eosinophil- ($x10^9$ cells/L) concentrations
- basophil ($x10^9$ cells/L) concentrations
- myeloperoxidase index (MPXI).

Platelet variables included:

- platelets (PLT, $x10^9$ cells/L)
- plateletcrit (PCT, %)
- mean platelet volume (MPV, fl)
- platelet distribution width (PDW, %)
- mean platelet concentration (MPC, g/dL)
- mean platelet mass (MPM, pg).

The assays were performed in the Clinical Pathology Laboratory, Faculty of Veterinary Science, University of Pretoria, South Africa.

If blood-borne parasites were observed on the blood smears, the percentage of infected cells was determined: blood smears were evaluated along both slide edges and the centre of the feathered edge. A minimum of 2500 single layered erythrocytes were evaluated per slide and the percentage of erythrocytes containing parasites calculated. The presence of *Theileria equi* and/or *Babesia caballi* was further confirmed using PCR methods previously described (Bhoora et al., 2010). All four horses were treated with imidocarb (2 mg/kg intramuscularly) and glycopyrrolate (0.0025 mg/kg intravenously) at 36 hours post infection when solitary piroplasms were observed on blood smears.

3.7 Serum biochemistry assays

Before infection, various assays which included total protein, albumin, creatine kinase, aspartate transaminase, urea, creatinine, alkaline phosphatase, gamma-glutamyl transferase, bilirubin, and glutamate dehydrogenase were performed on serum

samples. Assay specific kits were used according to the manufacturer's instructions on an automated analyser (Cobas Integra 400 plus, Roche, Switzerland), and were subject to daily internal quality control procedures. The assays were performed by the Clinical Pathology Laboratory, Faculty of Veterinary Science, University of Pretoria, South Africa.

3.8 Acute Phase Reactants

Serum amyloid A concentration was measured using the Eiken VET-SAA immunoturbidometric assay (Eiken Co., Tokyo, Japan) recently validated for use in horses (Jacobsen et al., 2019). Serum iron concentration was measured using the ferrozine method (Iron Gen.2, Roche, Basel, Switzerland). Both assays were performed on the Cobas Integra 400 Plus (Cobas Integra 400 Plus, Roche, Basel, Switzerland) in the Clinical Pathology Laboratory, Faculty of Veterinary Science, University of Pretoria, South Africa. For both assays, two levels of manufacturer-supplied quality control material were run before each batch of horse samples, and results fell within target ranges.

3.9 Cytokine Assay

Concentrations for cytokines IL-1α, IL-2, IL-6, IL-8, IL-10, IL-12, IL-17, IFN-γ, TNF-α, and monocyte chemoattractant protein (MCP)-1 were analysed in EDTA plasma using an equine-specific Milliplex® Map Magnetic Bead Panel (EMD Millipore, Billerica, MA, USA). These cytokines were chosen as they represented a mixture of mediators of innate and adaptive immunity.

The assay was performed according to the manufacturer's instructions and samples were measured in duplicate. Prior to the start of the assay, the 96-well assay plates were washed using kit wash buffer. Assay buffer (background), standard, and control reagents were added into the wells and diluted 1:1 with supplied serum matrix. In the next step, 25 µL of each sample and the magnetic beads coated with fluorescently labelled capture antibodies for each analyte were plated in sample wells, each sample was plated in duplicate. Plates were sealed and incubated on an orbital shaker for 16 hours at 4°C. Then the beads were washed followed by incubation with biotinylated detection antibodies and another incubation with streptavidin phycoerythrin. Beads were washed again prior to resuspension in drive fluid. Samples were analysed using a Luminex MagPix® instrument and Luminex xPONENT® software (Luminex, Austin, TX, USA). Data was analysed with Milliplex Analyst® software (EMD Millipore). A minimum count of 50 beads per well was used for inclusion in analysis. The mean of the duplicate samples was used for analysis. Values that fell below the lowest level of detection (LLOD) of the assay were assigned the lowest detectable concentration for that analyte, as determined by the Analyst® software. A 5-parameter logistical standard curve was calculated using the mean fluorescence intensity data from provided standards and used to calculate analyte concentration (Baud, 1992). The assay was performed at the Department of Immunology, Faculty of Health Sciences, University of Pretoria, South Africa. Due to logistical reasons, cytokine analysis in horse D was performed with reagents from a different batch from horse A, B, and C.

To confirm test functionality and to serve as positive controls, stored EDTA plasma samples (-80°C) from seven horses with severe systemic disease and known SAA concentrations of >1000 mg/L were included in the assay. These horses had

previously been presented as patients to the Onderstepoort Veterinary Academic Hospital (OVAH) and samples had been obtained for diagnostic purposes. The horses were diagnosed with severe gastrointestinal disorders (gastrointestinal perforation, hepatitis, colitis), (pleuro-) pneumonia, or neonatal sepsis.

3.10 Haemostasis assays

3.10.1 Thromboelastography

Thromboelastography was performed two hours from sample collection. A previous study has indicated that no statistically or clinically significant changes in TEG variables occur in citrated horse blood during the first two hours after collection (Lemon et al., 2021). Kaolin was used as activator, previously validated for use in horses (Epstein et al., 2009; Machackova et al., 2018; Macieira et al., 2007). Analyses were performed using the TEG® 5000 Thrombelastograph® Hemostasis System (Haemonetics., Boston, MA, USA) according to the manufacturer's instructions. Briefly, 1 mL of citrated whole blood was added into a kaolin-coated vial and gently mixed, after which 340 μL of the mixture was added to a preheated (37°C) cup in the TEG analyser, containing 20 μL of 0.2 M $CaCl_2$ to add up to a total of 360 μL. The cup was then carefully raised to the pin, measurement started and obtained for 120 minutes. The thromboelastogram was recorded electronically using dedicated software (TEG Analytical Software, Haemonetics, Boston, MA, USA).

Controls for level I (normal ranges) and level II (abnormal ranges) were performed on the TEG analysers on a weekly basis according to the manufacturer's recommendation. All analyses were performed by the principal investigator or a laboratory technician.

3.10.2 Prothrombin time and activated partial thromboplastin time

Citrated plasma (200 µL) was utilized to perform both assays on the ACL Elite analyser (Instrumentation Laboratory, USA). The PT was measured using the HemosIL RecombiPlasTin 2G assay, and the aPTT using the HemosIL SynthASil assay. Prothrombin time is a screening test for the extrinsic (tissue factor activated) pathway and aPTT is a screening test for the intrinsic (surface-induced) pathway. Both are screening tests for the common pathway.

3.10.3 D-dimer assay

Citrated plasma (50 µL) was used to determine the D-dimer concentration. The assay was performed on the on the ACL Elite (Instrumentation Laboratory, USA) using the HemosIL D-dimer assay. An increase in the D-dimer concentration indicate increased cross-linked fibrin formation and fibrinolysis (not fibrinogenolysis) or decreased clearance of FDPs by the liver and monocyte-phagocytic system.

3.10.4 Fibrinogen assay

Citrated plasma (100 µL) was utilized to perform the assay. The assay was performed on the ACL Elite (Instrumentation Laboratory, USA) using the HemosIL Fibrinogen C assay. Plasma fibrinogen concentration reflects the balance between production and consumption of fibrinogen. Accelerated consumption during a hypercoagulable state may be masked by increased production as fibrinogen is a positive APP that will increase during inflammation.

Two levels of manufacturer supplied control material were run prior to PT, aPTT, fibrinogen and D-dimer analysis, and results were within target ranges.

3.10.5 Antithrombin activity

Citrated plasma (200 µL) was utilised to perform the assay. Antithrombin activity in the plasma was measured on the Cobas Integra 400 Plus (Roche) using the Cobas Antithrombin assay. A normal pooled control sample, from 10 healthy horses, was run with each batch of tests. The horse's AT reading was normalized against the pooled samples which are presumed to have a 100% activity. PreciChrom I/II (Roche) was used as an assay control.

All haemostasis assays were performed in the Clinical Pathology Laboratory, Faculty of Veterinary Science, University of Pretoria, South Africa. Laboratory reference intervals are presented in table 3-2.

Table 3-2: Reference intervals for the Clinical Pathology Laboratory for thromboelastographic variables and classic coagulation assays

Variable	Reference interval	Unit
R	7-17	minutes
K	2-6	minutes
Angle	34-59	degrees
MA	48-61	millimetres
G	4.3-7.9	Kdynes/cm^2
LY30	0-1	%
LY60	0-6	%
CL30	Not available	%
CL60	Not available	%
Maximum rate of thrombus generation	Not available	mm/min
Time to maximum rate of thrombus generation	Not available	min
Thrombus generation	Not available	mm/min
Maximum rate of lysis	Not available	mm/min
Time to maximum rate of lysis	Not available	min
Lysis	Not available	mm/min
Prothrombin time (PT)	13.4-19.0	sec
Activated partial thromboplastin time (aPTT)	46.4-87.4	sec
D-dimer concentration	0-0.95	mg/L
Antithrombin (AT)	80-100	% activity
Fibrinogen concentration	0.9-2.8	g/L
Factor II	Not available	% activity
Factor VII	Not available	% activity
Factor IX	Not available	% activity
Factor X	Not available	% activity
Factor XII	Not available	% activity
Plasminogen	Not available	% activity
Plasminogen inhibitor	Not available	% activity

3.10.6 Haemostasis- and fibrinolytic factor assays

Citrated plasma was used to perform the assays. Factors II, VII, IX, X, XI, plasminogen/antiplasmin were measured according to the manufacturer's instructions using an automated coagulometric analyser (ACL Elite, Instrumentation Laboratory, Germany) and the HemosIL assays for factors II, VII, IX, X, XI, and

plasminogen/antiplasmin. The horse's coagulation factor activity reading was normalized against the pooled pre-infection samples which are presumed to have a 100% activity. Special Test Control Level 2 (ACL Elite) was used as the low abnormal control. The assay was performed in the Clinical Pathology Laboratory, Faculty of Veterinary Science, University of Pretoria, South Africa.

3.11 Histopathology

Tissue samples from lung, heart, and spleen from each horse were processed routinely, embedded in paraffin wax and cut into sections of 5-7 µm and stained with haematoxylin and eosin (H&E) using standard protocols (Bancroft and Layton, 2019). Samples were then examined microscopically for the presence of microthrombi. The assay was performed in the Pathology Department, Faculty of Veterinary Science, University of Pretoria, South Africa.

4 CHAPTER 4: TEMPORAL HAEMATOLOGICAL CHANGES AND ACUTE PHASE REACTANT RESPONSE IN HORSES EXPERIMENTALLY INFECTED WITH AFRICAN HORSE SICKNESS VIRUS

The results described in this chapter have been published as a research paper:

Experimental infection with African Horse Sickness Virus in horses induces only mild temporal hematological changes and acute phase reactant response

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4.1 Introduction

African Horse Sickness is a noncontagious infectious disease that is caused by the AHSV. The dsDNA Orbivirus is transmitted by insect vectors, specifically *Culicoides* midges. African Horse Sickness is endemic in sub-Saharan Africa and the virus primarily affects equids. In horses, infection results in four different clinical forms, the pulmonary ("dunkop"), cardiac ("dikkop"), fever, and mixed form (Coetzer and Guthrie, 2004; Theiler, 1921) with severe clinical signs which include dyspnoea, fever, and pulmonary oedema and result in high morbidity and mortality of up to 100% in naïve animals (Coetzer and Guthrie, 2004).

Haematological changes previously reported in horses with AHS include leukopenia, thrombocytopenia, and increased erythrocyte and HGB concentrations (haemoconcentration) (Skowronek et al., 1995). Variables reflecting platelet indices recognized as surrogate markers of platelet activation such as MPV, MPC, and MPM, have not been evaluated in AHS. In veterinary medicine, these variables have been demonstrated to be useful markers of platelet activation in dogs infected with canine parvovirus (Engelbrecht et al., 2021) and *Babesia rossi* (Goddard et al., 2015). Activated platelets change their shape which results in the release of the contents of their granules. Some of these contents also contribute to the host inflammatory and immune response following injury or infection, particularly by P-selectin-mediated binding to neutrophils or monocytes (Yun et al., 2016). The resulting changes in platelet indices are used as indicators for thrombopoiesis and platelet activation and have also been described in human diseases caused by endotheliotropic viruses such as Hantavirus (Laine et al., 2016), Denguevirus (Mukker and Kiran, 2018), Coronavirus (Liu et al., 2020), or Crimean-Congo fever (Yilmaz et al., 2016). In these

diseases, these indices have been reported as useful tools for diagnostic and prognostic purposes and are now considered important targets in the pathophysiology of many diseases (Yun et al., 2016) and might also be of use in AHS.

Infection with a pathogen generally results in an inflammatory response, with changes in the concentration of APR, such as iron and SAA (Eckersall and Bell, 2010). Serum amyloid A, a positive major APP, has become the APP of choice for detection of inflammation in equines over the last decade (Nolen-Walston, 2015). In contrast, serum iron is a negative APR and concentrations decrease during systemic inflammation, in order to limit iron availability for pathogen metabolism (Borges et al., 2007; Brosnahan et al., 2012; Ratledge and Dover, 2000).

While there are limited reports on some of the haematological changes observed in horses with AHS (Skowronek et al., 1995), changes in neither platelet indices nor concentrations of APR like SAA and iron have been evaluated to date. A better understanding of the haematological changes and the dynamics of the APR will improve our understanding of AHS and possibly allow for future identification of prognostic markers or therapeutics. Therefore, the objectives of this study were to describe the haematological changes throughout the disease course, incorporating newer variables, and to evaluate SAA and iron in horses experimentally infected with AHSV.

The study addressed the hypotheses that horses experimentally infected with AHSV would develop leukopenia, with neutropenia, and thrombocytopenia with concurrent platelet activation, indicated by changes in platelet indices associated with platelet

activation, and that SAA concentrations would increase markedly following infection while serum iron would decrease.

4.2 Materials and Methods

The study was designed as a prospective, longitudinal, experimental study which included four AHS-susceptible Boerperd cross horses.

4.2.1 Animals

Four AHS-susceptible horses that had tested negative for AHSV group-specific antibodies using a commercial competitive ELISA (Ingezim AHSV compac plus, Eurofins Technologies, Madrid, Spain) against all nine AHSV serotypes were included in this study (for details on animal selection, management, and testing see chapter 3.1). Prior to infection, peripheral blood smears were examined by the principal investigator, the veterinarian on site, and the clinical laboratory technologists for the presence of blood-borne parasites, specifically piroplasms. No piroplasms were observed. All four horses were also treated with imidocarb (2 mg/kg intramuscularly) and glycopyrrolate (0.0025 mg/kg intravenously) at 36 hours post infection when solitary piroplasms were observed on blood smears.

4.2.2 Study design

4.2.2.1 Experimental Infection

Low passage mouse brain suspension (5 mL), containing at least 10^5 mouse infective doses/mL of virulent AHSV serotypes was administered intravenously for infection. As different serovars were assessed for virulence during the vaccine trial, each horse was

infected with a different AHS serovar: horse 1, AHSV-2 (horse origin); horse 2, AHSV-4 (horse origin); horse 3, AHSV-6 (horse origin) and horse 4, AHSV-6 (dog origin).

4.2.2.2 Sampling procedures

Blood was collected from each horse prior to infection, then every 12 hours post-infection until the horses tested positive for AHS using a pan AHSV RT-PCR described in chapter 3.4. The RT-PCR for AHSV was performed daily for each horse, then twice daily once horses became febrile until viraemia was confirmed. Once positive on PCR, samples were obtained every 2 hours for 12 hours, followed by every 4 hours for another 12 hours. After this, every 12 hours sampling intervals were reinstituted. Sample acquisition was performed by venepuncture from the jugular vein directly into vacutainer tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and veins were alternated between samples. Blood was collected in specific sequence, through vacuum assistance, into a serum tube (4 mL), a 3.2% sodium citrate tube (4 mL), a heparin tube, and an EDTA tube (4 mL).

4.2.3 Diagnostic tests

Haematology was performed on all 12-hourly EDTA blood samples within two hours of collection and samples were kept at 8°C prior to analysis. The samples were evaluated on an automated haematology analyser (Advia 2120i, Siemens, Germany) (Chu and Stokol, 2021; Clark et al., 2002; Moritz, 2002) with manual blood smear evaluation. Evaluated erythrocyte variables included: HCT (L/L), HGB (g/L), RBC ($x10^9$ cells/L), RDW (%), MCV (fL), MCH (g/L), MCHC (g/L), and CHCM (g/L). A manual 100-cell leukocyte differential count was performed, and the resulting percentages used to calculate absolute leukocyte numbers, derived from the

automated white blood cell concentration. Leukocyte variables included: WBC ($x10^9$ cells/L), absolute mature neutrophil- ($x10^9$ cells/L), band neutrophil- ($x10^9$ cells/L), lymphocyte- ($x10^9$ cells/L), monocyte- ($x10^9$ cells/L), eosinophil- ($x10^9$ cells/L) and basophil ($x10^9$ cells/L) concentrations, and MPXI. Finally, platelet variables included: PLT ($x10^9$ cells/L), PCT (%), MPV (fL), PDW (%), MPC (g/dL), and MPM (pg).

Blood smears were further evaluated for the presence of blood-borne parasites. If blood-borne parasites were observed, the percentage of infected cells was determined: blood smears were evaluated along both slide edges, and the centre of the feathered edge. A minimum of 2500 single layered erythrocytes were evaluated per slide and the percentage of erythrocytes containing parasites calculated. The presence of *Theileria equi, Babesia caballi*, or both was confirmed using PCR methods previously described (Bhoora et al., 2010).

Serum amyloid A concentrations were measured using the Eiken VET-SAA immunoturbidometric assay (Eiken Co., Tokyo, Japan) (Jacobsen et al., 2019) and serum iron concentration was measured using the ferrozine method (Iron Gen.2, Roche, Basel, Switzerland). Both assays were performed on the Cobas Integra 400 Plus. (Cobas Integra 400 Plus, Roche, Basel, Switzerland)

4.2.4 Statistical Analysis

As the number of horses enrolled was small, changes occurring within a horse post infection could not be compared to one another. To determine changes of a variable within a subject over time, baseline values for all clinical pathology measurements were subtracted from all subsequent time points to remove inherent dependencies

due to the repeated measures sampling design. Data after subtractions were assessed for normality by plotting histograms, evaluating descriptive statistics, and performing the Anderson-Darling test in commercial software (MINITAB Statistical Software, Release 13.32, Minitab Inc, State College, PA, USA). Data that were not normally distributed were transformed using the natural logarithm or rank transformed when an appropriate transformation function could not be identified. Data were descriptively presented as the median and range per horse and time point. Changes in clinical pathology data over time were determined using a general linear model including natural logarithm of sampling time ("ln time") and "ln time squared". "ln time" allows for evaluation of linear changes over time, while a quadratic change over time ("ln time squared") allows for assessment of significance in values that do not follow a linear trajectory, i.e., increase first and then decrease or vice versa. A fixed effect was included for horse (serotype) and interaction terms included between horse and the time variables. Visual assessment was performed to evaluate the direction of the changes over time. Descriptive data were also presented as time before euthanasia because horses were euthanized at different time points after becoming PCR positive. Commercial software was used to fit statistical models and significance was set as $p<0.05$ (IBM SPSS Statistics Version 27, International Business Machines Corp., Armonk, NY, USA).

4.3 Results

Four Boerperd cross horses (A-D), including three fillies and one colt, between 18 and 24 months of age with body weights between 135 and 233 kg were enrolled in the study.

Clinical findings

The first febrile event (rectal temperature >38.5°C) occurred between 36 to 48 hours after infection (Table 4-1) and horses first tested positive for AHS on PCR from 72 to 96 hours post infection. Horses had increased respiratory rates from 152 hours post infection with horse A also developing mild swelling of the supraorbital fossae. Clinical signs in all horses were consistent with the pulmonary form of AHS and all horses were humanely euthanized between 180 to 228 hours post infection.

Table 4-1: Clinical progress in horses infected with African Horse Sickness Virus

	Fever (T >38.5°C)	**AHSV PCR positive**	**Euthanasia**
Horse A	48 hours	96 hours	228 hours
Horse B	48 hours	84 hours	192 hours
Horse C	48 hours	84 hours	180 hours
Horse D	36 hours	72 hours	180 hours

Descriptive results for the haematological variables, SAA, and serum iron concentrations for the group (median and range) are presented in Table 4-2 for illustrative purposes. The p-values for the haematological and APR variables for “ln time” and “ln time squared (in time^2)” were derived from the general linear model signifying whether variables changed significantly over the course of infection and whether they vary by serotype.

Changes of variables over time were evaluated. Transformed data are descriptively presented as the median and range per horse, and changes from the baseline value are indicated as increase (positive median) or decrease (negative median).

Significance ($p<0.05$) was determined either linear over time (in time) or as result of bidirectional changes over time (in time^2) evaluating possible interaction of the serotype.

Table 4-2: Summary of changes over time for all measured haematological variables and acute phase reactants.

	Serotype (each horse was infected with a different serotype)				Changes over time		Time by serotype interaction	
	A (n=19)	B (n=16)	C (n=15)	D (n=15)	ln time	(ln time)^2	ln time	(ln time)^2
Variable	**Median (range)**	**Median (range)**	**Median (range)**	**Median (range)**	**P value**	**P value**	**P value**	**P value**
Red blood cell concentration (x10^9/L) †	-0.60 (-1.44, 0.12)	-1.19 (-2.04, 2.62)	-0.87 (-1.74, 8.24)	-1.72 (-2.05, 0.89)	0.019	0.031	0.586	0.461
Haemoglobin concentration (g/L) †	-6 (-16, 4)	-15 (-27, 37)	-10 (-22, 119)	-28 (-34, 14)	0.006	0.008	0.566	0.446
Haematocrit (%)	-0.03 (-0.07, 0)	-0.05 (-0.08, 0.10)	-0.03 (-0.06, 0.39)	-0.08 (-0.09, 0.04)	0.003	0.003	0.196	0.146
Mean corpuscular volume (fL)	-0.70 (-1.70, -0.30)	-0.45 (-0.90, -0.20)	0 (-0.70, 4.00)	-0.50 (-0.80, 0.30)	0.194	0.218	0.064	0.036
Mean corpuscular haemoglobin (pg)	0.30 (-0.20, 1.20)	0.15 (-0.30, 0.40)	0.20 (-0.40, 0.50)	-0.3 (-1.30, 0.10)	0.012	0.008	0.147	0.115
White blood cell concentration (x10^9 cells/L)	-1.19 (-3.96, 1.01)	-3.25 (-6.98, 1.90)	-0.06 (-4.22, 2.68)	-1.32 (-3.75, 3.37)	0.303	0.026	0.036	0.034
Mature neutrophils (x10^9 cells/L)	-0.32 (-3.22, 1.29)	-0.33 (-2.86, 2.09)	-1.03 (-3.45, 1.12)	-0.67 (-1.48, 4.06)	0.936	0.661	0.102	0.084
Lymphocytes (x10^9 cells/L)	-0.48 (-2.35, 1.49)	-2.61 (-5.06, 0.25)	0.48 (-1.35, 2.29)	-0.59 (-2.24, 1.73)	0.613	0.188	0.865	0.760

Monocytes (x10^9 cells/L)	0.11 (-0.16, 0.28)	-0.02 (-0.19, 0.32)	0.10 (-0.13, 0.30)	-0.26 (-0.54, 0.38)	0.005	0.003	0.865	0.760
Eosinophil (x10^9 cells/L)	-0.51 (-0.64, -0.24)	-0.53 (-0.87, 0.02)	0 (-0.10, 0.24)	-0.12 (-0.21, 0.23)	0.137	0.047	0.887	0.754
Basophils (x10^9 cells/L)†	0 (0, 0.13)	0 (0, 0)	0 (0, 0.10)	-0.10 (-0.10, 0.08)	0.542	0.553	0.402	0.516
MPXI	-2.40 (-8.40, 0.90)	-0.95 (-6.20, 1.80)	-4.10 (-13.60, -0.30)	-0.70 (-6.90, 2.30)	0.001	0.001	0.981	0.965
Platelet concentration (x10^9 cells/L)	-50 (-68, -16)	-37 (-85, -6)	-83 (-199, 120)	-85 (-131, 122)	0.002	<0.001	0.071	0.009
Mean platelet volume (fL)	1.00 (0.30, 3.70)	0 (-0.40, 2.70)	-0.20 (-0.80, 1.00)	1.20 (-0.10, 3.20)	0.066	0.021	0.366	0.191
Platelet distribution width (%)†	-1.90 (-6.10, 41.00)	0.25 (-1.70, 32.60)	0.80 (-0.50, 31.40)	-2.20 (-12.10, 22.60)	0.007	0.001	0.496	0.514
Plateletcrit (%)	-0.03 (-0.04, 0)	-0.02 (-0.05, 0)	-0.07 (-0.15, 0.11)	-0.04 (-0.08, 0.06)	<0.001	<0.001	0.001	<0.001
Mean platelet component (g/dL)	-1.60 (-10.10, 0.80)	0.90 (-4.70, 2.10)	1.70 (-4.50, 3.30)	-3.00 (-9.70, 9.80)	0.060	0.030	0.540	0.390
Mean platelet mass (pg) †	0.23 (-0.10, 0.36)	0.08 (-0.02, 0.36)	-0.01 (-0.20, 0.19)	0.27 (-0.05, 0.51)	0.005	0.001	0.008	0.003
Serum iron (µmoL/L)	-18.40 (-26.8, 24.0)	-6.35 (-10.50, 10.40)	-1.40 (-5.50, 7.10)	-8.10 (-14.40, 2.70)	0.006	0.001	0.194	0.107
Serum amyloid A (mg/L) †	1 (0, 85)	0 (0, 24)	0 (0, 146)	2[a] (0, 91)	<0.001	<0.001	0.638	0.470

†Data rank transformed prior to statistical analysis

N = number of time points evaluated, g = grams, dL = decilitre, pg = picogram, mg = milligram, L = litre, µmoL = micromole, µL=microlitre, fL = femtolitre.

Temporal changes of haematological variables

Haematocrit (Figure 4-1A), HGB (Figure 4-1B), and RBC (Figure 4-1C) changed significantly over time but did not vary by serotype. Visually, these variables increased from 168 hours post infection in all horses following an initial decrease. Significant changes over time without variation by serotype were also recorded for RDW (Figure 4-1D), but no distinct pattern was discernible. For CHCM changes over time varied by serotype, visually, a mild increase was noted, with a severe decrease in one horse (Figure 4-1E). The remaining erythrocyte variables (MCV, MCHC) did not change significantly over time (Figures 4-1F-G). On blood smear evaluation, solitary piroplasms were observed in all horses intermittently; however, percentage of parasitized red blood cells were negligible (<0.001%). No piroplasms were observed in any horse from 132 hours post infection onward.

The WBC concentration changed significantly over time and varied by serotype. Upon visual inspection, WBC (Figure 4-2A) initially decreased, then increased from 156 hours onward, although little change was observed in one horse. Although there were no significant changes over time observed for the neutrophil (including band neutrophils) concentrations (Figure 4-2B), visual inspection of the segmented neutrophils revealed a similar pattern to WBC. Significant changes over time were observed in the monocyte (Figure 4-2C) and eosinophil (Figure 4-2D) concentrations but neither varied by serotype. No pattern was identified upon visual inspection of the monocyte changes, but the eosinophil concentrations decreased. No changes were observed for the basophils. The MPXI (Figure 4-2E) changed significantly but no variation by serotype was observed. Visually, a mild increase was followed by a

decrease in MPXI in two horses, in two horses MPXI decreased in the final disease phase.

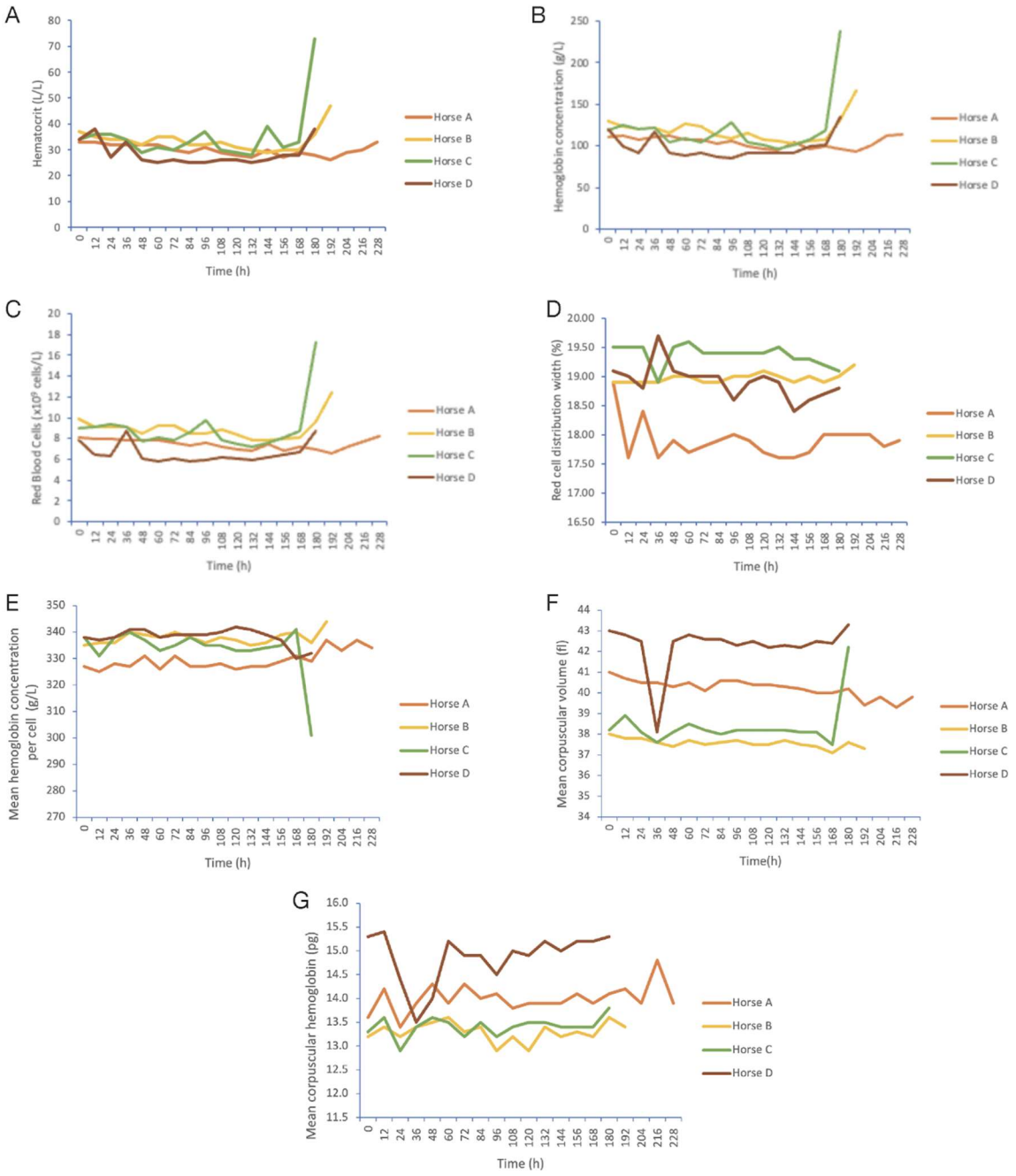

Figure 4-1: Line diagrams of changes in red blood cell variables.

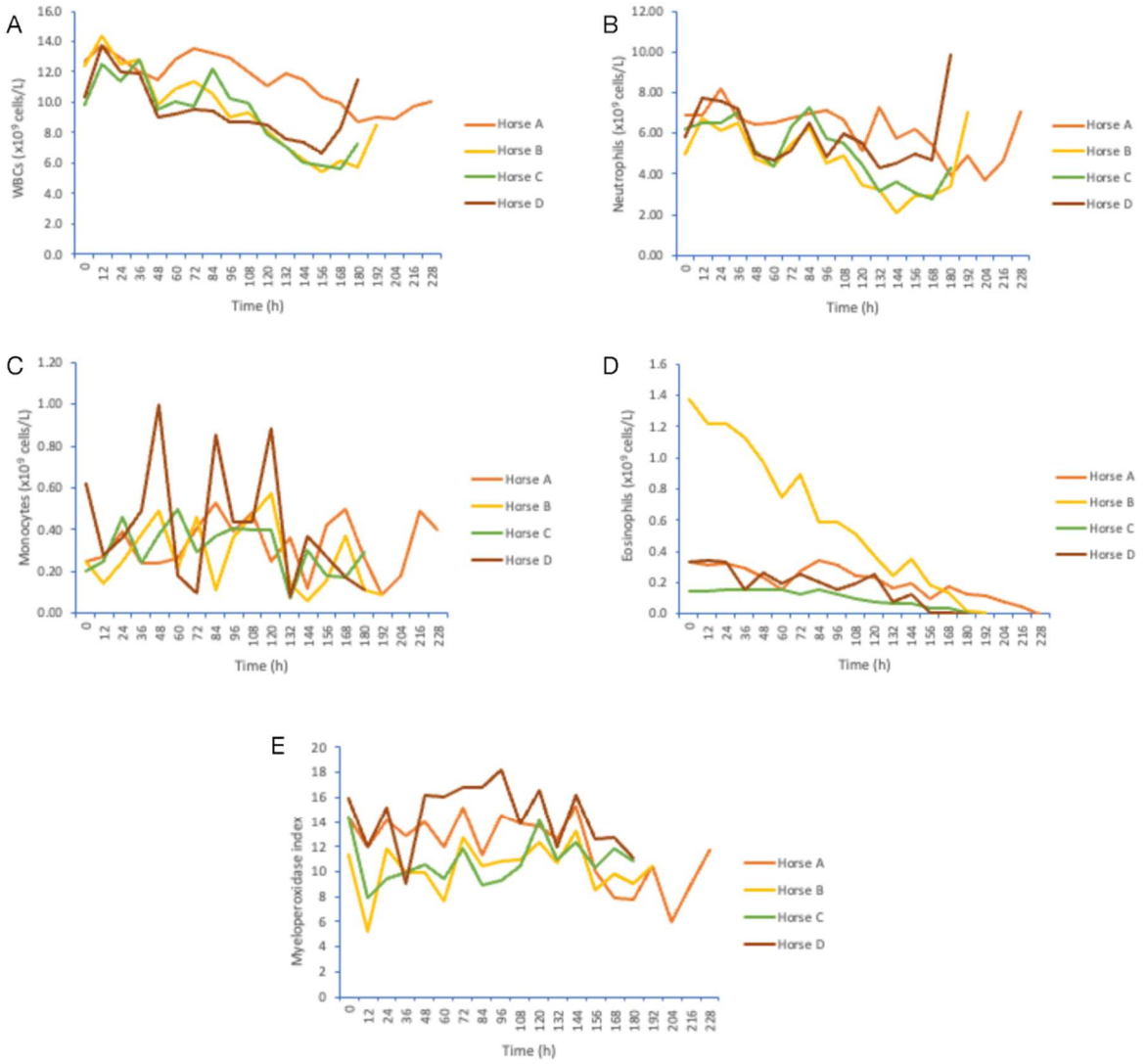

Figure 4-2: Line diagrams of changes in leukogram variables.

The PLT (Figure 4-3A) changed significantly over time and varied by serotype. Pseudothrombocytopenia caused by significant platelet aggregation was not observed. Visual inspection revealed more pronounced decreases in PLT in two horses. Plateletcrit (Figure 4-3B) changed significantly over time and varied by serotype, visual inspection revealed a more pronounced decrease in PCT in two horses. Platelet distribution width (Figure 4-3C) changed significantly over time but was not influenced by serotype and only increased at single time points to then return

to baseline values. Mean platelet volume (Figure 4-3D) and MPM (Figure 4-3E) also changed significantly over time with changes in MPM varying by serotype. Upon visual inspection, both MPV and MPM increased over time. Mean platelet component concentration (Figure 4-3F) changed significantly over time but was not influenced by serotype, visually, MPC mildly decreased during the disease course.

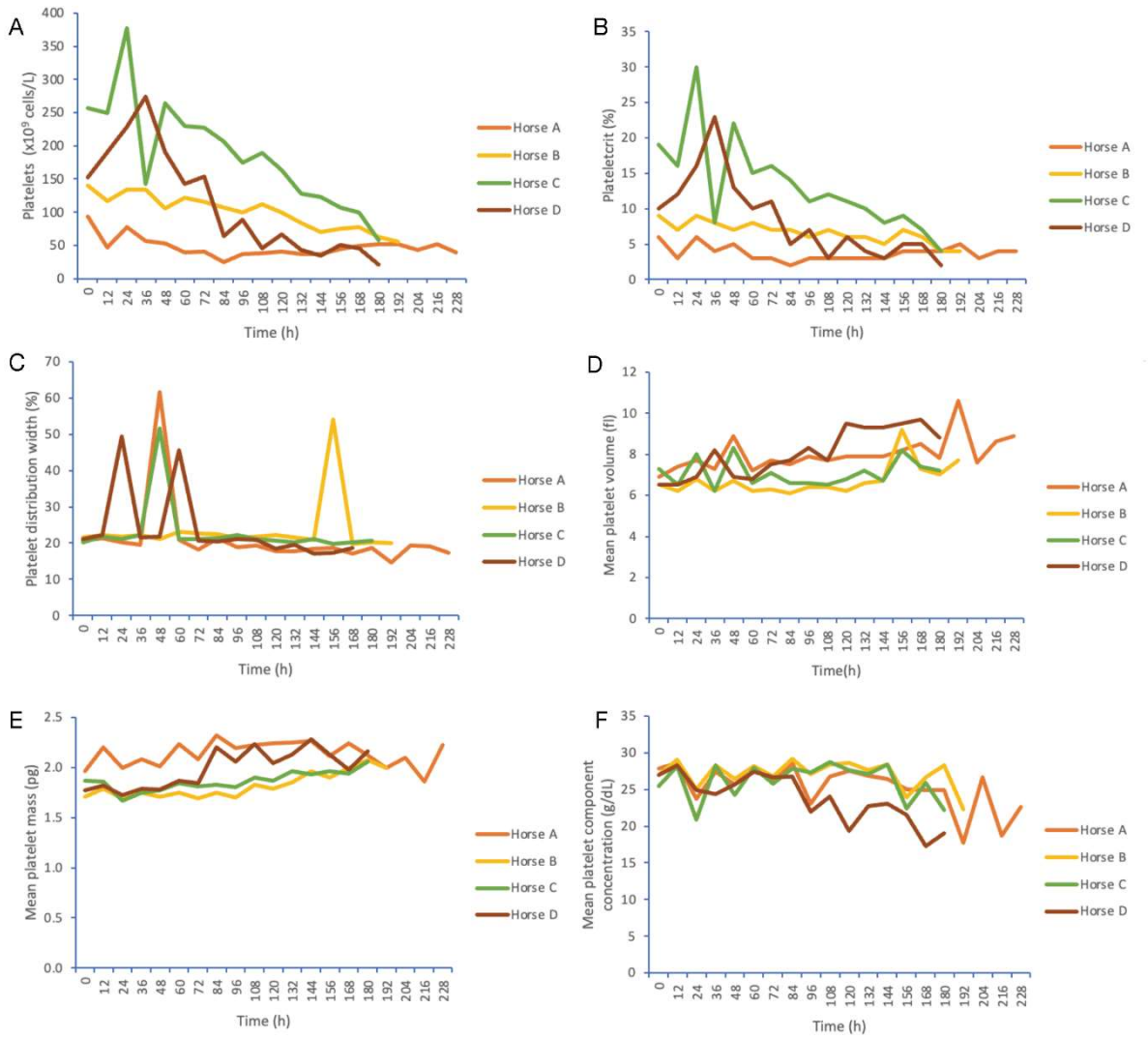

Figure 4-3: Line diagrams of changes in platelet variables.

Temporal changes of acute phase reactant variables

Visual inspection of SAA concentrations (Figure 4-4A) revealed an increase from 108h onward with the maximum concentration observed on the final samples, while serum iron concentrations decreased (Figure 4-4B). Changes over time were significant for both SAA and iron but changes did not vary by serotype.

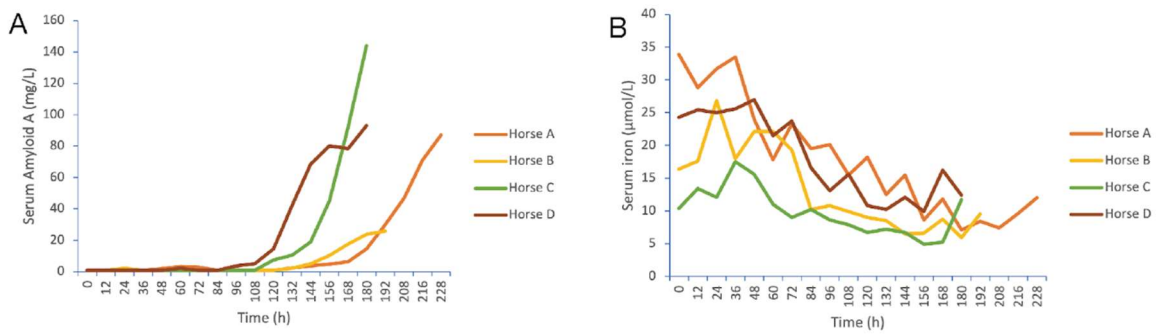

Figure 4-4: Line diagrams of changes in acute phase reactants.

Necropsy findings

Necropsy confirmed typical lesions of AHS in all horses, namely pulmonary oedema with frothy fluid in the airways as well as subcutaneous oedema in the head and neck, pleural and pericardial effusion, and gastrointestinal petechiae and haemorrhage.

4.4 Discussion

In this study, horses experimentally infected with AHSV subtypes demonstrated both expected and unexpected changes within the blood. Thrombocytopenia and haemoconcentration were expected and consistent with the observed clinical signs. Increasing platelet activation was observed throughout the course of the disease. Laboratory proxies of inflammation, specifically WBC, SAA and iron concentrations changed significantly; however, none of these variables changed as severely as

expected given the clinical picture of the infected horses. This raises the question about possible viral interference with the host's immune response and needs to be investigated further.

All infected horses developed typical signs of acute AHS ("dunkop" form) with high fever and respiratory distress in the late stages of the disease. Typical necropsy findings (Stern, 2011) were also identified, such as oedema and effusion, which are typically reported in horses with AHS and are thought to be caused by endothelial cell damage and increased vascular permeability (Zientara et al., 2015). The course of clinical disease and necropsy findings were similar among horses, irrespective of virus subtype. Previous reports suggested that disease severity is not linked to the serotype but rather to the virulence of the variant involved (Burrage and Laegreid, 1994; Laegreid et al., 1993), which is likely due to AHSV subtypes having variable tropism to cardiac and pulmonary endothelial cells.

Haemoconcentration during the late stage of the disease was likely the result of increased vascular permeability with leakage of plasma into the extravascular tissue. Severe oedema, most pronounced in the head and neck but also in pulmonary tissue, is a hallmark of the clinical forms of AHS (Gomez-Villamandos et al., 1999; Laegreid et al., 1992a). Splenic contraction due to adrenergic stimulation caused by pain and stress might also have contributed to the increases in these variables. Erythrocytosis (i.e. an absolute increase in red blood cell numbers), usually caused by hypoxia, was considered unlikely, as increases occurred within a few hours of onset of dyspnoea and the maturation time from reticulocytes into mature erythrocytes is 3-4 days (Gifford et al., 2006).

Horses enrolled in the study tested positive for *T. equi* on PCR. *Theileria equi* is endemic in South Africa and many horses will test positive, using sensitive tests like PCR, without having clinical signs. It is known that immunosuppression can result in recrudescence of acute piroplasmosis. For our study, parasitized erythrocytes were either not detected on examination of blood smears or observed in very low numbers (maximum 5 piroplasms in 3481 RBC <0.001%); however, clinical infections with *T. equi* are reported to have parasitaemia ranges from 1% to 7% (Friedhoff et al., 1990). It is therefore unlikely that changes observed in this study were caused by *T. equi* infection.

The decreased leukocyte count confirmed findings from an earlier study (Skowronek et al., 1995); however, contrary to previous results, our study did not observe clinically important leukopenia (<4.70 $x10^9$/L, University of Pretoria Faculty of Veterinary Science Clinical Pathology Laboratory) in any horse at any single point in time nor significant decreases in neutrophils or lymphocytes. Previous studies have suggested that differences in virulence in the same subtype can cause different clinical signs (Burrage and Laegreid, 1994; Laegreid et al., 1993); although all horses in this study developed similar clinical signs. Considering the severity of clinical and necropsy findings, changes consistent with a pronounced leukocyte response, namely a left shift with neutropenia and severe neutrophil toxic changes were expected in line with previous findings reported in severe cases of AHS (Skowronek et al., 1995). However, even in that previous study, the degree of leukopenia and left shift was, while present, only mild. The reason for this is not clear; but a possible explanation is viral immune escape mechanisms downregulating the host's response (Wall et al., 2021). A similar

observation has been made in sheep infected with the closely related BTV, where the animals do not develop consistent leukopenia (Ellis et al., 1990; Khorajiya et al., 2019).

Monocytes are a nonspecific marker of inflammation (van Furth, 1985) and different subpopulations with pro- and anti-inflammatory properties exist (Yang et al., 2014). During inflammation or infection, monocytes are recruited by chemokines into the tissues where they differentiate into macrophages and dendritic cells. Thus, they are important for the phagocytosis of pathogens but they also play an important role in the modulation of the innate and adaptive immune system by secreting cytokines including IL-1β, IL-6, TNF-α, which are involved in homeostasis (Foley and Conway, 2016a; Karlmark et al., 2012; Nathan, 2008). Monocytes can also migrate into peripheral tissues where they function as effector cells (Karlmark et al., 2012). African horse sickness virus has been shown to replicate in monocytes (Clift and Penrith, 2010), particularly targeting pulmonary intravascular macrophages (Carrasco et al., 1999; Clift and Penrith, 2010). Given the numerous tasks of monocytes, it is likely that monocytes migrated into the tissues, resulting in decreased numbers of monocytes in the peripheral circulation. Furthermore, during intracellular viral replication cell damage due to release of viral particles may occur and decrease the number of monocytes.

A decreased eosinophil concentration has been proposed as a marker for increased mortality in human sepsis and systemic inflammatory response syndrome. Eosinopenia has been associated with corticosteroid administration and endogenous cortisol and catecholamines secreted during inflammation (Harlow and Selye, 1937; Hills et al., 1948; Sabag et al., 1978). Eosinopenia can also be caused by chemotaxis

through margination or egression into tissues, with cortisol being one of the key drivers for migration of eosinophils into leukoid organs like the lymph nodes or the spleen (Bass, 1975, 1977; Bass et al., 1980). Reports about the cells observed in tissue histopathology in horses with AHS are sparse and to date, notable tissue accumulation of eosinophils has not been identified during infection with AHSV. Increases in endogenous cortisol may be the underlying driver for the eosinopenia observed in this study.

The MPXI, which decreased over the course of AHSV infection, represents the mean intracellular myeloperoxidase content in circulating neutrophils and is used as a marker of neutrophil activation (Moritz, 2002). A decrease in MPXI is an indication of widespread neutrophil degranulation and respiratory burst in response to systemic inflammation and have been reported in humans with bacterial sepsis, in dogs with severe systemic inflammation, in horses infused with endotoxin and in some horses with systemic inflammation due to other causes (Celliers et al., 2020; Klenner et al., 2010; Lilliehook et al., 2016; Schwarz et al., 2012; Yonezawa et al., 2010). In contrast, MPXI increased in people with non-septic bacterial infections and in septic foals and was unchanged in another group of horses with systemic inflammation and in humans with viral infections (Hooijberg et al., 2014; Piviani et al., 2011; Yonezawa et al., 2010). In horses with endotoxemia and systemic inflammatory response syndrome, the MPXI became negative, indicating marked activation of neutrophils with degranulation of myeloperoxidase into plasma (Schwarz et al., 2012; Wei et al., 2018). In the current study, although MPXI decreased, this change was not marked, suggesting that intravascular neutrophil activation and degranulation is not a prominent feature of this disease.

Thrombocytopenia was observed in all horses and progressed throughout the course of the disease. This is consistent with the clinical observation of bleeding tendencies following venepuncture in the later course of the disease and with haemorrhages and petechiae noted in the gastrointestinal tract on gross pathology. There are several possible causes for this decrease, with the most likely being platelet sequestration in the form of thrombin-induced platelet aggregation. This process can be triggered by endothelial inflammation and damage, resulting in exposure of TF (Jurk and Kehrel, 2005). This triggers a cascade leading to the cleavage of active thrombin from prothrombin and the activation of platelets (Coughlin, 2005; Eleftheriou et al., 2011; Emmi et al., 2015; Smith et al., 2012). With the endothelial damage observed in horses infected with AHS (Clift and Penrith, 2010; Gomez-Villamandos et al., 1999; Laegreid et al., 1992a), this seems probable. African horse sickness has also been reported to result in haemostasis abnormalities with prolonged clotting times manifested by increased aPTT, PT, TT, and FDPs, possibly indicating DIC (Skowronek et al., 1995; Van Amstel et al., 1987). Platelet consumption as it occurs during DIC, where platelets are incorporated into the fibrin network, can then lead to thrombocytopenia. Other mechanisms, such as a decrease in platelet production or platelet loss seem unlikely. The decrease in PLT is consistent with the decrease in PCT.

As platelets become activated, their shape changes to become more spherical, they increase in mass and volume, and degranulate with consequent adhesion and aggregation (and increased platelet-clumping) (Ruf and Patscheke, 1995). These changes result in increased MPV, MPM, and decreased MPC (Macey et al., 1999; Park et al., 2002; Segura et al., 2007). Increases in MPV and MPM with decreases in MPC were observed in the horses infected with AHSV in this study, and indicate

platelet activation. This is likely due to endothelial damage resulting in the exposure of TF and cleavage of prothrombin to thrombin activating platelets (Smith et al., 2012) described previously as well as the release of larger platelets from the bone marrow into the circulation. This is also supported by the increase in MPM commonly attributed to thrombopoiesis (Nam et al., 2018) resulting in the release of young, more active platelets (Thompson et al., 1984). Degranulation of activated platelets is consistent with the decreases in MPC observed in this study. MPC has previously been reported to decrease in sick foals especially with septic but also non-septic disease as well as in adult horses with severe inflammatory response syndrome, thrombocytopenia and colic due to obstruction and enteritis (Segura et al., 2006). Increases in MPV, indicating young and activated platelets, have also been reported in dogs with induced endotoxemia (Yilmaz et al., 2008), babesiosis (Goddard et al., 2015; Žvorc et al., 2010), parvo viral enteritis (Engelbrecht et al., 2021), or leishmaniosis (Temizel et al., 2011) as well as cattle infected with bovine viral diarrhoea virus (Kocatürk et al., 2010). Similar to AHS, these diseases cause inflammation of or damage to the endothelium with resulting changes in platelet production and activity. While further investigation of platelet activation in horses is required, the observed changes suggest platelet sequestration and activation in horses infected with AHS.

Both SAA and iron are used as inflammatory markers in horses. Decreases in serum iron were observed early in the course of the disease. Generally, circulating IL-6 and TNF-α stimulate the release of hepcidin by hepatocytes. This then induces apoferritin synthesis and sequestration within macrophages (Ganz, 2003; Nemeth et al., 2004) resulting in hypoferremia within 24 hours in the early stages of the disease, normalizing only when the inflammatory insult has resolved (Smith and Cipriano,

1987). In two horses iron concentration was below reported reference intervals at the beginning of the study. This might have been due to unrecognized inflammatory processes but might also be caused by diet or age with younger horses commonly having lower iron concentrations. However, with the intraindividual study design where each horse served as its own control, the decrease in iron concentration was controlled for in all analyses. Unexpectedly, while SAA concentration, a commonly used marker of acute inflammation in horses, increased over time, there was a significant delay before any increase was noted and it did not increase to clinically important levels. Increases in SAA are reported under physiological conditions including exercise (Cywinska et al., 2010; Cywinska et al., 2012; Cywinska et al., 2013) and parturition (Coutinho da Silva et al., 2013) as well as in numerous pathologies including surgical colic (Pihl et al., 2016), peritonitis (Pihl et al., 2013), bacterial pneumonia (Belgrave et al., 2013), and septic arthritis (Jacobsen et al., 2006). In horses infected with AHSV, despite severe clinical abnormalities, SAA concentrations did not increase to the extent commonly observed in patients with other severe systemic inflammatory diseases, such as colitis or bacterial pneumonia (Belgrave et al., 2013). SAA has a short half-life with increases commonly noted within 6-12 hours following the inflammatory insult (Tape and Kisilevsky, 1990); however, in these horses infected with AHS, increases were only noted from 96 hours post infection onward, 48 hours after fever was first noted and after seroconversion. This is surprising as SAA synthesis is induced by increases in circulating proinflammatory cytokines IL-1, IL-6, TNF-α, and IFN (Crisman et al., 2008), which are also involved in the mediation of fever (Netea et al., 2000). In contrast, significant decreases in serum iron, also mediated by circulating TNF-α and IL-6, were observed in earlier stages of

the disease, which suggests that other modulators might be involved in the sequestering of iron, making it a more sensitive APR in horses with AHS.

When evaluating the acute phase response to other orbiviruses, SAA concentrations increased 3.5 fold in sheep infected with BTV serotypes 1 and 8 (Sanchez-Cordon et al., 2013), below expected increases for a major APP (Ganz, 2003). The reason for the stunted response is unclear. In sheep, BTV has been reported to antagonize IFN synthesis and might be able to downregulate the innate immune response (Ratinier et al., 2016), possibly interfering with the synthesis of SAA. African horse sickness virus NS protein 4 has recently been demonstrated to contribute to potential mechanisms to overcome the host's antiviral response by colocalization with promyelocytic leukemia nuclear bodies (PML-NBs) (Boughan et al., 2020) and interference with the JAK/STAT pathway that is instrumental in the IFN pathway (Wall et al., 2021). Indeed, several viruses have evolved mechanisms to evade the innate immune system's response (Alcami, 2016). These evasion mechanisms might also contribute to the observed lack of a positive APP response in the face of severe systemic inflammation.

It is important to note that while SAA and iron concentrations are commonly thought of by clinicians merely as markers of inflammation, the physiological reasons for increases and decreases should not be forgotten. SAA plays a critical role in control and possibly propagation of the primordial acute phase response (Sack, 2018). For example, it has been proposed to modulate inflammation by inducing chemotaxis and migration in monocytes as well as stimulating cytokine release (Sack, 2018). It also inhibits lymphocyte proliferation, platelet aggregation, and phagocytosis but stimulates prostaglandin synthesis and metalloproteinase activation (Badolato et al., 2000;

Witkowska-Pilaszewicz et al., 2019). Iron is required for viral metabolism and a decrease in iron concentration assists the innate immune mechanism against invading pathogens (Schmidt, 2020). Considering these properties, dysregulation of these substances might enhance virus pathogenicity. Future studies in naturally infected horses should be performed to assess the prognostic value of these APR in horses infected with AHS.

The main limitation of this study is the small number of enrolled horses. However, as infection with AHSV is commonly fatal in naïve horses, sacrificing a larger number of horses was considered unethical. The horses used in this study were enrolled in a challenge study that served as prerequisite for a vaccine trial. All horses were infected with different AHVS serotypes. It has previously been shown that the virulence of the variant is more important than the serotype. For example, AHS/4SP caused development of the lung form while AHS/4PI only caused fever (Burrage and Laegreid, 1994; Laegreid et al., 1993). All infected horses in this study developed similar clinical signs consistent with the acute form (“dunkop”) of AHS and haematological changes were consistent between individuals. Thus, there was no evidence that the different serotypes used in this study significantly affected the clinical course of the disease.

In conclusion, the results of this study further highlight the temporal changes in haematological variables after infection with AHSV. The observed changes reflect the body’s response to infection; however, the changes in APR are only moderate. It is possible that derangements in the host’s immune response contribute to the observed dampened immune response in reaction to the inflammatory stimuli triggered by the

virus. Further studies investigating the host's immune response focusing on cytokine secretion patterns are required to better understand these changes.

5 CHAPTER 5: DESCRIPTION OF THE CHANGES IN SELECTED CYTOKINES IN HORSES EXPERIMENTALLY INFECTED WITH AFRICAN HORSE SICKNESS VIRUS

The results described in this chapter have been submitted for publication as a research paper:

Changes in selected plasma cytokines in horses experimentally infected with African Horse Sickness virus

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5.1 Introduction

African horse sickness is a highly infectious disease in equids resulting in high morbidity and mortality. It is caused by the Orbivirus AHSV that is endemic in sub-Saharan Africa and closely related to BTV which occurs in small ruminants. Both viruses are transmitted by insect vectors, particularly *Culicoides* midges.

Following infection, AHSV replicates in the regional lymph nodes, then disseminates in association with erythrocytes (Henning, 1956; Ozawa, 1972). Further replication occurs in endothelial cells pulmonary intravascular macrophages, interstitial macrophages, and fibroblasts (Laegreid et al., 1992a; Wohlsein et al., 1997a). The virus then enters its target organs, with tropism for the endothelial cells of the lungs, heart, spleen and lymphoid tissue (Clift and Penrith, 2010). The underlying disease mechanisms are however poorly understood.

During infection, pathogen-associated molecular patterns (PAMPs), which include viral nucleic acids, are recognized by host immune cells via pattern-recognition receptors (PRRs) (Werners and Bryant, 2012). Binding of pathogens to PRRs initiates transcription of various proinflammatory and anti-inflammatory cytokines. Key proinflammatory cytokines initiating an inflammatory response include TNF-α, IFN-γ, IL-1, IL-2, IL-6, IL-8, IL-12, IL-18, and MCP-1. Generally, virus detection by T lymphocytes initiates IFN-γ expression (Cavaillon and Adib-Conquy, 2002) which in turn activates immune cells including macrophages and natural killer cells. Macrophages subsequently produce TNF-α, IL-1, and IL-6 (Abbas AK, 2022; Brenner et al., 2015). TNF-α regulates the growth and function of various immune cells

including T- and B lymphocytes, natural killer cells, neutrophils, and dendritic cells (Akdis et al., 2011), thus stimulating production of further proinflammatory cytokines, particularly IL-1 and IL-6. Interleukin-1 is a leukocyte mediator involved in inducing the acute phase response (Kaneko et al., 2019). Interleukin-6 activates production of acute phase reactants (APR) and neutrophils and is important for the general coordination and development of the immune response. It promotes production of IL-2 and T lymphocyte differentiation (Gandhi et al., 2016; Heinrich et al., 2003). Key anti-inflammatory cytokines include IL-4, IL-10, and transforming growth factor (TGF)-β, which down-regulate proinflammatory cytokine gene expression (Malefyt et al., 1991; Romagnani, 1996; Seitz et al., 1995). Simultaneously to proinflammatory cytokines, IL-10 is produced by T lymphocytes and other immune cells (Saraiva et al., 2020) and inhibits the secretion of proinflammatory cytokines, including IL-1, IL-6, and TNF-α and regulates T lymphocyte, natural killer cell and macrophage function (de Waal Malefyt et al., 1991a; de Waal Malefyt et al., 1991b). Previous research has shown that dysregulation or sustained increased secretion of IL-10 indicates of poor prognosis as well as a higher risk for infection, multiple organ failure, and death in human patients (Gogos et al., 2000; Lu et al., 2021; Moore et al., 1993; Sornsenee et al., 2023; Tamayo et al., 2011).

Horses with systemic inflammation, specifically lipopolysaccharide-induced sepsis, have increased blood TNF-α, IL-1 (Cudmore et al., 2013; Holcombe et al., 2016; Morris et al., 2018), and IL-6 (Tadros and Frank, 2012). Compared to healthy foals, increased gene expression of IL-10 at the time of initial evaluation was associated with mortality in neonatal foals with sepsis (Pusterla et al., 2006). This may indicate sustained dysregulation of IL-10 secretion or be part of a pathological cytokine storm.

However, no data was available on the changes in these foals' cytokine profiles throughout the progression of the disease, so no definitive conclusions may be drawn. While the dynamics of these cytokines in the blood have not yet been investigated in AHS, in the previous chapter we described an unexpectantly blunted immune response with only mild changes in the leukogram and the APRs, SAA and iron, in horses experimentally infected with AHSV (Schliewert et al., 2022), suggesting downregulation of the innate immune response.

Cytokine production has been investigated in the closely related BTV which also induces endothelial cell damage. Upregulation of proinflammatory cytokines TNF-α, IL-1, and IL-6 was reported in vitro (Channappanavar et al., 2012; Rojas et al., 2017; Sanchez-Cordon et al., 2015; Umeshappa et al., 2012); however, there is increasing evidence that BTV inhibits the IFN response and interferes with cytokine signaling (Rojas et al., 2021), thus dampening the host immune response.

Despite one of the initial hypotheses of the broader study being that infection with AHSV initiates an overwhelming proinflammatory cytokine response, given the previous observation of a suppressed acute phase response with only mild leukogram changes in AHS, we now hypothesize that proinflammatory cytokine production is low, particularly IL-1, IL-6, and TNF-α (the inducers of the acute phase response), possibly due to viral interference with the host immune response. The objective of this study was to describe the kinetics of selected cytokines in plasma during experimental infection with AHSV.

5.2 Materials and Methods

This study was designed as a prospective, longitudinal, experimental study and included AHS-susceptible Boerperd cross horses that were experimentally infected with AHSV.

5.2.1 Animals

The study population included four AHS-susceptible Boerperd cross horses. The animals had tested negative for AHSV group-specific antibodies using a commercial competitive ELISA (INgezim AHSV compac plus, Eurofins Technologies, Madrid, Spain) against all nine AHSV serotypes (for details on animal selection, management, and testing see chapter 3.1).

5.2.2 Study Design

5.2.2.1 Experimental Infection

An infective dose of low passage mouse brain suspension (5 mL), containing at least 10^5 mouse infective doses/mL of virulent AHSV serotypes was administered intravenously. Different serovars were evaluated for virulence, and each horse was inoculated with a different AHSV serovar: horse 1, AHSV-2 (horse origin); horse 2, AHSV-3 (horse origin); horse 3, AHSV-6 (dog origin) and horse 4, AHSV-6 (horse origin).

5.2.2.2 Sampling procedures

Blood collection was performed prior to infection, then every 12 hours until each horse tested positive for AHS using PCR. For each horse, PCR for AHSV was performed every 24 hours, then every 12 hours once the horses became pyretic until confirmation

of viraemia using a method previously described (van Rijn et al., 2018b). Upon confirmation of viraemia, sampling frequency increased to every 2 hours for a total of 12 hours, then to every 4 hours for another 12 hours, then every 12 hours sampling intervals were reinstituted for the remainder of the trial. Samples were obtained via venepuncture from the jugular vein directly into vacutainer tubes (Becton, Dickinson and Company, USA) using alternate veins for each sample. Blood collection through vacuum assistance was in specific order into a serum tube (4 mL), a 3.2% sodium citrate tube (4 mL), a heparin tube and an EDTA tube (4 mL). Samples were kept at 8°C until analysis (EDTA whole blood) or stored at -80 °C following centrifugation and separation (EDTA plasma, heparin serum, citrate plasma).

Stored EDTA plasma samples (-80°C) from seven horses with severe systemic disease and serum SAA concentrations >1000 mg/L were included to confirm test functionality and served as positive controls. These horses had previously been presented as patients to the OVAH and samples had been obtained for diagnostic purposes. These patients were diagnosed with various severe gastrointestinal disorders (rectal perforation, hepatitis, colitis), (pleuro-) pneumonia, and neonatal sepsis.

5.2.3 Cytokine Assay

Concentrations for cytokines IL-1α, IL-2, IL-6, IL-8, IL-10, IL-12, IL-17, IFN-γ, TNF-α, and MCP-1 were analysed in EDTA plasma as described in Chapter 3.9 using an equine-specific Milliplex® Map Magnetic Bead Panel (Merck Millipore, Burlington, MA, USA) and a Luminex IS 100 instrument (Luminex Corp. Austin, TX, USA) per the

manufacturer's protocol. The cytokines were chosen as they represented a mixture of mediators of innate and adaptive immunity.

5.2.4 Statistical Analysis

Due to the small number of horses available and because many cytokine measurements were below detection limits, changes occurring within a horse post infection could not be compared to one another using meaningful statistical analysis. Changes are therefore described based on visual inspection.

5.3 Results

Clinical findings

Four Boerperd cross horses (A-D), including three fillies and one colt, between 18 and 24 months of age with body weights between 135 and 233 kg were enrolled in the study. The horses became pyretic (rectal temperature >38.5°C) between 36 to 48 hours post infection and AHS viraemia was first detected on PCR from 72 to 96 hours. Increased respiratory rates occurred from 152 hours post infection, and mild swelling of the supraorbital fossae was observed in horse A. Clinical signs during the end-stage of the disease were typical for the pulmonary form of AHS in all horses, and all horses were humanely euthanized between 180 to 228 hours post infection (Table 4-1).

Cytokine concentrations

Cytokine analysis returned appropriate standard curves on all test plates and acceptable readings were obtained. Upon visual inspection, substantial changes in cytokine concentration in the four experimentally infected horses were only observed in TNF-α and IL-10 (Table 5-1, Figure 5-1A and 5-1B). Tumour necrosis factor-α

increased up to 30-fold, and IL-10 up to 150-fold towards the end stage of the disease. All other cytokines remained under the lower limit of detection (LLOD) or only showed minor increases that were inconsistent over time and between horses. The LLOD (pg/mL) for the measured cytokines were: TNF-α = 3.91; IFN-γ = 438.41; MCP-1 = 341.94; IL-1α = 51.8; IL-2 = 14.37; IL-6 = 12.79; IL-8 = 58.58; IL-10 = 51.26; IL-12 = 39.22; IL-17 = 13.33.

Interleukin-1α (Figure 5-2B) and IL-6 (Figure 5-2D) increased in horse D only with a peak after viraemia was first confirmed, while IL-6 also increased very slightly in horse B at the time of euthanasia. Similarly, in horse D, IL-8 (Figure 5-2E) peaked after viraemia was first confirmed while MCP-1 peaked before viraemia, during initial viraemia and 24h before euthanasia. Interleukin-17 increased then decreased below the LLOD in horse D and increased slightly in horse B throughout the course of the disease while remaining below the LLOD in horse A and not displaying any changes in horse C (Table 5-1, Figure 5-1C).

For the positive control samples from clinical patients with SAA >1000 mg/L, most cytokine results were above the LLOD, apart from IL-1α, IL-12, and IL-17 which were below the LLOD for most sampling times in most horses. In these samples, IFN-γ increased up to 280-fold, TNF-α up to 500-fold, MCP-1 just above the LLOD, IL-1α up to 8-fold, IL-2 up to 80-fold, IL-6 up to 250-fold, IL-8 up to 30-fold, IL-10 up to 240-fold, IL-12 up to 2-fold, and IL-17 up to 13-fold. (Table 5-2).

Table 5-1: Changes in plasma concentrations of TNF-α, IL-10, and IL-17

		Horse A	Horse B	Horse C	Horse D
Hours post infection	**Cytokine**	**pg/mL**	**pg/mL**	**pg/mL**	**pg/mL**
0	TNF-α	<3.91	<3.91	<3.91	<3.91
	IL-10	<51.26	<51.26	113.88	76.33
	IL-17	<13.33	<13.33	34.71	31.56
12	TNF-α	<3.91	<3.91	<3.91	<3.91
	IL-10	<51.26	<51.26	157.57	64.48
	IL-17	<13.33	<13.33	35.02	33.46
24	TNF-α	<3.91	<3.91	<3.91	14.77
	IL-10	<51.26	<51.26	81.62	100.33
	IL-17	<13.33	<13.33	35.94	41.89
36	TNF-α	<3.91	<3.91	<3.91	<3.91
	IL-10	<51.26	<51.26	101.68	156.16
	IL-17	<13.33	<13.33	30.26	34.09
48	TNF-α	<3.91	<3.91	<3.91	63.3
	IL-10	<51.26	83.36	121.39	1968.66
	IL-17	<13.33	<13.33	30.26	34.09
60	TNF-α	<3.91	<3.91	<3.91	<3.91
	IL-10	<51.26	115.36	139.34	1476.2
	IL-17	<13.33	<13.33	36.25	<13.33
72	TNF-α	<3.91	<3.91	***<3.91****	***<3.91****
	IL-10	<51.26	58.78	***92.95****	***1495.93****
	IL-17	<13.33	<13.33	***34.71****	***<13.33****
74	TNF-α	n/s	n/s	<3.91	<3.91
	IL-10	n/s	n/s	69.73	<51.26
	IL-17	n/s	n/s	34.71	<13.33
76	TNF-α	n/s	n/s	<3.91	22.58
	IL-10	n/s	n/s	74.34	1446.54
	IL-17	n/s	n/s	33.15	<13.33
78	TNF-α	n/s	n/s	<3.91	<3.91
	IL-10	n/s	n/s	72.37	1396.9
	IL-17	n/s	n/s	35.33	<13.33
80	TNF-α	n/s	n/s	<3.91	<3.91
	IL-10	n/s	n/s	90.94	2184.66
	IL-17	n/s	n/s	36.25	<13.33
82	TNF-α	n/s	n/s	<3.91	38.72
	IL-10	n/s	n/s	74.34	1613.51
	IL-17	n/s	n/s	37.16	<13.33
84	TNF-α	<3.91	***<3.91****	<3.91	8.4
	IL-10	<51.26	***72.05****	72.37	1564.68

	IL-17	<13.33	***<13.33****	34.4	<13.33
86	TNF-α	n/s	<3.91	n/s	n/s
	IL-10	n/s	107.81	n/s	n/s
	IL-17	n/s	<13.33	n/s	n/s
88	TNF-α	n/s	<3.91	71.05	1652.42
	IL-10	n/s	53.55	n/s	n/s
	IL-17	n/s	<13.33	n/s	n/s
90	TNF-α	n/s	<3.91	n/s	n/s
	IL-10	n/s	204.09	n/s	n/s
	IL-17	n/s	<13.33	n/s	n/s
92	TNF-α	n/s	<3.91	<3.91	<3.91
	IL-10	n/s	198.55	83.61	1466.33
	IL-17	n/s	<13.33	38.96	<13.33
94	TNF-α	n/s	8.08	n/s	n/s
	IL-10	n/s	202.89	n/s	n/s
	IL-17	n/s	31.23	n/s	n/s
96	TNF-α	***<3.91****	<3.91	<3.91	<3.91
	IL-10	***<51.26****	199.96	69.08	2625.87
	IL-17	***<13.33****	35.02	32.2	<13.33
98	TNF-α	<3.91	n/s	n/s	n/s
	IL-10	<51.26	n/s	n/s	n/s
	IL-17	<13.33	n/s	n/s	n/s
100	TNF-α	<3.91	3.98	n/s	n/s
	IL-10	<51.26	166.79	n/s	n/s
	IL-17	<13.33	n/s	n/s	n/s
102	TNF-α	<3.91	n/s	n/s	n/s
	IL-10	<51.26	n/s	n/s	n/s
	IL-17	<13.33	n/s	n/s	n/s
104	TNF-α	<3.91	15.05	n/s	n/s
	IL-10	<51.26	261.25	n/s	n/s
	IL-17	<13.33	n/s	n/s	n/s
106	TNF-α	<3.91	n/s	n/s	n/s
	IL-10	<51.26	n/s	n/s	n/s
	IL-17	<13.33	n/s	n/s	n/s
108	TNF-α	<3.91	6.54	<3.91	24.53
	IL-10	55.28	236.25	76.33	1864.14
	IL-17	<13.33	35.94	32.2	<13.33
112	TNF-α	<3.91	n/s	n/s	n/s
	IL-10	59.37	n/s	n/s	n/s
	IL-17	<13.33	n/s	n/s	n/s
116	TNF-α	<3.91	n/s	n/s	n/s
	IL-10	<51.26	n/s	n/s	n/s

	IL-17	<13.33	n/s	n/s	n/s
120	TNF-α	<3.91	12.21	<3.91	<3.91
	IL-10	129.4	230.26	110.48	461.73
	IL-17	<13.33	35.33	37.76	<13.33
132	TNF-α	<3.91	21.36	<3.91	57.93
	IL-10	71.43	310.97	121.39	5338.25
	IL-17	<13.33	40.14	34.4	<13.33
144	TNF-α	<3.91	4.96	<3.91	82.66
	IL-10	101.73	268.14	214.66	5906.24
	IL-17	<13.33	32.83	30.59	<13.33
156	TNF-α	<3.91	17.27	9.87	94.77
	IL-10	115.36	517.99	470.71	12282.21
	IL-17	<13.33	32.83	30.59	<13.33
168	IL-10	146.74	982.84	413.61	8413.77
	TNF-α	<3.91	17	<3.91	84.4
	IL-17	<13.33	34.71	32.51	<13.33
180	IL-10	189.92	921.86	1430.3	8006.97
	TNF-α	<3.91	22.96	15.33	66.85
	IL-17	<13.33	32.2	35.33	<13.33
192	TNF-α	<3.91	35.38	n/s	n/s
	IL-10	195.4	1711.38	n/s	n/s
	IL-17	<13.33	34.09	n/s	n/s
204	TNF-α	<3.91	n/s	n/s	n/s
	IL-10	237.27	n/s	n/s	n/s
	IL-17	<13.33	n/s	n/s	n/s
216	TNF-α	<3.91	n/s	n/s	n/s
	IL-10	281.01	n/s	n/s	n/s
	IL-17	<13.33	n/s	n/s	n/s
228	TNF-α	<3.91	n/s	n/s	n/s
	IL-10	316.71	n/s	n/s	n/s
	IL-17	<13.33	n/s	n/s	n/s

pg, picogram; mL, milliliter; n/s, not sampled.

First timepoint positive for AHSV on PCR

Figure 5-1: Line graphs of cytokines with important changes in plasma concentrations

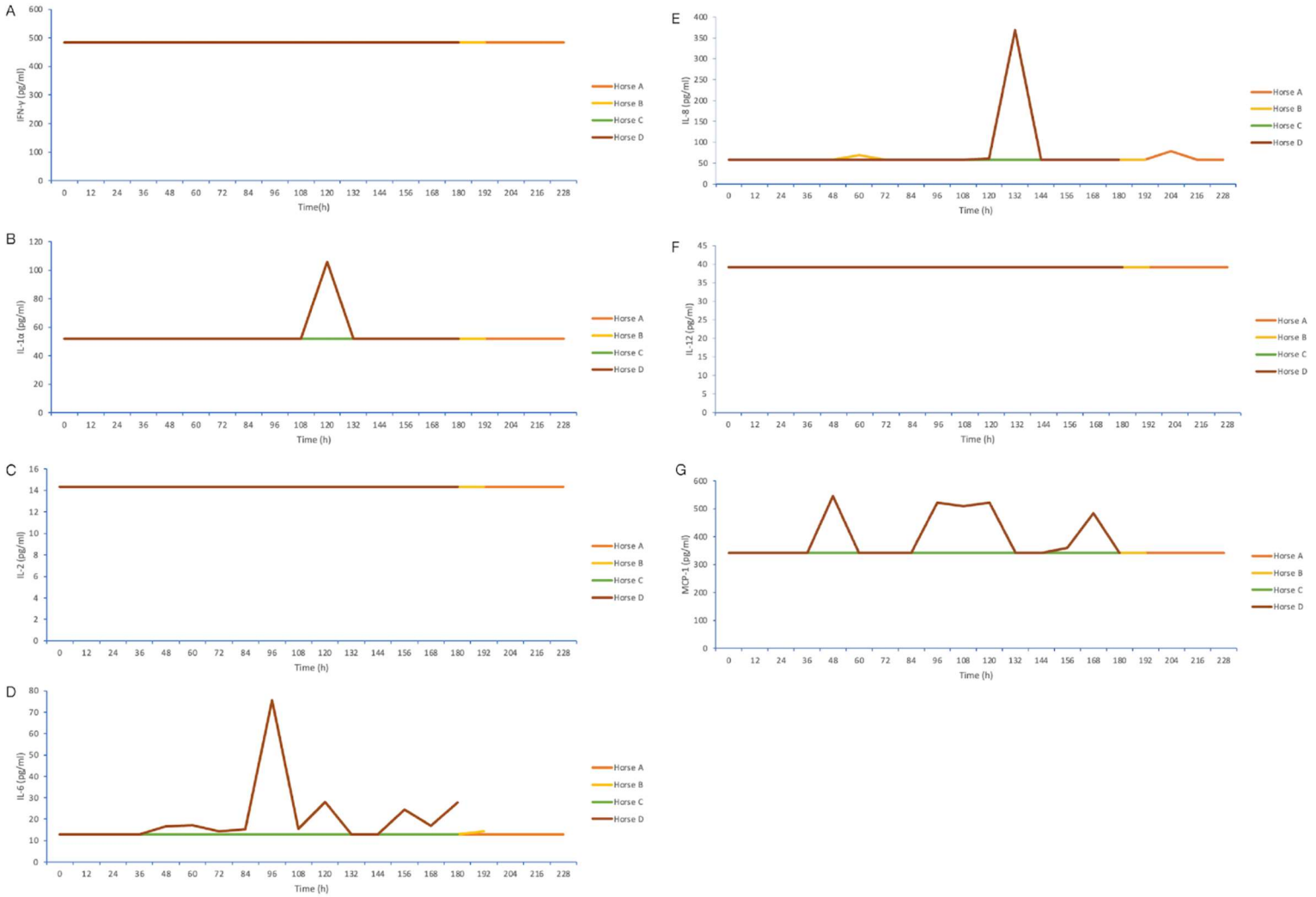

Figure 5-2: Line graphs of cytokines with no or variable changes in plasma concentrations

Table 5-2: Cytokine results in horses with other systemic diseases

Variable	Concentration	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7
		Colic	Hepatitis	Sepsis	Pneumonia	Peritonitis	Sepsis	Pleuropneumonia
SAA	mg/l	1213	1284	4287	2208	1122	1001	4750
IL-2	pg/mL	591	188	137	14	1182	14	91
IFN-γ	pg/mL	11648	5543	2456	483	22342	483	2640
IL-1a	pg/mL	194	52	52	52	412	52	52
IL-6	pg/mL	1962	850	345	13	3576	13	349
IL-8	pg/mL	1069	534	1168	358	1796	610	417
IL-10	pg/mL	8192	3021	6067	216	12456	1256	2213
IL-12	pg/mL	72	39	39	39	78	39	39
IL-17	pg/mL	172	13	13	13	90	13	13
TNF-α	pg/mL	1202	380	409	26	2018	51	269
MCP-1	pg/mL	375	342	342	342	342	342	360

5.4 Discussion

The almost complete absence of a detectable proinflammatory blood cytokine response in the face of severe clinical disease aligns with previous findings reporting an unexpectedly mild acute phase response in these horses (Schliewert et al., 2022). These findings suggest possible interference of AHSV with the innate immune response.

Hepatocyte synthesis of SAA is induced by proinflammatory cytokines IL-1, IL-6, TFN-α, and IFN (Mackiewicz et al., 1991), while sequestration of iron in tissues (resulting in hypoferremia) is facilitated by hepcidin, which is upregulated by IL-6 and TNF-α (Ganz, 2003; Nemeth et al., 2004). The observed lack of response in many of these cytokines here, particularly IL-6, explains the delayed acute phase response with mild changes in APR previously reported.

There was no detectable blood IFN-γ response in the AHSV-infected horses. This is an unusual finding, given the critical role that IFN-γ (a type-II IFN) plays in inhibiting

viral replication. Viral nucleic acids are recognized as PAMPs by PRRs on antigen presenting and natural killer cells. This leads to production of IFNs, both type I IFN-α and β (Koyama et al., 2008) and type II IFN-γ (Schroder et al., 2004), mainly via activation of the JAK/STAT signalling pathway on both virus-infected and uninfected cells. Interferons further initiate the upregulation of IFN-stimulated genes with antiviral, antiproliferative and immunomodulatory functions (de Veer et al., 2001). The resulting transcribed proteins directly inhibit viral replication (Weber et al., 2004). This is a key component of the host's response to pathogen invasion.

While many viruses have evolved strategies to disrupt these signalling pathways at various levels (Fleming 2016), recent discoveries have demonstrated how different Orbiviruses inhibit the IFN pathways. Bluetongue virus has been shown to interfere with the JAK/STAT response (Doceul et al., 2014) and alter the IFN-I response via its NS4 (Ratinier et al., 2016). Similarly, AHSV-NS4 interference with JAK/STAT signalling has been demonstrated (Wall et al., 2021); however, the exact mechanism is unclear to date. Additionally, AHSV-NS4 colocalizes with PML-NBs which regulate several diverse cellular functions in the nucleus of the host cell. The PML-NBs are involved in the antiviral innate immune defence through various mechanisms including their ability to improve IFN-γ signalling (El Bougrini et al., 2011) and IFN-β synthesis (El Asmi et al., 2014). Modification of PML-NBs by AHSV-NS4 may disrupt the host's ability to mount an antiviral response (Boughan et al., 2020) and suggests a potential mechanism by which AHSV evades the innate immune response. The significance of IFN-γ production in the cell-mediated immune response to AHSV viral protein VP2/VP5 was highlighted in a study comparing vaccinated to unvaccinated horses (El Garch et al., 2012) where IFN-γ was only detected in cells from vaccinated horses.

Additionally, research into the in vitro response of peripheral blood mononuclear cells to virulent AHSV4 documented interference in IFN pathways (Faber et al., 2021), which could explain why the expected increase in production of IFN-γ and further production of proinflammatory cytokines was not observed here. While there was a concurrent increase in transcriptors for proinflammatory cytokines in the in vitro model (Faber et al., 2021), this may not necessarily result in the production of the transcribed protein in vivo.

In the face of this suspected interference of AHSV with the IFN pathways, TNF-α and IL-10 were increased in the final stages of the disease. Tumour necrosis factor-α, a major proinflammatory cytokine, is mainly secreted by activated macrophages independently of IFNs (Abbas AK, 2022; Sedger and McDermott, 2014) which accounts for its increase here. Interleukin-10 is considered a major anti-inflammatory cytokine and is secreted by activated T-lymphocytes, B-lymphocytes, and monocytes/macrophages (Verma et al., 2016). Interleukin-10 production is not directly dependent on IFN; in fact IL-10 is able to suppress IFN synthesis (Ito et al., 1999). Downregulation of the inflammatory response through IL-10 may have contributed to the lack of response seen in some cytokines and to the clinical deterioration observed in the horses. However, it should be noted that IL-10 can act as a proinflammatory cytokine in certain contexts such as autoinflammatory diseases (Tilg et al., 2002), cancer (Naing et al., 2018; Naing et al., 2016) or Covid-19 caused by the SARS-CoV-2 (Lu et al., 2021), and it is possible that the increase in IL-10 observed in the AHSV infected horses represents a late proinflammatory response (Lauw et al., 2000). There were some changes in IL-17 in two horses. Although IL-17 synthesis is regulated by JAK/STAT pathways and thus subject to possible inhibition by AHSV NS4 (Wall et al.,

2021), upregulation of the IL-17 receptor has been observed in guinea pigs experimentally infected with AHS and a T-helper-17 cell response has been suggested as a key component in AHSV immunity (Fearon et al., 2021).

Interleukin-1α and IL-6 both showed minimal changes in AHSV-infected horses. Interleukin-1α is produced by blood monocytes including dendritic cells and tissue macrophages as well as endothelial cells (Di Paolo and Shayakhmetov, 2016). Activation requires intracellular cleavage of the precursor by a cysteine protease, caspase 1. Caspase 1 also needs to be activated, requiring prior assembly of an intracellular protein complex termed the inflammasome (Agostini et al., 2004; Martinon et al., 2009). Several viruses have been shown capable of inhibiting caspase-1 (Callus and Vaux, 2007), and it is possible that AHSV also interferes in this process. Production of the proinflammatory cytokine IL-6 is primarily associated with the JAK/STAT pathways (Wang et al., 2013) and AHSV NS4 interference in this pathway (Wall et al., 2021) may have prevented an increase here. The synthesis of IL-12, which was also not increased, is also regulated by JAK/STAT pathways (Seif et al., 2017).

Interleukin-2, secreted by activated T-lymphocytes, and IL-12, from monocytes/ macrophages, are both secreted very early in the immune response and have very short half-lives, therefore increases may simply have been missed (Bajetta et al., 1998; Hamza et al., 2010; Ross and Cantrell, 2018; Sojka et al., 2004). Monocyte chemotactic protein-1, which did not increase, is secreted by activated macrophages, these in turn require IFN-γ for activation (Leopold Wager and Wormley, 2014). As IL-8 synthesis is stimulated by IL-1 and TNF-α (Mukaida et al., 1991), it is not surprising

that an increase in IL-8 was not observed. To further elucidate the function and intricate interaction of these cytokines in AHS, more research is required.

It is important to consider that cytokines were measured in jugular blood samples, which may not be reflective of the local inflammatory response. Previous works have focussed on viral tropism to pulmonary macrophages during AHS infection (Carrasco et al., 1999; Clift and Penrith, 2010). It is possible that targeting of these cells by the AHSV results in a local response that is not reflective of the systemic inflammatory response. This is further supported by histopathological findings that are consistent with substantial inflammation in the lungs but not in other tissues in the body (Newsholme, 1983).

In this study, experimental infection with AHSV was evaluated. Natural infection occurs through bites of the *Culicoides* midge, therefore local immune responses may play an important role in host defense mechanisms (Lehiy et al., 2018). These were not triggered in our experimental infection model. Additionally, not only did the route of infection differ from natural infections but also the viral loads injected. It is possible that natural infection with fewer virus particles may allow the immune system to mount a more effective innate immune response. Further research in horses naturally infected with AHSV or the validation of an infection model that better replicates natural infection mechanisms is required to elucidate the host immune response in natural infections.

Another limitation of this study is that AHSV serotypes differed between animals. However, all four horses in our trial showed a similar cytokine pattern and it has been

demonstrated that the clinical course of the disease is not dependent on the serotype (AHSV 1-9) but rather the virulence of the variant present, as numerous variations of each serotype exist (Burrage and Laegreid, 1994; Laegreid et al., 1993). While the kinetics of the cytokine response were similar in all horses, cytokine concentrations in horse D were higher than in the other three horses. The reasons for this finding may be related to the virulence or different magnitude of immune interference of the virus used to infect horse D. Another plausible explanation for the different cytokine concentrations is a possible difference between the reagents/assays used in horses A-C and horse D or the assay performance between plates, as reagents from a different lot had to be used for the assay for horse D. However, standard curves were appropriate for all plates, indicating proper assay functionality and allowing for comparison between plates. Unfortunately, due to ethical reasons, infection of additional horses with identical serotypes was not possible, and investigations of possible differences in the host response to individual serotypes should be performed in future studies.

Overall, while there are still many unknown aspects of AHSV pathogenesis, these findings suggest interference of the virus with the host innate immune system. Further understanding of the mechanisms involved may improve our ability to develop a more effective vaccine or targeted treatment protocols. Specifically, the use of biological response modifiers, also known as immune response modifiers, may be of interest. These agents are currently used to treat autoimmune diseases and cancer; in horses, the immune response modifier imiquimod, an imidazoquinolinamine, is used to treat sarcoids (Nogueira et al., 2006). Imiquimod induces the production of IFN-α, IFN- γ,

and IL-12 (Dahl, 2000). Investigation of this drug or a similar compound for the treatment of AHS may be indicated.

The lack of an appropriate proinflammatory blood cytokine response in horses experimentally infected with AHSV supports findings from in vivo studies which have documented inhibition of the immune response by the virus – specifically IFN and JAK/STAT pathways. This knowledge may be useful to guide vaccine development to prevent AHS as well as provide pathways to improve treatment options for naturally infected animals.

Future research should include detailed investigation of the host immune response in naturally infected animals to further elucidate the inflammatory pathways.

6 CHAPTER 6: EXPERIMENTAL INFECTION WITH AFRICAN HORSE SICKNESS VIRUS RESULTS IN AN OVERT CONSUMPTIVE COAGULOPATHY

6.1 Introduction

Infection with AHSV commonly results in clinical signs consistent with hypocoagulation and submucosal petechiae, as well as prolonged bleeding following venepuncture, are commonly observed (Mellor and Hamblin, 2004b; Skowronek et al., 1995). Virus replication has been observed in endothelial cells and it is likely that both the direct endothelial cell damage and the ensuing loss of function result in the haemorrhages observed in the affected tissues on necropsy and histopathology (Gomez-Villamandos et al., 1999; Laegreid et al., 1992a; Newsholme, 1983). Precise regulation of haemostasis is vital to protect the body from injury and allow for repair of vascular lesions and prevent blood loss while simultaneously inhibiting blood from coagulating within the vasculature. The main inciting cause for initiation of haemostatic pathways is injury to the vascular endothelium with resulting exposure of TF to the bloodstream; however, it has been established that inflammation can also initiate haemostasis as proinflammatory cytokines IL-1, IL-6 and TNF (Grignani and Maiolo, 2000) promote TF expression on monocytes and other cells and as such propagate thrombin generation (Foley and Conway, 2016b), resulting in the conversion of fibrinogen to produce fibrin.

The endothelium, a single layer of cells that line the inner surface of blood vessels, has important functions in blood flow and haemostasis, and the regulation of vascular

tone. It also plays an important immunological role in the regulation of inflammation. Damage to the endothelium caused by trauma or an infectious agent can result in activation of the endothelial cells and inflammation (Stanek et al., 2018). Furthermore, endothelial injury often results in increased vascular permeability with subsequent leakage and tissue oedema (Prasad et al., 2021). While healthy endothelial cells have a vasodilatory action, vessel damage results in vasoconstriction mediated by locally synthesized vasoactive mediators like bradykinin, histamine, and vasopressin. Injury to the endothelium also exposes vWF (Savage et al., 1996) which consequently interacts with the GPIb/V/IX complex on the platelet resulting in platelet recruitment and aggregation onto the endothelial surface (Varga-Szabo et al., 2008). This is further aided by the reduction in blood flow resulting in an increased shear rate which facilitates this interaction. Exposed collagen also interacts with platelet receptors GPVI and $GP\alpha_2\beta_1$ (Cruz et al., 2005) which further facilitates platelet adhesion and thrombus formation (Neubauer and Zieger, 2022).

The endothelium has important anticoagulant properties. It acts as an anticoagulant boundary layer by secreting anticoagulant products with regulatory functions on platelet activity. For example, nitric oxide inhibits platelet adhesion and aggregation (Radomski et al., 1987), and prostacyclin acts directly on the platelet and interferes with the platelets' ability to respond to procoagulant stimuli which then prevents interactions between the platelet and the intact endothelium (Braune et al., 2020). Additionally, the endothelial surface activates the plasma protein AT by providing heparan sulphates which are part of the endothelial glycocalyx and thus promotes TAT complex formation (Bauer and Rosenberg, 1991). Moreover, healthy endothelial cells express TFPI, thus limiting excessive activation of factors VII and X via TF expression

(Girard and Broze, 1993). Furthermore, endothelial cells synthesize and express the membrane-bound thrombin receptor thrombomodulin (Hofsteenge et al., 1986). Expression of thrombomodulin not only decreases the amount of circulating thrombin but also increases thrombin affinity to protein C, which, once activated, forms a complex with protein S to inactivate factors Va and VIIIa (Stern et al., 1986). The thrombin that is bound to thrombomodulin is less effective in converting fibrinogen to fibrin and thus has reduced ability to promote platelet aggregation (Adams and Huntington, 2006).

During severe systemic disease, as a result of vascular endothelial damage, pathological activation of haemostasis can occur. Clinically, this can manifest as thromboembolic disease with microthrombi formation but also as severe haemorrhagic tendencies due to platelet depletion, consumption of coagulation factors, and acceleration of plasmin formation and fibrinolysis (Iba et al., 2022). If uncontrolled, this inappropriate activation of haemostasis may lead to the development of DIC and micro- and macrovascular thrombus formation with perfusion derangements resulting in multiple organ dysfunction and death (Costello and Nehring, 2022; Morris, 1988). Clinically, DIC can be classified as either overt, meaning with clinical signs of widespread haemorrhage, or non-overt, meaning the haemostatic system is still compensated without clinical signs of haemorrhage (Taylor et al., 2001). The diagnosis of DIC is made based on the presence of procoagulant activation, inhibitor consumption and increased fibrinolytic activity (Taylor et al., 2001). Early diagnosis of DIC allows for earlier treatment intervention, which is important as the prognosis for overt DIC is poor (Goggs et al., 2018; Wiinberg et al., 2008).

Very little is documented on the haemostatic changes observed in horses with AHS. Clinically, petechiae and prolonged bleeding after venepuncture are often observed in infected horses and one study reported the presence of thrombocytopenia with prolonged PT, aPTT, and TT as well as an increase in FDP (Skowronek et al., 1995). Investigations into the changes in other laboratory variables evaluating haemostasis, such as concentrations or activities of individual coagulation factors have not been described to date. Generally, while coagulation factor deficiency is uncommon in horses, in humans, cytokines and autoantibodies acting as acquired factor inhibitors and resulting disturbance of haemostasis have been described in viral infections (Bennett et al., 2021; Chuang et al., 2013) and may exacerbate the haemostasis abnormalities observed due to inflammation. While the traditional coagulation assays, namely PT and aPTT, are used to identify the cause of a coagulopathy affecting either the extrinsic or intrinsic coagulation cascade, they may not reflect the clinical picture. As these assays do not include cellular components integral to haemostasis, they have multiple limitations; for example, the endpoint of the PT and aPTT assays is clot initiation and these tests do not evaluate clot kinetics or clot stability. While a decreased PT and aPTT suggest hypercoaguable tendencies, these assays have been shown to have poor sensitivity for this purpose and the use of a global test of haemostasis such as TEG may prove to be beneficial to help distinguish between non-overt and overt DIC and allow for improved diagnosis and therapy of DIC in these horses.

Therefore, the objective of this study was to describe haemostatic changes occurring in horses experimentally infected with AHSV and to characterize the form of DIC expected in these horses using both plasma-based and viscoelastic assays. We

hypothesized that the infected horses would develop overt DIC with coagulation factor consumption.

6.2 Materials and Methods:

This study was a prospective, longitudinal, experimental study. Four AHS-susceptible Boerperd cross horses were included.

6.2.1 Animals

Four horses that had tested negative for neutralizing antibodies against all nine AHSV serotypes on a commercial competitive ELISA (INgezim AHSV compac plus, Eurofins Technologies, Hungary) were included in the study (please see chapter 3.1 for details on the selection of animals, their management, and testing).

6.2.2 Study Design

6.2.2.1 Experimental Infection

Horses were infected with an intravenous infective dose (5 mL) of low passage mouse brain suspension, containing a minimum of 10^5 mouse infective doses/mL of virulent AHSV serotypes. The vaccine trial assessed different serovars, so each horse was infected with a different AHSV serovar: horse 1, AHSV-2 (horse origin); horse 2, AHSV-4 (horse origin); horse 3, AHSV-6 (horse origin) and horse 4, AHSV-6 (dog origin).

6.2.2.2 Sampling procedures

Blood was obtained prior to infection, followed by blood sampling every 12 hours until PCR using a method previously described (van Rijn et al., 2018a) was positive for AHS. PCR was performed every 24 hours, then every 12 hours once horses were febrile until AHS viremia was confirmed. Once viraemia was confirmed, sampling frequency increased to every 2 hours for a total of 12 hours, then to every 4 hours for another 12 hours, after this every 12 hour sampling intervals were reinstituted for the remainder of the trial. Samples were obtained via venepuncture from the jugular vein directly into vacutainer tubes (Becton, Dickinson and Company, USA), through vacuum assistance, using alternate veins for each sampling. Blood collection was in specific order into a serum tube (4 mL), a 3.2% sodium citrate tube (4 mL), a heparin tube and an EDTA tube (4 mL). Samples were kept at 8°C until analysis (citrate) or stored at -80°C following centrifugation (EDTA plasma, serum, citrate plasma).

6.2.3 Haemostasis assays

Assays for haemostatic changes were performed on the 0-hour samples, then every 24 hours until humane euthanasia. Samples were kept at 8°C and processed within 2 hours after collection. Thromboelastography was performed 2 hours after collection (Lemon et al., 2021); remaining citrate blood was then centrifuged, and citrated plasma frozen at -80°C until batch analysis.

Kaolin-activated TEG was performed using citrated whole blood and measured variables included R, K, α-angle, MA, G, LY30, LY60, CL30 and CL60. A V-curve analysis was also performed and measured variables included MRTG, TMRTG, TG, MRL, TMRL and Lysis.

Citrated plasma was used to measure PT, aPTT, fibrinogen and D-dimer concentrations, AT activity, as well as activities of coagulation factors II (aFII), VII (aFVII), VIII (aFVIII), X (aFX), and XII (aFXII). Platelet concentration was measured in the EDTA whole blood. Detailed descriptions of the methodologies of the various haemostasis assays can be found in chapter 3.

6.2.4 Histopathology

Tissue samples from lung, heart, and spleen from each horse were processed routinely, embedded in paraffin wax and cut into sections of 5-7 µm and stained with haematoxylin and eosin (H&E) using standard protocols (Bancroft and Layton, 2019). Samples were then examined microscopically for the presence of microthrombi by a veterinary pathologist.

6.2.5 Statistical Analysis

Due to the small number of horses available, changes occurring within a horse post infection could not be compared to one another using meaningful statistical analysis. Changes are therefore described based on visual inspection.

6.3 Results

Clinical findings

Four Boerperd cross horses (A-D) were enrolled in the study. The horses developed pyrexia (rectal temperature >38.5°C) between 36 to 48 hours, then tested positive for AHS on PCR between 72 to 96 hours post infection. Respiratory rates increased from 152 hours post infection; mild swelling of the supraorbital fossae was observed in horse A. Prolonged bleeding from the venepuncture site was observed in all horses

towards the end of the study. No petechiae were observed on mucosal membranes. All horses developed typical clinical signs of the pulmonary form of AHS and were humanely euthanized between 180 to 228 hours post infection (Table 4-1). On gross necropsy, severe pulmonary oedema, tissue oedema, hydropericardium, and multifocal mucosal haemorrhage was observed in all horses.

Haemostasis assays

Samples were available for all horses every 24 hours from 0 hours to the time of euthanasia. For the TEG variables, the R (Fig 6-1A) decreased initially and then increased towards the end of the course of the disease; the K (Fig 6-1B) increased in one horse at the time of euthanasia but did not change upon visual inspection in the other three horses. The α-angle (Fig 6-1C) decreased in all horses towards the end of the disease. The MA decreased in all horses towards the end of the disease course (Fig 6-1D). The G value decreased in 3 horses towards the end of the disease (Fig 6-1E). Overall lysis after 30 and 60 minutes (LY30 and LY60, Fig 6-1F-G) as well as CL30 (Fig 6-1H) and CL60 (Fig 6-1I) varied widely between and within horses, however, at the time of humane euthanasia LY60 had decreased from baseline in all horse while CL60 increased in all animals. Overall, the TEG analyses revealed phases of both hyper- and hypocoagulability in all horses throughout the course of the disease. In two horses (C and D), the thromboelastograms were initially consistent with a hypercoagulable state (increased angle, MA and G above the upper reference limit) but these values normalized at the last sampling timepoint. The other two horses (A and B) had substantially decreased MA and G values consistent with hypocoagulation throughout the disease, while the angle was also below the lower reference limit towards the late stages of the disease.

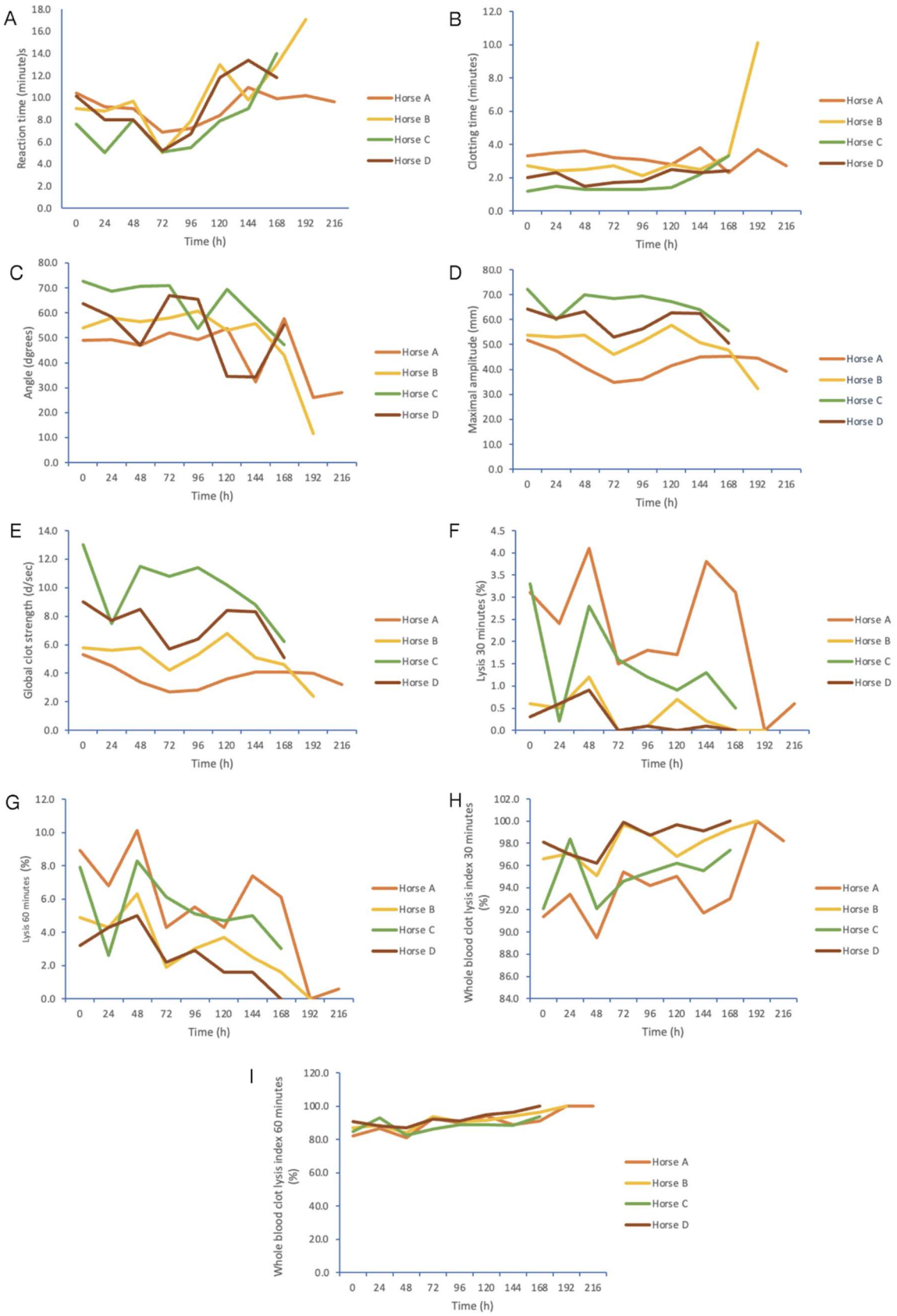

Figure 6-1: Line diagrams of the changes in thromboelastography variables

Analysis of the velocity curve revealed a decrease in MRTG (Fig 6-2A) over time, while TMRTG increased (Fig 6-2B). The TG (Fig 6-2C) decreased mildly throughout the course of the disease. No major changes were evident in visual inspection of fibrinolysis variables MRL (Fig 6-2D) and TMRL (Fig 6-2E), while overall lysis (Fig 6-2F) was decreasing towards the late disease stages. In three of the four horses no fibrinolysis was detected in the late stages of the disease.

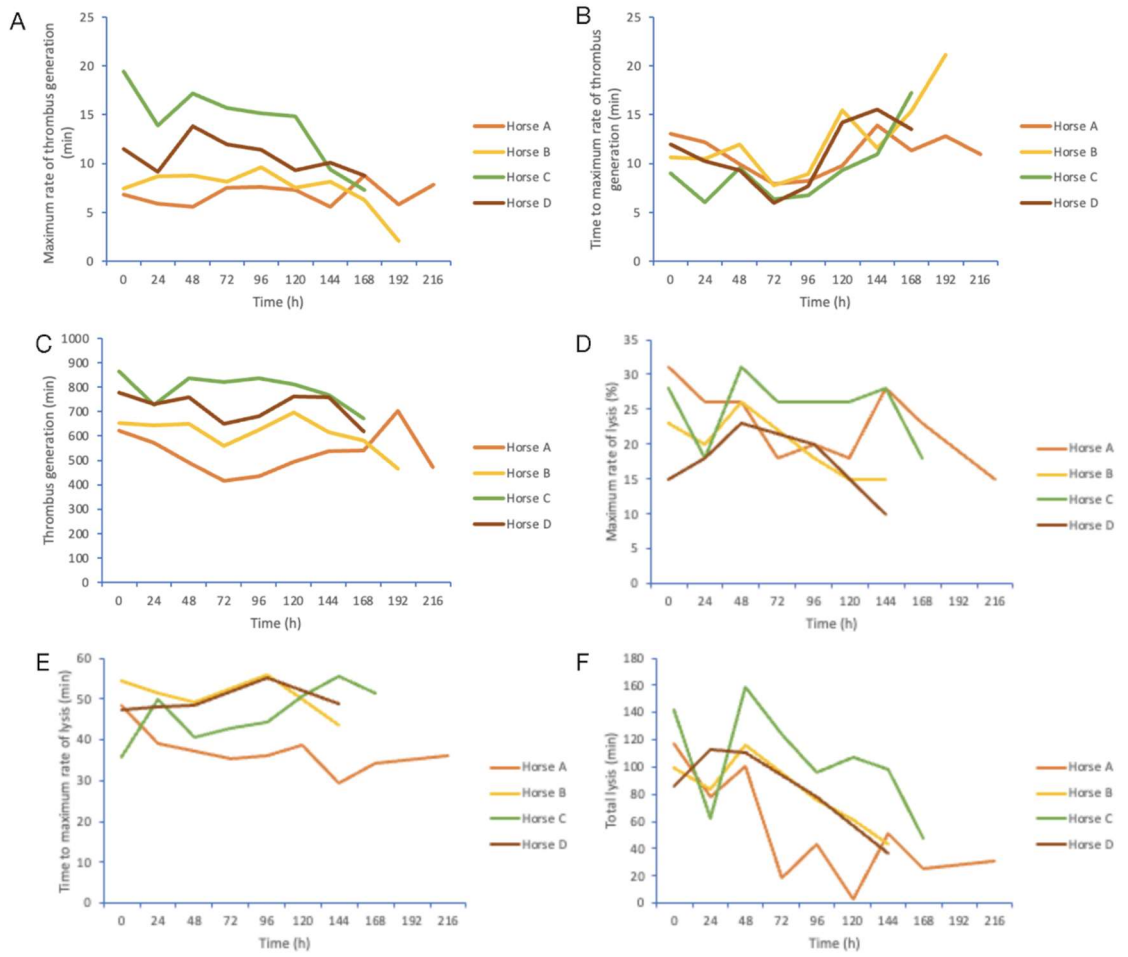

Figure 6-2: Line diagrams of the changes in clot velocity curve variables.

In all horses, hypocoagulation was also evident from the results of plasma-based coagulation assays, namely a substantial increase above the laboratory reference interval for PT (Figure 6-3A) and aPTT (Figure 6-3B), and a marked increase in D-

dimer concentrations (Figure 6-3D). Fibrinogen concentration increased mildly throughout the course of the disease but remained within the laboratory reference interval (Figure 6-3C). Initially, no overall change in AT activity was apparent; however, there was a sharp decrease for AT activity towards the end of the disease in three horses (Figure 6-3E). The platelet concentration decreased throughout the course of the disease (Figure 6-3F) and all horses developed severe thrombocytopenia.

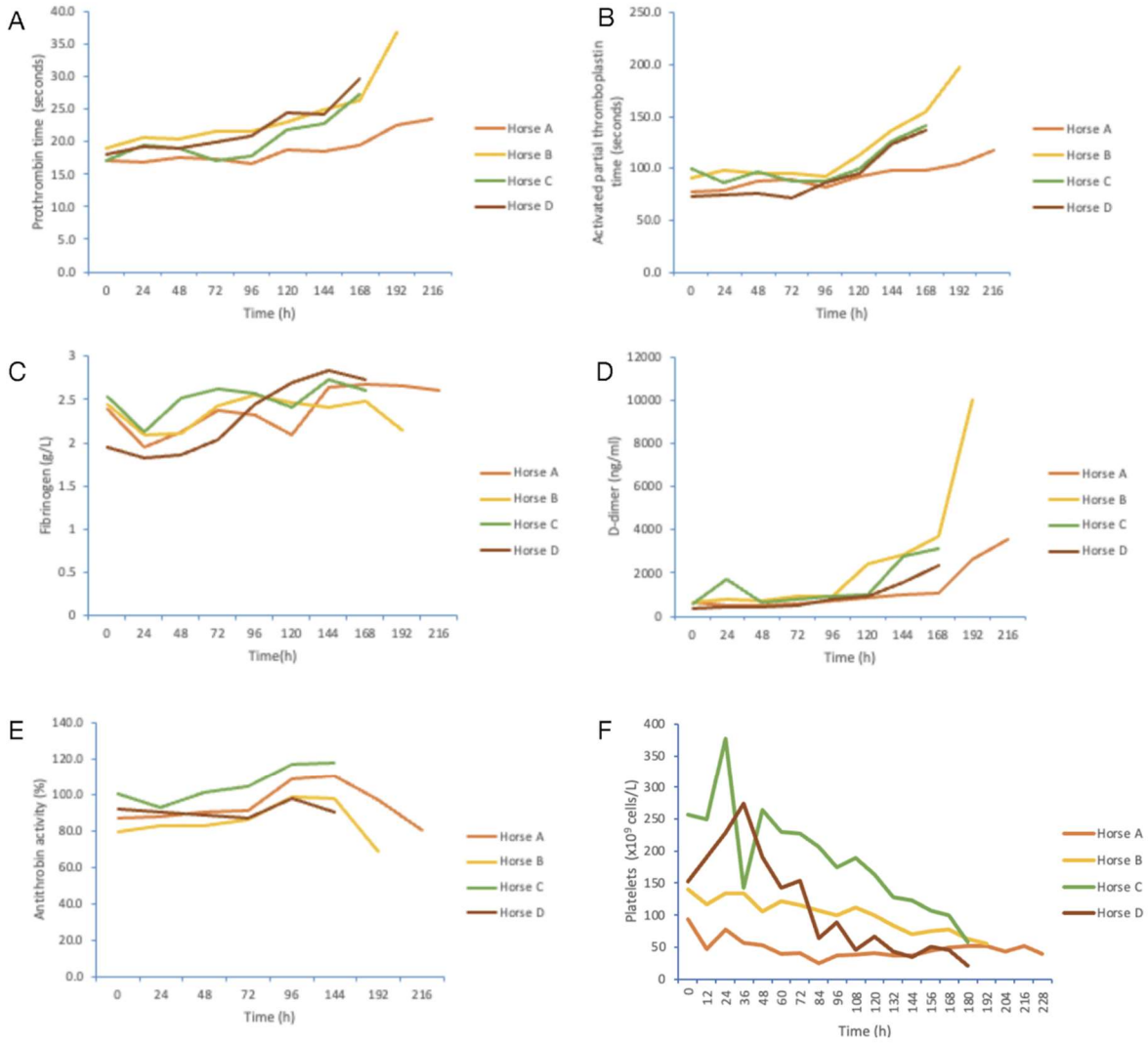

Figure 6-3: Line diagrams of the changes in plasma-based coagulation assays

The activities of coagulation factors II (Figure 6-4A), VII (Figure 6-4B), X (Figure 6-4C), and XII (Figure 6-4D) decreased from the baseline sample activities throughout the course of the disease with the lowest activity measured at the time of euthanasia. Activities for plasminogen (6-4E) and plasminogen inhibitor (6-4F) remained unchanged overall.

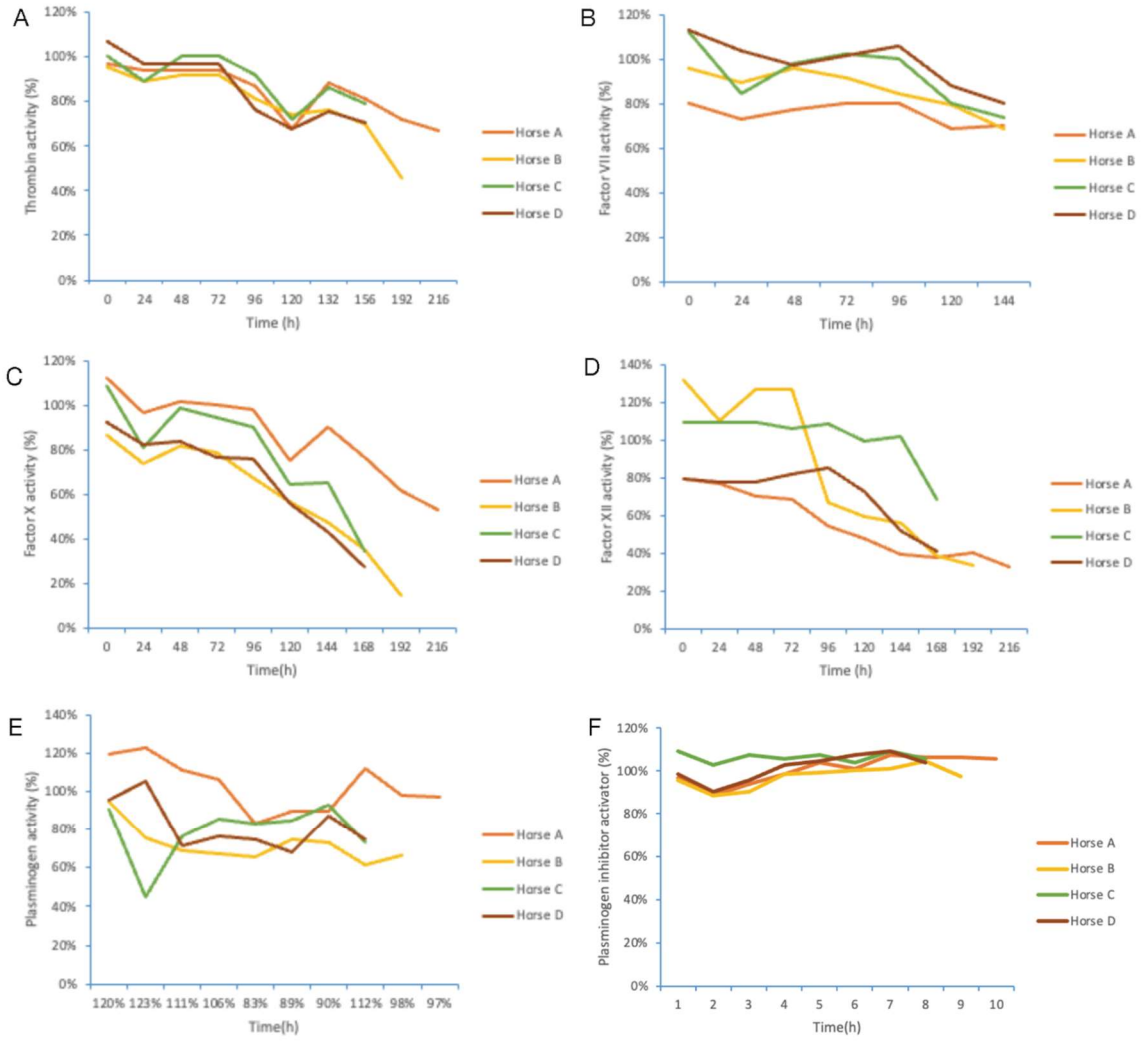

Figure 6-4: Line diagrams of the changes in coagulation and fibrinolysis factors

Gross necropsy revealed gastrointestinal petechiae and mucosal haemorrhages. Histopathology of the lung revealed moderate to severe variably proteinaceous oedema in the interstitium, the alveoli, and the vascular walls with mild to moderate mononuclear and, to a lesser extent, neutrophilic leukostasis, mononuclear vasculitis and perivasculitis. Mild to moderate vascular endothelial cell hypertrophy with mild multifocal fibrin exudation into alveolar lumens was also observed. Evaluation of the spleen revealed multifocal white pulp follicular lymphocytolysis with relatively mildly increased macrophages visible in these areas and mild white pulp atrophy while moderate to severe diffuse congestion of the red pulp was observed. Examination of the heart revealed mild/moderate diffuse congestion. Nuclear fragmentation and pyknosis was noted in the microvascular endothelial cells of the interstitial capillaries. No evidence of microthrombi was noted in any tissue.

6.4 Discussion

These findings confirm the development of a consumptive coagulopathy with haemorrhagic tendencies in horses experimentally infected with AHSV. The results of this study fulfil the criteria of DIC according to the ISTH guidelines for humans, namely activated procoagulant pathways with consumption of coagulation inhibitors and increased fibrinolysis (Goggs et al., 2018).

For all four horses participating in this study, both PT and aPTT were markedly increased, indicative of procoagulant activation. This was supported by the observed decreases in activities of coagulation factors II, VII, X, and XII. Prothrombin time is affected by changes in factor VII, while aPTT assesses activity of factors VIII, IX, XI and XII. Both PT and aPTT are affected by changes in the activity of factors X, V, II

and fibrinogen (Loizou et al., 2018). This finding indicates consumption of substrates required for coagulation and is the likely reason for the prolongation in PT and aPTT. The observed increase in the TEG R and the decrease in α-angle correlate with the observed decreases in coagulation factor activity and thus support procoagulant activation resulting in coagulation factor consumption. This is consistent with findings from a previous study in horses with gastrointestinal disorders and suspected coagulopathies where hypocoagulation resulted in increased R and K and decreased α-angle, MA, and G (Mendez-Angulo et al., 2010).

A mechanism that counteracts the effects of excessive consumption of coagulation factors is platelet activation together with sufficient quantities of fibrinogen (van Rooyen et al., 2019). Despite the marked thrombocytopenia noted, platelet activation was evident in these horses based on increases in MPV and MPM, and a decrease in MPC, surrogate markers for platelet activation (Schliewert et al., 2022). These parameters indicate changes which increase platelet surface (SangYun et al., 2019) which allows increased exposure of membrane receptors required for haemostasis (Moroi et al., 2020). The positive effect of platelet activation on haemostasis has been confirmed in previous studies (Barradas et al., 1994; Galt et al., 1991; Robless et al., 2003). Activated platelets upregulate integrin adhesion receptors, with conformation changes of the GPIIb/IIIa receptor (Hawiger, 1995) resulting in cross-linking between the receptors by fibrin or vWF. This propagates adhesion of more platelets and subsequent platelet aggregation (Yun et al., 2016). Furthermore, procoagulant factors including FV, fibrinogen, and vWF which stimulate activation of more platelets and thrombin formation are secreted by these activated platelets (DeNotta and Brooks, 2020). Platelet vWf, which is stored in the α-granules mediates platelet tethering via

interaction with GPIbα (Kulkarni et al., 2000). Through these mechanisms, platelet activation leads to the recruitment of more platelets. In addition, activated platelet express phosphatidylserine as well as FV and FX on their outer membrane. Phosphatidylserine binds FIX and FVIII form the tenase complex, which can activate FX. FV and FX form the prothombinase complex that converts prothrombin to thrombin (DeNotta and Brooks, 2020; Wang et al., 2022). As a result of these processes, large concentrations of thrombin are generated, allowing for formation of an insoluble, cross-linked fibrin clot (DeNotta and Brooks, 2020).

Fibrinogen, a minor acute phase protein, is upregulated during inflammation, and may affect TEG variables, specifically MA (Smith et al., 2012). While there was a mild increase observed in fibrinogen concentration throughout the course of the disease, concentrations remained within reference intervals and only decreased in all four horses shortly before euthanasia. It is possible that while fibrinogen production was increased, the concurrent consumption outweighed the increase.

As TEG evaluates the contribution of cellular components during haemostasis, the influence of other factors should be considered during evaluation of TEG. Moreover, the Partnership on Rotational Viscoelastic Test Standardization initiative recommends reporting the values for HCT, platelet count, and fibrinogen concentration when reporting TEG values to interpret the possible influence of these factors on the TEG values (Hanel et al., 2014). Platelet concentration and activation contribute to clot strength, therefore while thrombocytopenia will result in prolonged K, and decreased α-angle and MA (Nagler et al., 2013; Smith et al., 2012), platelet activation can positively affect K and MA (Bowbrick et al., 2003) Thromboelastography thus more

accurately represents coagulation status than the traditional haemostasis tests. Upon visual examination, neither K nor MA changed significantly in three of the infected horses during the course of the disease to indicate hypocoagulation, despite severe thrombocytopenia and decreased coagulation factor activity. It is therefore possible that platelet activation compensated for some of the hypocoagulable tendencies. This is supported by the observation that the changes observed in the plasma-based coagulation tests were more subjectively severe compared to the changes measured on TEG, where platelet dynamics are affecting haemostasis. Previous works have shown positive correlations between HCT and R or K, and negative correlations between HCT and α-angle, G, or MA (Brooks et al., 2014; Paltrinieri et al., 2008; Smith et al., 2012). This is likely because blood with a higher HCT contains more cells and less plasma containing concentrated coagulation proteins and enzymes compared to a sample with a lower HCT (McMichael et al., 2014; Smith et al., 2012). In the horses experimentally infected with AHSV, HCT increased substantially towards the final stages of the study, so it is possible that haemoconcentration may have affected the TEG measurements.

Thrombin (FII) is paramount for coagulation but also involved in anticoagulation and fibrinolysis by binding with thrombomodulin and at the same time blocking the sites on thrombomodulin which bind fibrinogen, platelets or FV thus affecting their procoagulant properties (Esmon, 1999). The thrombin-thrombomodulin complexes activate protein C, resulting in activation of the protein C anticoagulant pathway. Activated protein C has been described to be of particular importance in the microcirculation, due to the high numbers of endothelial cells present in the capillaries (Esmon, 1999). Additionally, thrombin-thrombomodulin complexes initiate negative

feedback pathways that limit coagulation (Wolberg et al., 2012). During coagulation, thrombin binds fibrinogen to form fibrin and is thus essential for the formation of a stable polymerized fibrin clot (Licari and Kovacic, 2009). Additionally, thrombin promotes further thrombin synthesis via a positive feedback mechanism including the production of FV, FVII, FVIII, and FXI, thus amplifying the coagulation cascade (Licari and Kovacic, 2009). Thrombomodulin-bound thrombin also inhibits fibrinolysis by activating thrombin-activable fibrinolysis inhibitor and thus interferes with plasminogen activation by fibrin (Declerck, 2011). Thrombus generation can be assessed by evaluation of the TEG velocity curve. The decreases in both MRTG and TMRTG observed in this study indicate longer time required for thrombus generation. In addition, the total amount of thrombus generated decreased over time. All of these calculated values are markers of procoagulant activity and indicate depletion of substrates required for thrombus generation.

All four horses showed a sharp decrease in AT activity towards the end of the disease course, indicative of consumption of coagulation inhibitors. Antithrombin is the primary anticoagulant in the body, comprising more than 70% of total anticoagulant activity, and inactivates thrombin and other coagulation factors (Bone, 1992; Green, 1988). Normally, AT inhibits overactivation of coagulation by inhibiting thrombin and prevents the generation of microthrombi (Iba and Saitoh, 2014). Antithrombin is a negative APP and during severe inflammation a decrease in AT concentrations occurs due to decreased production, and the degradation of AT by elastase secreted from activated neutrophils. Decreases in AT activity during inflammation may also be as a result of AT binding to receptors on the endothelial glycocalyx to modulate the inflammatory response of endothelial cells and leukocytes (Schlommer et al., 2021). However, as

severe inflammation was not observed in these horses, inflammation may have contributed somewhat to the observed decrease in AT activity but it is more likely that activation of haemostatic pathways and the continuous generation of thrombin resulted in increased consumption of AT which was most evident shortly before humane euthanasia (Levi et al., 2004). Additionally, in disease states with increased vascular permeability such as sepsis, extravasation of AT with subsequent cleavage has been described (Levy et al., 2016) This may be a factor in AHS, a disease that is clinically characterized by endothelial damage and extravasation of fluids into the tissues.

The presence of excessive fibrinolysis was evident in all four horses based on the sharp increase of D-dimer concentration at the end of the disease course. Fibrinolysis is initiated simultaneously with haemostasis to prevent excessive clot formation that could result in thrombosis. Plasminogen is converted to plasmin by tPA and uPA, and plasmin then cleaves fibrin at lysin residues (Longstaff and Kolev, 2015). The end-products of fibrinolysis are FDPs and D-dimer (May et al., 2021; Weitz et al., 2017). D-dimer is a more sensitive indicator of fibrinolysis because it is the breakdown product of lysis of crosslinked fibrin mediated by plasmin (Stokol et al., 2005). In horses, a cut-off value of 1000 ng/mL has been proposed for the diagnosis of DIC (Stokol et al., 2005) but sensitivity was only 50%; however, simultaneous evaluation of aPTT and AT allowed for identification of horses with a poorer prognosis. In the present study, D-dimer concentration increased to concentrations >2000 ng/mL in all horses in the end, indicating the presence of excessive fibrinolysis. Increased D-dimer concentrations have been observed in horses with gastrointestinal disease and severe systemic inflammation as well as in horses with thrombotic gastrointestinal disease

(Cesarini et al., 2010, 2014; Honore et al., 2022). This finding has been confirmed in horses with colic and related to diagnosis and outcome with poorer prognosis being associated with higher concentrations of D-dimer (Cesarini et al., 2016; Cesarini et al., 2010; Nikvand et al., 2019). Similarly, in foals with colic, activation of haemostasis and fibrinolysis leading to increased plasma D-dimer was also observed (Watts et al., 2011). Increased plasma D-dimer concentrations have also been reported in foals with sepsis, correlating with DIC noted on coagulation tests of these foals (Armengou et al., 2008). During experimental infection with equine herpes virus type 1, a virus that causes vasculitis of the endothelium, D-dimer concentration increased during viraemia, indicating activation of haemostasis and fibrinolysis (Goehring et al., 2013).

Although the D-dimer concentration increased significantly during the final stages of the disease process, excessive fibrinolysis could not be detected based on the TEG variables associated with fibrinolysis, such as increased LY30 and LY60 and decreased CL30 and CL60. It has been shown that exogenous activators, such as kaolin, overactivate the coagulation system, which may inhibit TEG sensitivity to fibrinolysis (Genet et al., 2012). In fact, it is recommended to add tPA to the TEG assay to improve the sensitivity for measuring fibrinolysis (Moore et al., 2017). It has also been reported in humans that traditional biomarkers for fibrinolysis, such as the plasmin α_2-AP complex, are more sensitive to detect fibrinolytic activation as TEG only detects fibrinolysis when tPA concentrations are fivefold increased (Raza et al., 2013). In addition, a false negative detection rate of fibrinolysis may occur due to rapid inhibition of tPA by PAI-1 (Leebeek and Rijken, 2015; Raza et al., 2013).

The haemostatic changes observed in these four horses fulfil the requirements for the diagnosis of DIC according to the ISTH guidelines, namely procoagulant activation, inhibitor consumption and excessive fibrinolysis. The associated bleeding tendencies that were observed clinically during the late stages of the disease further allow for the diagnosis of overt DIC. Previous work in people has shown that due to the dynamic nature of coagulopathies, TEG profiles can change over time with progressing DIC and that hypocoagulability is correlated with increased mortality risk (Tsantes et al., 2023). Histopathological examination of the spleen, the lung and the heart did not reveal any microthrombi, further confirming the presence of the overt form of DIC. This contradiction between haemostatic tests indicating DIC and the absence of thrombi on histopathological examination has previously been observed in humans (Katsumura and Ohtsubo, 1999; Wilde et al., 1988) but also been confirmed in horses diagnosed with DIC based on clinical and laboratory findings where no fibrin microthrombi were detected in post mortem tissues (Cesarini et al., 2016). It is important to identify the development of DIC early on in the disease process, as early intervention and treatment may improve therapy and outcome (Cesarini et al., 2014; Dolente et al., 2002). However, in our study, the horses infected with AHSV only developed pronounced changes of DIC during the later stages of the disease, making early diagnosis and intervention difficult.

Inflammation is closely intertwined with haemostasis and in many inflammatory diseases, haemostatic derangements occur as a result of inflammatory activation of haemostatic pathways via expression of TF on circulating immune cells (Foley and Conway, 2016b). However, an acute proinflammatory immune response was not detected in the horses in this study. It is therefore unlikely that proinflammatory

cytokines caused inordinate expression of TF on endothelial cells, macrophages, and monocytes leading to excessive thrombin formation. Consequently, it is likely that the severe endothelial damage that AHSV causes during its replication phase in the endothelium (Brown et al., 1994; Gomez-Villamandos et al., 1999; Laegreid et al., 1992b), with subsequent exposure of collagen and release of vWF in combination with platelet activation, resulted in activation of the haemostatic pathways (Neubauer and Zieger, 2022) and resulted in the development of DIC. Damage to the endothelium also results in dysfunction and imbalance of its anticoagulant properties which further exacerbates haemostatic derangements. While light microscopy of tissue from these horses did not reveal any endothelial changes other than mild to moderate endothelial cell swelling, possible alterations to the interendothelial junctions resulting in exposure of vWF could have been present and elucidated using electron microscopy. Other viruses, such as the BTV (Maclachlan et al., 2009), elephant endotheliotropic herpesvirus in Asian elephants (Guntawang et al., 2021), Dengue virus in humans (Jessie et al., 2004; Povoa et al., 2014), or Ebola and Marburg virus (Geisbert et al., 2003; Martines et al., 2015) are known to cause endothelial damage with haemostatic derangements making this a likely pathophysiology in AHS.

In conclusion, horses infected with AHSV develop severe overt consumptive coagulopathy with bleeding tendencies with plasma-based coagulation assays being more useful diagnostic tools compared to viscoelastic assays, especially in earlier stages of the disease process as platelet activation compensates for coagulation factor depletion. Activation of the haemostatic pathways resulting in DIC is likely caused by viral replication damaging the vascular endothelium, while inflammatory cytokines may play a secondary role. Further investigation of these pathways may

lead to a better understanding of the pathology and allow for earlier targeted intervention to prevent the progression into severe DIC resulting in multiorgan dysfunction and death in horses infected with AHS.

7 CHAPTER 7: GENERAL DISCUSSION

In sub-Saharan Africa, AHS is an important infectious viral disease in horses transmitted by arthropod vectors, especially *Culicoides* midges (Mellor et al., 2000). It is caused by the AHSV, a double-stranded RNA virus from the family of *Sedoreoviridae* (Calisher and Mertens, 1998; Matthijnssens et al., 2022). Since the first description of the three clinical forms in 1921 (Theiler, 1921), specifically the pulmonary ("dunkop"), cardiac ("dikkop") and fever forms, a fourth form with signs of the pulmonary and the cardiac form has been described (Coetzer and Guthrie, 2004). Infection of naïve horses results in mortality rates of up to 100% (Mellor and Hamblin, 2004b).

Despite the severe impact that the virus has both on the local economy as well as horse welfare (Vandenbergh, 2010) and the threat of incursion of AHSV into other territories (Schliewert, 2021), many details of the pathogenesis of AHS are still not understood. Following viraemia, the virus exhibits organotropism, particularly to the lungs, the heart, and the spleen (Coetzer and Guthrie, 2004). The virus replicates in endothelial cells, pulmonary intravascular macrophages, interstitial macrophages, and fibroblasts and damages these, this results in vascular leakage and the typical clinical signs of AHS (Carrasco et al., 1999; Gomez-Villamandos et al., 1999).

Chapter 1 provides background information on AHSV, specifically the viral composition, infection and replication, details on the clinical signs caused by infection, diagnostic tests and prevention and the known details of the pathogenesis of AHSV. While some haematological changes including leukopenia, thrombocytopenia, and increases in erythrocyte and HGB concentrations that occur during AHS have been

described previously (Skowronek et al., 1995), markers of platelet activation usually arising as a consequence of inflammatory mediators or activation of prothrombotic factors (Yun et al., 2016) have not been evaluated. Similarly, concentrations of APR such as SAA or serum iron, which change during inflammation, and have diagnostic and prognostic importance in other diseases (Long and Nolen-Walston, 2020), have not been evaluated during AHS. The study described in **Chapter 4** included four horses that were tested negative for antibodies against all 9 AHSV serotypes and infected with AHSV as part of a vaccine trial. Blood was obtained throughout the course of the disease until the horses developed severe clinical signs consistent with the pulmonary form of AHS and were humanely euthanized. Analysis revealed a significant increase in median HCT, as well as significant changes in the median WBC count, monocyte count, eosinophil count, and myeloperoxidase index in all horses over time. All horses developed marked thrombocytopenia and significant changes in markers of platelet activation were observed. However, the changes in leukocyte concentration, without a significant change in neutrophil concentration, as well as the changes in the SAA and iron concentrations, both of which are induced by proinflammatory cytokines IL-1, IL-6, and TNF-α (Crisman et al., 2008), were delayed (for SAA) and surprisingly mild considering the severity of clinical signs. The reasons for this blunted inflammatory response are unclear but we hypothesized that they may be caused by viral mechanisms to evade the innate immune system's response as has been shown for other viruses (Alcami, 2016) but more recently also been hypothesized for BTV (Rojas et al., 2021) and AHS (Wall et al., 2021). Detection of a pathogen via PRR triggers inflammatory pathways results in cytokine secretion to orchestrate the immune response. This leads to synthesis of APR and activates immune cells with the goal to eliminate the pathogen. Changes in selected cytokines

were examined in **Chapter 5** to further elucidate the host immune response. Changes in concentrations of a selection of cytokines commonly considered proinflammatory (IFN-γ, IL-1α, IL-2, IL-6, IL-8, IL-12, IL-17, TNF-α, and MCP-1) and the major anti-inflammatory cytokine, IL-10, were examined. Previous research in horses has shown that infectious stimuli result in increases in serum IL-1 and TNF-α (Cudmore et al., 2013; Holcombe et al., 2016; Morris et al., 2018), as well as increases in IL-6 (Ratinier et al., 2011) and IL-10 (Pusterla et al., 2006), which are described as prognosticators for nonsurvival. Substantial changes in cytokine concentration in AHSV-infected horses in were only observed in TFN-α and IL-10 while all other tested cytokines either remained under the LLOD or only displayed minor increases that were inconsistent over time and between horses. This finding of a nearly complete absence of a proinflammatory blood cytokine response during severe clinical disease was in agreement with the findings of **Chapter 4** and suggested interference of the AHSV with the innate immune response. In particular, the lack of an IFN-γ response indicates a possible virus evasion mechanism. African horse sickness has been reported to interfere with JAK/STAT signalling (Wall et al., 2021); an important pathway for initiation of the innate immune response. African horse sickness may further interfere with PML-NBs which are involved in the antiviral innate immune defence and the regulation of IFN-γ signalling (Boughan et al., 2020). These reports suggest possible methods that AHSV employs to evade the innate immune response and eventually overwhelm the host immune system.

Severe clinical signs such as subcutaneous oedema, vascular extravasation, and haemorrhages were reported on necropsy in horses infected with the AHSV (Newsholme, 1983). Bleeding tendencies along with increases in PT, aPTT, TT, and

FDPs have been reported (Skowronek et al., 1995), along with the severe thrombocytopenia which was also observed in the horses infected with AHSV in this study and described in **Chapter 4** suggesting coagulopathy. Haemostasis is a multifaceted process that is tightly controlled to prevent blood from clotting within the vessels. A fine balance between initiation, amplification, and propagation, the three stages of haemostasis, resulting in the production of thrombus formation of a stable clot and fibrinolysis, is required. Haemostasis is not only the product of the interaction of the various enzymes and molecules of the traditional coagulation cascade, but rather influenced greatly by trauma and inflammation. Exaggerated expression and increased exposure of TF to activated platelets is a known trigger for the progression into a prothrombotic state and once inhibitory regulatory factors become overwhelmed, may progress to DIC. Given the clinical findings in horses with AHS as well as the previous knowledge on changes in plasma-based coagulation tests, a detailed evaluation of the derangements evaluating plasma-based coagulation tests, coagulation factors, and thromboelastography is described in **Chapter 6.** Furthermore, post-mortem tissue sections were evaluated for evidence of fibrin. All horses displayed prolonged bleeding post venepuncture in the later stages of the disease and multifocal mucosal haemorrhage was observed. During the course of the disease marked hypocoagulation was evident in the plasma-based coagulation tests, specifically a substantial increase in PT, and PTT while increased D-dimer concentration indicated fibrinolysis. Simultaneously, decrease in activities of coagulation factors II, VII, X, and XII was evident, with the lowest activities measured at the time of euthanasia. Assessment of TEG revealed phases of hyper – and hypocoagulation throughout the course of the disease. Despite obvious development of a consumptive coagulopathy with haemorrhagic tendencies consistent with overt DIC, based on clinical findings and

laboratory tests (Taylor et al., 2001; Wiinberg et al., 2010), histopathological examination revealed no microthrombi. Due to the low concentration of proinflammatory cytokines in the blood, it is unlikely that the cytokines observed caused excessive expression of TF on endothelial cells, macrophages, and monocytes, resulting in exorbitant thrombin production. The inciting trigger for the development of DIC is suspected to be severe endothelial damage caused by virus replication and egression leading to exposure of TF (Brown et al., 1994; Gomez-Villamandos et al., 1999; Laegreid et al., 1992b) as well as the platelet activation described in chapter 4. However, the concentration of tissue cytokines and their potential effect on haemostasis, were not investigated.

The main limitation of this study is the small number of horses included. The horses included were essential for a virulence study which was a prerequisite for a vaccine trial. Due to the high mortality in naïve horses infected with AHSV, it was deemed unethical to consider more horses. While all horses were infected with a different AHSV serotype, it has previously been demonstrated that virulence of the specific variant of the serotype is more important than the serotype itself. However, all observations were similar between all four horses, making it unlikely that the course of the disease was significantly affected by the different serotypes that were used in this study. A limitation specific for the cytokine analysis was that the ideal timepoint for cytokine measurement remains unknown. A decision to increase sampling frequency once horses tested positive for AHSV on PCR was made, however, due to the commonly short-lived secretion and variable half-life of different cytokines (Liu et al., 2021), it is possible that peak concentrations were missed.

In conclusion, this study contributes to the knowledge of the host inflammatory response to AHSV and the possible mechanisms of viral interference with the host's innate immune response. It further elucidates the haemostatic derangements likely caused by viral replication in the vascular endothelium and possibly the inflammatory response and highlights the development of overt DIC with consumption of coagulation factors.

8 CHAPTER 8: CONCLUSIONS

Four horses were experimentally infected with AHSV and developed severe clinical signs consistent with AHS. Assessing haemostatic changes throughout the course of the disease, inflammatory changes represented by changes in WBC, SAA and iron were unexpectedly mild. This suggested possible downregulation of the innate immune response by viral mechanisms. Severe thrombocytopenia with significant platelet activation indicated activation of the haemostatic system.

Interference of the virus with the innate immune response was further demonstrated by the almost complete absence of a detectable proinflammatory blood cytokine response despite severe clinical disease. These results suggested that AHSV is capable of interfering with the innate immune response, possibly via interference with the JAK/STAT pathways or PML-NBs. This may explain the high morbidity and mortality of AHS in naïve horses.

Investigation of the haemostatic derangement occurring during AHS revealed development of overt DIC with coagulation factor consumption in all horses. Despite development of severe thrombocytopenia and depletion of coagulation factors throughout the course of the disease, clinical bleeding tendencies were only moderate, suggesting that platelet activation compensated for some of the deficiencies. Endothelial cell injury caused directly by the virus is suspected to be the main activator of the haemostatic system through exposure of TF to the bloodstream promoting haemostasis, proinflammatory cytokines inducing TF expression on intravascular cells

and monocytes likely only play a secondary role as increases in the concentration of TNF could only be observed in the late stages of the disease.

9 CHAPTER 9: FUTURE STUDIES

The results of this research pose further questions about the pathogenesis of the AHSV. As it has now been demonstrated that AHSV seems to interfere with the innate immune response, future research projects should aim to further investigate the exact pathways that the AHSV targets. Similar studies assessing the JAK/STAT pathways have been performed in cell culture for BTV, translation of these setups to AHSV should be possible. This approach has the benefit that no animal sacrifice is required to gain further insight into the immune response pathways before attempting to translate the result back into the animal model.

As IFN seems to be a target for viral interference, the possible therapeutic benefits of immune response modifiers such as imidiquod should be investigated. To date, systemic pharmacokinetics of these drugs have not been established in horses but in light of the potential benefit of these drugs, evaluation of these parameters may be justified.

Further research should also be aimed at the pathogenesis of the AHSV with an emphasis on endothelial damage caused by the virus. Endothelial injury is known to result in degradation of the glycocalyx, with resulting vascular hyperpermeability and consequentially tissue oedema, alterations of vasomotor tone regulation, derangements in leukocyte adhesion, and intravascular coagulation. To further elucidate the progression and severity of endothelial damage throughout the disease, presence and quantification of glycocalyx components in the blood can be determined

and potentially used as a prognosticator or to evaluate the success of therapeutic interventions.

To date, a reliable experimental infection model more closely mimicking natural infection i.e., intracutaneous deposition of the virus similar to the biting act of a vector, has not been established. As the local immune response may play an important role in the host defence, this would be an important step. Determination of the tissue cytokine concentrations, either in the target organs or the skin as the primary entry point for infection may be useful to further elucidate the host immune response. Similarly, future studies should evaluate the cytokine response in horses with natural infections to determine if differences in the immune response are present. Another important step in further determining possible protective cytokine patterns would be to assess the cytokine response in African donkeys or zebras, equids that do not usually develop clinic signs of AHS after infection with AHSV.

10 CHAPTER 10: REFERENCES

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11 CHAPTER 11: APPENDICES

11.1 ARRIVE guidelines

Table 11-1: ARRIVE guidelines
The ARRIVE guidelines include a list of information for inclusion in publications that describe in vivo experiments (Percie du Sert et al., 2020).

Item	Recommendation	Section/line number, or reason for not reporting
Study design	For each experiment, provide brief details of study design including: 1. The groups being compared, including control groups. If no control group has been used, the rationale should be stated. 2. The experimental unit (e.g., a single animal, litter, or cage of animals).	See Materials and methods section, chapter 3.3 1. No control group was included in the study. Each subject served as its own control. Furthermore, some results obtained were compared to established laboratory reference intervals. 2. Single animal
Sample size	1. Specify the exact number of experimental units allocated to each group, and the total number in each experiment. Also indicate the total number of animals used.	See Materials and methods section, chapter 3.1 1. Four horses were included. 2. The number of horses was based on the availability of horses enrolled in the virulence study. No sample

	2. Explain how the sample size was decided. Provide details of any *a priori* sample size calculation, if done.	size calculations were performed as the primary goal was to describe changes over time.
Inclusion and exclusion criteria	1. Describe any criteria used for including and excluding animals (or experimental units) during the experiment, and data points during the analysis. Specify if these criteria were established *a priori.* If no criteria were set, state this explicitly. 2. For each experimental group, report any animals, experimental units or data points not included in the analysis and explain why. If there were no exclusions, state so. 3. For each analysis, report the exact value of *n* in each experimental group.	See Materials and methods section, chapter 3.2 1. Chapter 3.2, criteria were established prior to inclusion of horses into the study. 2. Not all samples collected were examined in this study. All blood and tissue samples were also processed and stored and have been or will be used for multiple related studies on the pathogenesis of African Horse Sickness. 3. Chapter 3.2, 4 horses were included in all time points until euthanasia.
Randomisation	1. State whether randomisation was used to allocate experimental units to control and	See Materials and methods section, Chapter 3.4 1. No control group as each candidate served as its own control and some results obtained were

	treatment groups. If done, provide the method used to generate the randomisation sequence. 2. Describe the strategy used to minimise potential confounders such as the order of treatments and measurements, or animal/cage location. If confounders were not controlled, state this explicitly.	compared to established laboratory reference intervals. 2. Chapter 3.4, samples were always obtained in the same order.
Blinding	Describe who was aware of the group allocation at the different stages of the experiment (during the allocation, the conduct of the experiment, the outcome assessment, and the data analysis).	All experimental procedures were nonblinded.
Outcome measures	1. Clearly define all outcome measures assessed (e.g., cell death, molecular markers, or behavioural changes). 2. For hypothesis-testing studies, specify the primary outcome measure, i.e., the outcome measure that was used to determine the sample size.	See Materials and methods section Chapter 3.3.2 1. Chapter 3.3.2, horses were regularly evaluated by a veterinarian for any abnormalities. Horses that developed unbearable discomfort, including severe dyspnea, colic, dehydration, or any other condition, related or unrelated to the challenge, were humanely euthanized.

		2. Chapter 3.2, the number of horses was based on the availability of horses enrolled in the virulence study. No sample size calculations were performed as the primary goal was to describe changes over time.
Experimental animals	1. Provide details of the statistical methods used for each analysis, including software used. 2. Describe any methods used to assess whether the data met the assumptions of the statistical approach, and what was done if the assumptions were not met.	See Chapters 4.2.4, 5.2.4, 6.2.5 1. Chapters 4.2.4, 5.2.4, 6.2.5 describe the statistical methods in detail.
Experimental procedures	For each experimental group, including controls, describe the procedures in enough detail to allow others to replicate them, including: 1. What was done, how it was done and what was used. 2. When and how often. 3. Where (including detail of any acclimatisation periods).	See Materials and methods section Chapter 3.4 1. Chapter 3.3.1 describe the experimental infection, chapter 3.4 the sampling process and timing, chapters 3.5-3.11 describe the diagnostic tests performed in this study. 2. Chapter 3.4 describe the sampling times. 3. Chapter 3.2.1, horses were moved into the stables 14 days prior to the experiment.

	4. Why (provide rationale for procedures).	4. Chapter 1, samples were obtained to gain better insight into the pathophysiological changes occurring during experimental infection with African Horse Sickness.
Results	For each experiment conducted, including independent replications, report: 1. Summary/descriptive statistics for each experimental group, with a measure of variability where applicable (e.g., mean and SD, or median and range). 2. If applicable, the effect size with a confidence interval.	See Chapters 4.3, 5.3, 6.3 for detailed summary and statistics

11.2 Journal publication of work directly related to this thesis:

Schliewert EC, Hooijberg EH, Steyn JS, Potgieter C, Fosgate GT, Goddard A. Experimental infection with African Horse Sickness Virus in horses induces only mild temporal hematologic changes and acute phase reactant response. Am J Vet Res. 2022 Oct 13;83(11):1-11. doi: 10.2460/ajvr.22.08.0123. PMID: 36215210.

AJVR

AVMA

Experimental infection with African Horse Sickness Virus in horses induces only mild temporal hematologic changes and acute phase reactant response

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OBJECTIVE
African Horse Sickness (AHS) is a vector-borne disease endemic to sub-Saharan Africa caused by African Horse Sickness Virus (AHVS). Infections in naïve horses have high morbidity and mortality rates. AHS pathogenesis is not well understood; neither the hematologic changes nor acute phase response occurring during infection has been fully evaluated. The study's objective was to characterize the hematologic changes and acute phase response during experimental infection with AHSV.

ANIMALS
4 horses negative for AHSV group-specific antibodies.

PROCEDURES
In this prospective, longitudinal study conducted between November 23 and December 2, 2020, horses were experimentally infected with AHSV, and blood samples were obtained before inoculation and then every 12 hours until euthanasia. Hematologic changes and changes for serum amyloid A (SAA) and iron concentration were evaluated over time using a general linear model including natural logarithm of sampling time.

RESULTS
All horses were humanely euthanized due to severe clinical signs typical of AHS. Median Hct increased significantly, and the median WBC count, monocyte count, eosinophil count, and myeloperoxidase index changed significantly in all horses over time. Horses developed marked thrombocytopenia (median, 48 X 10^3 cells/μL; range, 21 X 10^3 to 58 X 10^3 cells/μL) while markers of platelet activation also changed significantly. Median SAA increased and serum iron concentration decreased significantly over time.

CLINICAL RELEVANCE
Results indicated severe thrombocytopenia with platelet activation occurs during infection with AHSV. Changes in acute phase reactants SAA and iron, while significant, were unexpectedly mild and might not be useful clinical markers.

African horse sickness (AHS) is an infectious disease with high mortality rates in affected equines that is caused by AHS Virus (AHVS). AHVS is a double-stranded RNA Orbivirus of the Reoviridae family and has 9 different serotypes.[1] The virus is transmitted by an insect vector, a *Culicoides* midge (*Culicoides imicola*), mainly during late summer and fall.

African horse sickness was first described by Sir Arnold Theiler in 1921 and classified into 3 forms: pulmonary ("dunkop"), cardiac ("dikkop"), and fever.[2] A fourth "mixed" form with clinical signs of both cardiac and pulmonary forms was later described.[3] While the detailed pathogenesis of AHS remains unknown, evidence of endothelial cell damage and loss of endothelial cell barrier function resulting in edema, effusion, and hemorrhage caused by the virus has been observed on necropsy and histopathology,[4,5] and virus or viral antigen is commonly found in microvascular endothelial cells.[6]

Hematologic changes that occur during AHS, including leukopenia, thrombocytopenia, and increased erythrocyte counts and hemoglobin concentration (hemoconcentration) have previously been described.[7] Variables reflecting platelet indices recognized as surrogate markers of platelet activation, such as mean platelet volume (MPV), mean

platelet component (MPC), and mean platelet mass (MPM), have not been evaluated in AHS. In veterinary medicine, these variables have been demonstrated to be useful markers of platelet activation in dogs infected with canine parvovirus[8] and *Babesia rossi*.[9] Changes in these platelet indices indicating thrombopoiesis and platelet activation have also been described in human diseases caused by endotheliotropic viruses where they have been reported as useful tools for diagnostic and prognostic purposes and might also be of use in AHS.

Infection with a pathogen generally results in an inflammatory response, with changes in the concentration of acute phase reactants (APRs), such as iron, and acute phase proteins (APPs), such as serum amyloid A (SAA) and fibrinogen.[10] Serum amyloid A, a positive major APP, has become the APP of choice for detection of inflammation in equines over the last decade.[11] In contrast, serum iron is a negative APR and concentrations decrease during systemic inflammation, in order to limit iron availability for pathogen metabolism.[12]

While there are limited reports on some of the hematologic changes observed in horses with AHS,[7] changes in neither platelet indices nor concentrations of APRs like SAA and iron have been evaluated to date. A better understanding of the hematologic changes and the dynamics of the APRs will improve our understanding of AHS and possibly allow for future identification of prognostic markers or therapeutics. The study's objective was to characterize the hematologic changes and acute phase response during experimental infection with AHSV.

We hypothesized that horses would develop leukopenia and thrombocytopenia with concurrent platelet activation and that SAA concentrations would increase markedly after infection while serum iron would decrease.

Materials and Methods

This was a prospective, longitudinal, experimental study that included 4 AHS-susceptible Boerperd cross horses and took place from November 23 to December 2, 2020. Research and animal ethics approval was granted by the Research Ethics Committee of the Faculty of Veterinary Science and Animal Ethics Committee of the University of Pretoria (REC 19-195). As this study involved the use of experimental animals, the study design and reporting of results were carried out in line with Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.[15]

Animals

Horses were obtained from a breeding facility and included in an independent AHSV virulence study required for a vaccine trial conducted by a pharmaceutical company (Deltamune [Pty] Ltd). To evaluate the efficacy of potential candidate vaccines, it is often necessary to conduct animal experiments, because no in vitro model is available. Infection with passaged AHSV subtypes commonly only results in mild clinical signs due to loss of virulence as a result of repeated passaging during the tissue culture process. Therefore, it is necessary to establish the serovars' virulence before using them in a clinical vaccine trial. The 4 horses used in our study were required to test the virulence of 4 strains of virulent field AHSV isolates (1 horse per serovar) which were available at the time. With the scarcity of knowledge on the detailed pathophysiologic changes caused by AHSV and the importance of AHS in South Africa, blood samples were obtained from these experimentally infected horses to reduce the number of animals sacrificed for future research purposes and maximize information gained from this sacrifice. All samples obtained are stored in a biobank to allow for inclusion in further research.

Horses were moved into individual stalls in a Bio-Safety Lab-2+ vector-free facility 14 days before commencement of the study to allow adaptation to the environment. Before infection, all the horses tested negative for AHSV group-specific antibodies using a commercial competitive ELISA (INgezim AHSV compac plus; Eurofins Technologies) against all 9 AHSV serotypes. Peripheral blood smears were examined by the principal investigator (ECS) and veterinarian on site (SS) for the presence of blood-borne parasites, specifically piroplasms. All 4 horses were also treated with imidocarb (2 mg/kg, IM) and glycopyrrolate (0.0025 mg/kg, IV) 36 hours after inoculation when solitary piroplasms were observed on blood smears.

Horses were fed *Eragrostis* hay-free choice and supplemented with a small amount of commercial concentrate product twice daily. Horses were allowed free access to water. Stalls were cleaned twice daily.

Physical examination and observation of attitude and appetite were performed twice daily on all horses. During the trial, horses were monitored regularly allowing for veterinary intervention should any abnormalities be observed. Horses that developed unbearable discomfort, including severe dyspnea, colic, dehydration, or any other condition, related or unrelated to the challenge, were humanely euthanized with sodium pentobarbital (200 mg/kg, IV). All euthanized animals were subjected to necropsy.

Experimental infection

Horses were inoculated IV with 5 mL of low passage mouse brain suspension, containing at least 10^9 mouse infective doses/mL of virulent AHSV serotypes. As different serovars were assessed for virulence during the vaccine trial, each horse was infected with a different AHS serovar: horse 1, AHSV-2 (horse origin); horse 2, AHSV-4 (horse origin); horse 3, AHSV-6 (horse origin); and horse 4, AHSV-6 (dog origin).

Sampling procedures

Blood was collected from each horse in the same order before inoculation (time of infection [time 0]) with AHSV and then every 12 hours afterward until the horses tested positive for AHS using a pan AHSV real-time (RT) PCR

assay. RT-PCR assay for AHSV was performed daily for each horse, then twice daily once horses became febrile until viremia was confirmed using a method previously described.[14] Briefly, the primers (F-pan-S4: TTAGGATGGAACCTTACGC and R-pan-S4: ATTCTGCCCCTCTCTAACCA) and probe (P-pan-S4: FAM-CTTTGAGTAGGTATTCGATCTCCTGCG-BBQ) used for testing were synthesized by Eurogentec and TIBMOLBIOL, respectively. RT-PCR assays (20 µL final volume) consisted of 800 nM primers each, 300 nM of probe, 2 mM MnO_2, 7.5 µL RT-PCR mix (Roche LC480 RNA Master HybProbes; Roche), and 5 µL of purified RNA. RT-PCR assay was performed in the LightCycler Nano (Roche): 98 °C for 20 seconds (dsRNA denaturation), 55 °C for 20 s and 61 °C for 10 min (reverse transcription), 95 °C for 30 seconds, and 40 cycles of 95 °C for 10 seconds, 55 °C for 10 seconds and 61 °C for 30 seconds (predenaturation, reverse transcription, target amplification). Purified genomic dsRNA (double-stranded RNA) extracted from cells infected with AHSV 32/62 (the OIE reference strain of AHSV4) was used as a positive control. Quantification cycle values of 37 to 39 were considered suspect, and quantification cycle values lower than 37 were positive. Once positive on RT-PCR assay, samples were obtained every 2 hours for 12 hours, followed by every 4 hours for another 12 hours. After this, 12-hour sampling intervals were reinstituted. Sample acquisition was performed by venipuncture from the jugular vein directly into vacutainer tubes (Becton, Dickinson and Company), and veins were alternated between samples. Blood was collected in specific sequence, through vacuum assistance, into a serum tube (4 mL), a 3.2% sodium citrate tube (4 mL), a heparin tube (4 mL), and an EDTA tube (4 mL).

Diagnostic tests

Hematology was performed on all 12-hourly EDTA blood samples within 2 hours of sampling and samples were kept at 8 °C prior to analysis. The samples were evaluated on an automated hematology analyzer[15-17] (ADVIA 2120i; Siemens) with manual blood smear evaluation. Evaluated erythrocyte variables included the following: RBC count (X 10^6 cells/µL), hemoglobin (HGB; g/L), Hct (%), MCV (fl), MCH (pg), MCHC (g/L), and mean of the optically measured HGB concentration in cells (CHCM; g/L). A manual 100-cell leukocyte differential count was performed, and the resulting percentages were used to calculate absolute leukocyte numbers that were derived from the automated WBC count. Leukocyte variables included concentrations of WBC (X 10^3 cells/µL), absolute mature neutrophils (X 10^3 cells/µL), band neutrophils (X 10^3 cells/µL), lymphocytes (X 10^3 cells/µL), monocytes (X 10^3 cells/µL), and eosinophils (X 10^3 cells/µL), and basophils (X 10^3 cells/µL) and myeloperoxidase index (MPXI). Finally, platelet variables included platelet concentration (PLT; X 10^3 cells/µL), plateletcrit (PCT; %), MPV (fL), platelet distribution width (PDW; %), MPC (g/dL), and MPM (pg).

Blood smears were further evaluated for the presence of intraerythrocytic parasites. If intraerythrocytic parasites were observed, the percentage of infected cells was determined: blood smears were evaluated along both slide edges and the center of the feathered edge. A minimum of 2,500 single-layered erythrocytes were evaluated per slide, and the percentage of erythrocytes containing parasites was calculated. The presence of *Theileria equi*, *Babesia caballi*, or both was confirmed using PCR methods previously described.[18]

Serum amyloid A concentrations were measured using the Eiken VET-SAA immunoturbidometric assay[19] (Eiken Co.), and serum iron concentration was measured using the ferrozine method (Iron Gen.2; Roche). Both assays were performed on the Cobas Integra 400 Plus (Cobas Integra 400 Plus; Roche).

Statistical analysis

As the number of horses enrolled was small, changes occurring within a horse postinfection (PI) could not be compared to one another. To determine changes in a variable within a subject over time, baseline values for all clinical pathology measurements were subtracted from all subsequent time points to remove inherent dependencies due to the repeated measures sampling design. Data after subtractions were assessed for normality by plotting histograms, evaluating descriptive statistics, and performing the Anderson-Darling test in commercial software (MINITAB Statistical Software, Release 13.32; Minitab Inc). Data that were not normally distributed were transformed using the natural logarithm or rank transformed when an appropriate transformation function could not be identified. Data were descriptively presented as the median and range per horse and time point. Changes in clinical pathology data over time were determined using a general linear model including natural logarithm of sampling time ("ln time") and "ln time squared". "ln time" allows for evaluation of linear changes over time, while a quadratic change over time ("ln time squared") allows for assessment of significance in values that do not follow a linear trajectory (ie, increase first and then decrease or vice versa). A fixed effect was included for horse (serotype) and interaction terms included between horse and the time variables. Visual assessment was performed to evaluate the direction of the changes over time. Descriptive data were also presented as time before euthanasia because horses were euthanized at different time points after testing positive on PCR assay. Commercial software (IBM SPSS Statistics Version 27; International Business Machines Corp) was used to fit statistical models and significance was set as $P < .05$.

Results

Four Boerperd cross horses (horse A through horse D) were included in the study. There were 3 fillies and 1 colt between 18 and 24 months of age with body weights between 135 and 233 kg.

Table 1—Median (range) results for selected hematologic variables and acute phase reactants for 4 Boerperd cross horses before experimental infection with African Horse Sickness Virus at time 0 (T0); when viremia was first detected (T PCR positive); at 36, 24, and 12 before euthanasia (TE-36, TE-24, and TE-12, respectively); and at the time of euthanasia (TE) between November 23 and December 2, 2020. Statistically significant changes over time or changes varying by serotype are denominated as Yes (present) or No (absent).

Variable	T0	T PCR positive	TE-36	TE-24	TE-12	TE	Changes over time	Time by serotype interaction
RBCs (X 10^3 RBCs/L)	8.51 (7.85–9.85)	8.00 (6.10–8.64)	8.00 (6.10–8.64)	7.10 (6.13–7.97)	8.22 (6.65–9.66)	10.61 (8.18–17.20)	Yes	No
Hemoglobin concentration (g/L)	119 (110–130)	110 (91–106)	98 (92–106)	104 (99–108)	116 (101–131)	150 (114–238)	Yes	No
Hct (%)	34 (33–37)	32 (26–30)	28 (26–30)	30 (28–31)	32 (38–36)	43 (33–73)	Yes	No
MCV (fl)	39.6 (38.0–43.0)	39.3 (37.6–42.6)	38.75 (37.4–42.2)	39.0 (37.1–42.5)	38.5 (37.5–42.4)	41.0 (37.3–42.4)	No	Yes
MCH (pg)	13.5 (13.2–15.3)	13.8 (13.4–14.9)	13.8 (13.3–15.9)	13.7 (13.2–15.2)	14.2 (13.4–15.2)	13.9 (13.4–15.3)	Yes	No
MCHC (g/L)	348 (333–356)	351 (251–356)	356 (351–361)	354 (349–357)	360 (358–376)	352 (328–359)	No	No
CHCM (g/L)	337 (327–338)	338 (334–339)	338 (327–339)	336 (334–339)	336 (333–340)	337 (330–341)	Yes	No
RBC distribution width (%)	19.0 (18.9–19.5)	19.0 (18.0–19.4)	18.7 (18.0–19.3)	18.8 (18.0–19.3)	18.9 (17.8–19.2)	18.9 (17.8–19.2)	Yes	No
WBCs (X 10^3 cells/µL)	11.4 (9.8–12.7)	11.4 (9.5–12.9)	6.7 (5.5–9.0)	6.4 (5.9–8.9)	7.0 (5.6–9.7)	9.3 (7.3–11.5)	Yes	Yes
Mature neutrophils (X 10^3 cells/µL)	6.0 (5.0–6.9)	6.7 (6.3–7.3)	3.6 (2.9–4.9)	3.1 (2.9–3.7)	3.1 (2.7–4.7)	5.7 (4.3–7.1)	No	No
Band neutrophils (X 10^3 cells/µL)	0.0 (0.0–0.0)	0.1 (0.0–0.1)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.1 (0.0–0.1)	No	No
Lymphocytes (X 10^3 cells/µL)	4.2 (3.3–6.3)	4.0 (3.1–5.2)	2.1 (2.0–3.9)	2.7 (2.7–5.0)	2.7 (2.2–4.5)	2.6 (1.3–2.6)	No	No
Monocytes (X 10^3 cells/µL)	0.2 (0.2–0.3)	0.3 (0.1–0.4)	0.2 (0.1–0.3)	0.2 (0.2–0.4)	0.2 (0.1–0.5)	0.3 (0.1–0.4)	Yes	No
Eosinophils (X 10^3 cells/µL)	0.4 (0.1–0.9)	0.1 (0.1–0.4)	0.1 (0.0–0.4)	0.1 (0.0–0.2)	0.0 (0.0–0.1)	0.0 (0.0–0.0)	No	No
Basophils (X 10^3 cells/µL)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.0 (0.0–0.1)	0.0 (0.0–0.1)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	No	No
Myeloperoxidase index	14.4 (11.4–15.9)	12.5 (8.9–16.8)	11.5 (8.5–16.2)	10.1 (6.0–12.6)	10.5 (8.9–12.8)	11.0 (8.9–12.8)	Yes	No
Platelets (X 10^3 cells/µL)	146 (93–257)	131 (37–207)	64 (35–123)	64 (43–107)	58 (64–100)	48 (21–58)	Yes	Yes
Plateletcrit (%)	0.09 (0.13–0.19)	0.06 (0.03–0.14)	0.06 (0.03–0.08)	0.06 (0.03–0.09)	0.05 (0.04–0.07)	0.04 (0.02–0.04)	Yes	Yes
Mean platelet volume (fl)	6.7 (6.5–7.3)	7.1 (6.1–7.9)	9.3 (6.7–10.6)	7.9 (7.3–9.5)	8.0 (7.0–9.7)	8.3 (7.2–8.9)	No	No
Platelet distribution width (%)	21.1 (20.3–22.9)	22.0 (18.8–45.5)	20.4 (14.6–54.1)	19.6 (17.1–19.8)	19.7 (17.2–19.8)	19.3 (17.4–20.6)	Yes	No
Mean platelet component (g/dL)	27.0 (25.4–27.8)	27.3 (23.1–29.1)	23.5 (17.7–28.4)	24.5 (21.5–26.6)	22.3 (17.3–28.3)	22.3 (19.0–22.6)	Yes	No
Mean platelet mass (pg)	1.82 (1.71–1.96)	1.84 (1.75–2.19)	1.96 (1.9–2.28)	2.05 (1.96–2.13)	1.96 (1.86–2.07)	2.11 (1.99–2.22)	Yes	Yes
Serum amyloid A (mg/L)	< 2.0	< 2.0	24.5 (10.5–68.5)	45.9 (17.7–80.3)	74.7 (23.9–92.0)	90.1 (25.8–144.1)	Yes	No
Serum iron (µmol/L)	20.4 (10.4–33.9)	15.2 (10.2–23.7)	7.6 (6.6–12.1)	8.1 (4.9–9.9)	7.8 (5.2–16.2)	11.9 (9.5–12.4)	Yes	No

CHCM = Mean of the optically measured hemoglobin concentration within cells.

Clinical findings

The first febrile event (rectal temperature > 38.5°C) occurred between 36 to 48 hours after inoculation, and the horses first tested positive for AHS on PCR assay from 72 to 96 hours PI. Horses had increased respiratory rates from 152 hours PI with horse A also developing mild swelling of the supraorbital fossae. Clinical signs in all horses were consistent with the pulmonary form of AHS, and all horses were humanely euthanized between 180 to 228 hours PI. Postmortem examination revealed pulmonary edema and multifocal hemorrhages on various mucosal surfaces in all horses.

The *P* values for hematologic and APR variables for "ln time" and "ln time squared" derived from the general linear model signify whether variables changed significantly over the course of infection and whether or not they vary by serotype **(Supplementary Table S1)**.

Hct, HGB, and concentration of RBCs changed significantly over time but did not vary by serotype **(Table 1; Figure 1; Supplementary Figure S1)**. Visually, these variables increased from 168 hours PI in all horses following an initial decrease. Significant changes over time without variation by serotype were also recorded for RDW but no distinct pattern was discernible. For CHCM, changes over time varied by serotype. Visually, a mild increase was noted, with a severe decrease of CHCM in 1 horse. The remaining erythrocyte variables (MCV, MCHC, MCH) did not change significantly over time. On blood smear evaluation, solitary piroplasms were

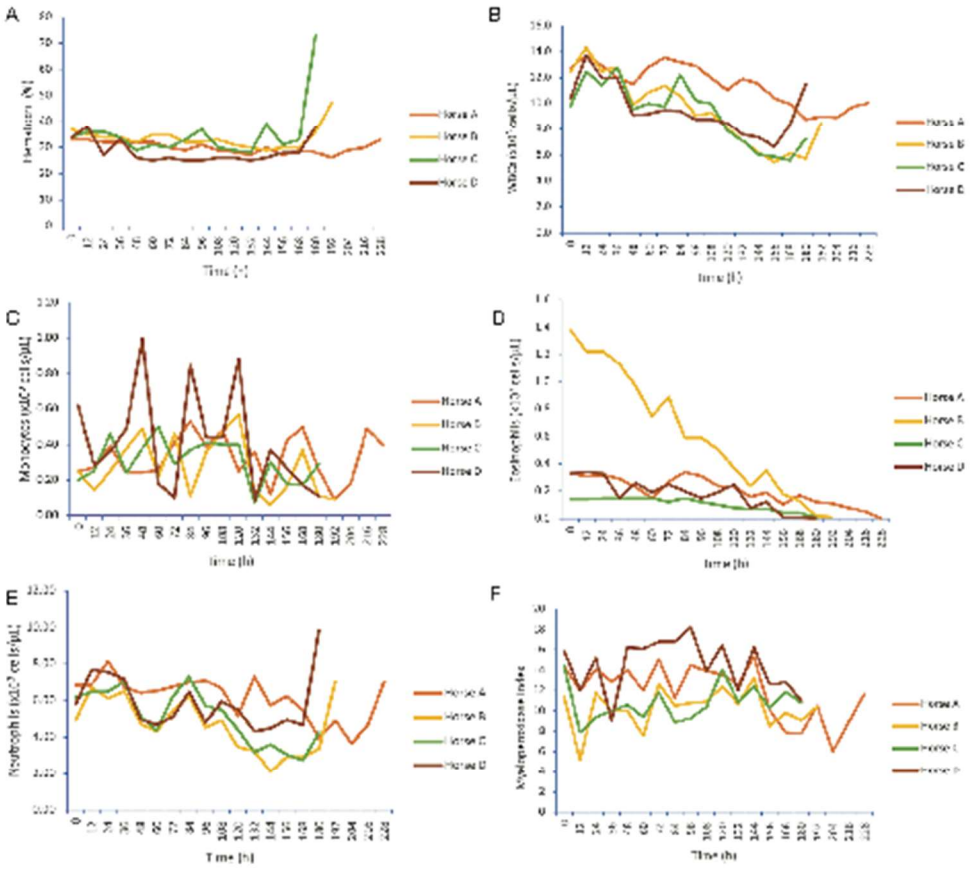

Figure 1—Line diagrams of changes in Hct (*P* = .003; A), WBC count (*P* = .026; B), monocyte count (*P* = .003; C), eosinophil count (*P* = .047; D), neutrophil count (*P* = .661; E), and myeloperoxidase index (*P* = .001; F) in 4 Boerperd cross horses (each represented by a different color) before experimental infection with African Horse Sickness Virus at time 0 and then every 12 hours throughout the course of the disease until euthanasia in a study conducted between November 23 and December 2, 2020.

observed in all horses intermittently; however, the percentage of parasitized RBCs was negligible (maximum 5 piroplasms in 3,481 RBC; < 0.001%). No piroplasms were observed in any horse from 132 hours postinfection onward.

The WBC concentration changed significantly over time and varied by serotype. Upon visual inspection (Table 1; Figure 1), WBC initially decreased and then increased from 156 hours onward, although little change was observed in 1 horse. Significant changes over time were observed in the monocyte and eosinophil concentrations but neither varied by serotype. No pattern was identified upon visual inspection of the monocyte changes, but eosinophils decreased. Although there were no significant changes over time observed for the neutrophil (including band neutrophils) or basophil concentrations, visual inspection of the segmented neutrophils revealed a similar pattern to WBC. The MPXI changed significantly but no variation by serotype was observed. Visually, a mild increase was followed by a decrease in MPXI in 2 horses and in 2 horses MPXI decreased in the final disease phase.

The platelet concentration changed significantly over time and varied by serotype. Pseudothrombocytopenia caused by significant platelet aggregation was not observed. Visual inspection revealed more pronounced decreases in PLT in

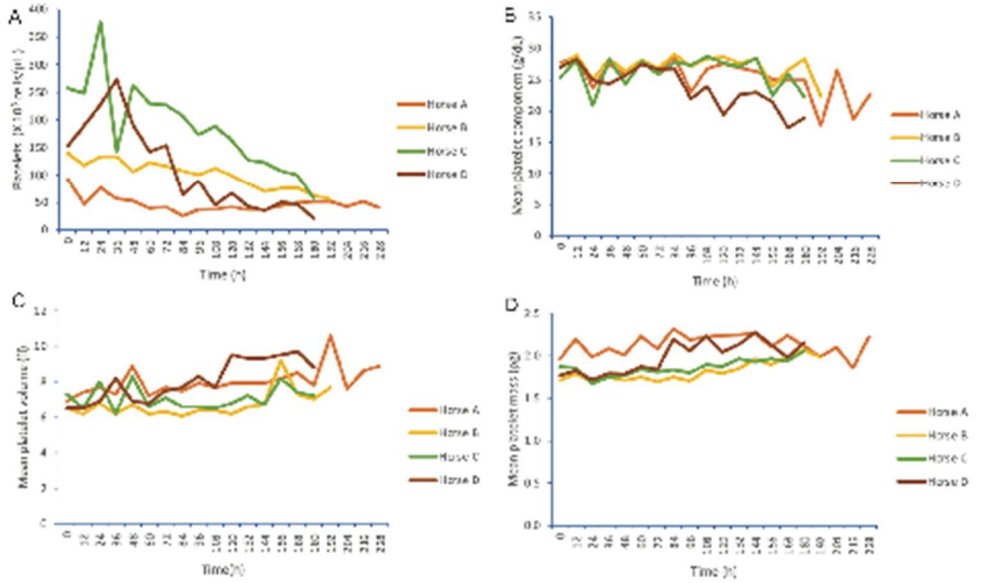

Figure 2—Line diagrams of changes in platelet count (P = .001; A), mean platelet component (P = .030; B), mean platelet volume (P = .021; C), and mean platelet mass (P = .001; D) for the horses described in Figure 1.

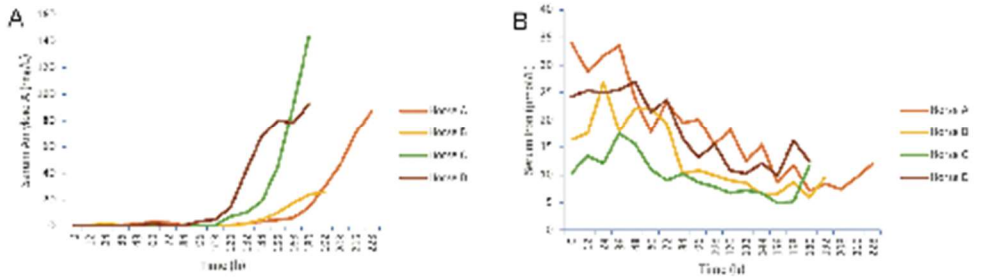

Figure 3—Serum concentrations of acute phase reactant serum amyloid A (P < .001; A) and iron (P = .001; B) in the 4 horses described in Figure 1.

2 horses; PCT changed significantly over time and varied by serotype, and visual inspection revealed a more pronounced decrease in PCT in 2 horses (Table 1; **Figure 2**; Supplementary Figure S1). Platelet distribution width changed significantly over time but was not influenced by serotype. PDW only increased at single time points to then return to baseline values. MPV and MPM also significantly changed over time with changes in MPM varying by serotype. Upon visual inspection, both MPV and MPM increased over time. MPC changed significantly over time but was not influenced by serotype, visually, MPC mildly decreased during the disease course.

Visual inspection of SAA concentrations revealed an increase from 108 h onward with the maximum concentration observed on the final samples, while serum iron concentrations decreased (Table 1; **Figure 3**). Changes over time were significant for both SAA and iron, but changes did not vary by serotype.

Necropsy confirmed typical lesions of AHS in all horses, namely pulmonary edema with frothy fluid in the airways as well as subcutaneous edema in the head and neck, pleural and pericardial effusion, and gastrointestinal petechia and hemorrhage.

Discussion

In this study, horses experimentally infected with AHSV subtypes demonstrated both expected and unexpected changes within the blood. Thrombocytopenia and hemoconcentration were expected and consistent with the observed clinical signs. Increasing platelet activation was observed throughout the course of the disease. Laboratory proxies of inflammation, specifically WBC, SAA, and iron concentrations changed significantly; however, neither variable changed as severely as expected given the clinical picture of the infected horses. This raises the question about possible viral interference with the host's immune response and needs to be investigated further.

All infected horses developed typical signs of acute AHS ("dunkop" form) with high fever and respiratory distress in the late stages of the disease. Typical necropsy findings were also identified; edema and effusion are typically reported in horses with AHS and are thought to be caused by endothelial cell damage and increased vascular permeability.[5] The course of clinical disease and necropsy findings were similar among horses, irrespective of virus subtype. Previous reports suggested that disease severity is not linked to the serotype but rather to the virulence of the variant involved,[20,21] which is likely due to AHSV subtypes having variable tropism to cardiac and pulmonary endothelial cells.

Hemoconcentration during the late stage of the disease was likely the result of increased vascular permeability with leakage of plasma into the extravascular tissue. Severe edema, most pronounced in the head and neck but also in pulmonary tissue, is a hallmark of the clinical forms of AHS.[4,5] Splenic contraction due to adrenergic stimulation caused by pain and stress might also have contributed to the increases in these variables. Erythrocytosis (ie, an absolute increase in RBC numbers), usually caused by hypoxia, was considered unlikely, as increases occurred within a few hours of the onset of dyspnea and the maturation time from reticulocytes into mature erythrocytes is about 3 to 4 days.

Horses enrolled in the study tested positive for *T. equi* on PCR assay. *Theileria equi* is endemic in South Africa, and many horses will test positive, using sensitive tests like PCR assays, without having clinical signs. It is known that immunosuppression can result in recrudescence of acute piroplasmosis. For our study, parasitized erythrocytes were either not detected on examination of blood smears or observed in very low numbers (maximum 5 piroplasms in 3,481 RBC, < 0.001%); however, clinical infections with *T. equi* are reported to have parasitemia ranges from 1% to 7%.[22] It is therefore unlikely that changes observed in this study were caused by *T. equi* infection.

The decreased leukocyte count confirmed findings from an earlier study;[7] however, contrary to previous results, our study did not observe clinically important leukopenia (< 4,700 cells/μL, University of Pretoria Faculty of Veterinary Science Clinical Pathology Laboratory) in any horse at any single point in time nor significant decreases in neutrophils or lymphocytes. Previous studies have suggested that differences in virulence in the same subtype can cause different clinical signs;[20,21] although all horses in this study developed similar clinical signs. Considering the severity of clinical and necropsy findings, changes consistent with a pronounced leukocyte response, namely a degenerative left shift and severe neutrophil toxic changes were expected in line with previous findings reported in severe cases of AHS.[7] However, even in that previous study, the degree of leukopenia and left shift was, while present, only mild. The reason for this is not clear, but a possible explanation is viral immune escape mechanisms downregulating the host's response.[23] A similar observation has been made in sheep infected with the closely related Bluetongue Virus (BTV), where the animals do not develop consistent leukopenia.[24]

Monocytes are a nonspecific marker of inflammation and different subpopulations with pro- and anti-inflammatory properties exist.[25] During inflammation or infection, monocytes are recruited by chemokines into the tissues where they differentiate into macrophages and dendritic cells. Thus, they are important for the phagocytosis of pathogens, but they also play an important role in the modulation of the innate and adaptive immune system by secreting cytokines including IL-1β, IL-6, TNF-α, which are involved in homeostasis.[26] Monocytes can also migrate into peripheral tissues where they function as effector cells.[26] AHSV has been shown to replicate in monocytes,[6] particularly targeting pulmonary intravascular macrophages.[6,27] Given the numerous tasks of monocytes, it is likely that monocytes migrated into the tissues, resulting in decreased

numbers of monocytes in the peripheral circulation. Furthermore, during intracellular viral replication, cell damage due to release of viral particles may occur and decrease the number of monocytes.

Decreased eosinophil counts have been proposed as a marker for increased mortality in human sepsis and systemic inflammatory response syndrome. Eosinopenia has been associated with corticosteroid administration and endogenous cortisol and catecholamines secreted during inflammation.[28] Eosinopenia can also be caused by chemotaxis through margination or egression into tissues, with cortisol being one of the key drivers for migration of eosinophils into leukemoid organs like the lymph nodes or the spleen.[29] Reports about the cells observed in tissue histopathology in horses with AHS are sparse, and to date, notable tissue accumulation of eosinophils has not been identified during infection with AHSV. Increases in endogenous cortisol may be the underlying driver for the eosinopenia observed in this study.

The MPXI, which decreased over the course of AHSV infection, represents the mean intracellular myeloperoxidase content in circulating neutrophils and is used as a marker of neutrophil activation.[17] A decrease in MPXI is an indication of widespread neutrophil degranulation and respiratory burst in response to systemic inflammation and has been reported in dogs with severe systemic inflammation, in horses infused with endotoxin, and in some horses with systemic inflammation due to other causes.[30–32] In contrast, MPXI increased in septic foals and was unchanged in another group of horses with systemic inflammation.[33,34] In horses with endotoxemia and systemic inflammatory response syndrome, the MPXI became negative, indicating marked activation of neutrophils with degranulation of myeloperoxidase into plasma.[32] In the current study, although MPXI decreased, this change was not marked, suggesting that intravascular neutrophil activation and degranulation is not a prominent feature of this disease.

Thrombocytopenia was observed in all horses and progressed throughout the course of the disease. This is consistent with the clinical observation of bleeding tendencies following venipuncture in the later course of the disease and with hemorrhages and petechia noted in the gastrointestinal tract on gross pathology. There are several possible causes for this decrease, with the most likely being platelet sequestration in the form of thrombin-induced platelet aggregation. This process can be triggered by endothelial inflammation and damage, resulting in exposure of tissue factor. This triggers a cascade leading to the cleavage of active thrombin from prothrombin and the activation of platelets.[35] With the endothelial damage observed in horses infected with AHS,[4–6] this seems probable. AHS has also been reported to result in coagulation abnormalities with prolonged clotting times manifested by increased activated partial thromboplastin time, prothrombin time, thrombin time, and fibrin degradation products, possibly indicating disseminated intravascular coagulation (DIC).[7,36] Platelet consumption as it occurs during DIC, where platelets are incorporated into the fibrin network, can then lead to thrombocytopenia. Other mechanisms, such as a decrease in platelet production or platelet loss, seem unlikely. The decrease in PLT is consistent with the decrease in PCT.

As platelets become activated, their shape changes to become more spherical, they increase in mass and volume and degranulate with consequent adhesion and aggregation (and increased platelet-clumping). These changes result in increased MPV and MPM and decreased MPC.[37–39] Increases in MPV and MPM with decreases in MPC were observed in the horses infected with AHSV in this study and indicate platelet activation.[35] This is likely due to endothelial damage resulting in the exposure of tissue factor and cleavage of prothrombin to thrombin activating platelets described previously as well as the release of larger platelets from the bone marrow into the circulation. This is also supported by the increase in MPM commonly attributed to thrombopoiesis[40] resulting in the release of young, more active platelets. Degranulation of activated platelets is consistent with the decreases in MPC observed in this study. MPC has previously been reported to decrease in sick foals, especially with septic but also nonseptic disease as well as in adult horses with severe inflammatory response syndrome, thrombocytopenia, and colic due to obstruction and enteritis.[41] Increases in MPV, indicating young and activated platelets, have also been reported in dogs with babesiosis,[9] parvo viral enteritis,[8] as well as cattle infected with bovine viral diarrhea virus.[42] Similar to AHS, these diseases cause inflammation of or damage to the endothelium with resulting changes in platelet production and activity. While further investigation of platelet activation in horses is required, the observed changes suggest platelet sequestration and activation in horses infected with AHS.

Both SAA and iron are used as inflammatory markers in horses. Decreases in serum iron were observed early in the course of the disease. Generally, circulating IL-6 and TNF-α stimulate the release of hepcidin by hepatocytes. This then induces apoferritin synthesis and sequestration within macrophages[43] resulting in hypoferremia within 24 hours in the early stages of the disease, normalizing only when the inflammatory insult has resolved. In 2 horses, iron concentration was below reported reference intervals at the beginning of the study. This might have been due to unrecognized inflammatory processes but might also be caused by diet or age with younger horses commonly having lower iron concentrations. However, with the intra-individual study design where each horse served as its own control, the decrease in iron concentration was controlled for in all analyses. Unexpectedly, while SAA concentration, a commonly used marker of acute inflammation in horses, increased over time, there was a significant delay before any increase was noted and it did not increase to clinically important levels. Increases in SAA are reported under physiologic conditions including exercise and parturition

as well as in numerous pathologies including surgical colic, peritonitis, bacterial pneumonia, and septic arthritis. In horses infected with AHSV, despite severe clinical abnormalities, SAA concentrations did not increase to the extent commonly observed in patients with other severe systemic inflammatory diseases, such as colitis or bacterial pneumonia.[11] SAA has a short half-life with increases commonly noted within 6 to 12 hours following the inflammatory insult[11]; however, in these horses infected with AHS, increases were only noted from 96 hours PI onward and 48 hours after fever was first noted and after seroconversion. This is surprising as SAA synthesis is induced by increases in circulating proinflammatory cytokines IL-1, IL-6, TFN-α, and interferon,[44] which are also involved in the mediation of fever. In contrast, significant decreases in serum iron, also mediated by circulating TNF-α and IL-6, were observed in earlier stages of the disease, which suggests that other modulators might be involved in the sequestering of iron, making it a more sensitive APR in horses with AHS.

When evaluating the acute phase response to other orbiviruses, SAA concentrations increased 3.5-fold in sheep infected with BTV serotypes 1 and 8,[45] below expected increases for a major APP.[43] The reason for the stunted response is unclear. In sheep, BTV has been reported to antagonize interferon synthesis and might be able to downregulate the innate immune response,[46] possibly interfering with the synthesis of SAA. African horse sickness virus nonstructural protein 4 has recently been demonstrated to contribute to potential mechanisms to overcome the host's antiviral response by colocalization with promyelocytic leukemia nuclear bodies[47] and interference with the JAK-STAT pathway that is instrumental in the interferon pathway.[23] Indeed, several viruses have evolved mechanisms to evade the innate immune system's response.[48] These evasion mechanisms might also contribute to the observed lack of a positive acute phase protein response in the face of severe systemic inflammation.

It is important to remember that while SAA and iron concentrations are commonly thought of by clinicians merely as markers of inflammation, the physiologic reasons for increases and decreases should not be forgotten. SAA plays a critical role in the control and possibly propagation of the primordial acute phase response.[49] For example, it has been proposed to modulate inflammation by inducing chemotaxis and migration in monocytes as well as stimulating cytokine release.[49] It also inhibits lymphocyte proliferation, platelet aggregation, and phagocytosis but stimulates prostaglandin synthesis and metalloproteinase activation.[50] Iron is required for viral metabolism, and a decrease in iron concentration assists the innate immune mechanism against invading pathogens.[12] Considering these properties, dysregulation of these substances might enhance virus pathogenicity. Future studies in naturally infected horses should be performed to assess the prognostic value of these APPs in horses infected with AHS.

The main limitation of this study is the small number of enrolled horses. However, as infection with AHSV is commonly fatal in naïve horses, sacrificing a larger number of horses was considered unethical. The horses used in this study were enrolled in a challenge study that served as a prerequisite for a vaccine trial. All horses were infected with different AHVS serotypes. It has previously been shown that the virulence of the variant is more important than the serotype. For example, AHS/4SP caused development of the lung form while AHS/4PI only caused fever.[20,21] All infected horses in this study developed similar clinical signs consistent with the acute form ("dunkop") of AHS, and hematologic changes were consistent between individuals. Thus, there was no evidence that the different serotypes used in this study significantly affected the clinical course of the disease.

In conclusion, the results of this study further highlight the temporal changes in hematologic variables after infection with AHSV. The observed changes reflect the body's response to infection; however, the changes in APPs are only moderate. It is possible that derangements in the host's immune response contribute to the observed dampened immune response in reaction to the inflammatory stimuli triggered by the virus. Further studies investigating the host's immune response focusing on cytokine secretion patterns are required to better understand these changes.

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Supplementary Materials

Supplementary materials are posted online at the journal website: avmajournals.avma.org

11.3 Research Ethics Approval

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
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Faculty of Veterinary Science

Research Ethics Committee 20 April 2020

CONDITIONALLY APPROVAL

Ethics Reference No	**REC195-19**
Protocol Title	**Investigation of hemostatic changes and markers of inflammation and endothelial activation or damage in horses experimentally and naturally infected with African Horse Sickness**
Principal Investigator	**Dr EC Schliewert**
Supervisors	**Prof A Goddard**

Dear Dr EC Schliewert,

We are pleased to inform you that your submission has been conditionally approved by the Faculty of Veterinary Sciences Research Ethics committee, subject to other relevant approvals.

Please note the following about your ethics approval:

1. Please use your reference number (REC195-19) on any documents or correspondence with the Research Ethics Committee regarding your research.
2. Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
3. Please note that ethical approval is granted for the duration of the research as stipulated in the original application for post graduate studies (e.g. Honours studies: 1 year, Masters studies: two years, and PhD studies: three years) and should be extended when the approval period lapses.
4. The digital archiving of data is a requirement of the University of Pretoria. The data should be accessible in the event of an enquiry or further analysis of the data.

Ethics approval is subject to the following:

1. The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.
2. **Applications using Animals:** FVS ethics recommendation does not imply that AEC approval is granted. The application has been pre-screened and recommended for review by the AEC. Research may not proceed until AEC approval is granted.

NOTES: Conditionally approved (pending obtaining all other relevant approvals).

We wish you the best with your research.

Yours sincerely

PROF M. OOSTHUIZEN
Chairperson: Research Ethics Committee

100 YEARS OF VETERINARY EDUCATION

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11.4 Animal Ethics Approval

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Animal Ethics Committee

11 May 2020

Approval Certificate
New Application

AEC Reference No.:	REC195-19
Title:	Investigation of hemostatic changes and markers of inflammation and endothelial activation or damage in horses experimentally and naturally infected with African Horse Sickness
Researcher:	Dr EC Schliewert
Student's Supervisor:	Prof A Goddard

Dear Dr EC Schliewert,

The **New Application** as supported by documents received between 2020-01-15 and 2020-05-04 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2020-05-04. Please note the following about your ethics approval:

1. The use of species is approved:

Species	**Number**
Boerperd Horses	4 (Deltamune/vaccine trial)
Samples Blood	Per horse: 27 samples x 35 ml each, (945 ml total) Live animals
Blood (Horses all breeds)	17 (Stored Historic/Retrospective) OVAH

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2021-05-11.
3. Please remember to use your protocol number (REC195-19) on any documents or correspondence with the AEC regarding your research.
4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.
Yours sincerely

Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee

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Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

11.5 Section 20 Permit

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/1/8 (1350)

Dr Eva-Christina Schliewert
Senior Lecturer in Equine Medicine
Department of Companion Animal Clinical Studies
University of Pretoria
Tel: 012 529 8137
Email: tine.schliewert@up.ac.za

Dear Dr Schliewert

RE: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984)

Your application dated 10 October 2019 requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers. I am pleased to inform you that permission is hereby granted to perform the following study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. This permission is given upon finding the biosecurity of the research project as described to be acceptable to DAFF. This permission does not serve as any approval or endorsement by DAFF for the commercial use or registration of any African horse sickness vaccine for any purpose in South Africa;
3. This permit is for the project entitled *"Investigation of haemostatic changes and markers of inflammation and endothelial activation or damage in horses experimentally and naturally infected with African Horse Sickness"* only;

4. The research project is approved as per the application form dated 10 October 2019 and the correspondence thereafter. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this research project under this Section 20 permit. Please apply in writing to HerryG@daff.gov.za;

5. If required, an application for an extension must be made by the responsible researcher at least one month prior to the expiry of this Section 20 permit. Please apply in writing to HerryG@daff.gov.za;

6. No part of this research project may begin until the valid ethical approval has been obtained in writing from the relevant South African authority;

7. Only the following samples may be used in this research project:

   7.1. Blood samples from the control group horses that will be infected with local South African strains of field AHS virus as part of the Deltamune project entitled "*Evaluation of the safety and immunogenicity of AHSV reassortant, for vaccine development against African Horsesickness*" with the Section 20 permit dated 18 September 2014 and the extension dated 14 November 2019. The samples must be collected in accordance with all biosafety and biosecurity measures as approved by the Section 20 permit dated 18 September 2014 ;

   7.2. Horse blood samples stored at the Clinical Pathology Laboratory at the Faculty of Veterinary Sciences, University of Pretoria;

   7.3. Tissue samples from horse carcasses at the Pathology Post Mortem Hall, Section of Anatomical Pathology, Faculty of Veterinary Sciences, University of Pretoria may be collected. Samples must be placed in 10% formalin immediately upon collection;

8. Samples must be packaged and transported in accordance with International Air Transport Association (IATA) requirements and/or the National Road Traffic Act, 1996 (Act No. 93 of 1996);

9. It is the responsibility of the researcher and relevant laboratory or facility managers to ensure that the human safety aspects of this research project are adequately addressed;

10. Any incidence or suspected incidence of a controlled or notifiable animal disease in terms of the Animal Diseases Act 1984 (Act no 35 of 84), must be reported immediately to the state veterinarian of the area;

11. All potentially infectious material utilised or generated during or by the research project is to be destroyed at completion of the research project;

12. Only a registered waste disposal company may be used for the removal of all potentially infectious waste from the research project. Records must be kept for five years for auditing purposes;
13. A dispensation for the storage of serum, plasma and blood cell samples from horses is attached.

Title of research/study: Investigation of haemostatic changes and markers of inflammation and endothelial activation or damage in horses experimentally and naturally infected with African Horse Sickness
Researcher: Dr Eva-Christina Schliewert
Institution: Deltamune (Pty) Ltd BSL 2+ Vector Free Stables, Roodeplaat, Pretoria; Clinical Pathology Laboratory, Onderstepoort Veterinary Academic Hospital; Pathology Post Mortem Hall, Section of Anatomical Pathology, Faculty of Veterinary Sciences, University of Pretoria
Permit Expiry date: 31 December 2020
Our ref Number: 12/11/1/1/8 (1350)
Your ref: REC 195-19

Kind regards,

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2019 -11- 27

SUBJECT: *PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 OF 1984) 12/11/1/1/8 (1350)*

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/1/8 (1350)

Dr Eva-Christina Schliewert
Senior Lecturer in Equine Medicine
Department of Companion Animal Clinical Studies
University of Pretoria
Tel: 012 529 8137
Email: tine.schliewert@up.ac.za

RE: DISPENSATION ON SECTION 20 APPROVAL IN TERMS OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984) FOR: "INVESTIGATION OF HAEMOSTATIC CHANGES AND MARKERS OF INFLAMMATION AND ENDOTHELIAL ACTIVATION OR DAMAGE IN HORSES EXPERIMENTALLY AND NATURALLY INFECTED WITH AFRICAN HORSE SICKNESS"

A dispensation is hereby granted on Point 11 of the Section 20 approval that was issued for the above mentioned study (attached):

i) Serum, plasma and blood cell samples from horses may be stored under access control at the Clinical Pathology Laboratory, Onderstepoort Veterinary Academic Hospital;

ii) Serum, plasma and blood cell samples from horses must not be outsourced or used for further research without prior written approval from the Director: Animal Health.

Kind regards,

DR. MPHO MAJA
DIRECTOR: ANIMAL HEALTH
Date: 2019 -11- 27