UNIVERSITY OF COPENHAGEN
FACULTY OR DEPARTMENT

Elucidating hemostatic aberrations in horses with migrating *Strongylus vulgaris* larvae with and without clinical disease

PhD thesis

Marie Louise Honoré Lytzhøft-Olsen, DVM

This thesis has been submitted to the Graduate School of Health and Medical Sciences

University of Copenhagen

5 February 2022

DEPARTMENT OF VETERINARY CLINICAL SCIENCES

FACULTY OF HEALTH AND MEDICAL SCIENCES

UNIVERSITY OF COPENHAGEN

Author: Marie Louise Honoré Lytzhøft-Olsen, DVM,
Department of Veterinary Clinical Sciences

Title: Elucidating hemostatic aberrations in horses with migrating *Strongylus vulgaris* larvae with and without clinical disease

Principal supervisor: Tina Holberg Pihl, DVM, PhD, Associate Professor
Department of Veterinary Clinical Sciences
Faculty of Health and Medical Sciences
University of Copenhagen, Denmark

Co-supervisor: Lise Nikolic Nielsen, DVM, CertSAM, PhD, Danish Specialist in Internal Medicine in Companion Animals, Professor (MSO) in Veterinary Clinical Pathology
Department of Veterinary Clinical Sciences
Faculty of Health and Medical Sciences
University of Copenhagen, Denmark

Assessment committee: Bettina Dunkel, DVM, PhD, Dip ACVIM, Dip ECEIM, Dip ACVECC, FHEA, MRCVS
Head of Equine and Professor in Equine Internal Medicine, Emergency and Critical Care
Department of Clinical Science and Services
The Royal Veterinary College, UK

Aage Kristian Olsen Alstrup, DVM, PhD, Danish specialist in Laboratory Animal Medicine, Associate Professor, Dr.Med.Vet
Department of Clinical Medicine - Nuclear Medicine and PET
Aarhus University, Denmark

Chair: Rikke Buhl, DVM, PhD, Professor
Department of Veterinary Clinical Sciences
Faculty of Health and Medical Sciences
University of Copenhagen, Denmark

Submitted: 5 February 2022

Table of contents

Acknowledgments

I owe a sincere thank you to my supervisor Associate Professor Tina H. Pihl and co-supervisor Professor Lise N. Nielsen, who have both been invaluable mentors each in their own way. I am thankful for the way they have introduced me to the world of research and have helped me find my way and formed me into a clinical researcher. Thank you for continuously providing me with inspirational research ideas, talks and feedback while giving me the freedom to make this project my own. You are both very empathic people and it has been a pleasure working under your wings.

I am very grateful to the all the clinical staff at the Large Animal Teaching Hospital at the University of Copenhagen for the substantial work involved in collecting samples for this project day and night over a period of three years. In connection with this, a big thank you goes to hospital director Susanne Nautrup Olsen for allowing me to collect samples at this state-of-the-art hospital. The owners who agreed to include their horses in this thesis also deserve a big thank you.

A special thank you to medical laboratory technologist Louise Bochsen and laboratory technicians Birgitte Klitgaard and Tina Roust from the Department of Veterinary Clinical Sciences. I have enjoyed working alongside you, and your help and guidance in the laboratory has been invaluable.

We have been fortunate enough to have a collaboration with one of the world's leading parasitologists, Professor Martin K. Nielsen and his skilled team at Gluck Equine Research Center at the University of Kentucky. I owe a substantial thank you to Martin and the undergraduate and graduate students at his laboratory for assisting with sample collection every other week for an entire year. In addition, thank you to the farm crew at the Barn 10 research herd for their great help in handling the horses. Unfortunately, Martin was in New Zealand during my stay at Gluck, but his team took great care of me and with that I owe a thank you to PhD candidate Jennifer Cain, Assistant Professor Ashley Steuer (now at Texas Tech University), Assistant Professor Jessica Scare (now at Eastern Kentucky University), and Research Analyst Holli Gravatte. They expanded my knowledge within parasitology tremendously and are all great people who made sure I had the best stay in Lexington.

Our Swedish collaborators Associate Professor Eva Týden, Assistant Professor Miia Riihimäki, DVM Ylva Hedberg-Alm, and DVM Cert. EM (IntMed) MRCVS Karin G. Anlén all deserve a big thank you for their great contribution to this project. I am also very grateful to the hospital staff at the University Animal Hospital at the Swedish University of Agricultural Sciences and Evidensia Helsingborg Equine Referral Hospital who contributed with invaluable sample collection, and to the Swedish horse owners who included their horses in this project.

I am very grateful to Associate Professor Peter Nejsum from Aarhus University who, during my stay at Gluck Equine Research Center, helped me over the phone when necessary during sample collections at odd times of the day due to the time difference. In addition, I very much appreciate that Peter and Professor Stig Thamsborg from the Department of Veterinary and Animal Sciences always contributed great input when discussing research ideas.

I am also very grateful to Professor Søren Saxmose Nielsen and Assistant Professor Jacob Stærk-Østergaard, who both contributed great statistical assistance and analysis.

I owe a big thank you to Peter Fischer from FischerDevices. Peter generously lent me two thromboelastography machines for my research stay at Gluck Equine Research Center and provided fantastic help with the paperwork required to get everything through customs in the USA. Kathryn Perrin, DVM, PhD also deserves thanks for lending me a transformer, ensuring that the thromboelastography machines worked in the USA and saving me from a lot of unnecessary trouble.

During this PhD, I have been fortunate enough to share an office with a great group of PhD students at the Department of Veterinary Clinical Sciences. Each and every one of them have been a great support throughout this process. We have been able to share successes, frustrations, and a lot of cake and coffee. We have had a great professional and social network. I will truly miss being a part of this team, known to some as Team Unicorn.

Thanks to our master's students (now all DVMs), who all worked very hard during their projects and helped ensure high quality research, and even made working with hemostasis musical. Through the work on their master's theses, Tanne Busk-Anderson and Laura Lind Flintrup contributed to Study I (Paper I), while Jenny Bladgren and Veronica Hallquist contributed to Study IV in this thesis.

Research is not possible without financial support. I owe a substantial thank you to all the funding sources behind this project. It would simply not have been possible without you. Thank you to the Horse Levy Foundation, the Augustinus Foundation, the KUSTOS Fund of 1881, the Swedish-Norwegian Foundation for Equine Research, Sveland, and the PhD school for supporting this project.

I want to say a big thank you to my family and friends for their never-ending love and support during this process. Even though I know that most of you have been struggling a little with my fascination with intestinal parasites, you have never ceased to listen, ask questions, and encourage me.

Above all, I am most grateful to my little family at home. My true pillars who have made this entire process possible. My husband Morten who has stood by me throughout this journey; listened during the highs and lows, encouraged and supported, and tolerated the degree of madness that this ride entails. Furthermore, I do not think I could have completed this thesis without the daily smiles, laughs, and hugs I am fortunate enough to get from my little boy Christian.

Marie Louise Honoré Lytzhøft-Olsen

Bistrup, February 2022.

List of papers

This PhD project has resulted in three papers. One paper is published in BMC Veterinary research. A second paper has been submitted and the third is ready for submission. The papers are enclosed at the end of the thesis.

Paper I: **Investigation of Two Different Human D-dimer Assays in The Horse**

Honoré, M.L., Pihl, T.H., Busk-Anderson, T.M., Flintrup, L.L, Nielsen, L.N.

Submitted to BMC Veterinary Research, July 2021.

Paper II: **A pilot study evaluating the Calibrated Automated Thrombogram assay and application of plasma-thromboelastography for detection of hemostatic aberrations in horses with gastrointestinal disease**

Honoré, M.L., Pihl, T.H., Nielsen, L.N.

BMC Veterinary Research, 2021, 17(1), p. 1-11.

Paper III: **Hemostatic changes identified in horses with migrating *Strongylus vulgaris* using plasma-thromboelastography and the Calibrated Automated Thrombogram assay**

Honoré, M.L., Nielsen, L. N., Stærk-Østergaard, J., Týden, E., Hedberg-Alm, Y., Riihimäki, M., Anlén, K. G., Nielsen, M. K., Pihl, T. H.

Manuscript in preparation for submission to BMC Veterinary Research.

Abbreviations

α	Angel degree (TEG)
aPTT	Activated partial thromboplastin time
AT	Anti-thrombin
AUC	Area under the curve
BW	Body weight
CAT	Calibrated Automated Thrombogram
CBC	Complete blood count
CI	Confidence interval
CMA	Cranial mesenteric artery
CV	Coefficients of variation
DDU	D-dimer units
DIC	Disseminated intravascular coagulation
DVT	Deep vein thrombosis
ELISA	Enzyme-linked immunosorbent assay
EPG	Eggs per gram
ETP	Endogenous thrombin potential (CAT)
FDP	Fibrin degradation products
FEU	Fibrinogen equivalent unit
f.V-f.XIII	Factor V, factor VII, and so forth
f.Va-f.XIIIa	Activated factor V, activated factor VII, and so forth
G	Shear elastic force (TEG)
GI	Gastrointestinal
HCT	Hematocrit
HR	Heart rate
ICCA	Ileocecocolic artery
K	Clotting time (TEG)
L1-5	First stage larvae, second stage larvae, and so forth
LOA	Limits of agreement
LY30 %	Fibrinolysis 30 min after MA (TEG)
LY60 %	Fibrinolysis 60 min after MA (TEG)
MA	Maximum amplitude (TEG)
miRNA	MicroRNA
MPC	Mean platelet component
MPV	Mean platelet volume
NSII	Non-strangulating intestinal infarction
NycoCard	NycoCard™ D-dimer assay
PLT	Platelet
PPP	Platelet poor plasma
PRP	Platelet rich plasma
PSGL-1	P-selectin glycoprotein ligand-1
PT	Prothrombin time
Q_1	1st quartile

Q_3	3rd quartile
R	Reaction time (TEG)
ROC	Receiver operating curve
RR	Respiration rate
SAA	Serum amyloid A
Se	Sensitivity
Sp	Specificity
SP	Split point (TEG)
Stago	STAGO STA-Liatest D-di+ assay
S. vulgaris	*Strongylus vulgaris*
TAT	*Thrombin–antithrombin*
TEG	Thromboelastography
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TP	Total protein
ttPeak	Time to peak (CAT)
TX	Thromboxane
vCAM-1	Vascular cell adhesion molecule 1
VTE	Venous thromboembolism
vWF	von Willebrand factor
WBC	White blood cell count

Summary

Strongylus vulgaris (bloodworm) is the most pathogenic intestinal parasite in horses. Its life cycle is approximately 6 months long, during which time it migrates in the cranial mesenteric artery and its branches. This migration causes local inflammation and damage to the endothelium, which initiates the activation of hemostasis, leading to verminous arteritis and thrombosis. In the worst case, these changes can lead to the formation of non-strangulating intestinal infarctions (NSII), which without acute surgical treatment will be fatal for the horse. However, not all horses infected with *S. vulgaris* develop NSII, and the reason for this is not yet fully understood. In addition, there currently is no optimal way to diagnose the presence of migrating *S. vulgaris* larvae or NSII.

Based on the damage caused by *S. vulgaris*, it is relevant to examine hemostatic markers as well as markers of endothelial damage. This will provide a better understanding of why some horses with *S. vulgaris* do not develop NSII, and will help to identify new diagnostic biomarkers.

The overall aim of this PhD was to investigate the hemostatic balance in horses with migrating *S. vulgaris* larvae with and without signs of clinical disease (NSII) in order to aid in the understanding of the development of NSII in some horses.

In Study I (Paper I), two human d-dimer assays, STAGO STA-Liatest D-di + (Stago) and NycoCardTM D-dimer assay (NycoCard), were investigated on citrated platelet poor plasma (PPP) in clinically healthy horses. They were subsequently compared across four groups of horses: clinically healthy horses, horses with gastrointestinal (GI) disease and low-grade systemic inflammation (serum amyloid A (SAA) 30-100 mg/L), horses with GI disease and severe systemic inflammation (SAA > 1000 mg/L), and horses with NSII. The results showed that both assays were applicable in horses, with acceptable validity and reliability. The high SAA group and the NSII group both had a significantly higher d-dimer concentration than the clinically healthy group. Both assays showed good agreement in classifying horses with a low or high d-dimer concentration, but results from the two assays cannot be compared one to one.

In Study II (Paper II), plasma-thromboelastography (TEG) and Calibrated Automated Thrombogram (CAT) assay were studied on citrated PPP. The CAT assay was first investigated in clinically healthy horses and then both assays were compared across three groups of horses: clinically healthy horses, horses with non-inflammatory and non-ischemic GI disease (mild GI disease), and horses with inflammatory and ischemic GI disease (severe GI disease). The results showed that the CAT assay had a high degree of validity and reliability. Both assays showed changes that could be interpreted as hypercoagulability in the horses with severe GI disease compared to the clinically healthy horses and the horses with mild GI disease.

In Study III (Paper III), clinical parameters, hematological and biochemical variables, *S. vulgaris* antibodies, d-dimer, plasma-TEG and CAT were used in six groups of horses: clinically healthy horses, horses with *S. vulgaris* without clinical disease (NSII negative), horses with *S. vulgaris* with clinical disease (NSII positive), horses with idiopathic peritonitis, horses with enterocolitis, and horses with strangulating intestinal lesions. The results showed that horses with migrating *S. vulgaris* larvae without and with clinical disease (NSII) had significant hemostatic changes compared to the remaining groups. A model including inflammatory markers (iron and SAA) and global hemostatic markers (angle (plasma-TEG) and endogenous thrombin potential (ETP; CAT)) was, to some degree, able to differentiate horses with NSII caused by *S. vulgaris* from the remaining groups.

In Study IV, plasma-TEG and CAT were measured on citrated plasma collected every two weeks for one year in horses with migrating *S. vulgaris* larvae without clinical disease. Seasonal hemostatic changes were observed, but without any unique pattern.

In Study V, two human endothelial markers, vascular cell adhesion molecule 1 (vCAM-1) and P-selectin, were investigated on serum from four groups of horses: clinically healthy horses, horses with migrating *S. vulgaris* larvae with clinical disease (NSII positive), horses with enterocolitis, and horses with strangulating intestinal lesions. Both assays produced satisfactory standard curves, but measurements from the four groups of horses were below the lower level of detection for both assays.

Based on the results of this PhD project, it can be concluded that human d-dimer assays (Stago and NycoCard) and plasma-based global hemostatic tests (plasma-TEG and CAT) can be used on citrated plasma from horses. Horses with migrating *S. vulgaris* larvae both without and with clinical disease (NSII) exhibit hemostatic changes compared to clinically healthy horses and horses with other inflammatory and/or ischemic GI diseases. Combining inflammatory markers (iron and SAA) and global hemostatic tests (angle and ETP) can, to some degree, distinguish horses with NSII caused by *S. vulgaris* from clinically healthy horses and horses with other inflammatory and/or ischemic GI diseases. This seems to support the idea that horses can cope with a *S. vulgaris* infection as long as they do not experience concomitant systemic inflammation.

Sammendrag (Danish summary)

Strongylus vulgaris (blodorm) er hestens mest patogene indvoldsorm. Den har en ca. 6 måneder lang livscyklus, hvor den undervejs migrerer i den kraniale mesenterielle arterie og dens forgreninger. Migrationen forårsager lokal inflammation og skade på endothelet, hvilket initierer en aktivering af hæmostasen. Samlet leder dette til verminøs arteritis og trombose. I værste fald kan disse forandringer føre til dannelsen af non-strangulerende intestinale infarkter (NSII), som uden akut kirurgisk behandling vil være dødelig. Det er dog ikke alle heste inficeret med *S. vulgaris,* som udvikler NSII. Årsagen til dette er endnu ikke fuldt forstået. Derudover findes der på nuværende tidspunkt ikke nogen optimal måde at diagnosticere tilstedeværelsen af migrerende *S. vulgaris* larver eller NSII.

På bagrund af de skader *S. vulgaris* medfører, er det relevant at undersøge hæmostasemarkører samt markører for endothelskade. Dette for at få en bedre forståelse for, hvorfor ikke alle heste med *S. vulgaris* udvikler NSII, samt for at identificere nye diagnostiske biomarkører.

Hovedformålet med dette ph.d.-projekt var at undersøge den hæmostatiske balance i heste med migrerende *S. vulgaris* larver med og uden tegn på klinisk sygdom (NSII). Dette for at forbedre forståelsen for hvorfor kun nogen heste inficeret med *S. vulgaris* udvikler NSII.

I Studie I (Paper I) blev to humane d-dimer assays, STAGO STA-Liatest D-di+ (Stago) og NycoCard™ D-dimer assay (NycoCard), undersøgt på citratstabiliseret trombocytreduceret plasma i klinisk raske heste. Siden blev de sammenlignet i fire grupper af heste; klinisk raske, heste med gastrointestinal (GI) sygdom og lavgradig systemisk inflammation (serum amyloid A (SAA) 30-100 mg/L), heste med GI sygdom og svær systemisk inflammation (SAA > 1000 mg/L) og heste med NSII. Resultaterne viste, at begge assays var applicerbare hos hest med acceptabel præcision og pålidelighed. Gruppen med høj SAA og NSII gruppen blev begge fundet at have en signifikant højere d-dimer koncentration end den klinisk raske gruppe. Begge assays var overordnet enige om klassificeringen af heste i forhold til lav og høj d-dimer koncentration, men resultaterne kunne ikke sammenlignes en til en.

I Studie II (Paper II) blev plasma-tromboelastografi (TEG) og Calibrated Automated Thrombogram (CAT) assay undersøgt på citratstabiliseret trombocytreduceret plasma. For CAT først via en validering i klinisk raske heste og siden blev begge assays sammenlignet i tre grupper af heste; klinisk raske, heste med non-inflammatorisk og non-iskæmisk GI sygdom (mild GI sygdom) og heste med inflammatorisk og iskæmisk GI sygdom (svær GI sygdom). Fra resultaterne sås, at CAT viste høj grad af præcision og pålidelighed. Begge assays fandt tegn på hyperkoagulabilitet i hestene med svær GI sygdom sammenlignet med de klinisk raske og hestene med mild GI sygdom.

I Studie III (Paper III) blev kliniske parametre, hæmatologiske og biokemiske variable, *S. vulgaris* antistoffer, d-dimer, plasma-TEG og CAT appliceret i seks grupper af heste; klinisk raske, heste med *S. vulgaris* uden klinisk sygdom (NSII negative), heste med *S. vulgaris* med klinisk sygdom (NSII positive), heste med idiopatisk peritonitis, heste med enterocolitis og heste med strangulerende intestinale læsioner. Resultaterne herfra viste, at heste med migrerende *S. vulgaris* larver uden og med klinisk sygdom (NSII) havde hæmostatiske forandringer i sammenligning med de andre grupper. En model indeholdende inflammatoriske markører (jern og SAA) samt globale hæmostase markører (angle (plasma-TEG) og endogent trombinpotentiale (ETP) (CAT)) var i nogen grad i stand til at differentiere heste med NSII forårsaget af *S. vulgaris* fra de resterende grupper.

I Studie IV blev plasma-TEG og CAT målt på citratstabiliseret plasma indsamlet hver anden uge over et år hos heste med migrerende *S. vulgaris* larver uden klinisk sygdom. Det viste, at der var sæsonbetingede hæmostatiske forandringer. Dog uden noget entydigt mønster.

I Studie V blev to humane endothelmarkører, vascular cell adhesion molecule 1 (vCAM-1) og P-selectin, undersøgt på serum fra fire grupper af heste; klinisk raske, heste med migrerende *S. vulgaris* larver og klinisk sygdom (NSII positive), enterocolitis og strangulerende intestinale læsioner. Begge assays producerede tilfredsstillende standardkurver, men målingerne på prøverne fra de fire grupper af heste var under den nedre detektionsgrænse for begge assays.

Baseret på resultaterne fra dette ph.d.-projekt kan det dermed konkluderes at humane d-dimer assays (Stago and NycoCard) og plasmabaserede globale hæmostasetest (plasma-TEG og CAT) kan anvendes på citratstabiliseret plasma i hest. Heste med migrerende *S. vulgaris* larver både uden og med klinisk sygdom (NSII) udviser hæmostatiske forandringer i sammenligning med klinisk raske heste og heste med anden form for inflammatorisk og/eller iskæmisk GI sygdom. Ved at kombinere systemiske inflammationsmarkører (jern og SAA) og globale hæmostase test (angle og ETP) er det i nogen grad muligt at skelne heste med NSII forårsaget af *S. vulgaris* fra klinisk raske heste og heste med anden form for inflammatorisk og/eller iskæmisk GI sygdom; hvilket understøtter hypotesen om, at heste kan håndtere en infektion med *S. vulgaris,* så længe de ikke udsættes for samtidig systemisk inflammation.

1 Introduction

Grazing horses are ubiquitously infected with gastrointestinal (GI) parasites (1), with *Strongylus vulgaris* (*S. vulgaris*) commonly considered the most pathogenic equine intestinal parasite (2). During its life cycle, *S. vulgaris* migrates through the mesenteric arteries and its branches and causes verminous arteritis and thrombosis (2,3). While some horses can be infected with *S. vulgaris* without displaying overt signs of clinical disease (4,5), in other horses the migration of *S. vulgaris* will cause non-strangulating intestinal infarctions (NSII), which is a life-threatening condition requiring immediate surgical intervention for the horse to survive (6,7).

The introduction of macrocyclic anthelmintic formulations in the 1970s and 80s lead to a marked reduction in the prevalence of *S. vulgaris* (8–13). However, this resulted in widespread resistance among other equine intestinal parasites (14–21). To delay the development of resistance, a targeted selective treatment strategy was implemented and prescription-only legislation requiring a parasitological diagnosis or the veterinarian having a thorough knowledge of the management practices at the farm was implemented in Denmark in 1999 and Sweden in 2007. This has been suggested to have caused an increase in the prevalence of *S. vulgaris* due to the challenge of diagnosis during the migrating stages. *S. vulgaris* is now endemic in Denmark and Sweden (22,23), and the frequency of horses presenting with NSII in Denmark has increased in recent decades (7).

There are currently no highly sensitive or specific diagnostic tests that can diagnose NSII caused by *S. vulgaris* in horses prior to an exploratory laparotomy or post mortem examination (7), and horses with NSII often mimic horses with idiopathic peritonitis and enterocolitis (24–26). In addition, there is no certain way of diagnosing the presence of *S. vulgaris* while it migrates in the cranial mesenteric artery and its branches in horses without clinically overt disease, and new and improved ways to diagnose horses with migrating *S. vulgaris* with and without clinical disease are therefore urgently needed.

The migration of *S. vulgaris* is known to cause local inflammation, alterations to the vasculature with damaged endothelial lining, activation of hemostasis, and subsequent thrombosis (2,27,28). The d-dimer concentration, which is a good rule-out marker for thrombosis (29–31), has been found to be associated with the migration of *S. vulgaris* in naturally infected foals (32). It might therefore be of interest to explore the use of d-dimer further in horses with migrating *S. vulgaris* and to apply markers of vascular endothelial damage. Global hemostatic tests such as thromboelastography (TEG) and thrombin generation time as assessed by the Calibrated Automated Thrombogram (CAT) assay are surperior to the conventional hemostatic markers, and are used in human medicine to diagnose, monitor, and treat hemostatic aberrations (33,34). Whole blood-TEG has been used in other cases of equine GI disease (35–38), and plasma-TEG has been used in humans (39,40), dogs (41), and pigs (42). The CAT assay has yet to be investigated in horses. It would be relevant to investigate these global hemostatic tests in horses with migrating *S. vulgaris* with and without clinical disease as they assess the entire hemostatic process from initiation to clot formation and fibrinolysis. They might aid in the diagnostic workup and help to fill the knowledge gap in terms of the complete pathophysiological process caused by *S. vulgaris,* as it is still unclear why only some horses develop NSII. Inflammation is known to affect hemostasis (43), and it could therefore be speculated that horses that develop NSII have migrating *S. vulgaris* and are simultaneously affected by inflammation of another cause, thus further

triggering hemostasis and tipping the balance toward a pathological hypercoagulable state. However, this still needs to be fully elucidated.

1.1 Aim, objectives and hypotheses

The overall aim of this PhD was to investigate the hemostatic balance in horses with migrating *S. vulgaris* larvae with and without signs of clinical disease in order to increase our understanding of the development of NSII in some horses.

The main hypotheses were:

- Human d-dimer assays can be used on equine citrated platelet poor plasma (PPP) with a high degree of validity and reliability, and horses with NSII have increased d-dimer concentrations compared to clinically healthy horses, horses with GI disease and mild systemic inflammation, and horses with GI disease and strong systemic inflammation (Study I, Paper I).
- Global hemostatic tests can be used on equine citrated PPP with a high degree of validity and reliability and are able to detect hemostatic changes in horses with GI disease compared to clinically healthy horses (Study II, Paper II).
- Horses with migrating *S. vulgaris* larvae with and without clinical signs of disease (NSII) show hemostatic changes that can be interpreted as hypercoagulable when compared to clinically healthy horses and horses with other severe GI disorders (Study III, Paper III).
- Horses with migrating *S. vulgaris* larvae without clinical disease have seasonal hemostatic changes correlated with the life cycle of *S. vulgaris* (Study IV, Section 3.8 and 4.5).
- Markers of endothelial damage are increased in serum from horses infected with *S. vulgaris* with clinical disease (NSII) compared to clinically healthy horses, horses with enterocolitis, and horses with strangulating lesions (Study V, Section 3.9 and 4.6).

To test these hypotheses, five studies were performed with the following objectives:

Study I (Paper I): To evaluate the STAGO STA-Liatest D-di+ assay and the NycoCard™ d-dimer assay on equine citrated PPP in clinically healthy horses, horses with GI disease and a mild systemic inflammatory response, horses with GI disease and a strong systemic inflammatory response, and horses with GI disease caused by *S. vulgaris* (NSII).

Study II (Paper II): To evaluate the two global hemostatic tests CAT and plasma-TEG on equine citrated PPP in clinically healthy horses, horses with mild GI disease, and horses with severe GI disease.

Study III (Paper III): To investigate whether horses with *S. vulgaris* with and without clinical disease (NSII) had hemostatic changes measurable with d-dimer, plasma-TEG, and CAT, and to

compare these to clinically healthy horses and horses with severe GI diseases (idiopathic peritonitis, enterocolitis, and strangulating intestinal lesions) with similar clinical signs and laboratory findings as horses with NSII.

Study IV: To investigate whether horses with migrating *S. vulgaris* larvae without clinical disease had hemostatic changes detectable with plasma-TEG and CAT and to assess whether these hemostatic changes were seasonal in relation to the life cycle of *S. vulgaris*.

Study V: To investigate whether two human endothelial markers (vascular cell adhesion molecule 1 (vCAM-1) and P-selectin) measured with human enzyme-linked immunosorbent assays (ELISA) were measurable and significantly different in clinically healthy horses, horses with migrating S. vulgaris with clinical disease (NSII), horses with enterocolitis, and horses with strangulating lesions.

The first three studies are described in Papers I, II, and III. Study IV are described in Section 3.8 and 4.5. Study V are described in Section 3.9 and 4.6.

2 Background

2.1 Strongylus vulgaris in the horse

Grazing horses are ubiquitously infected with intestinal parasites (22), with the most common group, Strongylidae, consisting of two subgroups – the small strongyles (cyathostominae) and the large strongyles (strongylinae) (44). *Strongylus vulgaris* belongs to the group of large strongyles and is considered to be the most pathogenic intestinal parasite in the horse (45,46), most often occurring in a co-infection with small strongyles (44).

2.1.1 Life cycle of *Strongylus vulgaris*

The life cycle of *S. vulgaris* is seasonal (2,47–49) and large numbers of infective larvae are ingested by the horse during the warmer months of spring and summer, resulting in an increase in the adult worm population in the horse during winter months (47,48). The life cycle is direct and includes a free-living phase and a parasitic phase. Transmission is fecal-oral (49,50), with infected horses shedding eggs to the pasture through feces. The eggs rely on an optimal temperature (20°C to 35°C) to hatch to first stage larvae (L1). At this temperature optimum hatching takes approximately one to two days (51). The L1 larvae develop to second stage larvae (L2) and then to the infective third stage larvae (L3) (49). At 20°C to 35°C, this development takes approximately three to seven days (51). To protect themselves from the environment during their free-living phase, the L3 larvae are enclosed in their L2 cuticle, which they shed once the L3 larvae are ingested by a horse (49). The L3 larvae then penetrate the mucosa and submucosa of the ileum, cecum, and/or colon (3,49). The L3 larvae reside in the submucosae for two to three days and then molt to fourth stage larvae (L4). Within seven to nine days post infection, the L4 larvae then penetrate the arteries and arterioles of the submucosae (3). They migrate in and on the tunica intima of the arteries and arterioles against the blood flow (3,49). They continue their migration via the arterial system until they reach the cranial mesenteric artery (CMA) and its branches approximately two weeks post infection (3,52). The L4 larvae then stay here for three to four months, during which time they grow and finally moult to fifth stage larvae (L5), which returns to the large intestine (3). From here, the L5 larvae are encapsulated in small nodules in the intestinal wall (3). When the nodules rupture, adult worms are released into the intestinal lumen. It then takes an additional six to eight weeks for the adult worms to become sexually mature and able to produce eggs. The total life cycle thus takes six to seven months to complete (49) (**Figure 1**).

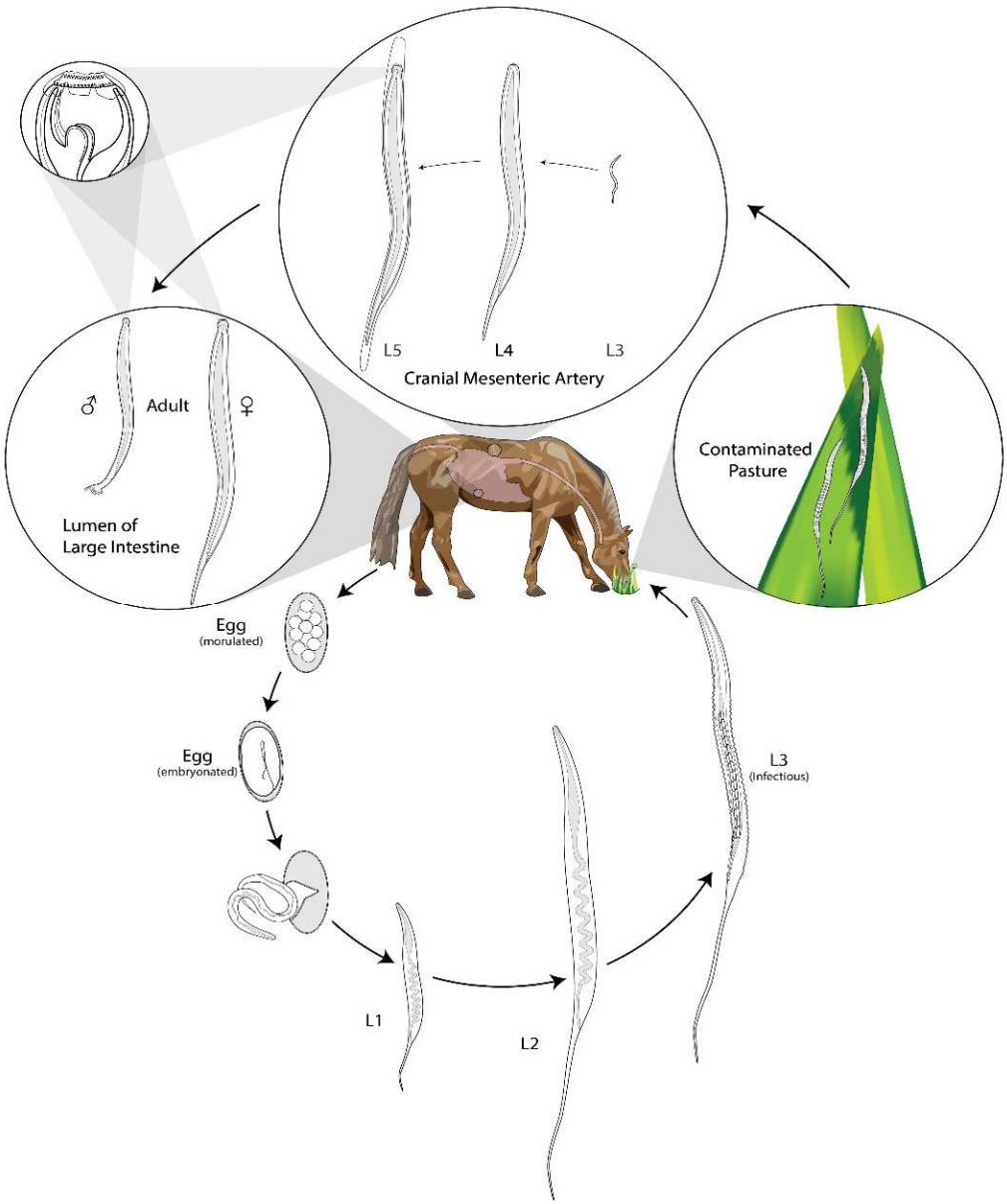


Figure 1. The lifecycle of *Strongylus vulgaris*. The free-living stage showing the development from eggs shed with feces to infective third stage larvae (L3) being ingested by the horse from contaminated pasture. The parasitic phase with the migration and development of the L3 larvae to adult worms after six to seven months. So*urce*: Reprinted from *M.K. Nielsen: Diagnosing equine parasites: The challenges of a moving target*. Doctoral Dissertation submitted to the Faculty of Health and Medicine, University of Copenhagen in March 2021. With permission from Martin K. Nielsen and Jamie K. Norris.

2.1.2 Pathogenesis and clinical implications of *Strongylus vulgaris*

As *S. vulgaris* migrates through the intestinal vasculature, it wreaks havoc resulting in severe alterations to the vasculature and affecting the hemostasis of the horse (3,27,32). This can ultimately lead to the occurrence of NSII, which can be fatal to the horse if left untreated (7).

2.1.2.1 Hemomelasma ilei

When the L3 larvae of *S. vulgaris* penetrate the intestinal mucosal lining as early as two days post infection, it causes a grossly visible subserosal hemorrhage also known as hemomelasma ilei. After a few days there microscopically are signs of a local inflammatory response with the infiltration of leukocytes at the site of these lesions (3,46).

2.1.2.2 Verminous arteritis and thrombosis

Arteritis and thrombosis develop in the small submucosal arteries of the intestine due to the presence of L4 larvae (3,46). The L4 larvae continue their migration toward their predilection site, the CMA and its branches and/or the ileocecocolic artery (ICCA) (3,53–58). Macroscopically visible tortuous migratory fibrin tracks and plaques are present in the arterial intima of the CMA and ICCA (3,46,59,60) and can also be seen in the aorta (46,57,61) and renal arteries (57) in some cases. The migration of the L4 larvae disrupts the endothelial lining of the arteries leaving a denuded intimal surface (3,46,54,60,62,63). Subendothelial collagen is exposed, which initiates platelet activation and adhesion and the release of coagulation factors (27,60). The damaged endothelium can produce a large quantity of procoagulant factors (60) and the damage cause changes in the shape of endothelial cells, with nuclear bulging, rounding up, cell separation, and protrusion into the arterial lumen leading to changes in blood flow (27,64). Red blood cells, fibrin aggregates, and white blood cells are attached to the surface in areas with thrombus formation. Platelets adhere to the red blood cells and fibrin strands (27). Once platelets adhere to the damaged vessel wall, they undergo membrane transformation. This leads to the release of mediators, such as serotonin, calcium, and adenosine diphosphate (ADP), encouraging further platelet aggregation and platelet plug formation (65,66). This is a self-reinforcing effect that leads to the additional stimulation of platelet aggregation (65–68). This means that once a thrombus has formed, the subsequent endothelial damage and altered blood flow will through a vicious cycle cause the thrombus to increase in size (27,60). Live larvae are found both in the lumen of the CMA and incorporated in thrombus material (3,27,53,54,57,60). In addition, arterial wall thickening occurs. Typically, the CMA and its branches and the ICCA are enlarged with a tough, thickened arterial wall due to disposition of collagen and fibrosis, with an irregular endothelial lining and a narrowed lumen due to thrombi formation (3,46,69–72,54–57,60–63). Microscopically this is characterized by a markedly thickened and uneven intima with infiltration of plasma cells, lymphocytes, macrophages, neutrophils, eosinophils, and fibrous tissue proliferation (27,46,55,57,60). These findings are particularly apparent at the sub-intimal level, and decrease in intensity toward the sub-serosa (57). The characteristic lesions seen in the mesenteric arteries in response to the migration of *S. vulgaris* larvae are defined as verminous arteritis (49). The lesions caused by the L4 larvae progress (27,46,54), and begin to involve the tunica media with fibrous thickening and loss of the internal elastic membrane (46,70), and the infiltration of plasma cells and lymphocytes (46,57). The affected arteries are enlarged due to an increased luminal size with thrombosis and due to a thickened arterial wall caused by cellular infiltration and fibrosis (49,73). It is therefore debatable whether the enlargement of the affected arteries can be defined as an aneurysm, which typically shows thinning of the arterial wall (49). However, extensive and long-lasting damage to the arterial tunica media can in some cases lead to the development of a true aneurysm (46). In some cases, extensive collateral circulation are found surrounding the CMA and its branches (56,61). Fibrosis, thickening and perivascular infiltration mainly with eosinophils in the arterial wall is seen in the vasa vasorum and the lumen is obliterated. This can cause secondary damage to the arteries due to interference with the nutritional supply (46,60).

Not all horses with migrating *S. vulgaris* larvae develop clinically overt disease with NSII. In some cases, the only noticeable finding nine months post infection is the presence of a healed arterial lesion with an even endothelial lining with no indications of thrombosis. In these cases, when the infection subsides, the thrombus material is incorporated into the arterial wall causing intimal fibrosis, which is then covered by a normal endothelium. Microscopically, the intima of the arterial wall is fibrotic and all three layers of the arterial wall are infiltrated with macrophages and lymphocytes (46).

2.1.2.3 Non-strangulating intestinal infarcts

The presence of migrating *S. vulgaris* larvae in the CMA and its major branches is associated with the occurrence of NSII (6,7,45,46,52,53,74–77). Different theories behind the development of NSII have been proposed in the literature (78–80). The term thromboembolic colic has previously been used due to the finding of arteries and/or arterioles completely occluded by thrombi or emboli that had detached and moved downstream in the vessel tree, causing local ischemia and infarction of segments of the intestinal wall (28,46,56,74,81,82). However, it has subsequently been shown that thrombi or emboli are not always present in the peripheral vessels in cases of local intestinal ischemia (72), which is why NSII seems to be a more fitting description. In cases where there is no evidence of thrombi or embolisms in the peripheral vessels, it has been suggested that infarctions are the result of reduced blood flow due to a partial occlusion of the CMA and its branches causing hypoxia and subsequently infarctions (72,77,83). The infarcted part of the intestine undergoes necrosis, resulting in septic peritonitis due to the compromised intestinal barrier. If left untreated, this can be fatal due to a potential rupture of the affected intestine resulting in septic shock (6,7,84).

On post mortem examination of parasite-naïve foals given high doses of L3 larvae, NSII of the small and large intestine were noted as black to red areas clearly demarcated and raised above the unaffected intestine (46,81,85). Similar findings were made in horses naturally infected with *S. vulgaris* presenting at a hospital with clinical disease, with the affected intestine having a clearly demarcated area, most often in the pelvic flexure or the caecum, with edema, severe discoloration, and in some cases fibrin deposits on the serosa (32).

The exact mechanisms behind the development of NSII are still not fully understood. In some horses, collateral blood supply has been found to form prior to the development of necrosis and gangrene (76).

2.1.3 Prevalence of *Strongylus vulgaris* and NSII

In the 1960s and earlier, *S. vulgaris* was highly prevalent with a farm-level prevalence of 80-100% (86–90). In the 1970s and 80s, anthelmintics containing macrolides, which are effective against the migrating stages of *S. vulgaris,* became available and were usually administered prophylactically multiple times a year to interrupt the lifecycle of *S. vulgaris* (8–13). As a result, the prevalence of *S. vulgaris* was drastically reduced to below 5% (91–94). A downside of this was the occurrence of widespread anthelmintic resistance among cyathostomin parasites (14–17) and *Parascaris equorum* (18–21). In order to delay the development of further resistance, prescription-only legislation requiring a parasitological diagnosis or the veterinarian having an in-depth knowledge of the management practices at the farm was implemented in Denmark in 1999 and in Sweden in 2007. This prohibited the use of prophylactic treatments and a selective treatment strategy was widely implemented in both countries over the following decades (22,95). The selective strategy is based on fecal examination of all horses twice a year to identify the high egg shedders. Most commonly, only horses with high egg counts (> 200 or 500 eggs per gram (EPG)) are treated, meaning that many horses are left

untreated (95). This seems to have resulted in an increased prevalence of *S. vulgaris,* with an individual prevalence of 12.2% and a farm level prevalence of 64.3% in Denmark (22), and an individual prevalence of 28% and a farm level of 61% in Sweden (23). This increase cannot be explained by the occurrence of resistance as a significant association has been found between the occurrence of *S. vulgaris* and selective treatment. When using selective treatment, the individual- and farm-level prevalence of *S. vulgaris* was found to be nearly twice as high compared to farms not using fecal egg counts (22).

NSII is diagnosed with increasing frequency in horses referred to the Large Animal Teaching Hospital at the University of Copenhagen (6,7) and to the Veterinary University Hospital in Uppsala (84,96). In a 2018 study from Copenhagen (7), there was a 33% survival-to-discharge rate for horses that had an intestinal resection. For horses receiving medical treatment, the mortality rate was 100 %. In a follow up study at the same facility, the survival-to-discharge rate of horses that underwent surgery increased to 80%, while the mortality rate for horses receiving medical treatment remained the same. The increased survival rate seemed to result from a significant decrease in the time from admission to surgery (97). In a recent study conducted in Sweden, 50% of horses that had an intestinal resection survived to discharge. The lower survival rate compared to the Danish study could be due to three out of five of the horses not having surgery until three to seven days after admission. In this study, the mortality rate was also 100% for NSII horses receiving medical treatment (84). It therefore seems that survival increases with a rapid and accurate diagnosis resulting in the initiation of the correct treatment. This highlights the urgent need for diagnostic and prognostic biomarkers of *S. vulgaris.*

2.1.4 Diagnosis of *Strongylus vulgaris*

Diagnosing *S. vulgaris* is challenging for various reasons (7,98). Eggs are not excreted with the feces during the long migratory phase, and even after this stage when adult worms do excrete eggs with the feces, they are difficult to identify and differentiate from the remaining strongyle eggs (44). It is also challenging to diagnose horses with clinical overt disease (NSII) caused by *S. vulgaris* as it mimics other types of severe GI disease (7,26). More reliable diagnostic techniques aimed at identifying these extraintestinal larvae are thus needed due to the severe pathology associated with the migration of *S. vulgaris* larvae in the mesenteric arteries (99).

2.1.4.1 Fecal diagnosis

The most common way to diagnose the presence of intestinal parasites is with fecal examination. Numerous methods have been developed aimed at quantifying the number of eggs (100–104). One major drawback of these tests is that they are incapable of distinguishing between the different types of strongyle eggs (44), thus necessitating an additional diagnostic step. A larval culture that facilitates the development of L3 larvae can be used (99,105–108). A sensitivity of 73% and a specificity of 84% has been found for the larval culture to diagnose an infection with *S. vulgaris*. However, no correlation between the number of larvae in the culture and the number of adult worms in the intestine has been found (109). The larval culture is time-consuming as it takes approximately two weeks for the eggs to develop to the L3 stage (110). A semi quantitative real-time PCR analysis has been developed as an alternative to the larval culture (110) and has been found to identify a significantly higher proportion of *S. vulgaris* positive horses (99). The semi-quantitative real-time PCR assay is able to detect less than or equal to 0.5 *S. vulgaris* eggs with no immediate cross-reactivity (110). One major limitation common to all these methods is that they are unable to detect *S. vulgaris* during its long prepatent period when it migrates in the CMA.

2.1.4.2 Serological diagnosis

The potential to develop a serological diagnostic test for the migrating *S. vulgaris* larvae has been investigated multiple times, as *S. vulgaris* spends several months in the CMA and its branches (111–116). Most of these tests did not have a complete antigen characterization and were not fully evaluated or validated as diagnostic tests (111–115). However, one promising *S. vulgaris* specific antibody ELISA test against migrating *S. vulgaris* larvae has been developed and validated (116). This test uses the recombinant SvSXP protein, which is highly immunogenic with IgG(toxin (T)) antibodies specific for recombinant SvSXP (116,117). A cut-off for horses older than 7 months was found to be 13.47% of the positive control (PP) with a diagnostic sensitivity of 65.5% (95% confidence interval (CI): 51.9-77.5%) and a diagnostic specificity of 82.1% (95% CI: 63.1-93.9%) (116). The serum antibody concentration was found to be positively correlated with the arterial *S. vulgaris* burden (118). In addition, horses with NSII were found to have a significantly higher antibody titer than horses with other causes of colic (6,7). However, it is important to consider the diagnostic drawbacks of this antibody ELISA test when interpreting the results. For example, false negative samples are possible as an elevated antibody titer primarily occurs five months post infection (119), so a negative antibody titer does not necessarily mean that a horse is not infected with *S. vulgaris* and/or suffer from NSII. There is also a risk of false positive samples as it can also take five months for the antibody concentration to normalize again post treatment, and the antibody concentration can even increase further following ivermectin treatment (119). A positive antibody test should therefore be interpreted either as a current or recent infection (119).

2.1.4.3 Rectal examination

The potential to diagnose the presence of *S. vulgaris* via rectal examination has been investigated in the literature. Rectal palpation can be used to assess the presence of changes in the CMA and its major branches caused by the migration of *S. vulgaris.* A mass palpable per rectum in the lumbosacral region has been found to be indicative of verminous arteritis (120,121), and palpation can also be used to detect the infarcted area of the intestine, which presents as a firm and sometimes painful rectal mass (7,84,122).

Previous studies have evaluated transrectal ultrasonography as a method for diagnosing migrating *S. vulgaris* (70,123,124). These studies assessed the appearance of the intimal surface, the arterial wall thickness and echogenicity, and the presence or absence of a mass in the lumen of the CMA (70,123,124). A good correlation was found between the ultrasonographic findings and postmortem results, and the technique seems to be useful in diagnosing arteritis caused by migrating *S. vulgaris* (70). Although practical and financial aspects make this technique unsuitable for herd-level screening (98), it could be useful for selected cases within a hospital setting (7).

2.1.4.4 Arteriographic diagnosis

Contrast arteriography has been used successfully to determine the presence of lesions in the CMA and its branches following inoculation with *S. vulgaris* larvae (56,61,125,126) and to evaluate the degree of resolution of these lesions post treatment (61,125,126). However, this method is only relevant as a research tool as it requires general anesthesia of the horse (56,61,125,126) and would therefore not be a useful diagnostic tool in a clinical setting.

2.1.4.5 Laboratory parameters

An acute systemic inflammatory response has been found in horses with NSII associated with *S. vulgaris,* including an increased fibrinogen and serum amyloid A (SAA) concentration, and a decreased white blood

cell count (WBC), iron concentration (7), and platelet count (127). Horses with colitis (128) and to some extent idiopathic peritonitis (26) have similar laboratory findings and these should therefore be considered as differential diagnoses (7) and the laboratory variables alone cannot differentiate horses with NSII. Eosinophilia is well described in many helminth infections and has been found in horses with migrating *S. vulgaris* in some studies (62,129) but not in others (7).

Due to the inconsistent signs of NSII, an assessment of the peritoneal fluid is crucial as it can contribute important information and avoid serious intra-abdominal pathology being overlooked. Peritonitis has been identified in 90% of horses with NSII characterized by an increased total protein (TP) and WBC. Abdominocentesis is easily performed and can aid in identifying horses with NSII (7). The identification of peritonitis, however, is not unique for horses with NSII caused by *S. vulgaris* (26,130,131) and cannot exclusively diagnose these cases.

An additional challenge in a country such as Sweden is that cases with idiopathic peritonitis responding well to medical treatment predominate over cases with NSII (84). It therefore might not be realistic to recommend an exploratory laparotomy in all cases presenting with septic peritonitis with no known origin in areas where *S. vulgaris* is endemic (7,84). A recent study suggested that horses with septic peritonitis with no known cause presenting during the winter months with a palpable mass/thickening of the colon, neutropenia, hyperfibrinogenemia, a high peritoneal TP, and persistence of colic signs beyond 48 hours of antimicrobial treatment strongly indicates the presence of NSII (84). The only finding unique to NSII compared to idiopathic peritonitis was the finding of a rectally palpable mass/thickening of the colon (84).

2.2 The effects of *Strongylus vulgaris* on hemostasis

As described in detail in Section 2.1.2 the migration of *S. vulgaris* larvae causes marked macroscopic and microscopic alterations to the vasculature, which affects the hemostasis.

It seems, that the migration of the L4 larvae is most important in the pathogenesis of verminous arteritis and thrombosis of the CMA, and its branches, and the ICCA (3,53,54,60,69,132,133), as the L4 larvae causes a denuded surface of the arterial intima, which is one of the most important factors in thrombogenesis (3,54,60,62,63). One question that remains is whether the larvae themselves stimulate thrombus formation or if it is the result of the arterial wall reaction in response to the larvae (27).

Lesions caused by *S. vulgaris* are progressive with alterations in the arterial blood flow and an environment for continual thrombus formation (27). Thromboxane A2 (TXA_2), which is a product from the platelet membrane, is a potent stimulator of further platelet aggregation and a potent vasoconstrictor (66), and as platelets have been found to be an important component of thrombi associated with arterial damage it is likely that TXA_2 is increased in cases of verminous arteritis (27,69). Single daily measurements of TXA_2's stable metabolite, thromboxane B2 (TXB_2) were shown to be of no diagnostic value in horses with migrating *S. vulgaris* larvae, as the systemic concentration was not consistently elevated. Perhaps, due to the very short half-life of TXB_2 of 20 min. Nevertheless, local production of TXA_2 at the damaged vasculature cannot be excluded (69).

When assessing hemostatic parameters in foals with migrating *S. vulgaris,* the d-dimer concentration has been found to be significantly associated with the number of *S. vulgaris* larvae in the CMA, and both d-dimer and fibrinogen were positively associated with *S. vulgaris* antibody values. A significant increase in d-dimer,

prolonged prothrombin time (PT), and decreased antithrombin (AT) activity has been identified around week 12 to 15 post infection with *S. vulgaris,* and these hemostatic changes thus correspond with the migration of *S. vulgaris.* This indicates that the migrating larvae pays a part in a mild initiation of coagulation and fibrinolysis (32). In addition, decreased hemoglobin, hematocrit (HCT) (121), and RBC (57), and increased fibrinogen have also been identified (7,84). Despite these findings, the diagnostic value of hematology appears to be poor (45,57).

2.3 Hemostasis

2.3.1 A cell-based hemostatic model

The coagulation cascade model or waterfall model dealing with an intrinsic, extrinsic, and common pathway has for a long time shaped our understanding of coagulation. This model was based on coagulation being a series of steps where the activation of one clotting factor led to the activation of another, finally leading to a burst of thrombin generation (134,135). However, this could not explain the coagulation *in vivo* but only *in vitro* (136). It is now believed that hemostasis *in vivo* occurs in a stepwise manner regulated by cellular components (137) as an interaction between platelets, endothelial cells, and a balance of activating and inhibiting coagulation, anti-coagulation and fibrinolytic factors (138). This is known as the cell-based model of hemostasis (136,137,139,140).

In this cell-based model, hemostasis is believed to occur in three overlapping phases (137). The first phase is **the initiation phase**, which takes place on the surface of tissue factor (TF)-bearing cells such as fibroblasts (137). Tissue factor is the main initiator of coagulation and is synthesized and localized on the cell surface (141,142). In inflammatory conditions, it can be found on monocytes and endothelial cells. When the wall of a blood vessel is damaged, plasma will come into contact with TF-bearing extravascular cells. This will allow factor VII (f.VII) in the plasma to bind to the cellular TF, which forms a tight complex and f.VII will be activated (f.VIIa) (137). The f.VIIa/TF complex then proceeds to activate factor X (f.Xa) and factor IX (f.IXa), and f.Xa subsequently activates factor V (f.Va) (143). Tissue factor pathway inhibitor (TFPI) or AT inhibits the activated f.X when leaving the protected cell surface environment. Nonetheless, the remaining f.Xa on the cell surface combines with f.Va, producing a small amount of thrombin (137,144). This plays a crucial role in the activation of platelets and factor VIII (f.VIII) during the next phase.

The next phase is **the amplification phase,** during which platelets and co-factors are amplified in preparation for thrombin generation (137). The damaged vessel wall allows platelets to interact with extravascular tissue. Platelets are partially activated by binding to matrix proteins or localization near TF exposure. The small amount of thrombin generated in the initiation phase now amplifies the pro-coagulant signal by enhancing platelet adhesion and activation of platelets, f.V, f.VIII, and f.XI (144,145). When activated, platelets release partially activated f.V, which is then completely activated by thrombin or f.Xa (143). A complex is formed between von Willebrand factor (vWF) and f.VIII, and this complex binds to the platelet surface and is cleaved by thrombin, activating f.VIII and releasing it from vWF (146).

With platelets activated and f.Va and f.VIIIa bound to their surface, the gathering of pro-coagulant complexes and extensive thrombin production can begin (137). This happens during **the propagation phase, during which** the tenase-complex and prothrombinase-complex are assembled on the platelet surface (137). The tenase-complex (f.IXa/f.VIIIa) assembles when f.IXa reaches the platelet surface, and can then activate f.X. The activated f.X then forms a complex with f.Va, known as the prothrombinase-complex. This complex

cleaves prothrombin and then produces the burst of thrombin needed to form a hemostatic fibrin clot (137) (**Figure 2**).

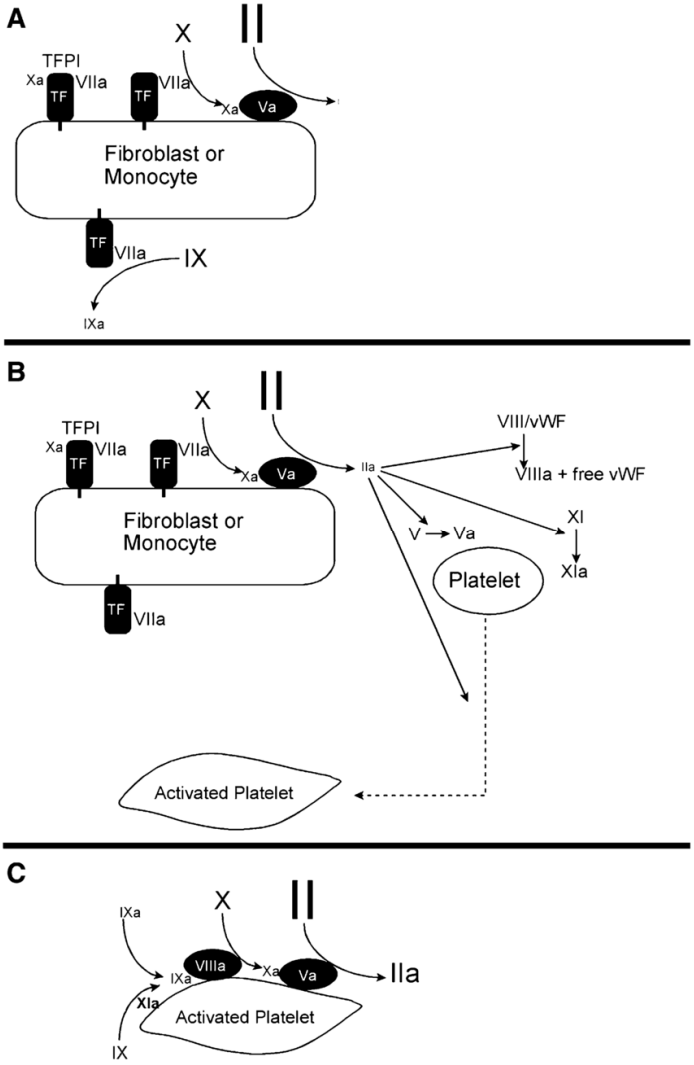


Figure 2. The cell-based coagulation cascade with the A) initiation phase, B) amplification phase, and C) propagation phase. *Source:* Reprinted with permission from Hematology/Oncology Clinics of North America, Volume: 21, Issue: 1, Pages: 1-11, First published: February 2007, DOI: (10.1016/j.hoc.2006.11.004).

Several mechanisms serve to prevent inappropriate coagulation. Endothelial cells express antithrombotic features that prevent the initiation of coagulation when the endothelium is intact. Plasma protease inhibitors inhibit active proteases that diffuse into the fluid phase by localizing reactions to cell surfaces, and the propagation and inactivation steps are confined to different cell surfaces (137).

Antithrombin is a natural anticoagulant that prevents excessive coagulation (147). It primarily circulates as a monomer in plasma but is also found on the surface of platelets and endothelial cells (148). AT is a specific inhibitor of thrombin but also functions to inhibit f.XIIa, f.XIa, f.IXa, and f.Xa (149–151).

D-dimer is the end product of fibrinolysis when cross-linked fibrin is degraded (152). The generation of d-dimer entails the sequential activity of thrombin, f.XIIIa, and plasmin. The process begins when thrombin

transforms soluble fibrinogen to fibrin monomers (153). The fibrinogen molecules are symmetrical dimers consisting of three pairs of three entwined polypeptide chains. The three chains are linked by disulfide bonds and the fibrinogen molecules consist of a central E domain and two peripheral D domains (154). Thrombin cleaves short peptides from the fibrinogen molecule to form fibrin monomers, which polymerize end-to-end in an overlapping manner in order to produce double-stranded fibrin protofibrils. The strength of the fibrin network is enhanced by f.XIII, which is activated by thrombin and cross-links the D domains of fibrin monomers (155). The construction of cross-linked fibrin, which is the base for d-dimer formation entails activation of hemostasis with thrombin generation, alteration of fibrinogen to fibrin monomers, the formation of fibrin polymers and the cross-linking of these by f.XIIIa. D-dimer is created when cross-linked fibrin is degraded by plasmin. This process begins when plasminogen is converted to plasmin by tissue plasminogen activator, which is released from endothelial cells in response to injury. The activation of plasminogen, and thus plasmin formation, is localized to the fibrin surface (156,157). Plasmin decomposes the fibrin network into soluble fragments (158) releasing d-dimer in to the circulation (159). D-dimer is therefore only created when cross-linked fibrin is degraded.

2.3.1 Vascular endothelium

Vascular endothelial cells are highly specialized to be anti-thrombotic. In many ways, endothelial cells are closely related to platelets, expressing a variety of the same surface receptors (137). In the case of injury or inflammation, the endothelial cells will, however, alter their surface expression in order to facilitate hemostasis (160). These mechanisms may however in various disease states facilitate in thrombosis (137,161–163).

Vascular cell adhesion molecules regulate a multitude of adhesive and signaling events (164). They are of interest due to their role in a range of pathological and physiological processes such as inflammation (161–163,165), hemostasis (166,167), and thrombosis (161–163,166,167). Vascular cell adhesion molecule 1 and P-selectin derived from endothelial cells have been shown to be altered in patients with deep vein thrombosis (DVT) (168–172).

2.3.1.1 Vascular cell adhesion molecule 1

Vascular cell adhesion molecule 1 is a cell surface glycoprotein predominantly expressed in endothelial cells (173,174) and plays an important role in inflammation (175). Its expression is activated at sites of inflammation by pro-inflammatory cytokines and shear stress (176). In chronic conditions or during severe inflammation, vCAM-1 can also be expressed on the surface of other cells such as tissue macrophages and dendritic cells (177,178).

Vascular cell adhesion molecule 1 is significantly increased in patients with thrombosis (179) and correlates with the extent of atherosclerosis (162). Even though vCAM-1 is increased in humans with DVT (163,180) it cannot substitute or aid d-dimer in the diagnosis of DVT (180). However, it is still relevant to further investigate its role in DVT (180).

Equine vCAM-1 is an essential marker of the inflammatory activation of endothelial cells (181) but does not otherwise seem to have been widely assessed in equine medicine.

2.3.1.2 P-selectin

P-selectin is a cell surface glycoprotein. Endothelial cells and platelets harbor a pre-formed state, which can be mobilized to cell surfaces in the case of an inflammatory or thrombogenic event (182–184). P-selectin is

located on the cell surface and is found in a soluble form as it is shed from the surface of platelets following activation (185). The main counter-receptor for P-selectin is P-selectin glycoprotein ligand-1 (PSGL-1) (186,187). Through interactions with PSGL-1, P-selectin mediates leukocyte trafficking, leukocyte-vascular endothelium adhesion, and leukocyte-platelet adhesion (187–189). The interaction of P-selectin with PSGL-1 also plays a vital part in inflammatory, thrombotic, and ischemic conditions (190–192). P-selectin is considered a marker of platelet activation (164,193) and, is used as a disease marker (190,194,195), and as a therapeutic target in humans (196–198). P-selectin was significantly increased in human patients with thrombosis (163,179,180,199) but does not seem to add any significant diagnostic value to d-dimer (179,180,200). It has been suggested that P-selectin concentrations depend on the degree of thrombosis (179), which might explain why not all studies have found P-selectin to be indicative of DVT (201).

Equine P-selectin and equine PSGL-1 have been characterized (164,186,202–205), laying the groundwork for future studies assessing the potential diagnostic and therapeutic role of P-selectin in equine medicine. Equine platelets expressing P-selectin (203) were bound well by human P-selectin antibodies (205), which may indicate that human P-selectin assays could be applied to equine samples.

2.3.2 Conventional hemostatic markers

Routine coagulation testing in veterinary medicine often consists of platelet (PLT) count, PT, activated partial thromboplastin time (aPTT), fibrinogen, and fibrin degradation products (FDPs) (206). Although the commonly used static coagulation tests in many ways do not mirror the complexity of the hemostatic process in vivo, this does not mean that they are without value. It is nonetheless important to understand what can be deducted from them (136). It is important with an early and accurate diagnosis of hemostatic abnormalities. It however seems that once the conventional hemostatic tests are markedly abnormal it often is too late to intervene (206).

2.3.2.1 Prothrombin time and activated partial thromboplastin time

Activated partial thromboplastin time was traditionally believed to assess the intrinsic pathway, while PT was believed to assess the extrinsic pathway (207). These tests should however not be considered redundant in the cell-based hemostasis model, in which it is thought that PT evaluates the level of procoagulant factors during the initiation phase, whereas aPTT reflects the level of procoagulant factors produced during the propagation phase (140). PT and aPTT provide information about the plasma level of soluble factors necessary for hemostasis. When there is a deficiency in one or more of the soluble coagulation factors, these screening tests will be abnormal (136), though they do not fully reflect the complexity of in vivo hemostasis as they do not reflect the role of inhibitors (136). They are nonetheless able to detect deficiencies in one or more coagulation factors in patients with a bleeding tendency, yet they may not necessarily identify the clinical risk of bleeding (136). Two patients with identical aPTT and PT values can have a completely different risk of bleeding, and the clinical implications of these tests must always be considered within a larger context (136).

Both aPTT and PT have been used and assessed in multiple equine studies. Horses with colitis and clinicopathologic evidence of disseminated intravascular coagulation (DIC) have been found to have a prolonged aPTT compared to horses with colitis without signs of DIC (208) and clinically healthy horses (38). Horses with colitis also showed a prolonged PT compared to clinically healthy horses. These findings support a diagnosis of hypocoagulability (38). In horses with colic, aPTT and PT values increased during hospitalization, regardless of the diagnosis (209). In one study, mortality in horses with GI disease was significantly associated with PT at admission and on day two and three of hospitalization, as well as a prolonged aPTT at admission and on

day two (37). A prolonged aPTT and PT was shown to be indicative of a poor prognosis and predictive of mortality in horses with colic (35,210,211), yet some of the non-survivors had PT values within the reference range, indicating that PT alone does not have a good predictive value for non-survival (210). An abnormal aPTT value is more commonly seen in horses with strangulating, inflammatory, and/or ischemic GI lesions compared to horses with non-inflammatory and non-ischemic lesions (35,36). Prolonged PT and aPTT were found in horses with SIRS compared to horses without SIRS and in non-survivors compared to survivors. In addition, aPTT was prolonged in horses with ileus (36). In foals naturally infected with *S. vulgaris,* PT has been found to be prolonged at approximately three months of age (32). PT and aPTT can often be measured in larger laboratory facilities, where these assays are best at detecting hypocoagulability and are not considered suitable for detecting hypercoagulability (138).

2.3.2.2 Antithrombin

There is a clear relationship between systemic inflammation and the hemostatic system in patients with sepsis, where AT plays an important role in the pathogenesis (148). The AT level has been found to drop in cases of sepsis and DIC due to consumption exceeding production, and the AT level serves as a predictor of the outcome (212). AT functions as a negative acute phase protein (213) and can potentially be used as a therapeutic agent (148). Measurement of the AT level has proven to be useful in terms of diagnosing DIC and determining when the hemostatic imbalance ends (214).

Multiple studies have assessed and applied AT in both dogs and horses. Dogs with hyperadrenocorticism have been found to have decreased AT activity (215). In dogs infected with *Angiostrongylus vasorum,* no difference in AT was found between those with bleeding diatheses and those without. Hypocoagulable dogs did not show a specific pattern in AT activity, and AT did not differ between those that were hypocoagulable on TEG and those that were not (216).

Decreased AT activity was found in foals naturally infected with *S. vulgaris* (32) and in horses with acute GI disease (206). One study on equine colic found the incidence of decreased AT levels to be 8.6 times higher in non-surviving compared to surviving patients (217), while AT levels that remained below baseline values postoperatively were associated with mortality in horses with large colon volvulus (218), suggesting that AT has a good prognostic value (217,218). Hypoproteinemia in horses with colitis has been linked to a decrease in AT activity 48 hours after admission. Low plasma AT activity can therefore indicate non-specific protein loss, which can contribute to hemostatic aberrations (208). Altogether assessing the AT activity seems to be a good predictor of equine colic and might also prove useful in monitoring therapeutic responses (206,217,218).

2.3.2.3 D-dimer

D-dimer is a global marker of the hemostatic and fibrinolytic system, and an indirect marker of thrombotic activity (153). Various d-dimer assays are commercially available (219) including immunofluorescent assays, latex agglutination assays, and ELISAs. Each type of assay has advantages and disadvantages and should be evaluated accordingly (152,220–222). D-dimer can be measured in either whole blood, plasma, or serum. Monoclonal antibodies will recognize a specific epitope that is present on cross-linked d-dimer molecules but absent on the d-domain of non-cross-linked fibrinogen and fibrin monomers (220). The different d-dimer assays are not directly comparable and there is no established reference standard (219,223) as the results will not necessarily be reported using the same units. Some assays use fibrinogen equivalent units (FEUs), which relate the d-dimer mass to the mass of fibrinogen. Other assays use d-dimer units (DDU), which only

relates to the d-dimer mass. Testing the same sample with an FEU assay and a DDU assay would give a 1.75-fold discrepancy in the result (220).

D-dimer is a valuable test for detecting hemostatic abnormalities and is vital for assessing and diagnosing patients with intravascular thrombosis (224–227). D-dimer has a high negative predictive value with negative values effectively excluding venous thrombosis and positive values being indicative but not confirming of thrombosis (29–31,224–226,228). The circulating d-dimer concentration can be increased in a number of conditions besides DVT (229–235). The elevation of d-dimer in these situations however is less specific than it is for DVT (227). In humans, d-dimer also increases with advancing age (221,236).

In dogs a low d-dimer concentration has likewise been found to be a good rule-out marker for thrombosis (237).

Plasma d-dimer has been investigated in horses with diseases predisposing for hemostatic imbalances. In particular horses with GI disease and a marked systemic inflammatory response may develop hemostatic changes (209–211). D-dimer measurements at the time of admission and during hospitalization can be used to monitor the treatment response for medical and surgical colic patients (209), and as a prognostic marker of survival (210,211,238,239). An increased d-dimer concentration in horses was associated with a hypercoagulable state leading to DIC in cases with severe GI disease and sepsis (240). Ischemic and inflammatory GI disorders were associated with increased d-dimer concentrations at admission compared to horses with medical obstructive disorders (209). Horses suffering from severe disorders such as ischemic lesions requiring intestinal resection with high d-dimer concentrations had an increased risk of mortality (210). The d-dimer concentration has been suggested as the most sensitive test for diagnosing DIC, the presence of a hypercoagulable condition, and thromboembolic disease (210,239–242) in horses. D-dimer was significantly correlated with the number of *S. vulgaris* larvae in the CMA, the *S. vulgaris* antibody level, and fibrinogen concentration in foals (32), suggesting that the migration of *S. vulgaris* larvae results in a mild activation of coagulation and subsequent fibrinolysis in horses (32).

2.3.3 Global hemostatic tests

The conventional static hemostatic markers only assess part of the full hemostatic process, making it difficult to recognize complex coagulopathies and mainly hypercoagulation in patients in clinical practice (138). Assays such as PT and aPTT are primarily used to detect hypocoagulation, as their ability to detect hypercoagulation is inadequate (138). Global hemostatic tests such as TEG and CAT are considered superior to the conventional hemostatic test as they evaluate the entire hemostatic process from initiation to clot formation and fibrinolysis (243,244). As with any test, it is important to view the results in a clinical context and ensure interpretation by skilled personnel (136).

2.3.3.1 Thromboelastography

While conventional hemostatic tests only assess the hemostasis until the first fibrin strands are created, TEG assesses the entire hemostatic process (245) and provides information about the quality of the clot and the dynamics of its creation (246). Thromboelastography was first described in 1948 (247) and was developed for whole blood analysis (147). When a TEG analysis is run, a variety of variables are recorded. The first is the split point (SP), which is the time to initial conversion of fibrinogen to fibrin (245). Next is the reaction time (R), which is the time from test start to initial fibrin formation, which evaluates the activity of plasma coagulation factors (245,248). Third is the clotting time (K), which is the time until a pre-set level of clot strength is

achieved; normally an amplitude of 20 mm (245). Studies have found a modest correlation of PT and aPTT with R and K in horses (38,249). The angle (α) measures the speed of fibrin build-up and cross-linking, which is an evaluation of the rate of clot formation that can indicate fibrinogen deficiencies (245,248). Maximum amplitude (MA) is a measurement of the bonding of fibrin and platelets representing the maximum strength of the fibrin clot, which relates to the platelet function (245,248) (**Figure 3**). The shear elastic force (G) is a linear function of the MA and a measure of global clot strength (245). The degree of fibrinolysis is measured 30 minutes (LY30) and 60 minutes (LY60) post MA (245,248). In human medicine, a coagulation index has been created based on R, K, MA and angle. It is an indicator of coagulation and assess whether an individual is normal, hypo-, or hypercoagulable (147,245). Hypercoagulability is defined as a shorter R and K value and an increased angle, MA, and G with one or more altered variable, while the opposite is defined as hypocoagulability (36,244,245,250).

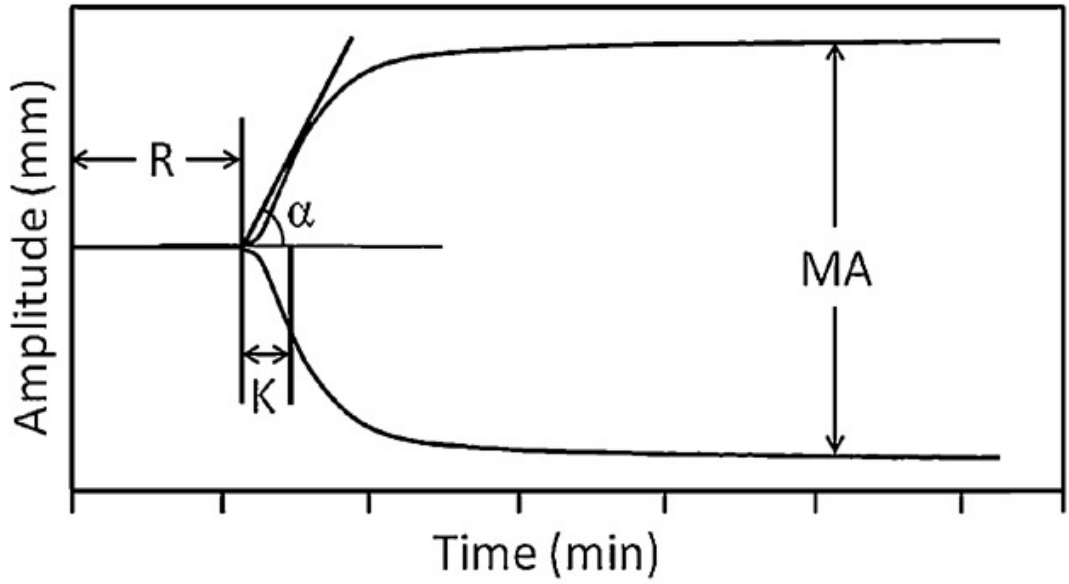


Figure 3. An outline of a tracing generated by thromboelastography (TEG) displaying the reaction time (R), clotting time (K), angle (α), and maximum amplitude (MA). *Source:* Reprinted with permission from: Plasma Processes and Polymers, Volume: 10, Issue: 9, Pages: 817-828, first published: 09 July 2013, DOI: (10.1002/ppap.201300027).

TEG can be performed both with and without the use of an activator (249,251), where the most commonly used are kaolin or TF (251–253). The use of an activator has been found to speed up the initiation of clot formation and lower the test variability, resulting in a more robust assay (249,254). Tissue factor has been applied as an activator in humans (255) and dogs (256). In horses, using TF as the activator has been associated with a shortened R and K time and an increased angle when compared with TEG without an activator. In addition, TF decreased variability in measurements. A disadvantage of using an activator is that hypocoagulable conditions might be masked by the artificial shortening of clotting times and rates (249). Kaolin has likewise been applied as an activator in humans (252) and dogs (253,257). A native, TF-, and kaolin-activated TEG have been compared in horses. There was a significant difference between the activated and non-activated assays, with the non-activated assay showing the highest degree of variation. It therefore seems that an activator should be used in order to minimize the degree of variation when using TEG in clinical practice. This will reduce susceptibility to pre-analytical factors and increase sensitivity for intra-individual changes.

No significant difference was found between kaolin- and TF-activated TEG, and dilute TF-TEG was found to be useful with the lowest analytical and intra-individual variation (251).

Whole blood-TEG is a time sensitive test, which in human studies have been found to be applicable on fresh whole blood within four to six minutes of sampling, and within 120 minutes of sampling on citrated whole blood with Kaolin or TF as an activator (252,258). For equine samples, a fixed storage time of 30 minutes post sampling has been proposed in order to avoid unreliable results (259). In recent years, a couple of studies have however looked at the use of TEG on citrated plasma (39–42). In humans, plasma-TEG has previously been used in a study examining the TEG assay and in a study of experimental human endotoxemia (39,40). In dogs plasma-TEG was successfully applied when looking at biological variation (41). In septic pigs, plasma-TEG displayed a similar pattern to whole blood-TEG, showing an increased hemostatic response (42). The advantage of plasma-TEG is that samples can be stored for longer periods prior to analysis, thus increasing the practicability of the test.

In human medicine, TEG, especially hypocoagulable tracings, appears to be prognostic of mortality in patients with sepsis, and may aid in the diagnosis of DIC (260). In experimentally induced endotoxemia, TEG has shown a hypercoagulable state (40). Thromboelastography may provide valuable and detailed information about dysfunctional aspects of the hemostatic process, resulting in a more targeted transfusion strategy (261). Whole blood-TEG has also been investigated in equine medicine. One study in horses with acute GI disease found that TEG performed at admission was able to identify the presence of inflammatory lesions and risk of fatality more frequently than conventional hemostatic markers (36). However, another study found the opposite to be true (37). Potential explanations for this discrepancy include the use of different inclusion criteria and that TEG results can be influenced by a variety of factors, thus highlighting the complexity of viscoelastic coagulation testing (37). This is further supported by TEG showing a tendency towards a hypercoagulable state in horses with ischemic and/or inflammatory GI lesions in one study (35) and hypocoagulable in another (38). There was a marked overlap with the normal range in both studies, indicating that TEG might not be sufficient as the sole test (35,38).

2.3.3.2 Thrombin generation test

Thrombin plays a vital role in hemostasis and thrombosis (262), as no hemostatic pathway bypasses thrombin, meaning that thrombin generation essentially reflects all thrombotic-hemostatic functions of the blood (263). The CAT assay measures the activity of free thrombin made during coagulation (263) in a more physiological setting than the conventional coagulation tests (263–265). The CAT assay generates four variables. The lag time is the time it takes until 1/6 of the maximum peak thrombin concentration is reached and this correlates well with the plasma clotting time. The endogenous thrombin potential (ETP) is often considered the parameter with the highest predictive value in terms of bleeding/thrombosis risk for the CAT assay. The ETP, or area under the curve, expresses the total enzymatic work by thrombin while active. The peak is the maximal thrombin concentration measured. Peak and ETP normally correlate well, but peak is not as easily saturated with increasing concentrations of coagulation factors and triggers, and might therefore prove to be a more sensitive measure of the plasma thrombin generation capacity. Finally, the time to peak (ttPeak) is the time until the peak concentration is reached (266,267) (**Figure 4**).

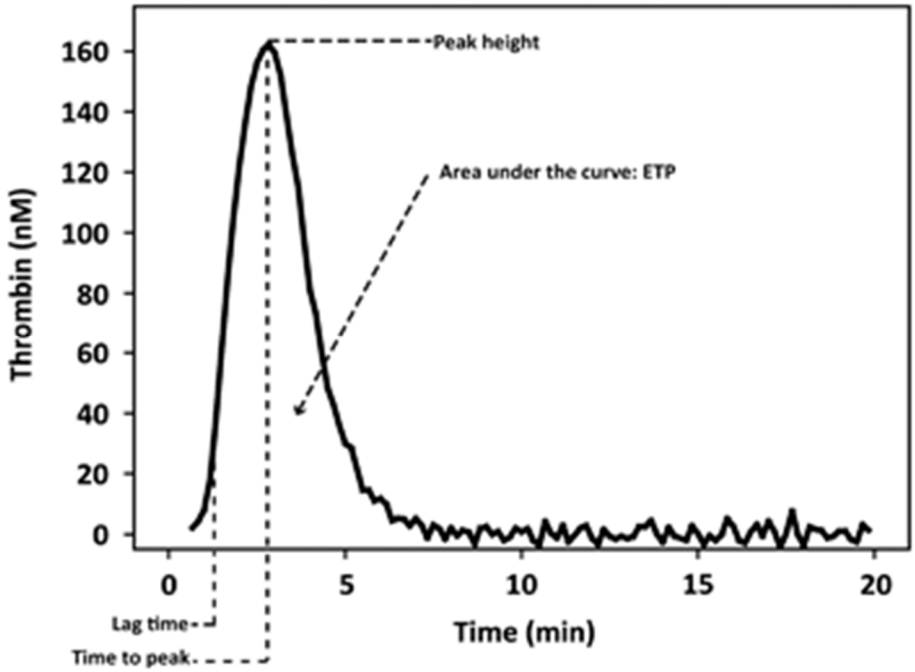


Figure 4. An outline of a tracing generated by the Calibrated Automated Thrombogram (CAT) assay displaying the lag time, time to peak, endogenous thrombin potential (ETP), and peak. *Source:* Reprinted with permission from: Veterinary Clinical Pathology, Volume: 40, Issue: 1, Pages: 24-31, First published: 07 February 2011, DOI: (10.1111/j.1939-165X.2011.00282.x).

In human medicine, the CAT assay has been used with both hypo- and hypercoagulable conditions (263–265). A hypercoagulable condition is indicated by a shortened lag time and ttPeak, and a higher ETP and peak with one or more variables being altered. Hypocoagulability is characterized by the opposite findings (268). Studies have found that an increased peak concentration (269) and ETP measurement are associated with an increased risk of a venous thrombotic event (270–273). Conversely, the CAT assay can also help identify patients with a low risk of reoccurring venous thromboembolism (VTE) (274). In addition, the CAT assay is valuable in assessing the prognosis of patients with acute ischemic stroke undergoing thrombolysis; patients with a low ETP value at admission have an increased risk of a poor therapeutic outcome (275). The CAT assay has been investigated for use in veterinary medicine in dogs (276–279) and cats (280). Using feline plasma samples, the CAT assay showed adequate repeatability and was found to be suitable for measuring the ETP (280). The CAT assay has also been found to work well with canine plasma, with good analytical reproducibility, and might be useful when assessing the use of anticoagulant therapy in dogs (277). A hypercoagulable state was identified in dogs with hyperadrenocorticism by an increased ETP when dogs were analyzed as a group (279). Low doses of prednisolone have been found to increase ETP in dogs (278). The CAT assay has previously been shown to be a robust assay with reliable monitoring of thrombin generation that is able to distinguish hemophilic dogs from healthy dogs (276). To the best of the author's knowledge, the CAT assay has not been investigated for use in horses.

2.4 The suggested interplay between *Strongylus vulgaris* and hemostasis with the relevance of the global hemostatic tests

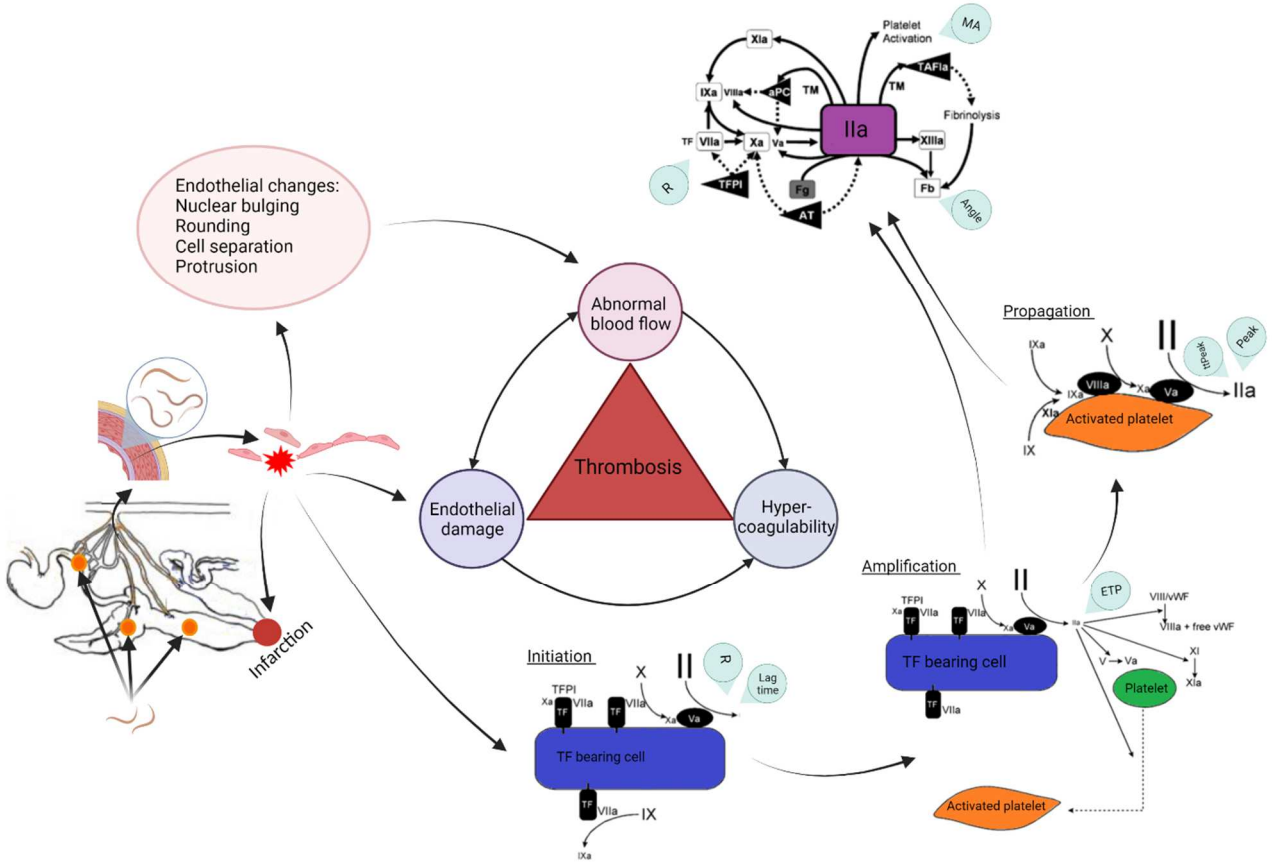


Figure 5. An illustration of the suggested effects of *Strongylus vulgaris* on the hemostasis with the hypothesized relevance of the global hemostatic tests pinpointed. Third stage larvae (L3) penetrate the ileum, cecum, and/or colon. After moulting to fourth stage larvae (L4) they migrate in the tunica intima of the arteries against the blood flow until they reach the cranial mesenteric artery and its branches. This migration leads to a denuded endothelial surface and exposure of the subendothelial collagen. This initiates platelet adhesion and production of procoagulant factors, and the endothelial cells change in shape, causing changes in blood flow, contributing to thrombus formation and initiating a vicious cycle. It is important to note that the hemostatic process should not be viewed as a separate stepwise process, but more as a simultaneous self-reinforcing process. Hence, the allocation of variables from the global hemostatic tests (thromboelastographic reaction time (R), angle and maximum amplitude (MA), calibrated automated thrombogram lag time, time to peak (ttPeak), peak and endogenous thrombin potential (ETP)) in the figure should also be viewed as an approximation since most represent more than one location.
Source: Created with BioRender by Honoré, M. L., 2022. Modified from the following figures: Hoffman & Monroe, 2007, figure 1; Smith, 2009, figure 3; https://www.cram.com/flashcards/parasitology-10810-horse-strongyles-2nd-year-fall-1492750, downloaded the 6th of January 2022. Reprinted with permission from: Hematology/Oncology Clinics of North America, Volume: 21, Issue: 1, Pages: 1-11, First published: February 2007, DOI: (10.1016/j.hoc.2006.11.004), and from Journal of Veterinary Emergency and Critical Care, Volume: 19, Issue: 1, Pages: 3-10, First published: February 2009, DOI: (10.1111/j.1476-4431.2009.00389.x.).

3 Materials and methods

This thesis is based on five studies. A detailed description of the materials and methods for Studies I, II, and III are available in Papers I, II, and III. Studies IV and V are not covered in any of the papers and will therefore be described in more detail.

3.1 Sampling centers

This thesis was an international multicenter study with samples collected at four different facilities (Table 1). The necessary approvals were obtained from the ethical board of the Swedish university of Agricultural Sciences, Evidensia Helsingborg Equine Referral Hospital, Gluck Equine Research Center at the University of Kentucky, and the Department of Veterinary Clinical Sciences at the University of Copenhagen. All relevant guidelines and regulations were followed. Written consent (Appendix I) was acquired for client-owned horses in Denmark and Sweden.

Table 1. Sampling centers included in this project.

Sampling center	Sampling period	Sample population	Study
Large Animal Teaching Hospital, University of Copenhagen, Denmark	December 2017 – March 2021	Clinically healthy horses owned by the hospital All horses admitted with acute abdominal pain and/or peritonitis	Study I Study II Study III Study IV Study V
University Animal Hospital, Swedish University of Agricultural Sciences, Sweden	January 2018 – March 2021	All horses admitted with peritonitis and/or surgical colic	Study III Study V
Evidensia Helsingborg Equine Referral Hospital, Sweden	January 2018 – March 2021	All horses admitted with peritonitis and/or surgical colic	Study III Study V
Gluck Equine Research Center, University of Kentucky, USA	January 2018 – February 2019	All 18 adult horses in the herd [a] were sampled once every other week for one year	Study III Study IV

[a] The Barn 10 research herd at the University of Kentucky's Maine Chance Farm (281).

3.2 Horses

All clinically healthy horses underwent a thorough clinical examination performed by ML Honoré. The remaining horses admitted to the Large Animal Teaching Hospital at the University of Copenhagen, the University Animal Hospital at the Swedish University of Agricultural Sciences, and Evidensia Helsingborg Equine Referral Hospital were examined by the veterinarian in charge at admission. All clinical variables and blood parameters used were taken at the time of admission. The final diagnoses were based on all information available in the horses' records, including clinical variables, laboratory variables, response to treatment, and potential surgical and postmortem findings. The horses from the Barn 10 research herd at the University of Kentucky's Maine Chance Farm were semi feral and it was therefore not possible to perform a clinical examination, but these horses were monitored several times a day by trained personnel and no clinical signs of disease were reported.

An overview of the specific inclusion criteria for each group of horses included in Studies I–V is given in Table 2. Horses were excluded if they were below one year of age or dead on arrival to the hospital.

Table 2. The inclusion criteria for each group of horses in this thesis. *S. vulgaris*: *Strongylus vulgaris*, NSII: non-strangulating intestinal infarction

Groups of horses	*Abbreviated group names*	*Inclusion criteria*	*Study*

Clinically healthy horses		• Considered healthy based on the following being within normal reference intervals:
		  • Clinical examination
		  • Complete blood count (CBC)
		  • Serum biochemistry profile including serum amyloid A (SAA) and fibrinogen
		• No history or clinical or laboratory signs of colic or acute or chronic systemic inflammatory disease.
		• Treated with ivermectin or moxidectin between one or two weeks prior to examination and sample collection.
			Study I
			Study II
			Study III
			Study IV
			Study V

Horses with GI disease and a mild systemic inflammatory response	• Low SAA (Study I)	• GI disease and SAA between 30-100 mg/L at admission.	Study I
Horses with GI disease and a strong systemic inflammatory response	• High SAA (Study I)	• GI disease and SAA > 1,000 mg/L at admission.	Study I

Horses with mild GI disease	• Mild GI disease	• Non-strangulating intestinal obstructions or displacements without signs of secondary inflammation or ischemia of the intestines based on:
		  • Clinical signs
		  • CBC
		  • Serum biochemistry including SAA and fibrinogen
		  • Rectal palpation, naso-gastric intubation, and where appropriate abdominocentesis, transabdominal ultrasonography, surgery, and postmortem findings.
			Study II

Horses with severe GI disease	• Severe GI disease	• Inflammatory or strangulating intestinal diseases with ischemia of the intestine based on:
		  • Clinical signs
		  • CBC
		  • Serum biochemistry including SAA and fibrinogen
		  • Rectal palpation, nasogastric intubation, and where appropriate abdominocentesis, transabdominal ultrasonography, surgery, and postmortem findings.
			Study II

Horses with migrating *S. vulgaris* without clinical disease	• *S. vulgaris* NSII negative (Study III)	• Part of the well-documented Barn 10 research herd at the University of Kentucky's Maine Chance Farm (281). • The presence of *S. vulgaris* was confirmed via regular larval cultures and a positive *S. vulgaris* antibody titer (>13.47%) (116).	Study III Study IV

Horses with migrating *S. vulgaris* with clinical disease (NSII)	• NSII (Study I)
	• *S. vulgaris* NSII positive (Study III)
		• NSII was as previously described (7) confirmed at surgery or postmortem with a finding of one or more areas of localized intestinal infarction without signs of strangulation such as a clear volvulus or enterocolitis, and either:
		  • Signs of migrating *S. vulgaris* larvae, seen as thrombosis and arteritis in the CMA and/or its branches, present at post-mortem examination.
		  • Signs of arteritis and/or larvae on histology of the resected intestine from surviving horses.
			Study I
			Study III
			Study V

Horses with idiopathic peritonitis		• Peritoneal fluid sample with a white blood cell count (WBC) > $10x10^9$ cells/L and a peritoneal total protein > 20 g/L that responded to medical treatment, with survival to discharge or with no apparent cause identified at surgery or necropsy.	Study III

Horses with enterocolitis		• Peracute and acute colitis:
		  • Acute abdominal pain or diarrhea of maximum 48 hours duration prior to admission or identified with diarrhea within 48 of admission.
		  • Horses without diarrhea but a very short, intense course of disease and a postmortem diagnosis of enterocolitis or typhlocolitis.
		  • Based on macroscopic signs of generalized intestinal inflammation (hyperemia, necrosis, and edema) with no signs of strangulation.
		• Duodenitis-proximal jejunitis:
		  • Excessive gastric reflux (> 20 L) over > 24 hours that responded to medical treatment or where no concomitant mechanical obstruction was identified at surgery or postmortem.
			Study III
			Study V

Horses with strangulating lesions		• One or more strangulating intestinal lesions in the ileum, jejunum, and/or ascending colon confirmed at either surgery or postmortem.	Study III Study V

Power calculations were not performed for Studies I, II, IV, or V. The aim was to include around ten horses in each group in order to show a potential difference between groups but at the same time not include too many in case some of the assays or methods were not applicable in horses. In Study III (Paper III), power calculations were based on the results of Study II, and we found that around 20 horses in each group would be sufficient to show a relevant difference.

3.3 Clinical variables

An overview of the recorded clinical variables for each horse included in Studies I–V is given in Table 3.

Table 3. Clinical variables registered for each horse. * Not recorded for the horses from the Barn 10 research herd at the Gluck Equine Research Center at the University of Kentucky.

	Variable	Description
Demographics	Age	Age in years
	Body Weight (BW)*	BW in kg
	Sex	Mare, gelding, or stallion
Clinical parameters	Breed	Breed of horse
	Heart rate (HR)*	Beats pr. min.
	Respiratory rate (RR)*	Breaths pr. min.
	Rectal temperature*	Degrees in Celsius
	Borborygmia*	Normal, decreased, or increased intestinal sounds on auscultation
	Mucus membranes*	Normal (pink), pale, hyperemic (red), or cyanotic (purple)
Disease	Affected portion of GI	Stomach, small intestine, cecum, colon, peritoneum, extraenteral, unknown
	Disease process	Simple obstruction, strangulating obstruction, non-strangulating infarction, enterocolitis, peritonitis, ulcera, perforation, no pathology seen, other, unknown

3.4 Blood sampling

As described in Papers I, II and III, blood samples were taken by jugular venipuncture with a 21 g needle and vacutainer system. Blood samples were collected prior to catheter placement and any potential treatment (apart from those given by the referring veterinarian). In the rare case that catheter placement could not be delayed due to a need for rapid medical intervention, samples were collected via intravenous catheter. Initially, the sodium citrate tubes were filled (the first tube was discarded), then serum separator tubes, and finally EDTA tubes. Sodium citrate tubes (3.2% 0.109M) were used for hemostatic tests, serum tubes were used for biochemical analysis, and EDTA tubes were used for CBC. At Gluck Equine Research Center, the blood samples were collected in glass tubes (the sodium citrate tubes were coated internally with silicone) in contrast to the other sampling centers where they were collected in plastic tubes. Serum and EDTA samples were stored at 4°C prior to analysis. Sodium citrate tubes were centrifuged at 2,000 *g* for 15 min to achieve a platelet count below 10 × 109/L (282) within a maximum of 4 hours. The citrated plasma was transferred to multiple cryotubes with 1 ml in each and stored at -80° until analysis.

3.5 Hematology and biochemistry

Hematological and biochemical blood analysis was performed for all horses.

For horses admitted to the Large Animal Teaching Hospital at the University of Copenhagen, this was part of the routine diagnostic workup at admission, and analyses were run on fresh sample material as soon as possible after sampling. For clinically healthy horses from the same facility, analyses were also run on fresh sample material directly after sampling.

For horses admitted to the two hospitals in Sweden, this was also part of the routine diagnostic workup at admission and analyses were run on fresh sample material. However, iron, SAA, and fibrinogen were not part of the routine blood analyses and were not measured in all horses at admission. In case of missing values, these were measured in batches in Denmark from frozen sample material shipped from Sweden. For SAA, all samples were rerun in Denmark to ensure comparable results due to the different assays used. MPV and MPC were not measured for the samples from Sweden. In addition, the platelet (PLT) concentration was not part of the routine blood analyses and was not measured for all horses from Sweden. This could not be done at a later stage as only plasma and serum samples were stored.

A routine hematology analysis was performed on fresh samples from the Barn 10 research herd horses directly after sampling. However, PLT, MPV, and MPC were not measured for the samples from the USA. The routine biochemical analysis was performed in batches on frozen sample material shipped from the USA to Denmark.

Not all hematological or biochemical variables were included for analysis; the variables selected for further evaluation are shown in Table 4.

Table 4. Blood variables included for analysis in this thesis. The country stated indicates in which country the specific machine/assay was used. WBC: white blood cell count, HCT: hematocrit, PLT: platelet, MPV: mean platelet volume, MPC: mean platelet component, SAA: Serum amyloid A.

Variable	Unit	Machine	Assay/Method/Comments
WBC (Blood)	mia./L	Advia 2120i [I] (Denmark)	The analyses were done on fresh samples at the sampling center where samples were collected.
		Advia 2120i [I] (Sweden)	
		Idexx Procyte Dx [II] (Sweden)	
		Genesis Hematology Analyzer [III] (USA)	
HCT (Blood)	%	Advia 2120i [I] (Denmark)	The analyses were done on fresh samples at the sampling center where samples were collected.
		Advia 2120i [I] (Sweden)	
		Idexx Procyte Dx [II] (Sweden)	
		Genesis Hematology Analyzer [III] (USA)	
PLT (Blood)	mia./L	Advia 2120i [I] (Denmark)	The analyses were done on fresh samples at the sampling center where samples were collected. Was not measured in all horses from the Swedish sampling centers and was not measured in samples from USA.
		Advia 2120i [I] (Sweden)	
		Idexx Procyte Dx [II] (Sweden)	
MPV (Blood)	fl	Advia 2120i [I] (Denmark)	Only measured on samples collected in Denmark.
MPC (Blood)	g/L	Advia 2120i [I] (Denmark)	Only measured on samples collected in Denmark.
Iron (Serum)	umol/L	Advia 1800 [I] (Denmark)	Colorimetric spectrophotometric measurement. Samples from all four sampling centers were analyzed in Denmark.
SAA (Serum)	mg/L	Advia 1800 [I] (Denmark)	LZ SAA EIKEN automated immunoturbidimetric assay [IV]. Samples from all four sampling centers were analyzed in Denmark.
Fibrinogen (Citrated plasma)	g/L	ACL Top 500 [V] (Denmark)	PT-based automated coagulometric assay with HemosIL RecombiPlastin. All samples from USA were analyzed in Denmark, and 12/32 samples from Sweden were analyzed in Denmark.
		KoneLab Prime 30i [VI] (Sweden)	Quantitative immunoturbidimetric assay [VII].

[I] Siemens Health Care Diagnostics Inc., IL, USA. [II] IDEXX, Hoofddorp, The Netherlands. [III] Oxford Science Inc., Oxford, CT, USA. [IV] EIKEN Chemical Co. Ltd., Tokyo, Japan. [V] ILS Danmark, Allerød, Denmark. [VI] Thermo Fisher Scientific, Waltham, U.S.A. [VII] DiaSystem Scandinavia AB, Huskvarna, Sweden.

3.6 *Strongylus vulgaris* specific antibodies

The concentration of *S. vulgaris* specific antibodies was used in Studies III (Paper III) and IV. Fresh serum samples collected from the Barn 10 research herd were analyzed shortly after collection. Serum samples collected in Denmark and Sweden were collectively shipped frozen to the University of Kentucky and analyzed in a batch. Samples were analyzed for *S. vulgaris*-specific antibodies with an indirect antibody ELISA using recombinant SvSXP protein as antigen as previously described (Paper III) (6,116). Results were expressed as the normalized value, percentage of a positive control (PP) (116).

3.7 Hemostatic assays

All hemostatic analyses (Tables 5 and 6) were performed on whole blood or thawed citrated plasma at the University of Copenhagen by trained personnel and on the same machines in an attempt to ensure that any differences found were the result of pathological differences between the groups of horses. A more detailed description of the applied protocols can be found in Papers I, II, and III.

Table 5. The conventional hemostatic variables measured in this thesis.

Variable	Unit	Assay/Machine	Method/Comments
Activated partial thromboplastin time (aPTT)	Sec	STA-Cephascreen with kefalin [a]	Automated coagulometric method
Prothrombin time (PT)	Sec	STA-NeoPTimal with rabbit thromboplastin [a]	Automated coagulometric method
Antithrombin (AT)	%	STA-Stachrom ATIII with bovine thrombin [a]	Automated chromogenic method
D-dimer	mg/L	STA-Liatest D-Di+ with murine d-dimer antibodies [a]	Automated photometric antibody-antigen method
	mg/L	NycoCard with monoclonal antibodies [b]	Manual immunometric flow through assay

[a] STAGO STA Satellite coagulation analyzer (Triolab, Brøndby, Denmark)

[b] NycoCard™ D-dimer assay (Abbott Laboratories, Copenhagen, Denmark)

Table 6. The variables measured for the applied global hemostatic tests (whole blood- and plasma-thromboelastography (TEG) and the Calibrated Automated Thrombogram (CAT)) in this thesis.

Method/variable		Unit	Description	Assay/Machine
TEG	Split point (SP)	min.	Time to initial conversion of fibrinogen to fibrin	Diluted TF, TEG 5000 Haemostasis Analyzer [a]
	Reaction time (R)	min.	Time to initial fibrin clot formation	
	Clot formation time (K)	min.	Time from initial clot formation to reaching a preset level of clot strength (20 mm)	
	Angle	Degrees	Speed of fibrin build up and cross-linking	
	Maximum amplitude (MA)	mm.	Maximum clot strength	
	Shear elastic force (G)	(dynes/cm^2)	A linear function of the MA and a measure of global clot strength	
	Lysis at 30 min. (Ly30)	%	Degree of fibrinolysis 30 minutes after MA	
	Lysis at 60 min. (Ly60)	%	Degree of fibrinolysis 60 minutes after MA	
CAT	Lag time	min.	Time until 1/6 of the total thrombin concentration is reached	PPP reagent (5 pM TF), Thrombinoscope BV [b]
	Endogenous thrombin potential (ETP)	nM*min.	The total amount of thrombin generated	
	Peak	nM	Maximal thrombin concentration	
	Time to peak (ttPeak)	min.	Time to maximal thrombin concentration	

[a] TEG 5000 Haemostasis Analyzer, Haemoscope Corporation, Illinois, USA

[b] Thrombinoscope BV, Maastricht, The Netherlands

3.8 Seasonal hemostatic changes correlated with *Strongylus vulgaris* (Study IV)

Blood samples were collected every other week from January 2018 to February 2019 from the adult horses at the Barn 10 research herd at the University of Kentucky's Maine Chance Farm (i.e. horses with migrating *S. vulgaris* without clinical disease), which is described in more detail in Section 3.2 and Paper III. At the same time points, fecal samples were collected for larval culture and the *S. vulgaris* antibody concentration in serum was measured. Blood samples taken every other week for one year from the six horses with the highest concentration of *S. vulgaris* antibodies on average were included in the study. Blood samples from 10 clinically healthy horses (described in detail in Section 3.2 and Papers I, II, and III) collected in the spring of 2018 were also included. Collection, handling, and analysis of blood samples were as described in Sections 3.4, 3.5, 3.6, and 3.7. Plasma-TEG and CAT variables were compared with results from July to assess any differences in variables over the year.

3.9 Endothelial markers (Study V)

Serum samples from four groups of horses (clinically healthy horses, horses with migrating S. vulgaris with clinical disease (*S. vulgaris* NSII positive), horses with enterocolitis, and horses with strangulating lesions) described in more detail in Section 3.2 and Paper III were analyzed with two human ELISAs for vCAM-1 and P-selectin and then compared across groups. Collection, handling, and analysis of blood samples were as described in Sections 3.4 and 3.5.

3.9.1 Vascular cell adhesion molecule 1

The concentration of serum vCAM-1 was analyzed using a commercially available human quantitative sandwich ELISA. All reagents were supplied with the kit and the assay was performed according to the manufacturer's instructions (Human sVCAM-1/CD106 ELISA Kit, PromoCell GmbH, Heidelberg, Germany). All samples, standards, and blanks were assayed in duplicate. The assay procedure can be found in Appendix II, Table 14.

3.9.2 P-selectin

The concentration of serum P-selectin was analyzed using a commercially available human quantitative sandwich ELISA immunoassay. All reagents were supplied with the kit and the assay was performed according to the manufacturer's instructions (Human P-Selectin ELISA Kit, OriGene Technologies, Inc., Rockville, MD, USA). All samples, standards, and blanks were assayed in duplicate. The assay procedure can be found in Appendix II, Table 15.

3.10 Statistical analyses

Statistical analysis was performed in Microsoft Excel 2016 (Washington, USA; Studies I and II), GraphPad Prism 8.3.0 (San Diego, CA, USA; Studies I–V), and R (R Core Team (2020) R: A Language and Environment for Statistical Computing, Addison R Foundation for Statistical Computing, Vienna, Austria; Studies I–V). The level of significance was set to 0.05.

Normal distribution was assessed visually (Studies I, III, IV, and V), using the D'Agostino-Pearson K-squared test (Study II), or the Shapiro-Wilk test (Study III). If variables were not normally distributed, a log or log2 transformation was applied or variables were analyzed with non-parametric methods if normality could not be achieved.

In Study I (Paper I), the d-dimer assays were evaluated by inter- and intra-coefficients of variation (CVs) and linearity under dilution, and groups were compared using the Kruskal-Wallis test, while Dunn's multiple comparisons test was applied *post hoc*. The two methods were compared using a spaghetti plot, a Passing-Bablok regression, Spearman's correlation test, and a Bland-Altman plot.

In Study II (Paper II), CAT was evaluated by inter- and intra-CVs. Groups were compared using a one-way ANOVA or Kruskal-Wallis test for TEG and CAT. Dunn's or Tukey's multiple comparisons tests were applied *post hoc*. Spearman's correlation was used to evaluate the correlation between fibrinogen and MA and G (plasma-TEG).

In Study III (Paper III), variables were compared using a one-way ANOVA, and Tukey's multiple comparisons test was applied *post hoc*. A logistic regression analysis was used to assess potential differences in the diagnostic variables for the six groups of horses. Some data imputation was performed to compensate for a few

missing values (see statistical appendix in Paper III). A logistic regression model was applied to try and distinguish between the *S. vulgaris* NSII positive group (y = 1) and the remaining groups (y = 0). Based on the model, two receiver operating curves (ROC) were generated. One included all data while the other was based on a cross-validation. A multinomial regression was used to assess the differences across all six groups.

In Study IV as described in Section 3.8 and 4.5, a one-way ANOVA with a Geisser-Greenhouse correction was used to assess whether there were significant differences in CAT and plasma-TEG variables over the year, with July functioning as reference month.

In Study V as described in Section 3.9 and 4.6, groups were compared using a one-way ANOVA.

4 Results

A summary of the most important results from Studies I–III is shown in this section; additional details are outlined in Papers I–III. Results for Studies IV–V are shown in full here.

4.1 Inclusion and exclusion of horses

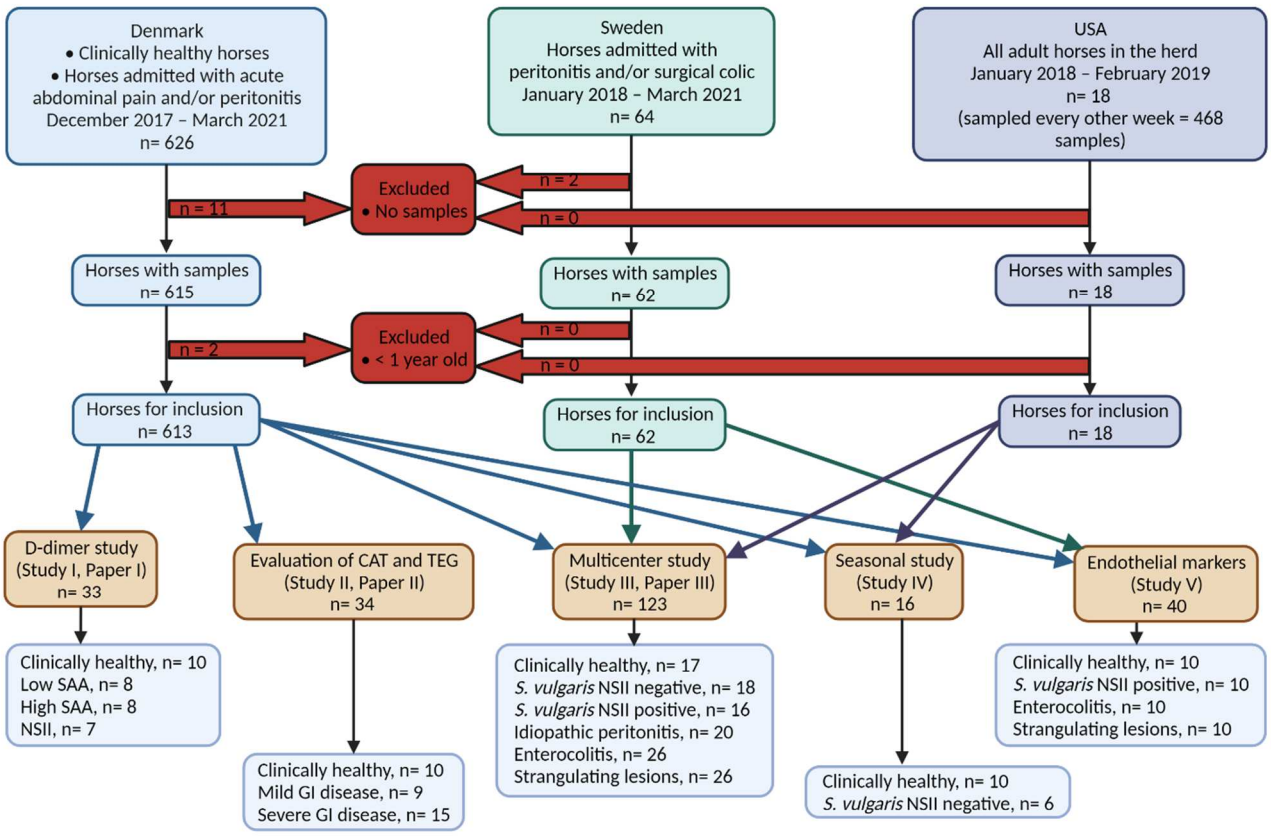


Figure 6. The overall inclusion and exclusion process of horses in this thesis. The exclusion process specific for each study is not included in the figure for simplistic reasons. SAA: serum amyloid A, NSII: non-strangulating intestinal infarction, GI: gastro intestinal, *S. vulgaris*: *Strongylus vulgaris*. Created with BioRender.

4.2 Study I (Paper I) - Investigation of human d-dimer assays in the horse

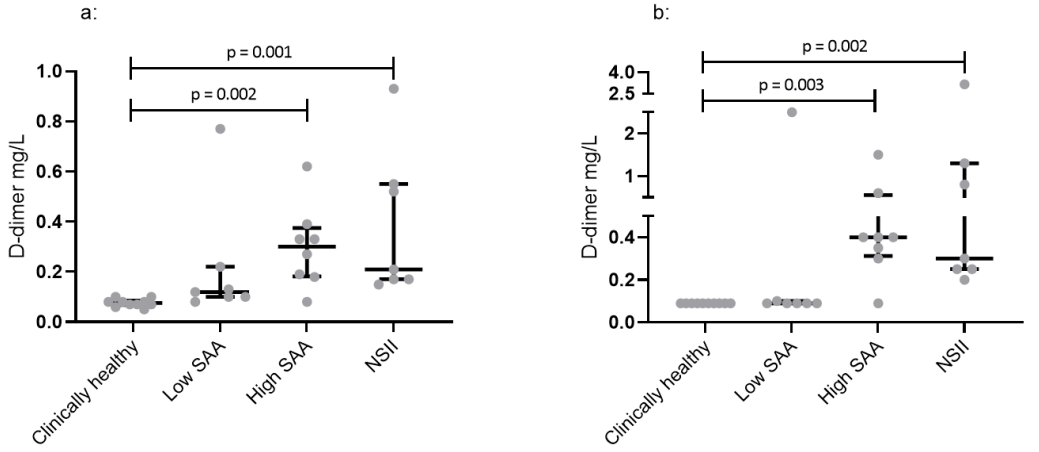
4.2.1 Test evaluation

As described in Paper I, the precision measurements (intra- and inter-CVs) for the Stago were all below the accepted level of 5%, apart from the low pool inter-CV (5.98%). All CVs for the NycoCard were above the accepted 10% level.

A linearity under dilution test showed compliance values between the observed and expected values of 89% and 70% for the Stago, and 97% and 65% for the NycoCard, respectively.

4.2.2 Group comparison

As demonstrated in Paper I, both Stago and NycoCard showed an overall statistically significant difference among the four groups (p = 0.0003), with the *post hoc* results displayed in Figure 7.



	Stago
	D-dimer (mg/L)
Clinically healthy	0.075 (0.07-0.09)
Low SAA	0.12 (0.1-0.22)
High SAA	0.3 (0.18-0.38)
NSII	0.21 (0.17-0.55)

	NycoCard
	D-dimer (mg/L)
Clinically healthy	< 0.1 (< 0.1-< 0.1)
Low SAA	< 0.1 (< 0.1-0.1)
High SAA	0.4 (0.31-0.55)
NSII	0.3 (0.25-1.3)

Figure 7. Group comparison across the four groups of horses using the a) STAGO STA-Liatest D-di+ (Stago) and b) NycoCard™ D-dimer assays (NycoCard). Horizontal lines and values in the tables represent the median and interquartile range. Note that the y-axis differs between the graphs. SAA: Serum amyloid A, NSII: non-strangulating intestinal infarction.

4.2.3 Method comparison

D-dimer concentrations measured with the NycoCard assay seemed generally higher than the concentrations measured with the Stago assay. Nonetheless, there seemed to be good agreement between the two assays, particularly for the lower concentrations (Figure 8).

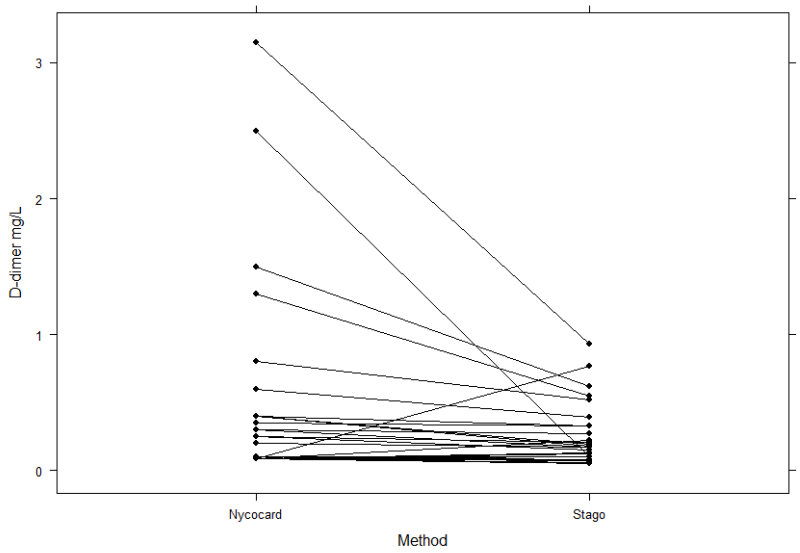


Figure 8. Spaghetti plot displaying the subject-wise trends for the d-dimer (mg/L) concentration for each horse measured using the STAGO STA-Liatest D-di+ (Stago) and NycoCard™ D-dimer assay (NycoCard).

All but two measurements in the Bland-Altman plot were within the 95% LOA of -2.9 – 2.11 mg/L (log2) with a mean of -0.4 mg/L (log2) and a median of -0.3 mg/L (log2) (Figure 9).

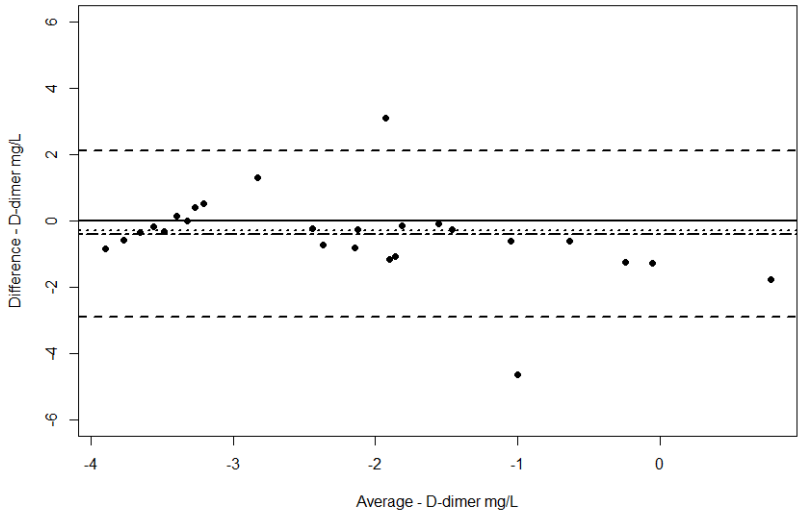


Figure 9. Bland-Altman plot displaying the difference plotted against the average for log2 transformed STAGO STA-Liatest D-di+ (Stago) and NycoCard™ D-dimer assay (NycoCard) measurements including 95% limits of agreement (LOA). Mean: dot/dashed line, median: dotted line, LOA: dashed lines.

4.2.4 Sample handling

As described in Paper I, two out of 33 horses (both in the NSII group) had samples collected in 3.8% (rather than 3.2%) sodium citrate tubes.

4.3 Study II (Paper II) - Evaluation of the Calibrated Automated Thrombogram and plasma-thromboelastography in horses

4.3.1 Test evaluation (Calibrated Automated Thrombogram)

As described in Paper II, the precision measurements (intra- and inter-CVs) for the CAT assay were all below the accepted 5%, and increasing concentrations of unfractionated heparin resulted in decreased thrombin generation.

4.3.2 Group comparison (Calibrated Automated Thrombogram and plasma-thromboelastography)

The CAT assay found an overall significant difference between the three groups of horses for peak (p = 0.04) and ttPeak (p = 0.01), with *post hoc* results displayed in Figure 10. The severe GI disease group was found to be hypercoagulable when compared to the clinically healthy horses and horses with mild GI disease (Paper II).

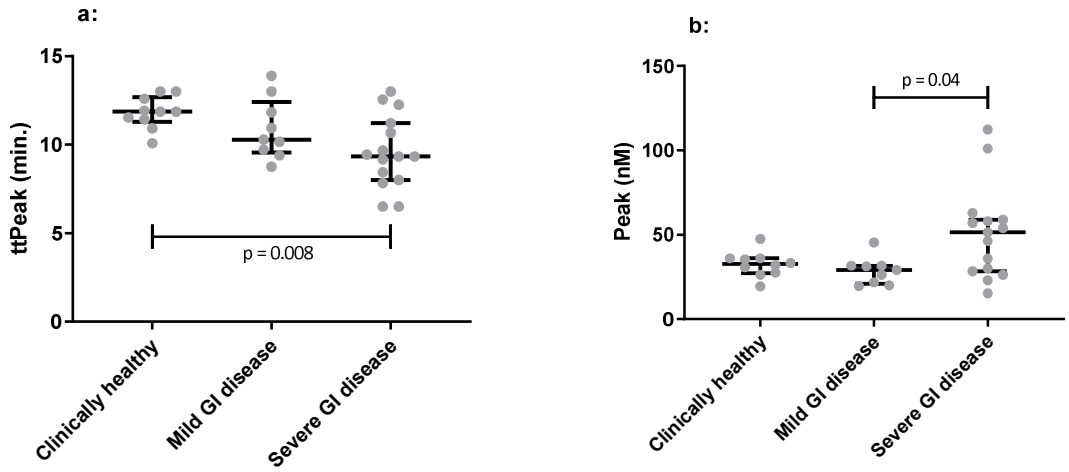


Figure 10. Group comparison across the three groups of horses (clinically healthy, mild and severe gastrointestinal (GI) disease) for the Calibrated Automated Thrombogram (CAT) assay. a) time to peak (ttPeak), b) peak. The horizontal bars display the median, 1st, and 3rd quartile.

Plasma-TEG ran successfully on equine PPP. An overall significant difference was found in angle (p = 0.016), MA (p = 0.023), and G (p = 0.022) among the three groups, with *post hoc* results displayed in Figure 11, showing that the severe GI disease group were hypercoagulable when compared to the clinically healthy horses (Paper II).

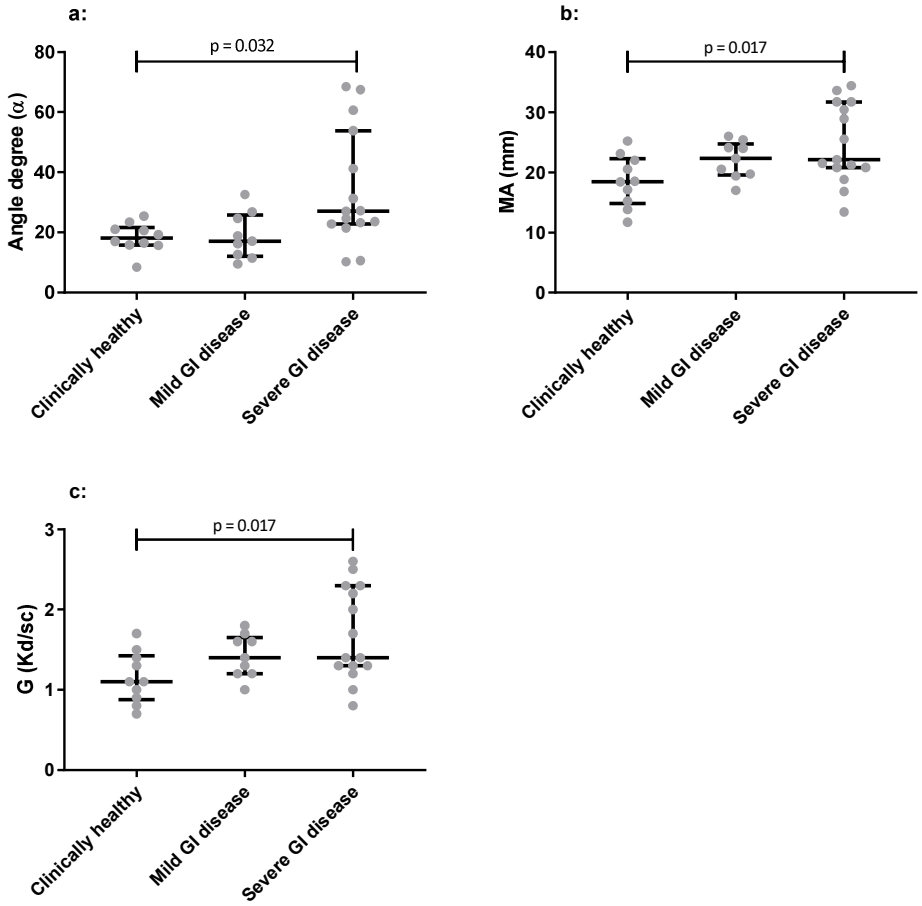


Figure 11. Group comparison across the three groups of horses (clinically healthy, mild and severe gastrointestinal (GI) disease) for plasma thromboelastography (plasma TEG). a) angle degree, b) maximum amplitude (MA), c) shear elastic force (G). The horizontal bars display the median and 1st and 3rd quartiles.

4.3.3 Group comparison (whole blood-thromboelastography and conventional hemostatic markers)

As described in Paper II, no significant difference was found in whole blood-TEG among the three groups of horses. Significantly lower angle ($p = 0.003$), MA ($p < 0.0001$), and G ($p < 0.0001$) were found with plasma-TEG compared to whole blood-TEG.

In terms of fibrinogen concentration, aPTT, PT, and AT, no significant differences were found among the three groups, while horses with severe GI disease had a significantly higher d-dimer concentration than horses with mild GI disease ($p = 0.0084$) and clinically healthy horses ($p < 0.0001$) on *post hoc* analysis.

4.4 Study III (Paper III) – Identification of hemostatic changes in horses with migrating *Strongylus vulgaris*

4.4.1 Group comparison (plasma-thromboelastography)

The *S. vulgaris* NSII negative group and the groups with severe GI disease (*S. vulgaris* NSII positive, idiopathic peritonitis, enterocolitis, and strangulating intestinal lesions) had hemostatic changes when compared to the clinically healthy horses without *S. vulgaris* (Figure 12).

The *S. vulgaris* NSII negative group had a significantly increased angle ($p = 0.0004$), MA ($p = 0.01$), and G ($p = 0.003$) compared to the clinically healthy horses without *S. vulgaris*. The *S. vulgaris* NSII positive group had a significantly longer R time ($p = 0.04$) compared to the horses with strangulating lesions. Furthermore, the *S. vulgaris* NSII positive group had a significantly higher MA than the enterocolitis group ($p = 0.02$) and strangulating lesions group ($p = 0.02$), as well as a significantly higher G compared to the enterocolitis ($p = 0.03$) and strangulating lesions ($p = 0.03$) groups (Figure 12).

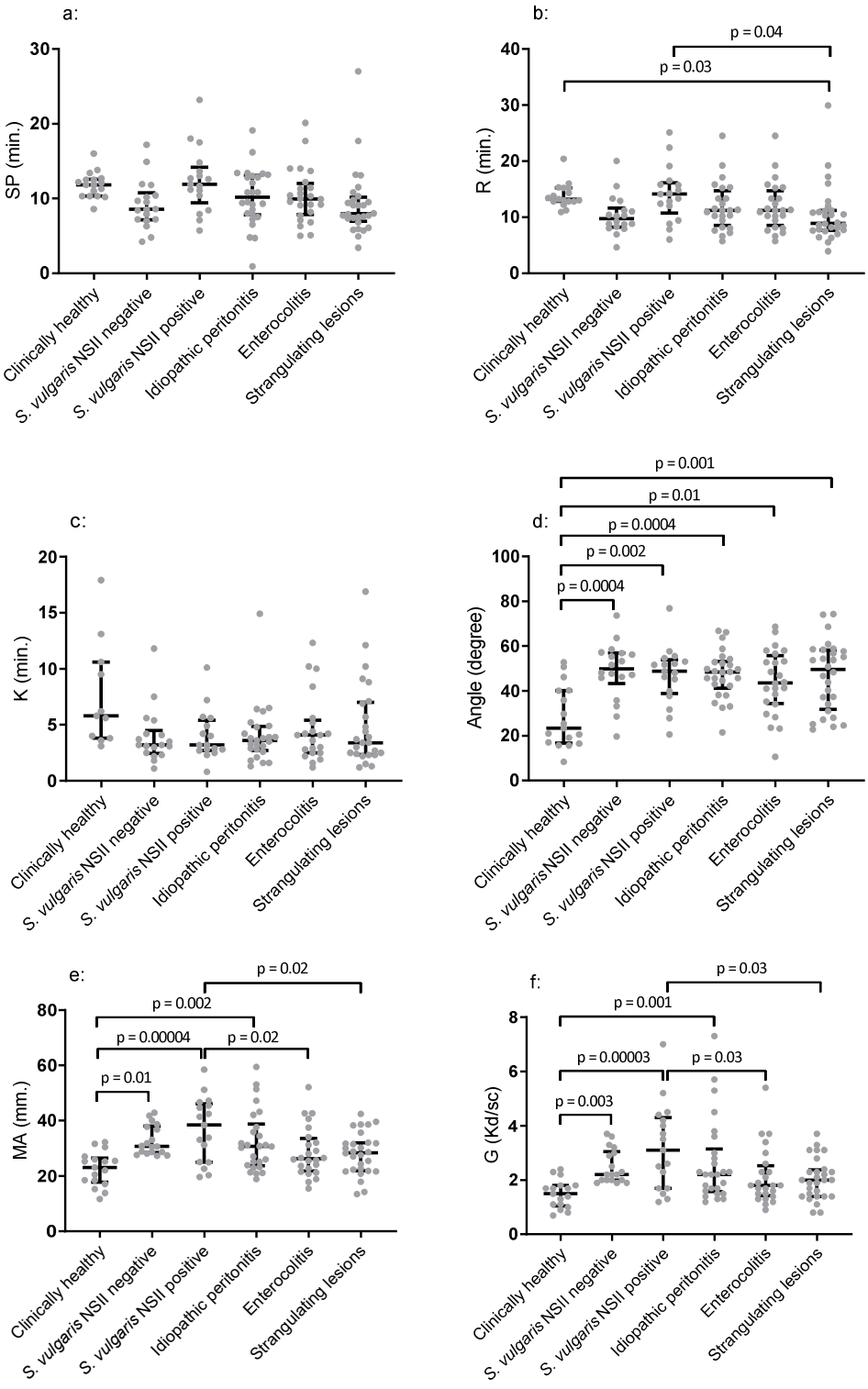


Figure 12. Group comparison for plasma-thromboelastography (TEG) across the six groups of horses (clinically healthy without *Strongylus vulgaris*, *S. vulgaris* non-strangulating intestinal infarction (NSII) negative, *S. vulgaris* NSII positive, idiopathic peritonitis, enterocolitis, and strangulating lesions). Lines show the median and interquartile range. SP: split point, MA: maximum amplitude, G: shear elastic force.

4.4.2 Group comparison (Calibrated Automated Thrombogram)

The *S. vulgaris* NSII negative group and the groups with severe GI disease (*S. vulgaris* NSII positive, idiopathic peritonitis, enterocolitis, and strangulating intestinal lesions) had hemostatic changes when compared to the clinically healthy horses without *S. vulgaris* (Figure 13).

The *S. vulgaris* NSII negative group had a significantly longer lag time (p = 0.02) than the clinically healthy horses without *S. vulgaris*. The *S. vulgaris* NSII positive group had a significantly longer lag time than the horses with enterocolitis (p = 0.01) and strangulating lesions (p = 0.001). In addition, the *S. vulgaris* NSII positive group had a significantly longer ttPeak than the enterocolitis group (p = 0.002) and horses with strangulating lesions (p = 0.002) (Figure 13).

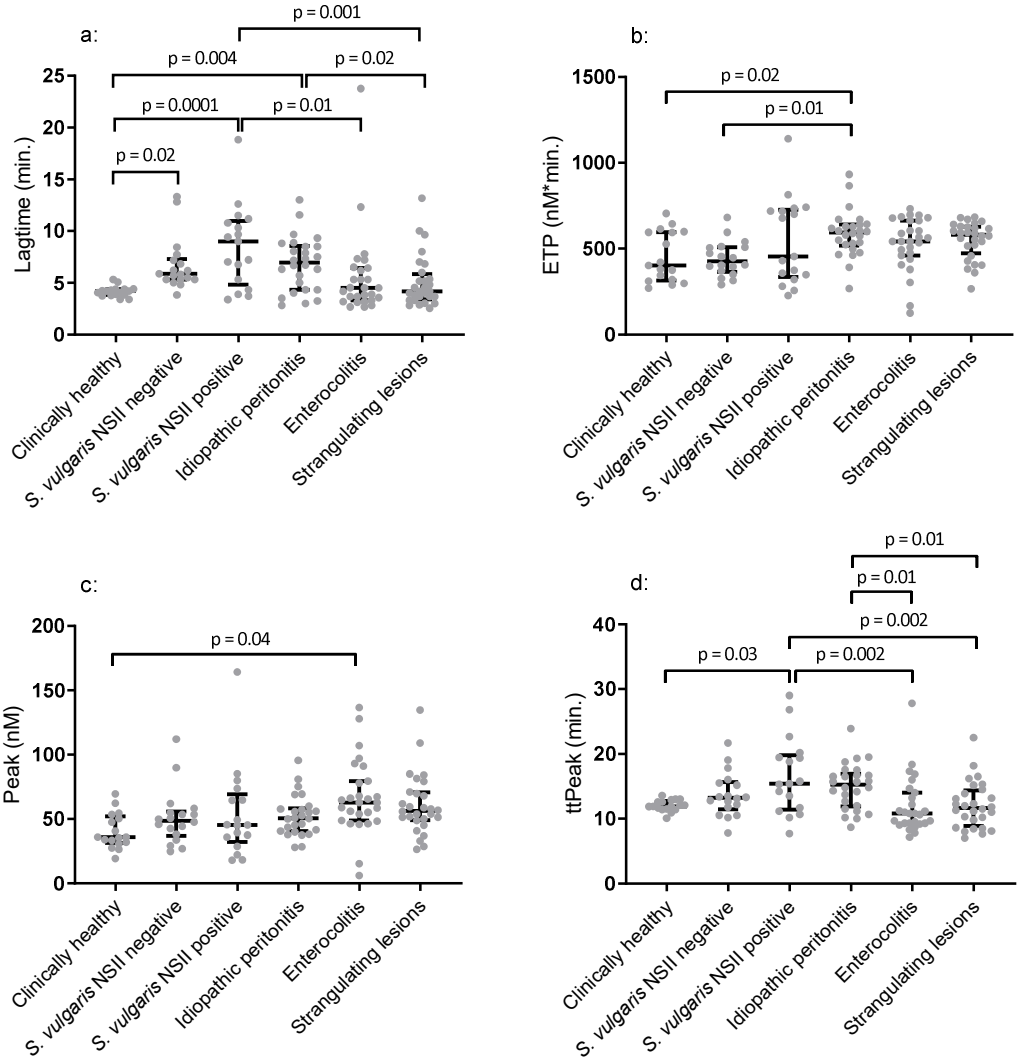


Figure 13. Group comparison for the Calibrated Automated Thrombogram (CAT) across the six groups of horses (clinically healthy without *Strongylus vulgaris*, *S. vulgaris* non-strangulating intestinal infarction (NSII) negative, *S. vulgaris* NSII positive, idiopathic peritonitis, enterocolitis, and strangulating lesions). Lines show the median and interquartile range. ETP: endogenous thrombin potential, ttPeak: time to peak.

4.4.3 Group comparison (clinical and laboratory variables)

As further described in Paper III, the *S. vulgaris* NSII positive group was characterized by one or more of the following findings: increased rectal temperature, decreased iron concentration, increased SAA, and increased fibrinogen and d-dimer concentration. In addition, all horses had increased *S. vulgaris* antibody concentration (> 13.47%).

4.4.4 Distinction of *Strongylus vulgaris* associated non-strangulating intestinal infarction positive horses

A multivariate logistic regression model including iron, SAA, angle (plasma-TEG) and ETP (CAT) were to some degree able to classify horses as belonging to the *S. vulgaris* NSII positive group or not (Table 7).

Table 7. The model for predicting *Strongylus vulgaris* associated non-strangulating intestinal infarctions (NSII) included iron, serum amyloid A (SAA), angle (plasma-thromboelastography) and endogenous thrombin potential (ETP) (Calibrated Automated Thrombogram).

	Estimate	Standard Error	P-value
Intercept	1.74	1.58	0.27
Iron (mmol/L)	-0.14	0.06	0.01
SAA (mg/L)	0.0008	0.0002	0.0002
Angle (degree)	0.05	0.03	0.14
ETP (nM*min.)	-0.01	0.004	0.002

The model showed that a decreased iron concentration, increased SAA concentration, increased angle (plasma TEG), and decreased ETP (CAT) were indicative of being classified in the *S. vulgaris* NSII positive group (Table 8).

Table 8. The odds and 95% confidence interval (CI) for iron, serum amyloid A, angle, and endogenous thrombin potential (ETP) of being classified in the *Strongylus vulgaris* NSII positive group. The considered change for SAA is +/- 1,000, for the remaining variables it is +/- 1.

	Odds	95% CI
Iron (mmol/L)	0.87	0.78 - 0.97
SAA (mg/L)	2.31	1.48 - 3.60
Angle (degree)	1.05	0.99 - 1.11
ETP (nM*min.)	0.99	0.98 - 0.99

Using a selected cut-off of 0.4, the model correctly classified 10/16 horses in the *S. vulgaris* NSII positive group, while 6/16 as false negatives. At the same time, 5/102 horses were classified as false positives (Figure 14), as described in Paper III.

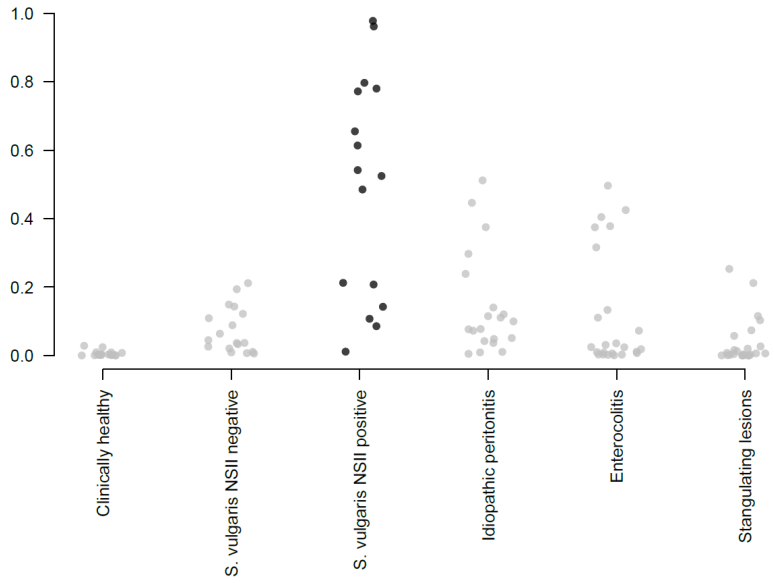


Figure 14. The predicted probability of being classified in the *Strongylus vulgaris* non-strangulating intestinal infarctions (NSII) positive group.

A cross-validated ROC curve as described in Paper III, showed an area under the curve (AUC) of 0.83 and a cut-off of 51.2% had an accuracy of 90.0%, with 2/103 horses classified as false positives and 10/16 as false negatives.

A multinomial regression showed that clinically healthy horses without *S. vulgaris* were classified with 100% accuracy by the model, and the *S. vulgaris* NSII negative group was also classified accurately. The severe GI disease groups showed a more marked overlap in their predicted classification probabilities; the *S. vulgaris* NSII positive group in particular showed a marked overlap with the idiopathic peritonitis group, while the enterocolitis group showed a marked overlap with the strangulating lesions group (Figure 15).

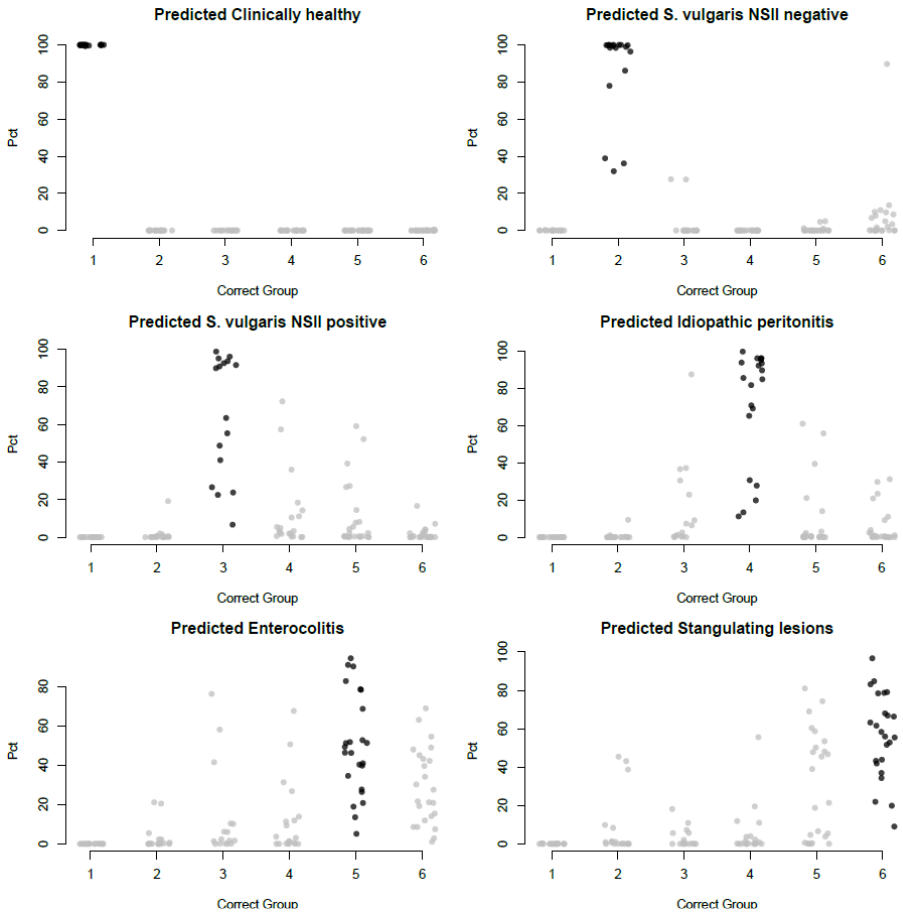


Figure 15. A multinomial regression dot plot showing the predicted probability of being classified in each of the six groups. NSII: non strangulating intestinal infarction, *S. vulgaris*: *Strongylus vulgaris*.

4.4.5 Sample handling

In Paper III, 6/123 horses: 3/16 in the *S. vulgaris* NSII positive group, 2/26 in the enterocolitis group, 1/26 with strangulating intestinal lesions had samples collected in 3.8% sodium citrate tubes, rather than 3.2%.

A platelet count > 10x10^9/L for the citrated plasma samples was found in 37/123 horses: 9/16 in the *S. vulgaris* NSII positive group, 4/20 with idiopathic peritonitis, 13/26 in the enterocolitis group, and 11/26 with strangulating intestinal lesions (Table 9).

Table 9. Median and minimum–maximum platelet (PLT) counts for the citrated plasma samples with a count > 10x10^9/L. *S. vulgaris*: *Strongylus vulgaris*, NSII: non-strangulating intestinal infarction.

	PLT count (mia./L)	
	Median	Min. - max.
***S. vulgaris* NSII positive**	15	12 - 46
Idiopathic peritonitis	13	11 - 17
Enterocolitis	15	11 - 93
Strangulating lesions	18	11 - 32

4.5 Study IV – Seasonal hemostatic changes correlated with *Strongylus vulgaris*

Both plasma-TEG and CAT identified seasonal hemostatic changes in the *S. vulgaris* NSII negative group when using July as the reference month.

4.5.1 Seasonal changes in plasma-thromboelastography

When compared to July, a significantly shorter R time was seen in August ($p = 0.02$) and December ($p = 0.002$) for the *S. vulgaris* NSII negative group. Furthermore, angle was significantly increased in April ($p = 0.02$) and December ($p = 0.01$). The MA and G had a similar seasonal pattern. A significantly increased MA and G were found in February ($p = 0.002$ and 0.002), March ($p = 0.02$ and 0.02), April ($p = 0.002$ and 0.004), June ($p = 0.005$ and 0.01), September ($p = 0.02$ and 0.01), November ($p = 0.02$ and 0.02), and December ($p = 0.01$ and 0.02) when compared to July in the *S. vulgaris* NSII negative group (Figure 16). Medians and 1st and 3rd quartiles can be found in Appendix III, Table 16.

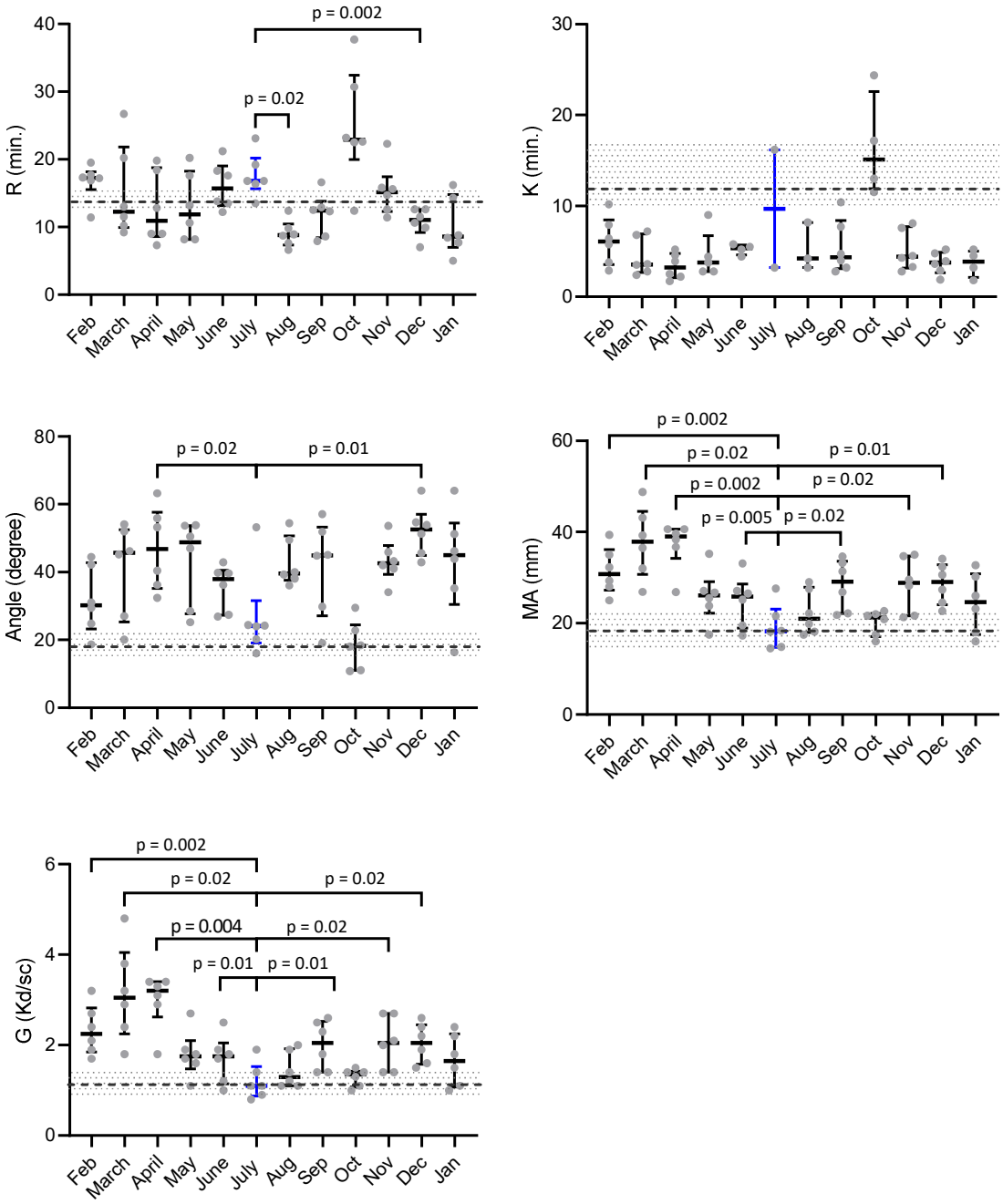


Figure 16. Plasma-thromboelastography variables compared across months for the *Strongylus vulgaris* non-strangulating intestinal infarction (NSII) negative horses, with July as the reference month. Bars in the dot plots indicate the median with 1st and 3rd quartiles. The shaded area indicates the 1st and 3rd quartiles with the median as a dashed line, for the clinically healthy horses without *S. vulgaris*.

4.5.2 Seasonal changes in the Calibrated Automated Thrombogram

A significantly shorter lag time was found in December (p = 0.02) and January (p = 0.02) for the *S. vulgaris* NSII negative group when compared to July. The ETP was significantly increased in June (p = 0.01), August (p = 0.004), September (p = 0.004), November (p = 0.01), December (p = 0.01), and January (p = 0.02) when compared to July in the *S. vulgaris* NSII negative group. Showing a similar pattern, a significantly higher peak

was found in August (p = 0.004), September (p = 0.01), November (p = 0.001), December (p = 0.01), and January (p = 0.01) when compared to July for the *S. vulgaris* NSII negative group. A significantly shorter ttPeak was seen in August (p = 0.03), December (p = 0.001), and January (p = 0.01) when compared to July in the *S. vulgaris* NSII negative group (Figure 17). Medians and 1st and 3rd quartiles can be found in Appendix III, Table 17.

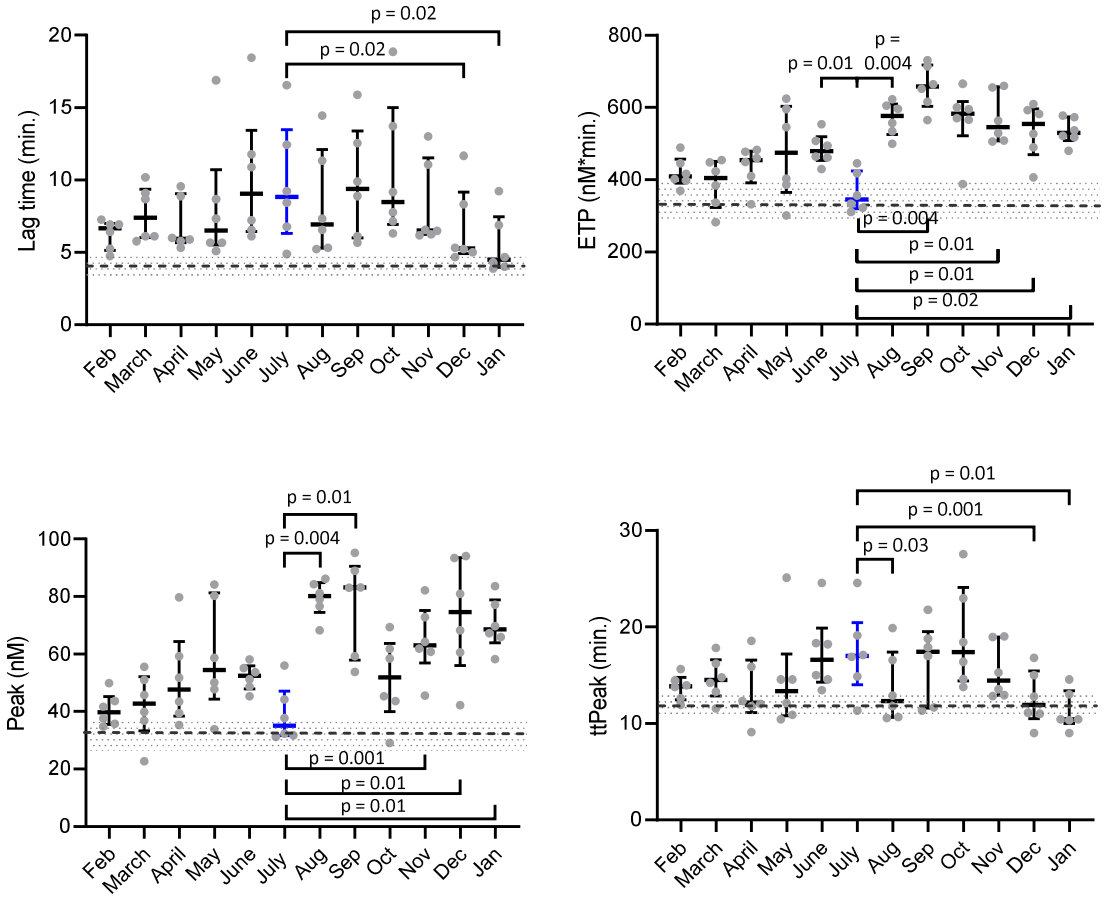


Figure 17. Calibrated Automated Thrombogram variables compared across months for the *Strongylus vulgaris* non-strangulating intestinal infarction (NSII) negative horses, with July as the reference month. Bars in the dot plots indicate the median with 1st and 3rd quartiles. The shaded area indicates the 1st and 3rd quartiles with the median as a dashed line, for clinically healthy horses without *S. vulgaris*.

4.5.3 Sample handling

Samples collected in February, March, and April were not handled according to the initial protocol. These samples were instead left to sediment with the citrated plasma then being transferred to cryotubes and stored at -80°C. After thawing and prior to plasma-TEG and CAT analyses, these plasma samples were centrifuged at 2,000 *g* for 15 min. with the supernatant being transferred to new cryotubes and then analyzed.

4.6 Study V – Investigation of two human endothelial marker assays in horses

4.6.1 Investigation of vascular cell adhesion molecule 1

The human vCAM-1 ELISA produced an acceptable standard curve with acceptable coefficients of variation, with the exception that standard sample 1 was above 5% (Figure 18, Table 10).

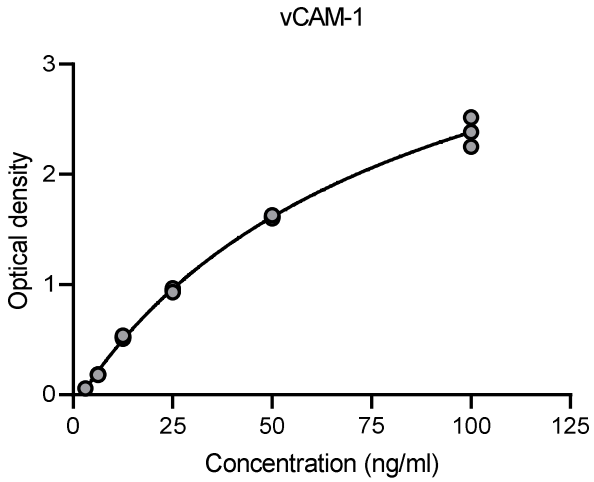


Figure 18. Standard curve for the human vascular cell adhesion molecule 1 (vCAM-1) ELISA showing duplicate measurements and their averages at 100%, 50%, 25%, 12.5%, 6.3%, and 3.1% concentration.

Table 10. Standard sample measurements for the human vascular cell adhesion molecule 1 (vCAM-1) ELISA including the coefficient of variation (CV). M1: measurement 1, M2: measurement 2.

Sample	Concentration	Optical density			CV %
		M1	M2	Average	
Standard sample 1	100% (100 ng/ml)	2.25	2.51	2.38	7.9
Standard sample 2	50% (50 ng/ml)	1.6	1.63	1.61	1.2
Standard sample 3	25% (25 ng/ml)	0.97	0.93	0.95	2.6
Standard sample 4	12.5% (12.5 ng/ml)	0.51	0.54	0.52	3.6
Standard sample 5	6.3% (6.3 ng/ml)	0.19	0.18	0.19	4.3
Standard sample 6	3.1% (3.1 ng/ml)	0.06	0.06	0.06	4.5

Measurements for vCAM-1 were below the detection limit of the assay in all four groups of horses (Table 11).

Table 11. Vascular cell adhesion molecule 1 (vCAM-1) ELISA measurements for the four groups of horses (clinically healthy, *Strongylus vulgaris* non-strangulating intestinal infarction (NSII) positive, enterocolitis, and strangulating intestinal lesions). The lower level of detection was 1.88 ng/mL.

	Optical density	
	Median	1st - 3rd quartile
Clinically healthy	-0.0297	-0.0327 – -0.0224
***S. vulgaris* NSII positive**	-0.0227	-0.0283 – -0.0164
Enterocolitis	-0.0178	-0.0318 – -0.0093
Strangulating lesions	-0.0095	-0.0212 – 0.0034

4.6.2 Investigation of P-selectin

The human P-selectin ELISA produced an acceptable standard curve with acceptable coefficients of variation, with the exception that standard samples 3 and 5 were above 5% (Figure 19, Table 12).

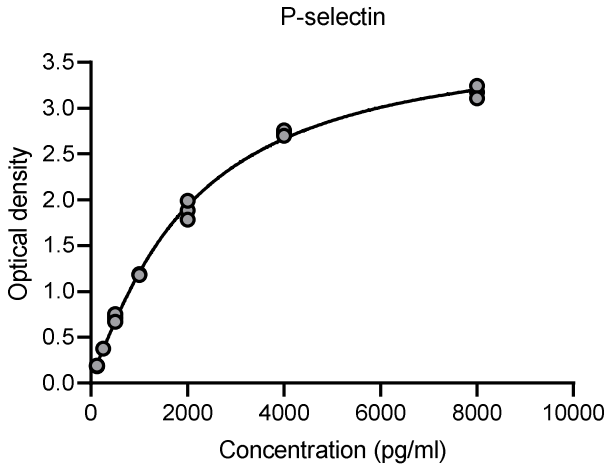


Figure 19. Standard curve for the human P-selectin ELISA showing duplicate measurements and their averages at 100%, 50%, 25%, 12.5%, 6.3%, 3.1%, and 1.6% concentration.

Table 12. Standard sample measurements for the P-selectin ELISA including the coefficient of variation (CV). M1: measurement 1, M2: measurement 2.

Sample	Concentration	Optical density			CV %
		M1	M2	Average	
Standard sample 1	100% (8,000 pg/ml)	3.24	3.11	3.17	3.01
Standard sample 2	50% (4,000 pg/ml)	2.76	2.70	2.73	1.6
Standard sample 3	25% (2,000 pg/ml)	1.99	1.78	1.89	7.8
Standard sample 4	12.5% (1,000 pg/ml)	1.20	1.18	1.19	0.97
Standard sample 5	6.3% (500 pg/ml)	0.75	0.67	0.71	8.2
Standard sample 6	3.1% (250 pg/ml)	0.38	0.37	0.38	1.2
Standard sample 7	1.6% (125 pg/ml)	0.18	0.19	0.19	3.2

Measurements for P-selectin were below the detection limit of the assay for all four groups of horses (Table 13).

Table 13. P-selectin ELISA measurements for the four groups of horses (clinically healthy, *Strongylus vulgaris* non-strangulating intestinal infarction (NSII) positive, enterocolitis, and strangulating intestinal lesions). The lower level of detection was 62 pg/mL.

	Optical density	
	Median	1st - 3rd quartile
Clinically healthy	-0.0091	-0.0127 – -0.002
***S. vulgaris* NSII positive**	-0.0042	-0.0089 – 0.0015
Enterocolitis	-0.0078	-0.0104 – -0.0037
Strangulating lesions	-0.0023	-0.0092 – 0.0102

5 Discussion

Overall, this thesis provided novel results with regard to hemostasis in horses with migrating *S. vulgaris* larvae with and without clinical disease (NSII), which can aid in the understanding of the pathophysiology and development of NSII and diagnostic workup. In addition, this thesis has shown that plasma-based global hemostatic tests can provide valuable information in horses with various kinds of severe GI disease.

5.1 The applicability of plasma-based global hemostatic tests in horses

Both CAT and plasma-TEG were applicable in equine citrated plasma (Studies II, III, and IV). Based on results obtained by applying all three available triggering agents (low PPP, PPP, and high PPP) on samples from the clinically healthy horses (Study II), and what has been done in human (283) and canine (279) studies, the triggering agent with a concentration of 5pM TF was used for CAT (Studies II, III, and IV). It might, however, be relevant for future studies to investigate the triggering agents in more depth on samples from horses with clinical disease. For example, the low PPP might reveal more subtle hemostatic changes among disease groups, and it may be relevant to apply different triggering agents depending on the suspected hemostatic change being either hypo- or hypercoagulable.

Plasma-TEG failed to reach a K time in all horses (Studies II, III, and IV) due to the K time being preset to the time taken until the clot reaches 20 mm (245,248,284). This was established for human whole blood samples and is therefore not directly transferable to equine plasma samples. In order to continue using plasma-TEG, new standards would need to be established.

Plasma-TEG was able to identify significant differences between horses with severe GI disease and clinically healthy horses that were not found using the whole blood TEG (Study II). Plasma-TEG and whole blood-TEG should not be directly compared as being the same assay as the composition of PPP and whole blood is significantly different, especially in terms of platelet count. It would, however, be interesting to assess whether plasma-TEG is a more sensitive assay than whole blood-TEG.

5.1 The lack of platelets in global plasma hemostasis testing

Platelets play a substantial role in hemostasis (136,137,207). One disadvantage of plasma-based hemostatic tests is that they do not evaluate platelet function, and it could therefore be debated whether they can still be classed as global hemostatic tests. Thromboelastography was originally developed as a whole blood assay, and MA in particular is a reflection of platelet count and platelet activity (245,247). In Study II (Paper II), the plasma-TEG readings had a lower MA and G compared to whole blood-TEG, which may reflect the lack of platelets. Meanwhile, the CAT assay, which is also considered a global hemostatic test, has been developed for use with both PPP, platelet rich plasma (PRP), and whole blood (265,285).

When removing the platelets from the TEG analysis, one could argue that MA and G only represent an elaborate analysis of the fibrinogen function and concentration. In Study II (Paper II), a positive correlation was found between fibrinogen, MA, and G. However, only MA and G were able to detect a significant difference among the three groups of horses. In Study III (Paper III), fibrinogen, MA, and G showed varying differences across the groups of horses. These findings indicate that MA and G from plasma-TEG are more than just advanced fibrinogen assays and are of clinical relevance in equine medicine.

One function of global hemostatic tests is to mimic the complexity of *in vivo* hemostasis as much as possible. However, even when platelets are included, the contribution of TF-bearing cells and local tissue conditions

are not reflected (136), meaning that it is not possible to fully recreate the conditions of *in vivo* coagulation in this set up.

5.2 Plasma-based global hemostatic tests as markers of migrating *Strongylus vulgaris*

Accurately diagnosing the presence of migrating *S. vulgaris* larvae in a clinical setting has been a continual challenge in equine clinical practice (98). Study III (Paper III) and Study IV demonstrated that horses with migrating *S. vulgaris* larvae with no signs of clinical disease (*S. vulgaris* NSII negative group) showed hemostatic changes that can be interpreted as both hypercoagulability and hypocoagulability detected using plasma-TEG and CAT when compared to clinically healthy horses without *S. vulgaris* and when the *S. vulgaris* NSII negative group was assessed over a year. The model developed in Study III (Paper III) including SAA, iron, angle (plasma TEG), and ETP (CAT) could fully distinguish clinically healthy horses without *S. vulgaris* from horses with migrating *S. vulgaris* without clinical disease (NSII). Study IV indicates that there are seasonal hemostatic aberrations in horses infected with *S. vulgaris* without clinical disease. It therefore seems that horses with migrating *S. vulgaris* larvae suffer from hemostatic changes even when they show no signs of clinical disease, indicating that global hemostatic tests could aid in diagnosing the presence of migrating *S. vulgaris*. However, due to the relatively large overlap for most variables between clinically healthy horses without migrating *S. vulgaris* and horses with migrating *S. vulgaris* without clinical disease, these global hemostatic tests cannot be used alone in diagnosing an infection with *S. vulgaris*.

In addition, the horses with migrating *S. vulgaris* without clinical disease from the Barn 10 research herd have conversely to horses not kept for research purposes been infected with large numbers of *S. vulgaris* and other parasites throughout their entire lives and are constantly reinfected (4,5). This means that they most likely suffer from more substantial chronic lesions compared to horses with a more natural infection. It would be relevant to investigate these findings in naturally infected horses with a lower *S. vulgaris* burden, where the life cycle is occasionally interrupted by anthelmintic treatment. It would also be interesting to investigate how long the hemostatic changes persist after the infection is cleared and what level of infection is necessary for the changes to occur.

In Study IV, July was chosen as the reference month as this was the only month during which no NSII cases have been observed (7). In addition, the temperature-dependent seasonal life cycle of *S. vulgaris* will most likely ensure a lower less progressed worm burden in the horse during the warm summer months (47,48), with less damage to the CMA and its branches. No completely distinct seasonal pattern was observed. Nonetheless even with the inclusion of only six horses it was clear that variations in hemostasis did occur throughout the year. It is not possible to determine from this study whether the observed seasonal hemostatic changes were caused by the migration of *S. vulgaris* alone. Other internal and external factors such as reproductive hormones (286–288) change over the course of a year and could therefore also influence hemostasis. These findings thus should be explored in more detail in future studies.

5.3 Where are we now in diagnosing non-strangulating intestinal infarctions

Making an accurate and timely diagnosis of NSII caused by *S. vulgaris* prior to an exploratory laparotomy or postmortem examination has always been a challenge in a clinical setting and is of vital importance for the horse's survival (7). One reason is that horses presenting with NSII caused by *S. vulgaris* mimics horses with

other types of severe GI disease such as idiopathic peritonitis (26,84,122), enterocolitis (24,25), and strangulating lesions. Another explanation is that horses can often suffer from more than one disease process at a time. In this thesis and in other similar studies (84,122), horses tend to be excluded if they suffer from more than one disease process, so as to differentiate more accurately between horses. However, in a clinical setting, horses may suffer from both NSII caused by *S. vulgaris* and e.g. enterocolitis, and it could be speculated that some horses presenting with idiopathic peritonitis have migrating *S. vulgaris* but without developing fulminant NSII. The exact parasitological status of all the horses with idiopathic peritonitis, enterocolitis, and strangulating lesions in Study III (Paper III) was not always known. This means that these horses could be infected with *S. vulgaris* while being NSII negative. As the focus was on distinguishing horses with NSII from the remaining severe GI disease groups, this was not considered problematic.

The variables applied in Study III (Paper III) can to some degree distinguish horses with migrating *S. vulgaris* with and without clinical disease from the remaining groups. As described, a model including iron, SAA, angle (plasma-TEG), and ETP (CAT) had a marked predictive power for distinguishing horses in the *S. vulgaris* NSII positive group. This model resulted in a relatively large proportion of false negative horses with NSII caused by *S. vulgaris*. One could debate whether a higher proportion of false negatives or false positives are worse. While false positives will result in horses undergoing an unnecessary exploratory laparotomy, which entails the risks associated with an unnecessary general anesthesia and recovery, as well as a prolonged rehabilitation period and considerable expense for the owner, false negatives result in horses that need an exploratory laparotomy receiving the wrong treatment, which in the case of a NSII will be fatal for the horse and thus seems indisputably worse. This model can therefore not stand alone in diagnosing horses with NSII.

A recent Swedish study comparing horses with idiopathic peritonitis and horses with NSII found that if the horses presented with a septic peritonitis and a palpable rectal mass during the winter months, they were likely to suffer from NSII caused by *S. vulgaris* (84). These findings are supported by two Danish studies from the Large Animal Teaching Hospital at the University of Copenhagen (7,122). The combination of the model developed in Study III (Paper III) and the additional information (the horse being admitted during winter, having septic peritonitis and an abdominal mass on rectal examination) could potentially ensure a lower rate of false negatives and thus be an appropriate new way to diagnose horses with NSII caused by *S. vulgaris*. In case of doubt (7,122) or in cases where signs of colic and/or fever persist beyond 48 hours after admission despite medical treatment, it seems reasonable to suggest an exploratory laparotomy as previously recommended (84,289).

Vascular endothelial markers have been shown to be suitable disease markers in human medicine, where they are increased in patients with thrombosis, atherosclerosis, (162,163,179,180,199), and in case of platelet activation (164,193). As *S. vulgaris* is known to cause endothelial damage, activation of hemostasis, and thrombosis (27), human vCAM-1 and P-selectin was investigated as potential diagnostic markers and to gain further understanding of the pathophysiological process of NSII. The ELISAs performed well with the standard samples but were not suitable for use with equine serum in this study. However, it may be relevant for future studies to use alternative ELISAs or produce ELISA assays *de novo* for equine-specific antibodies.

5.4 Understanding the development of non-strangulating intestinal infarctions

The exact pathogenesis behind the thrombosis caused by *S. vulgaris* migration has not yet been fully elucidated. Mechanical endothelial damage with the exposure of subendothelial collagen seems to play a major role (27,55,60). However, it also seems likely that *S. vulgaris* excrete mediators that might affect hemostasis as has been seen for *Angiostrongylus vasorum* in dogs (290), which can contribute to the development of verminous arteritis and thrombosis and potentially affect the immune system causing inflammation, which in turn can affect hemostasis.

Not all horses infected with migrating *S. vulgaris* larvae develop clinical disease and NSII (4,5) and the exact reason for this is still unknown. The inclusion of horses with migrating *S. vulgaris* larvae without clinical disease is unique. It provides valuable information and supports our understanding of the hemostatic pathophysiological changes caused by the parasite without the presence of systemic inflammation. In this thesis it was found that horses with migrating *S. vulgaris* larvae without clinically overt disease show hemostatic changes with the use of d-dimer, plasma-TEG, and CAT when compared to clinically healthy horses without migrating *S. vulgaris* larvae (Study III) and when assessed seasonally (Study V). This population is known to harbor high numbers of intestinal parasites including *S. vulgaris* (4,5) and it is interesting to investigate why these horses with large numbers of *S. vulgaris* and clear hemostatic changes (Studies III and IV) do not develop clinical disease (NSII) caused by *S. vulgaris*, with one hypothesis being that these horses experience minimal external stressors. External stressors can compromise the immune system thus increasing the risk of systemic inflammation (291,292), which can activate procoagulant factors even further (43). It seems possible that horses can cope with an *S. vulgaris* infection as long as they are not stressed beyond capacity, and NSII might therefore develop in horses with migrating *S. vulgaris* larvae that have encountered one or more external stressors such as being moved or having a concomitant disease with a systemic inflammation. This might tip the balance where the horse's regulatory and inhibitory systems can no longer compensate, and a thrombotic event with the formation NSII will occur as a consequence.

5.5 The relevance of conventional hemostatic markers and common laboratory variables

As described in Study II (Paper II), no significant differences were found among the three groups of horses (clinically healthy, mild GI disease, severe GI disease) for PT, aPTT, or AT. Theses markers do not adequately reflect the complexity of the hemostatic process (136) and aPTT and PT are best suited for diagnosing hypocoagulability (138). Consequently, PT, aPTT, and AT measurements were not included in Study III (Paper III) and are not believed to add any valuable information with regard to diagnosing the presence of *S. vulgaris* or NSII.

In contrast, d-dimer was significantly different among the groups of horses, as shown in Studies I, II, and III (Papers I, II, and III). In Study I (Paper I), both d-dimer assays for the high SAA group and the *S. vulgaris* NSII positive group showed a significantly higher d-dimer concentration than in the clinically healthy group. In Study II (Paper II), the severe GI disease group had a significantly higher d-dimer concentration than the mild GI disease and clinically healthy groups. D-dimer was therefore included in Study III (Paper III), in which a significantly higher d-dimer concentration was found in the *S. vulgaris* NSII positive group, idiopathic peritonitis, and enterocolitis groups compared to the clinically healthy horses.

D-dimer is known as a valuable rule-out marker for intravascular thrombosis (29–31,224–226,228) and is associated with hypercoagulable conditions leading to DIC, and human studies have shown that vascular endothelial markers do not add any diagnostic value to the d-dimer concentration (179,180,200). However, the final model described in Study III (Paper III) does not include d-dimer. This might indicate that the global hemostatic tests are superior to d-dimer, yet d-dimer is currently more readily available as a stall-side test, especially with the use of NycoCard, and could be a valuable diagnostic marker in smaller clinical settings without access to global hemostatic tests in horses with suspected NSII.

A decreased serum iron concentration seems to be a recurring finding in horses with migrating *S. vulgaris* and NSII as seen in Study III (Paper III) and other previous publications (7,122,293). A low systemic iron concentration is a sensitive indicator of systemic inflammation in horses, and a continued decrease in iron during hospitalization is indicative of a poor prognosis (294).

In situations without access to the more advanced global hemostatic tests, it seems that an increased d-dimer as seen in Studies I and III (Papers I and III) and decreased iron concentration as seen in Study III (Paper III), which both are more readily available test, can be used to indicate the occurrence of NSII. This is especially true when identified during the winter months in horses with septic peritonitis and a palpable rectal mass (84).

5.6 Methodological considerations

5.6.1 Study population

Finding the optimal way to group horses for a particular study is not always straightforward but can have great impact on the results and conclusions.

Establishing a control group or a group of clinically healthy animals is often more difficult than it sounds. In this thesis, the same inclusion criteria were used and the same ten animals constituted the clinically healthy group in Studies I–V. In Study III (Paper III), ten additional animals were sampled, seven of which were included. It was important that the clinically healthy horses were free of migrating *S. vulgaris* larvae to avoid a potential effect on the hemostasis and presence of thrombosis when assessing plasma-TEG and CAT, and so that this group could be used as a reference for the groups with migrating *S. vulgaris* larvae with and without clinical disease. However, samples collected one to two weeks post anthelmintic treatment showed an antibody PP value > 13.47% in all but two of the clinically healthy horses, which would be considered positive for a current or recent (within 5 months) infection with *S. vulgaris* (116,119). This is not unexpected as *S. vulgaris* is considered endemic in Denmark (22) and the antibody level has been shown to be increased for up to five months post infection and even to increase further post treatment with ivermectin (119). The increased antibody concentration in the clinically healthy horses is therefore most likely to indicate a recent infection with *S. vulgaris* and might be further increased by treatment with ivermectin or moxidectin. Regardless of the antibody level, the clinically healthy horses were still considered free of *S. vulgaris* post treatment as to the author's knowledge, there is no current indication of resistance in *S. vulgaris* toward the macrolides, and these have been found to be effective against migrating *S. vulgaris* larvae (119,295–297). However, we cannot know for certain that all clinically healthy horses were completely free of *S. vulgaris*. In addition, we do not currently know how long it takes for hemostasis to normalize after an infection with *S. vulgaris*, and this requires further investigation. However, it was not possible to wait longer than one to two weeks post treatment to collect the blood samples due to the risk of reinfection.

With regard to the various disease processes included in the thesis, horses were excluded if blood samples were not collected at admission or if samples were handled incorrectly after sampling. This could potentially create some degree of selection bias, as some disease processes may not be fully represented. However, no specific patterns were observed in the excluded samples.

For Study I (Paper I), horses with GI disease were grouped according to the degree of systemic inflammation as being low or high, based on the SAA concentration. In Study II (Paper II), horses were grouped based on having a mild GI disease with no ischemia and/or inflammation or having severe GI disease with ischemia and/or inflammation. This method of classification in both Study I and Study II (Papers I and II) might be problematic as the disease processes within each group could vary markedly, making it difficult for the relevant tests to differentiate between the groups. This method of grouping was chosen as the aim was to assess whether the hemostatic tests could detect an overall difference between the groups. On the other hand, classifying horses based on the disease process as in Study III (Paper III) and Study V might also be problematic as the severity of the disease process could still vary substantially within each group, giving markedly different results despite the same overall diagnosis. This method of dividing into groups was used as it transfers well to a clinical setting with the aim of distinguishing horses with NSII from horses with other severe GI disease processes.

Overall, the low number of horses included in each group is a limitation of this thesis. However, the majority of the work was conducted for the first time and should be considered exploratory, thus laying the groundwork for further studies. Furthermore, despite the relatively small number of horses, it was possible to find significant differences among groups and show the relevance of the applied global hemostatic tests.

In Study III (Paper III) and Study IV, horses with migrating *S. vulgaris* without clinical disease were compared to clinically healthy horses without *S. vulgaris*. These two groups of horses share some similarities in that they are not exposed to any major external stressors, they are subject to the same management routines, they are not transported or moved, and they are not exercised. However, they live in different areas of the world, albeit with a somewhat comparable climate, and one herd are semi-feral, whereas the other is stabled most of the time. Furthermore, the horses with migrating *S. vulgaris* without clinical disease are exposed to high numbers of parasites including *S. vulgaris,* and are continuously infected and have been for their entire lives without being treated with anthelmintics (4,5). It might therefore be interesting to compare clinically healthy horses without migrating *S. vulgaris* to horses with migrating *S. vulgaris* without clinical disease from the same geographic region, kept under the same conditions and with a less substantial worm burden.

5.6.2 Sample handling

Hemostatic markers are relatively sensitive with regard to sample handling (282,298,299). This means that it is important to standardize sample handling procedures as far as possible, especially if serial samples are to be collected from the same individual and compared (298). The samples for Studies I and II were all collected and handled by one person. In Studies III, IV, and V, samples were collected by a number of personnel with different professional backgrounds (veterinarians, nurses, and students) at different facilities with different laboratory facilities available (e.g. different centrifuges). This might be seen as a limitation of the study as completely uniform sample handling is not possible with this set up. However, the advantage is that if these tests are to be applied in a clinical setting, this method in many ways mimics sample collection within different clinical facilities and in the field by different personnel. If the tests are so sensitive that they are not

applicable under these conditions, then they will not be relevant in equine practice. The small variations in sample handling in his thesis did not seem to have an influence on the clinical distinction of horses.

Sodium citrate tubes are available with a concentration of either 3.2% or 3.8%. The concentration of anticoagulant has been shown to affect the results of the hemostatic markers with an increased clotting time depending on the reagent used, with the use of 3.8 % due to a dilution effect (300). However, this difference in clotting time was found to be clinically irrelevant in dogs (301), and in humans, the d-dimer concentration was found to be unaffected by the sodium citrate concentration (302). Nonetheless, it is recommended for laboratories to adhere to one of the concentrations to avoid variability, and the concentration of 3.2% is recommended for coagulation testing (282,300,301). The aim was to adhere to the use of 3.2% sodium citrate tubes, but 2/33 samples in Study I (Paper I) and 6/123 samples in Study III (Paper III) were collected in 3.8% sodium citrate tubes. It was however decided to include these as discussed in Study I (Paper I) as the potential difference was not thought to have any clinical impact or affect the classification of horses in the different disease categories.

Although the aim was to collect all samples via direct venipuncture with the use of a vacutainer as recommended (282), some of the samples were collected via an intravenous catheter as some horses needed more rapid medical intervention and the health of the horse was prioritized over sample handling. These samples were included as this was considered to have no clinical relevance (303).

The samples collected at the Barn 10 herd at Gluck Equine Research Center were all collected in glass blood collection tubes as opposed to the plastic tubes used at the other facilities. This had no impact on the interpretation of results from Study IV, which compared results from the research herd throughout the year. However, it may lead to some variation in the results of the hemostatic tests in Study III, where horses from the Barn 10 herd were compared to the remaining groups of horses from the other facilities. Glass is known to speed up clotting time, but a human study found the difference to be clinically insignificant (304), and the sodium citrate blood tubes were coated internally with silicone to avoid contact activation.

Samples collected from the Barn 10 research herd in February, March, and April for study IV were not centrifuged in accordance with the protocol. Instead, they were left to sediment, with the plasma then being transferred to cryotubes and frozen. For these samples, the plasma was then centrifuged after being thawed and the supernatant was transferred to new cryotubes. When left to sediment, the platelets and procoagulant factors have a longer time to interact with one another, which can influence the subsequent results of the hemostatic analysis. The samples were nonetheless included in the study, but if the results are to be explored further uniform sample handling should be ensured.

The aim was to produce PPP with a platelet count < 10x10^9/L for all citrated plasma samples as recommended (282). However, due to different personnel with different training and professional backgrounds collecting samples for Study III (Paper III), and potentially due to the use of different centrifuges, 37/123 samples in Study III (Paper III) had a platelet count > 10x10^9/L. The only existing definitions for plasma samples used for hemostatic testing are PPP and PRP, which contain substantially higher platelet concentrations than seen in the plasma in this study post centrifugation (305,306). The use of PRP as opposed to PPP will affect the variables in the hemostatic tests (306). Our samples are closest to the definition of PPP and it was concluded that the slight deviations from the target platelet count would not have any clinically relevant effect.

The above demonstrates the challenge of performing a large multicenter study in which multiple people are responsible for sample handling. At the same time, this type of study is valuable and contributes a substantial amount of knowledge, and samples should be included as long as the challenges do not compromise the integrity of the study.

6 Conclusions

This thesis has shown that human d-dimer assays (NycoCard and Stago), plasma-TEG and CAT are valid and reliable for use on equine citrated plasma. It has provided knowledge about the hemostatic balance in horses with migrating *S. vulgaris* larvae with and without NSII, which has improved our understanding of the development of NSII. It was found that a model including both inflammatory and hemostatic markers could, to some degree, distinguish horses with NSII from the remaining groups, which seems to support the idea that horses can cope with a *S. vulgaris* infection as long as they do not experience concomitant systemic inflammation.

The main findings in relation to the objectives of the five studies were:

Study I (Paper I):

The Stago and NycoCard assays were both found to be applicable for use on equine PPP, with an acceptable level of precision and good reliability. Both assays revealed significantly higher d-dimer concentrations in the high SAA group and NSII group compared to the clinically healthy horses. D-dimer concentrations measured using the Stago and NycoCard assays could not be compared directly. However, it seemed that the two assays showed good agreement in classifying horses with either a low or high d-dimer concentration, with the best agreement in the lower d-dimer concentrations. As a result, serial measurements in the same horse should be made using the same assay. The NycoCard assay was considered to be a potentially useful stall-side test for horses suspected to be at risk of thrombotic disease, due to it being very user friendly.

Study II (Paper II):

Plasma-TEG and CAT were applicable for use on equine citrated PPP. The CAT assay was found to have a high degree of precision and reliability. With both tests, horses with severe GI disease could be interpreted as being hypercoagulable compared to the clinically healthy horses and, to some degree, horses with mild GI disease.

Study III (Paper III):

Significant hemostatic changes were found in the *S. vulgaris* NSII negative and positive groups using plasma-TEG and CAT. The hemostatic changes for both groups could be interpreted as both hypo- and hypercoagulability when compared to the clinically healthy horses (without *S. vulgaris*) and the horses with other severe GI diseases (idiopathic peritonitis, enterocolitis, and strangulating lesions). The *S. vulgaris* NSII positive group could, to some degree, be distinguished from the remaining groups using a model that included inflammatory markers (decreased iron, increased SAA) and hemostatic markers (increased angle, decreased ETP). In general, global hemostatic tests can aid in establishing the horse's hemostatic balance and assess the risk of thrombotic disease.

Study IV:

The *S. vulgaris* NSII negative group showed seasonal hemostatic changes over the year using plasma-TEG and CAT. However, no clear seasonal pattern was observed. These findings should be confirmed in future studies with more horses and potentially horses with a more natural infection profile.

Study V:

The human endothelial marker assays for vCAM-1 and P-selectin used in this thesis performed well, creating a satisfactory standard curve. However, they were not applicable on equine serum samples in this thesis for any of the investigated groups (clinically healthy, *S. vulgaris* NSII positive, enterocolitis, and strangulating lesions), giving values below the lower level of detection for both assays. However, it still seems relevant to investigate these and other endothelial markers in future studies, perhaps with the use of alternative ELISAs or different methodologies. It is likely that they will improve our understanding of the development of NSII and potentially function as diagnostic markers.

7 Perspectives

This PhD project has provided invaluable new knowledge regarding plasma-based hemostatic tests in horses with GI disease. It has suggested a model that can, to some degree, distinguish horses with NSII caused by *S. vulgaris*, and it has provided a better understanding of the hemostatic balance in horses with *S. vulgaris* with and without clinical disease (NSII). It therefore offers a missing piece in the puzzle of understanding why only some horses with *S. vulgaris* develop clinical disease. However, additional studies and further work are still needed.

This thesis includes relatively small groups of horses as much of the work was conducted for the first time, thus being explorative and laying the groundwork for future studies. The results from this thesis should therefore be confirmed in studies that include larger groups of horses.

Study III (Paper III) identified a model including both inflammatory and hemostatic markers that could, to some degree, distinguish horses with NSII caused by *S. vulgaris* from the remaining groups. However, the application of this model alone resulted in some false negative horses. This means that these horses would not receive the correct treatment, most likely resulting in a fatal outcome. Laboratory variables can seldom stand alone in the diagnostic workup and must be combined with the animal's medical history and clinical findings. Recent studies have shown that horses with NSII caused by *S. vulgaris* are often admitted during the winter with septic peritonitis and an abdominal mass on rectal examination (84,122). Future studies should thus focus on combining the laboratory variables from this project with the clinical variables from these other studies and assess the diagnostic potential of a model that includes medical history, clinical parameters, and laboratory variables. This could prove to be the best way to diagnose NSII caused by *S. vulgaris*.

As shown in this thesis, the d-dimer concentration was significantly increased, while the iron concentration was significantly decreased in horses with NSII (Studies I and III). Future studies might look into the use of these measurements as diagnostic markers for NSII as a more readily available and cost-efficient alternative to the global hemostatic tests. Alternatively, it would be relevant for future studies to assess the use of the Viscoelastic Coagulation Monitor (VCM-Vet), a novel portable viscoelastic analyzer that is easy to use as a point-of-care assay in a variety of clinical settings to monitor hemostasis. It uses untreated whole blood that is applied directly, with contact activation initiating the hemostatic process (307). In human medicine, the VCM has been shown to make rapid viscoelastic measurements of hemostasis in native blood samples (308). The VCM-Vet has been evaluated in healthy adult dogs and cats, in which reference intervals have been established. The test was found to be applicable as a semi-quantitative coagulation assay in both species (307,309). The VCM-Vet has also been found to be reliable in healthy adult horses (310). Results generated by the VCM-Vet are not directly comparable to results obtained using TEG (307,309). However, the obtained information is similar, and analogous conclusions can be drawn using each assay (307). The VCM-Vet might therefore prove to be a valuable tool in future equine clinical practice to assess the hemostatic balance. In general, future studies should help to establish whether the global hemostatic markers could help improve treatment and thus prognosis for horses with severe GI disease.

The horses in this study with *S. vulgaris* without clinical disease (NSII negative) all came from a unique herd that harbors large quantities of intestinal parasites, including *S. vulgaris*. They have never received anthel-

mintic treatment and are kept as semi-feral. It would therefore be relevant to confirm and explore the findings of hemostatic aberrations in this population further in horses that are more representative of the average privately owned horse, for instance horses with a lower, less chronic parasitic infection that are stabled, transported, and ridden, and have received anthelmintic treatments, etc.

Even though the human vascular endothelial marker assays used were not applicable on equine serum in this study (Study V), it would still be relevant to work toward the identification of alternative assays due to the endothelial damage that *S. vulgaris* is known to cause. This could help with a better understanding of the pathophysiological process in horses with *S. vulgaris* and NSII, while potentially functioning as diagnostic biomarkers.

From a veterinary perspective, the most important end goal is to continuously improve and ensure the welfare and wellbeing of our animals, in this case horses. To the best of the author's knowledge, there is currently no documentation of anthelmintic resistance in *S. vulgaris*. However, it is likely to be only a matter of time before this arises. The ultimate long-term focus should therefore be on improving the diagnostic possibilities during the early infective stages (L3 and L4) and effectively inhibiting the progression of the *S. vulgaris* life cycle with alternatives to our current anthelmintic products.

One way to progress would be to add further to our knowledge of the pathophysiological process in the migration of *S. vulgaris* and the development of NSII. This could be achieved by using organoids, in which some initial groundbreaking work already has been done in horses (311,312). The organoids could be used to gain an improved understanding of the penetration of the intestinal mucosa by the L3 larvae and an improved understanding of the L4 larvae within the tunica intima and the endothelium of the arterial wall.

It also seems relevant to strive for a better understanding of the mediators excreted by *S. vulgaris,* including microRNAs (miRNA). It could be speculated that *S. vulgaris* excrete miRNAs that affect the immune system and potentially the hemostasis of the horse. This could also improve our understanding of why only some horses develop NSII. In addition, the miRNAs could function as diagnostic markers and therapeutic targets through the development of a vaccine, which could address the L3 or L4 stages and inhibit the life cycle of *S. vulgaris* or attenuate the severity of the pathologic changes it causes. However, developing a vaccine against a complex and moving organism is likely to be challenging.

8 References

1. Reinemeyer CR, Nielsen MK. Parasitism and Colic. Vet Clin North Am Pract. 2009 Aug;25(2):233–45.
2. Duncan JL. *Strongylus vulgaris* infection in the horse. Vet Rec. 1974;95(2):34–7.
3. Duncan JL, Pirie HM. The life cycle of *Strongylus vulgaris* in the horse. Res Vet Sci. 1972;13(4):374–9.
4. Lyons ET, Tolliver SC, Stamper S, Drudge JH, Granstrom DE, Collins SS. Transmission of some species of internal parasites in horses born in 1990, 1991, and 1992 in the same pasture on a farm in central Kentucky. Vet Parasitol. 1994;52(3–4):257–69.
5. Lyons ET, Tolliver SC, Collins SS, Drudge JH, Granstrom DE. Transmission of some species of internal parasites in horses born in 1993, 1994, and 1995 on the same pasture on a farm in central Kentucky. Vet Parasitol. 1997;70:225–40.
6. Nielsen MK, Jacobsen S, Olsen SN, Bousquet E, Pihl T. Nonstrangulating intestinal infarction associated with *Strongylus vulgaris* in referred Danish equine cases. Spec Focus Parasitol. 2016;48(3):376–9.
7. Pihl TH, Nielsen MK, Olsen SN, Leifsson PS, Jacobsen S. Nonstrangulating intestinal infarctions associated with *Strongylus vulgaris*: Clinical presentation and treatment outcomes of 30 horses (2008-2016). Equine Vet J. 2017;1–7.
8. Love S, Duncan JL. Could the worms have turned? Equine Vet J. 1991;23(3):152–4.
9. Drudge JH, Lyons ET. Control of internal parasites of the horse. J Am Vet Med Assoc. 1966;148(4):378–83.
10. Garber L. Equine 1998, Part III: Management and Health of Horses, 1998. Natl Anim Heal Monit Syst. 1998;1–60.
11. Lloyd S, Smith J, Connan RM, Hatcher MA, Hedges TR, Humphrey DJ, et al. Parasite control methods used by horse owners: Factors predisposing to the development of anthelmintic resistance in nematodes. Vet Rec. 2000;146(17):487–92.
12. Earle CG, Kington HA, Coles GC. Helminth control used by trainers of thoroughbreds in England. Vet Rec. 2002;150(13):405–8.
13. Matthee S, Dreyer FH, Hoffmann WA, van Niekerk FE. An introductory survey of helminth control practices in south africa and anthelmintic resistance on Thoroughbred stud farms in the Western Cape Province. J S Afr Vet Assoc. 2002 Dec;73(4):195–200.
14. Kaplan RM. Drug resistance in nematodes of veterinary importance: a status report. Trends Parasitol. 2004;20(10):477–81.
15. Lyons ET, Tolliver SC, Collins SS. Probable reason why small strongyle EPG counts are returning “early” after ivermectin treatment of horses on a farm in Central Kentucky. Parasitol Res. 2009;104(3):569–74.
16. Lyons ET, Tolliver SC, Kuzmina TA, Collins SS. Critical tests evaluating efficacy of moxidectin against small strongyles in horses from a herd for which reduced activity had been found in field tests in Central Kentucky. Parasitol Res. 2010;107(6):1495–8.
17. Lyons ET, Tolliver SC, Collins SS, Ionita M, Kuzmina TA, Rossano M. Field tests demonstrating reduced activity of ivermectin and moxidectin against small strongyles in horses on 14 farms in Central Kentucky in 2007–2009. Parasitol Res. 2011;108(2):355–60.
18. Boersema JH, Eysker M, Nas JWM. Apparent resistance of Parascaris equorum to macrocylic lactones. Vet Rec. 2002 Mar 2;150(9):279.
19. Hearn FPD, Peregrine AS. Identification of foals infected with Parascaris equorum apparently resistant to ivermectin. J Am Vet Med Assoc. 2003;223(4):482–5.
20. Veronesi F, Moretta I, Moretti A, Fioretti DP, Genchi C. Field effectiveness of pyrantel and failure of Parascaris equorum egg count reduction following ivermectin treatment in Italian horse farms. Vet Parasitol. 2009;161(1):138–41.
21. Näreaho A, Vainio K, Oksanen A. Impaired efficacy of ivermectin against Parascaris equorum, and both ivermectin and pyrantel against strongyle infections in trotter foals in Finland. Vet Parasitol. 2011;182(2):372–7.

22. Nielsen MK, Vidyashankar AN, Olsen SN, Monrad J, Thamsborg SM. *Strongylus vulgaris* associated with usage of selective therapy on Danish horse farms-Is it reemerging? Vet Parasitol. 2012;189(2–4):260–6.
23. Tydén E, Enemark HL, Franko MA, Höglund J, Osterman-Lind E. Prevalence of *Strongylus vulgaris* in horses after ten years of prescription usage of anthelmintics in Sweden. Vet Parasitol. 2019;2(January):1–7.
24. Staempfli HR, Townsend HG, Prescott JF. Prognostic features and clinical presentation of acute idiopathic enterocolitis in horses. Can Vet J. 1991;32(4):232–7.
25. Saville WJ, Hinchcliff KW, Moore BR, Kohn CW, Reed SM, Mitten LA, et al. Necrotizing enterocolitis in horses: a retrospective study. J Vet Intern Med. 1996;10(4):265–70.
26. Odelros E, Kendall A, Hedberg-Alm Y, Pringle J. Idiopathic peritonitis in horses: A retrospective study of 130 cases in Sweden (2002-2017). Acta Vet Scand. 2019;61(1):1–8.
27. White NA, Moore JN, Douglas M. SEM study of *Strongylus vulgaris* larva-induced arteritis in the pony. Equine Vet J. 1983;15(4):349–53.
28. White NA. Intestinal infarction associated with mesenteric vascular thrombotic disease in the horse. J Am Vet Med Assoc. 1981;178(3):259–62.
29. Wells PS, Brill-Edwards P, Stevens P, Panju A, Patel A, Douketis J, et al. A novel and rapid whole-blood assay for D-dimer in patients with clinically suspected deep vein thrombosis. Circulation. 1995 Apr;91(8):2184–7.
30. Freyburger G, Trillaud H, Labrouche S, Gauthier P, Javorschi S, Bernard P, et al. D-dimer strategy in thrombosis exclusion--a gold standard study in 100 patients suspected of deep venous thrombosis or pulmonary embolism: 8 DD methods compared. Thromb Haemost. 1998 Jan;79(1):32–7.
31. Brill-Edwards P, Lee A. D-dimer testing in the diagnosis of acute venous thromboembolism. Thromb Haemost. 1999 Aug;82(2):688–94.
32. Pihl TH, Nielsen MK, Jacobsen S. Changes in Hemostatic Indices in Foals Naturally Infected With *Strongylus vulgaris*. J Equine Vet Sci. 2017;54:1–7.
33. Lancé MD. A general review of major global coagulation assays: Thrombelastography, thrombin generation test and clot waveform analysis. Thromb J. 2015;13(1):1–6.
34. Spahn DR, Bouillon B, Cerny V, Coats TJ, Duranteau J, Fernández-mondéjar E, et al. Management of bleeding and coagulopathy following major trauma: an updated European guideline. Critcal care. 2013;17:1–45.
35. Dunkel B, Chan DL, Boston R, Monreal L. Association between Hypercoagulability and Decreased Survival in Horses with Ischemic or Inflammatory Gastrointestinal Disease. J Vet Intern Med. 2010;24:1467–74.
36. Epstein KL, Brainard BM, Barton MH, Moore JN, Gomez-Ibanez SE, Lopes MAF, et al. Thrombelastography in Horses with Acute Gastrointestinal Disease. J Vet Intern Med. 2011;25(2):307–14.
37. Epstein KL, Brainard BM, Giguere S, Vrono Z, Moore JN. Serial viscoelastic and traditional coagulation testing in horses with gastrointestinal disease. J Vet Emerg Crit Care. 2013;23(5):504–16.
38. Mendez-angulo JL, Mudge MC, Vilar- P, Stingle N, Couto CG. Thromboelastography in healthy horses and horses with inflammatory gastrointestinal disorders and suspected coagulopathies. J Vet Emerg Crit Care. 2010;20(5):488–93.
39. Contreras-García A, Merhi Y, Ruiz JC, Wertheimer MR, Hoemann CD. Thromboelastography (TEG) cups and pins with different PECVD coatings: Effect on the coagulation cascade in platelet-poor blood plasma. Plasma Process Polym. 2013;10(9):817–28.
40. Ostrowski SR, Berg RMGG, Windeløv NA, Meyer MASS, Plovsing RR, Møller K, et al. Discrepant Fibrinolytic Response in Plasma and Whole Blood during Experimental Endotoxemia in Healthy Volunteers. PLoS One. 2013;8(3):1–9.
41. Wiinberg B, Jensen AL, Kjelgaard-Hansen M, Rojkjaer R, Johansson PI, Gade LP, et al. Study on biological variation of haemostatic parameters in clinically healthy dogs. Vet J. 2007;174(1):62–8.

42. Krogh AK, Brunse A, Thymann T, Kristensen AT, Nielsen LN. Plasma Thromboelastography in Septic Preterm Pigs and Full-Term Pigs at Birth and 11 Days Post-Partum. In: Joint Congress of Veterinary Pathology and Veterinary Clinical Pathology 25-28 September 2019 | Arnhem, The Netherlands. 2019. p. 287.
43. Esmon CT. The interactions between inflammation and coagulation. Br J Haematol. 2005;131:417–30.
44. Lichtenfels JR, Kharchenko VA, Dvojnos GM. Illustrated identification keys to strongylid parasites (strongylidae: Nematoda) of horses, zebras and asses (Equidae). Vet Parasitol. 2008;156(1–2):4–161.
45. Drudge, J.H.; Lyons, E.T.; Szanto J. Pathogenesis of Migrating Stages of Helminths, with Special Reference to *Strongylus vulgaris*. 1966;199–214.
46. Duncan RB, Duncan JL, Pirie HM. The pathogenesis of single experimental infections with *Strongylus vulgaris* in foals. Res Vet Sci. 1975;18(1):82–93.
47. Ogbourne CP. Studies on the epidemiology of *Strongylus vulgaris* infection of the horse. Int J Parasitol. 1975;5(4):423–6.
48. Ogbourne CP. Variations in the fecundity of strongylid worms of the horse. Parasitology. 1971;63(2):289–98.
49. McCraw BM, Slocombe JO. *Strongylus vulgaris* in the horse: a review. Can Vet J. 1976;17(6):150–7.
50. Ogbourne CP. Observations on the free-living stages of strongylid nematodes of the horse. Parasitology. 1972;64(3):461–77.
51. Mfitilodze MW, Hutchinson GW. Development and survival of free-living stages of equine strongyles under laboratory conditions. Vet Parasitol. 1987;23(1–2):121–33.
52. Enigk K. The development of the three species of strongylus of the horse during the prepatent period. Equine Infect Dis Conf. 1970;259–68.
53. Ottaway CW, Bingham ML. Further Observations on the Incidence of Parasitic Aneurysm in the Horse. Vet re. 1946;58:155–9.
54. Poynter D. The arterial lesions produced by *Strongylus vulgaris* and their relationship to the migratory route of the parasite in its host. Res Vet Sci. 1960;1(205):205–17.
55. Morgan SJ, Stromberg PC, Storts RW, Sowa BA, Lay JC. Histology and morphometry of *Strongylus vulgaris*-mediated equine mesenteric arteritis. J Comp Pathol. 1991;104(1):89–99.
56. Slocombe JO, Rendano VT, Owen RR, Pennock PW, McCraw BM. Arteriography in ponies with *Strongylus vulgaris* arteritis. Can J Comp Med Rev Can Med Comp. 1977;41(2):137–45.
57. Pilo C, Altea A, Varcasia A, Scala A, Pirino S, Nicolussi P, et al. *Strongylus vulgaris* (Looss, 1900) in horses in Italy: Is it still a problem? Vet Parasitol. 2012;18:161–7.
58. Duncan JL. The Life Cycle, Pathogenisis and Epidemiology of *S. vulgaris* in the Horse. Equine Vet J. 1973;5(1):20–5.
59. Enigk K. Further investigations on the biology of *S. vulgaris* in the host animal. Z Tropenmed Parasitol. 1951;2(4):247–63.
60. Marinković D, Sanja A-K, Krstić V, Milijana K. Morphological findings in the cranial mesenteric artery of horses with verminous arteritis. Acta Vet Brno. 2009;59(2–3):231–41.
61. Rendano VT, Georgi JR, White KK, Sack WO, King JM, Bianchi DG, et al. Equine verminous arteritis. An arteriographic evaluation of the larvicidal activity of albendazole. Equine Vet J. 1979;11(4):223–31.
62. Bailey M, Kent J, Martin SC, Lloyd S, Soulsby EJ. Haematological and biochemical values in horses naturally infected with *Strongylus vulgaris*. Vet Rec. 1984;115(7):144–7.
63. DeLay J, Peregrine AS, Parsons DA. Verminous arteritis in a 3-month-old thoroughbred foal. Can Vet J. 2001;42(4):289–91.
64. Gau GS, Ryder TA, Mackenzie ML. The effect of blood flow on the surface morphology of the human endothelium. J Pathol. 1980;131(1):55–64.
65. Mustard JF, Packham MA, Kinlough-Rathbone RL. Platelets and thrombosis in the development of atherosclerosis and its complications. Adv Exp Med Biol. 1978;102:7–30.
66. Smith JB, Ingerman CM, Silver MJ. Drug Effects on Platelet Aggregation and Prostaglandin Formation BT - Drugs, Lipid Metabolism, and Atherosclerosis. Kritchevsky D, Paoletti R, Holmes WL, editors.

Drugs, Lipid Metab Atheroscler. 1978;239–51.
67. Hamberg M, Svensson J, Wakabayashi T, Samuelsson B. Isolation and Structure of Two Prostaglandin Endoperoxides that Cause Platelet Aggregation. Natl Acad Sci. 1974;71(2):345–9.
68. Moncada S, Vane JR. Pharmacology and endogenous roles of prostaglandin endoperoxides, thromboxane A2, and prostacyclin. Pharmacol Rev. 1978 Sep;30(3):293–331.
69. Cambridge H, Reynoldson JA, Dunsmore JD. Plasma thromboxane B2 levels in horses experimentally infected with *Strongylus vulgaris*. Equine Vet J Suppl. 1989;(7):14–8.
70. Wallace KD, Selcer BA, Tyler D, Brown J, Becht JL. Transrectal ultrasonography of the cranial mesenteric artery of the horse. Am J Vet Res. 1989 Oct;50(10):1699–703.
71. Klei TR, French DD, Chapman MR, McClure JR, Dennis VA, Taylor HW, et al. Protection of yearling ponies against *Strongylus vulgaris* by foalhood vaccination. Equine Vet J. 1989;21(7 S):2–7.
72. White NA. Intestinal infarction associated with thrombotic disease in the horse. J Am Vet Med Assoc. 1981;178(3):259–62.
73. Drudge JH. Clinical Aspects of *Strongylus vulgaris* Infection in the Horse. Vet Clin North Am Large Anim Pract. 1979;12:251–65.
74. Enigk K. Die Pathogenese der thrombotisch-embolische Kolik des Pferdes. Monatsh Tierheilkd. 1951;3:65–74.
75. Curtis RA. Mesenteric Aneurism in a Horse. Can Vet J = La Rev Vet Can. 1964;5(2):36–8.
76. Wright AI. Verminous Arteritis as a Cause of Colic in the Horse. Equine Vet J. 1972;4(4):169–74.
77. Sellers AF, Lowe JE, Drost CJ, Rendano VT, Georgi JR, Roberts MC. Retropulsion-propulsion in equine large colon. Am J Vet Res. 1982;43(3):390–6.
78. Olt A. Verminous Aneurysms of the Horse and their Unknown Connection with Colic. Dtsch tierärztl Wschr. 1932;40:326.
79. Ershov V. The Biology of Horse Strongyles and the Allergic Reactions caused by them. Proc 2nd int Conf Equine Infect Dis, Paris 1969. 1970;304.
80. Skalinskii E. Toxic action of Larvae of *Strongylus Vulgaris* on Horses. Vet Bull Weybridge. 1957;29:137.
81. Enigk K. Zur Entwicklung von Strongylus vulgaris (Nematodes) im Wirtstie. Z Tropenmed Parasitol. 1950;2:287–306.
82. Enigk K. Further Investigations on the Biology of *Strongylus Vulgaris* (Nematoda) in the Host Animal. Zeitschrift fiir Tropenmedizin und Parasitol. 1951;2(4):247–63.
83. Slocombe JOD, Owen R, Pennock PW, McCraw BM. Arteriographic presentation of the early development of vascular lesions in ponies infected with *Strongylus vulgaris*. Proceedings, 23rd Annu Meet, Am Assoc Equine Pr. 1978;305–11.
84. Hedberg-Alm Y, Tydén E, Tamminen L-M, Linström L, Anlén K, Svensson M, et al. Clinical Features and Treatment Response to Differentiate Idiopathic Peritonitis From Non- strangulating Intestinal Infarction Associated With *Strongylus Vulgaris* Infection in the Horse. BMC Vet Res. 2021;In review:1–23.
85. Klei TR, Torbert BJ, Ochoa R, Bello TR. Morphologic and clinicopathologic changes following *Strongylus vulgaris* infections of immune and nonimmune ponies. Am J Vet Res. 1982 Jul;43(7):1300–7.
86. Bollinger O. Die Kolik der Pferde und das Wurmaneurysma der Eingeweidearterien: Eine pathologisch-anatomische und klinische Untersuchung. Akad Wiss Munchen Sitzber. 1870;1:539–44.
87. Robertson D. Intestinal parasites of Shetland ponies in the north of Scotland. Vet Rec. 1939;51(25):779–81.
88. Slocombe JO, McCraw BM. Gastrointestinal nematodes in horses in Ontario. Can Vet J = La Rev Vet Can. 1973 May;14(5):101–5.
89. Tolliver SC, Lyons ET, Drudge JH. Prevalence of internal parasites in horses in critical tests of activity of parasiticides over a 28-year period (1956-1983) in Kentucky. Vet Parasitol. 1987;23(3–4):273–84.
90. Nilsson O, Andersson T. *Strongylus vulgaris* hos hast-epizootoologi och profylax. Sven Vet Tidn. 1979;31:148–56.
91. Höglund J, Ljungström B-L, Nilsson O, Lundquist H, Osterman E, Uggla A. Occurrence of Gasterophilus

intestinalis and some Parasitic Nematodes of Horses in Sweden. Acta Vet Scand. 1997;38(2):157–66.
92. Craven J, Bjørn H, Henriksen SA, Nansen P, Larsen M, Lendal S. Survey of anthelmintic resistance on Danish horse farms, using 5 different methods of calculating faecal egg count reduction. Equine Vet J. 1998 Jul 1;30(4):289–93.
93. Lind EO, Höglund J, Ljungström B-L, Nilsson O, Uggla A. A field survey on the distribution of strongyle infections of horses in Sweden and factors affecting faecal egg counts. Equine Vet J. 1999 Jan 1;31(1):68–72.
94. Boxell AC, Gibson KT, Hobbs RP, Thompson RCA. Occurrence of gastrointestinal parasites in horses in metropolitan Perth, Western Australia. Aust Vet J. 2004 Jan 1;82(1–2):91–5.
95. Nielsen MK, Monrad J, Olsen SN. Prescription-only anthelmintics - A questionnaire survey of strategies for surveillance and control of equine strongyles in Denmark. Vet Parasitol. 2006;135(1):47–55.
96. Hedberg-Alm Y, Penell J, Riihimäki M, Osterman-Lind E, Nielsen MK, Tydén E. Parasite occurrence and parasite management in swedish horses presenting with gastrointestinal disease-a case-control study. Animals. 2020;10(4).
97. Honoré ML, Nielsen MK, Olsen SN, Leifsson PS, Jacobsen S, Pihl TH. Study on survival of nonstrangulating intestinal infarctions associated with *Strongylus vulgaris*: Treatment outcomes of 39 horses (2008–2019). Danish Soc Parasitol Danish Soc Trop Med Int Heal – Jt Spring Symp 2019. 2019;34.
98. Andersen U V, Howe DK, Olsen SN, Nielsen MK. Recent advances in diagnosing pathogenic equine gastrointestinal helminths: The challenge of prepatent detection. Vet Parasitol. 2013 Feb;192(1–3):1–9.
99. Kaspar A, Pfister K, Nielsen MK, Silaghi C, Fink H, Scheuerle MC. Detection of *Strongylus vulgaris* in equine faecal samples by real-time PCR and larval culture - method comparison and occurrence assessment. BMC Vet Res. 2017;13(1):1–9.
100. Cain JL, Peters KT, Suri P, Roher A, Rutledge MH, Nielsen MK. The effect of analyst training on fecal egg counting variability. Parasitol Res. 2021;120(4):1363–70.
101. Cain JL, Slusarewicz P, Rutledge MH, McVey MR, Wielgus KM, Zynda HM, et al. Diagnostic performance of McMaster, Wisconsin, and automated egg counting techniques for enumeration of equine strongyle eggs in fecal samples. Vet Parasitol. 2020;284(June):1–8.
102. Noel ML, Scare JA, Bellaw JL, Nielsen MK. Accuracy and Precision of Mini-FLOTAC and McMaster Techniques for Determining Equine Strongyle Egg Counts. J Equine Vet Sci. 2017;48:182-187.e1.
103. Presland SL, Morgan ER, Coles GC. Counting nematode eggs in equine faecal samples. Vet Rec. 2005;156(7):208–10.
104. Cringoli G. FLOTAC, a novel apparatus for a multivalent faecal egg count technique. Parassitologia. 2006 Sep;48(3):381–4.
105. Nikolic Nielsen L, Wiinberg B, Kjelgaard Hansen M, Jensen AL, Kristensen AT. Prolonged activated prothromboplastin time and breed specific variation in haemostatic analytes in healthy adult Bernese Mountain dogs. Vet J. 2011;190(1):150–3.
106. Bevilaqua CML, Rodrigues M de L, Concordet D. Identification of infective larvae of some common nematode strongylids of horses. Rev Med Vet (Toulouse). 1993;144(12):989–95.
107. Bellaw JL, Nielsen MK. Evaluation of Baermann apparatus sedimentation time on recovery of *Strongylus vulgaris* and S. edentatus third stage larvae from equine coprocultures. Vet Parasitol. 2015;211(1–2):99–101.
108. Nielsen MK, Facison C, Scare JA, Martin AN, Gravatte HS, Steuer AE. Diagnosing *Strongylus vulgaris* in pooled fecal samples. Vet Parasitol. 2021;296(June):1–4.
109. Nielsen MK, Baptiste KE, Tolliver SC, Collins SS, Lyons ET. Analysis of multiyear studies in horses in Kentucky to ascertain whether counts of eggs and larvae per gram of feces are reliable indicators of numbers of strongyles and ascarids present. Vet Parasitol. 2010;174(1–2):77–84.
110. Nielsen MK, Peterson DS, Monrad J, Thamsborg SM, Olsen SN, Kaplan RM. Detection and semi-quantification of *Strongylus vulgaris* DNA in equine faeces by real-time quantitative PCR. Int J

Parasitol. 2008;38(3–4):443–53.
111. Wynne E, Slocombe JOD, Wilkie BN. Antigenic analyses of tissues and excretory and secretory products from *Strongylus vulgaris*. Can J Comp Med. 1981;45(3):259–65.
112. Klei TR, Chapman MR, Torbert BJ, McClure JR. Antibody responses of ponies to initial and challenge infections of *Strongylus vulgaris*. Vet Parasitol. 1983;12(2):187–98.
113. Nichol C, Masterson WJ. Characterisation of surface antigens of *Strongylus vulgaris* of potential immunodiagnostic importance. Mol Biochem Parasitol. 1987;25(1):29–38.
114. Adeyefa CAO. Precipitin response of the mitogen produced by *Strongylus vulgaris* arterial larvae. Vet Parasitol. 1992;43(3–4):243–7.
115. Hassan SE, Ghazy AA, Abdel-Rahman EH. Isolation and Characterization of Immunodiagnostic Antigen from *Strongylus vulgaris* Infecting Horses. World Appl Sci J. 2010;8(2):235–40.
116. Andersen U V., Howe DK, Dangoudoubiyam S, Toft N, Reinemeyer CR, Lyons ET, et al. SvSXP: A Strongylus vulgaris antigen with potential for prepatent diagnosis. Parasites and Vectors. 2013;6(1):1–13.
117. Ek N. Serum Levels of the Immunoglobulins IgG and IgG(T) in Horses. Acta Vet Scand. 1974;15(4):609–19.
118. Nielsen MK, Vidyashankar AN, Gravatte HS, Bellaw J, Lyons ET, Andersen UV. Development of *Strongylus vulgaris*-specific serum antibodies in naturally infected foals. Vet Parasitol. 2014;200(3–4):265–70.
119. Nielsen MK, Vidyashankar AN, Bellaw J, Gravatte HS, Cao X, Rubinson EF, et al. Serum *Strongylus vulgaris*-specific antibody responses to anthelmintic treatment in naturally infected horses. Parasitol Res. 2014;114(2):445–51.
120. Coffman JR, Carlson KL. Verminous arteritis in horses. J Am Vet Med Assoc. 1971;158(8):1358–60.
121. Vibe-Petersen G, Nielsen K. Verminous enteritis and thrombo-embolic colic in the horse. A description of 36 cases (author's transl). Scand J Vet Sci Vibe-Petersen,. 1979;31(9):385–91.
122. Poulsen L, Henriksson FV, Dahl M, Honoré ML, Olsen SN, Leifsson PS, et al. Differentiating *Strongylus vulgaris*-associated, non-strangulating intestinal infarctions from idiopathic peritonitis and acute colitis. Equine Vet J. 2021;(In review).
123. Wallace KD, Selcer BA, Tyler DE, Brown J. In vitro ultrasonographic appearance of the normal and verminous equine aorta, cranial mesenteric artery, and its branches. Am J Vet Res. 1989;50(10):1774–8.
124. Wallace KD, Selcer BA, Becht JL. Technique for transrectal ultrasonography of the cranial mesenteric artery of the horse. Am J Vet Res. 1989 Oct;50(10):1695–8.
125. Holmes R, Klei T, Mcclure J, Watters J. Sequential mesenteric arteriography in pony foals during repeated inoculations of *Strongylus vulgaris* and treatments with ivermectin. Am J Vet Res. 1990;51(4):661–5.
126. Slocombe JO, McCraw BM, Pennock PW, Ducharme N, Baird JD. *Strongylus vulgaris* in the tunica media of arteries of ponies and treatment with ivermectin. Can J Vet Res. 1987;51(2):232–5.
127. Greatorex JC. Diagnosis and treatment of "verminous aneurysm" formation in the horse. Vet Rec. 1977;101:184–7.
128. Pihl TH, Scheepers E, Sanz M, Goddard A, Page P, Toft N, et al. Acute-phase proteins as diagnostic markers in horses with colic. J Vet Emerg Crit care. 2016;26(5):664–74.
129. Dennis VA, Klei TR, Miller MA, Chapman MR, McClure JR. Immune responses of pony foals during repeated infections of Strongylus vulgaris and regular ivermectin treatments. Vet Parasitol. 1992;42(1–2):83–99.
130. Dyson S. Review of 30 cases of peritonitis in the horse. Equine Vet J. 1983;15(1):25–30.
131. Henderson ISF, Mair TS, Keen JA, Shaw DJ, Mcgorum BC. Study of the short- and long-term outcomes of 65 horses with peritonitis. Vet Rec. 2008;163:293–7.
132. Slocombe JO, McCraw BM. Evaluation of pyrantel pamoate, nitramisole and avermectin B1a against migrating *Strongylus vulgaris* larvae. Can J Comp Med Rev Can Med Comp. 1980;44(1):93–100.

133. Slocombe JO, McCraw BM, Pennock PW, Baird JD. Effectiveness of fenbendazole against later 4th-stage *Strongylus vulgaris* in ponies. Am J Vet Res. 1983;44(12):2285–9.
134. Macfarlane RG. An enzyme cascade in the blood clotting mechanism, and its function as a biochemical amplifier. Nature. 1964 May;202:498–9.
135. Davie EW, Ratnoff OD. Waterfall Sequence for Intrinsic Blood Clotting. Science (80-). 1964;145(3638):1310–2.
136. Hoffman M, Monroe DM. Coagulation 2006: A Modern View of Hemostasis. Hematol Oncol Clin North Am. 2007;21(1):1–11.
137. Hoffman M, Monroe DM. A cell-based model of hemostasis. Thromb Haemost. 2001;85(6):958–65.
138. Dunkel B. Hypercoagulation and thrombosis associated with infection in horses. Equine Vet Educ. 2021;1–3.
139. Smith SA. The cell-based model of coagulation: State-Of-The-Art Review. J Vet Emerg Crit Care. 2009;19(1):3–10.
140. Ferreira CN, Sousa M de O, Dusse LMS, Carvalho M das G. A cell-based model of coagulation and its implications. Rev Bras Hematol Hemoter. 2010;32(5):416–21.
141. Martin DM, Boys CW, Ruf W. Tissue factor: molecular recognition and cofactor function. FASEB J Off Publ Fed Am Soc Exp Biol. 1995 Jul;9(10):852–9.
142. Banner DW, D'Arcy A, Chène C, Winkler FK, Guha A, Konigsberg WH, et al. The crystal structure of the complex of blood coagulation factor VIIa with soluble tissue factor. Nature. 1996;380(6569):41–6.
143. Monkovic DD, Tracy PB. Activation of human factor V by factor Xa and thrombin. Biochemistry. 1990 Feb;29(5):1118–28.
144. Monroe DM, Hoffman M, Roberts HR. Transmission of a procoagulant signal from tissue factor-bearing cell to platelets. Blood Coagul fibrinolysis an Int J Haemost Thromb. 1996 Jun;7(4):459–64.
145. Díaz-Ricart M, Estebanell E, Lozano M, Aznar-Salatti J, White JG, Ordinas A, et al. Thrombin facilitates primary platelet adhesion onto vascular surfaces in the absence of plasma adhesive proteins: studies under flow conditions. Haematologica. 2000 Mar;85(3):280–8.
146. Hultin MB. Modulation of thrombin-mediated activation of factor VIII:C by calcium ions, phospholipid, and platelets. Blood. 1985 Jul;66(1):53–8.
147. Donahue SM, Otto CM. Thromboelastography: A tool for measuring hypercoagulability, hypocoagulability, and fibrinolysis. J Vet Emerg Crit Care. 2005;15(1):9–16.
148. Opal SM, Kessler CM, Roemisch J, Knaub S. Antithrombin, heparin, and heparan sulfate. Crit Care Med. 2002;30(5):325–31.
149. Bock SC, Harris JF, Balazs I, Trent JM. Assignment of the human antithrombin III structural gene to chromosome 1q23-25. Cytogenet Cell Genet. 1985;39(1):67–9.
150. Levi M, Ten Cate H. Disseminated intravascular coagulation. N Engl J Med. 1999 Aug;341(8):586–92.
151. Vervloet MG, Thijs LG, Hack CE. Derangements of coagulation and fibrinolysis in critically ill patients with sepsis and septic shock. Semin Thromb Hemost. 1998;24(1):33–44.
152. Adam SS, Key NS, Greenberg CS. D-dimer antigen: current concepts and future prospects. Am Soc Hematol. 2009;113(13):2878–87.
153. Weitz JI, Fredenburgh JC, Eikelboom JW. A Test in Context: D-Dimer. J Am Coll Cardiol. 2017;70(19):2411–20.
154. Hoeprich PD, Doolittle RF. Dimeric half-molecules of human fibrinogen are joined through disulfide bonds in an antiparallel orientation. Biochemistry. 1983 Apr 26;22(9):2049–55.
155. Mosesson MW, Siebenlist KR, Hainfeld JF, Wall JS. The Covalent Structure of Factor XIIIa Crosslinked Fibrinogen Fibrils. J Struct Biol. 1995;115(1):88–101.
156. Longstaff C, Kolev K. Basic mechanisms and regulation of fibrinolysis. J Thromb Haemost. 2015 Jun 1;13(S1):98–105.
157. Francis CW, Marder VJ, Barlow GH. Plasmic Degradation of Crosslinked Fibrin: Characterization of new macromolecular soluble complexes and a model of their structure. J Clin Invest. 1980;66(5):1033–43.
158. Gaffney PJ, Joe F. The lysis of crosslinked human fibrin by plasmin yields initially a single molecular

complex, D dimer-E. Thromb Res. 1979;15(5):673–87.
159. Hager K, Platt D. Fibrin Degeneration Product Concentrations (D-Dimers) in the Course of Ageing. Gerontology. 1995;41(3):159–65.
160. Moore KL, Andreoli SP, Esmon NL, Esmon CT, Bang NU. Endotoxin enhances tissue factor and suppresses thrombomodulin expression of human vascular endothelium in vitro. J Clin Invest. 1987 Jan;79(1):124–30.
161. Eppihimer MJ, Schaub RG. P-Selectin-Dependent Inhibition of Thrombosis During Venous Stasis. Arter Thromb Vasc Biol. 2000;20:2483–8.
162. Peter K, Weirich U, Nordt TK, Ruef J, Bode C. Soluble vascular cell adhesion molecule-1 (VCAM-1) as potential marker of atherosclerosis. Thromb Haemost. 1999 Sep;82 Suppl 1:38–43.
163. Smith A, Quarmby JW, Collins M, Lockhart SM, Burnand KG. Changes in the levels of soluble adhesion molecules and coagulation factors in patients with deep vein thrombosis. Thromb Haemost. 1999 Dec;82(6):1593–9.
164. Xu J, Cai J, Anderson B, Wagner B, Albrecht R, Peek SF, et al. Cloning and functional characterization of recombinant equine P-selectin. Vet Immunol Immunopathol. 2007;116:115–30.
165. Luster AD, Alon R, von Andrian UH. Immune cell migration in inflammation: Present and future therapeutic targets. Nat Immunol. 2005;6(12):1182–90.
166. Shebuski R, Kilgore K. Role of inflammatory mediators in autoimmune disease. J Pharmacol Exp Ther. 2002;300(3):65–77.
167. Levi M, Van Der Poll T, Ten Cate H. Tissue factor in infection and severe inflammation. Semin Thromb Hemost. 2006;32(1):33–9.
168. Aken BE Van, Heijer M Den, Bos GMJ, Deventer SJH Van, Reitsma PH. Recurrent Venous Thrombosis and Markers of Inflammation. 2000;4–7.
169. van Aken BE, Reitsma PH, Rosendaal FR. Interleukin 8 and venous thrombosis: evidence for a role of inflammation in thrombosis. Br J Haematol. 2002;116:173–7.
170. Matos MF, Lourenço DM, Orikaza CM, Bajerl JAH, Noguti MAE, Morelli VM. The role of IL-6 , IL-8 and MCP-1 and their promoter polymorphisms IL-6 -174GC , IL-8 -251AT and MCP-1 -2518AG in the risk of venous thromboembolism: A case-control study. Thromb Res. 2011;128(3):216–20.
171. Poredos P, Kaja M. In patients with idiopathic venous thrombosis , interleukin-10 is decreased and related to endothelial dysfunction. 2011;596–602.
172. Kamphuisen PW, Eikenboom JCJ, Vos HL, Pablo R, Sturk A, Bertina RM, et al. Increased Levels of Factor VIII and Fibrinogen in Patients with Venous Thrombosis Are not Caused by Acute Phase Reactions. Thromb Haemost. 1999;81:680–3.
173. Osborn L, Hession C, Tizard R, Vassallo C, Luhowskyj S, Chi-Rosso G, et al. Direct expression cloning of vascular cell adhesion molecule 1, a cytokine-induced endothelial protein that binds to lymphocytes. Cell. 1989 Dec;59(6):1203–11.
174. Rice GE, Bevilacqua MP. An inducible endothelial cell surface glycoprotein mediates melanoma adhesion. Science. 1989 Dec;246(4935):1303–6.
175. Kong D, Kim YK, Kim MR, Jang JH, Lee S. Emerging Roles of Vascular Cell Adhesion Molecule-1 (VCAM-1) in Immunological Disorders and Cancer. Int J Mol Sci. 2018;19:1–16.
176. Cook-mills JM, Marchese ME, Abdala-valencia H. Vascular Cell Adhesion Molecule-1 Expression and Signaling During Disease : Regulation by Reactive Oxygen Species and Antioxidants. Antioxid Redox Signal. 2011;15(6):1607–38.
177. van Oosten M, van de Bilt E, de Vries HE, van Berkel TJC. Vascular Adhesion Molecule - I and Intercellular Adhesion Molecule-1 Expression on Rat Liver Cells After Lipopolysaccharide Administration In Viuo. Hepatology. 1995;22(5):1538–46.
178. Sharma R, Sharma R, Khaket TP, Dutta C. Breast cancer metastasis : Putative therapeutic role of vascular cell adhesion molecule-1. Cell Oncol. 2017;40:199–208.
179. Mosevoll KA, Lindås R, Tvedt THA, Bruserud O, Reikvam H. Altered plasma levels of cytokines, soluble adhesion molecules and matrix metalloproteases in venous thrombosis. Thromb Res. 2015;136(1):30–

9.
180. Božič M, Blinc A, Stegnar M. D-dimer, other markers of haemostasis activation and soluble adhesion molecules in patients with different clinical probabilities of deep vein thrombosis. Thromb Res. 2002;108(2–3):107–14.
181. Gregg AJ, Schenkel AR. Cloning and structural analysis of equine platelet endothelial cell adhesion molecule (PECAM , CD31) and vascular cell adhesion molecule-1 (vCAM-1, CD106). Vet Immunol Immunopathol. 2008;122:295–308.
182. Furie B, Furie BC. Role of platelet P-selectin and microparticle PSGL-1 in thrombus formation. Trends Mol Med. 2004;10(4):171–8.
183. Polgar J, Matuskova J, Wagner DD. The P-selectin, tissue factor, coagulation triad. J Thromb Haemost. 2005;3(8):1590–6.
184. Geng J-G, Chen M, Chou K-C. P-selectin cell adhesion molecule in inflammation, thrombosis, cancer growth and metastasis. Curr Med Chem. 2004 Aug;11(16):2153–60.
185. Hartwell DW, Mayadas TN, Berger G, Frenette PS, Rayburn H, Hynes RO, et al. Role of P-selectin cytoplasmic domain in granular targeting in vivo and in early inflammatory responses. J Cell Biol. 1998;143(4):1129–41.
186. Xu J, Cai J, Suresh M, Peek SF, Darien BJ. Equine PSGL-1 modifications required for P-selectin binding. Vet Immunol Immunopathol. 2015;131(2009):33–43.
187. Yang J, Furie BC, Furie B. The biology of P-selectin glycoprotein ligand-1: its role as a selectin counterreceptor in leukocyte-endothelial and leukocyte-platelet interaction. Thromb Haemost. 1999 Jan;81(1):1–7.
188. Cummings RD. Structure and function of the selectin ligand PSGL-1. Brazilian J Med Biol Res. 1999;32(5):519–28.
189. McEver RP, Cummings RD. Perspectives series: cell adhesion in vascular biology. Role of PSGL-1 binding to selectins in leukocyte recruitment. J Clin Invest. 1997 Aug;100(3):485–91.
190. Cambien B, Wagner DD. A new role in hemostasis for the adhesion receptor P-selectin. Trends Mol Med. 2004;10(4):179–86.
191. Egami K, Murohara T, Aoki M, Matsuishi T. Ischemia-induced angiogenesis: role of inflammatory response mediated by P-selectin. J Leukoc Biol. 2006 May;79(5):971–6.
192. Liu L, Kubes P. Molecular mechanisms of leukocyte recruitment: Organ-specific mechanisms of action. Thromb Haemost. 2003;89(2):213–20.
193. Ferroni P, Pulcinelli FM, Lenti L, Gazzaniga PP. Is soluble P-selectin determination a more reliable marker of in vivo platelet activation than CD62P flow cytometric analysis? Vol. 81, Thrombosis and haemostasis. Germany; 1999. p. 472–3.
194. Kappelmayer J, Nagy B, Miszti-Blasius K, Hevessy Z, Setiadi H. The emerging value of P-selection as a disease marker. Clin Chem Lab Med. 2004;42(5):475–86.
195. Merten M, Thiagarajan P. P-selectin in arterial thrombosis. Z Kardiol. 2004;93(11):855–63.
196. Constantin G. PSGL-1 as a novel therapeutic target. Drug News Perspect. 2004 Nov;17(9):579–86.
197. Farmer DG, Anselmo D, Da Shen X, Ke B, Carmody IC, Gao F, et al. Disruption of P-selectin signaling modulates cell trafficking and results in improved outcomes after mouse warm intestinal ischemia and reperfusion injury. Transplantation. 2005 Sep;80(6):828–35.
198. Hansen A, Kumar A, Wolf D, Frankenbergerova K, Filusch A, Gross M-L, et al. Evaluation of cardioprotective effects of recombinant soluble P-selectin glycoprotein ligand-immunoglobulin in myocardial ischemia-reperfusion injury by real-time myocardial contrast echocardiography. J Am Coll Cardiol. 2004 Aug;44(4):887–91.
199. Piccin A, Murphy WG, Smith OP. Circulating microparticles: pathophysiology and clinical implications. Blood Rev. 2007;21:157–71.
200. Vandy F, Stabler C, Eliassen A, Hawley A, Guire K, Myers D, et al. Soluble P-selectin for the diagnosis of lower extremity deep venous thrombosis. J Vasc Srg Venous Lymphat Disord. 2013;1(2):1–21.
201. Bucek RA, Reiter M, Quehenberger P, Minar E. The role of soluble cell adhesion molecules in patients

with suspected deep vein thrombosis. Blood Coagul Fibrinolysis. 2003;14:653–7.
202. Sayasith K, Bouchard N, Boerboom D, Brown KA, Doré M, Sirois J. Molecular characterization of equine P-selectin (CD62P) and its regulation in ovarian follicles during the ovulatory process. Biol Reprod. 2005;72(3):736–44.
203. Lalko CC, Deppe E, Ulatowski D, Lutgen A, Hart AP, Patton EA, et al. Equine platelet CD62P (P-selectin) expression: a phenotypic and morphologic study. Vet Immunol Immunopathol. 2003;91:119–34.
204. Lasry B, Svaren J, Wagner B, Darien BJ, Xu J. Identification of equine P-selectin glycoprotein ligand-1 (CD162). Mamm Genome. 2005;16:66–71.
205. Monreal L, Pino M, Ordinas A, Brugue R, White JG. Assessment of Platelet Function in Horses: Ultrastructure, Flow Cytometry, and Perfusion Techniques. J Vet Intern Med. 2006;20:581–8.
206. Dallap BL. Coagulopathy in the equine critical care patient. Vet Clin North Am - Equine Pract. 2004;20(1):231–51.
207. Hoffman M. Remodeling the Blood Coagulation Cascade. J Thromb Thrombolysis. 2003;16:17–20.
208. Dolente BA, Wilkins PA, Boston RC. Clinicopathologic evidence of disseminated intravascular coagulation in horses with acute colitis. J Am Vet Med Assoc. 2002;220(7):1034–8.
209. Cesarini C, Monreal L, Armengou L, Delgado MÁ, Ríos J, Jose-Cunilleras E. Progression of plasma D-dimer concentration and coagulopathies during hospitalization in horses with colic. J Vet Emerg Crit Care. 2014;24(6):672–80.
210. Cesarini C, Monreal L, Armengou L, Delgado MÁ, Ríos J, Jose-Cunilleras E. Association of Admission Plasma D-dimer Concentration with Diagnosis and Outcome in Horses with Colic. J Vet Intern Med. 2010;24:1490–7.
211. Nikvand AA, Jalali SM, Ghadrdan Mashhadi A, Razi Jalali M, Hassanpour Amirabadi S. Clinical, hematologic, hemostatic, and serum biochemical findings related to survival in Arabian horses with colic. Vet Clin Pathol. 2019;48(3):441–8.
212. Mammen EF. Antithrombin: its physiological importance and role in DIC. Semin Thromb Hemost. 1998;24(1):19–25.
213. Niessen RW, Lamping RJ, Jansen PM, Prins MH, Peters M, Taylor FBJ, et al. Antithrombin acts as a negative acute phase protein as established with studies on HepG2 cells and in baboons. Thromb Haemost. 1997 Sep;78(3):1088–92.
214. Bick RL. Disseminated intravascular coagulation and related syndromes: A clinical review. Semin Thromb Hemost. 1988;14(4):299–338.
215. Jacoby RC, Owings JT, Ortega T, Gosselin R, Feldman EC. Biochemical basis for the hypercoagulable state seen in Cushing syndrome. Arch Surg. 2001;136(9):1003–7.
216. Adamantos S, Waters S, Boag A, Services LV, House L, College RV, et al. Coagulation status in dogs with naturally occurring Angiostrongylus vasorum infection. J Small Anim Pract. 2015;56(August):485–90.
217. Johnstone IB, Crane S. Haemostatic abnormalities in horses with colic - Their prognostic value. Equine Vet J. 1986;18(4):271–4.
218. Holland M, Kelly AB, Snyder JR, Steffey EP, Willits N, McNeal D. Antithrombin III activity in horses with large colon torsion. Am J Vet Res. 1986 Apr;47(4):897–900.
219. Longstaff C, Adcock D, Olson JD, Jennings I, Kitchen S, Mutch N, et al. Harmonisation of D-dimer - A call for action. Vol. 137, Thrombosis research. United States; 2016. p. 219–20.
220. Olson JD. D-dimer: An Overview of Hemostasis and Fibrinolysis, Assays, and Clinical Applications. Adv Clin Chem. 2015;69:1–46.
221. Tripodi A. D-dimer Testing in Laboratory Practice. Clin Chem. 2011 Sep 1;57(9):1256–62.
222. Thachil J, Lippi G, Favaloro EJ. D-Dimer Testing: Laboratory Aspects and Current Issues. Methods Mol Biol. 2017;1646:91–104.
223. Rodger MA, Le Gal G, Langlois NJ, Gin B, Mallick R, Giulivi A, et al. "HERDOO2" clinical decision rule to guide duration of anticoagulation in women with unprovoked venous thromboembolism. Can I use any d-Dimer? Thromb Res. 2018 Sep;169:82–6.

224. Wells PS, Anderson DR, Rodger M, Forgie M, Kearon C, Dreyer J, et al. Evaluation of D-dimer in the diagnosis of suspected deep-vein thrombosis. N Engl J Med. 2003;349(13):1227–35.
225. Wells PS. Integrated strategies for the diagnosis of venous thromboembolism. J Thromb Haemost. 2007 Jul;5 Suppl 1:41–50.
226. Di Nisio M, Squizzato A, Rutjes AWS, Büller HR, Zwinderman AH, Bossuyt PMM. Diagnostic accuracy of D-dimer test for exclusion of venous thromboembolism: a systematic review. J Thromb Haemost. 2007 Feb;5(2):296–304.
227. Johnson ED, Schell JC, Rodgers GM. The D-dimer assay. Am J Hematol. 2019;94(7):833–9.
228. Bick RL. Disseminated intravascular coagulation. Hematol /Oncology Clin North Am. 1992;6(6):1259–85.
229. Weber T, Högler S, Auer J, Berent R, Lassnig E, Kvas E, et al. D-dimer in acute aortic dissection. Chest. 2003 May;123(5):1375–8.
230. Li Y, Qi X, Li H, Dai J, Deng H, Li J, et al. D-dimer level for predicting the in-hospital mortality in liver cirrhosis: A retrospective study. Exp Ther Med. 2017 Jan;13(1):285–9.
231. Rodelo JR, De G, Valencia ML, Ospina S, Arango CM, Gómez CI, et al. D-dimer is a significant prognostic factor in patients with suspected infection and sepsis. Am J Emerg Med. 2012;30(9):1991–9.
232. AlKhalfan F, Kerneis M, Nafee T, Yee MK, Chi G, Plotnikov A, et al. D-Dimer Levels and Effect of Rivaroxaban on Those Levels and Outcomes in Patients With Acute Coronary Syndrome (An ATLAS ACS-TIMI 46 Trial Substudy). Am J Cardiol. 2018 Nov;122(9):1459–64.
233. Feinstein DI. Disseminated intravascular coagulation in patients with solid tumors. Oncology (Williston Park). 2015 Feb;29(2):96–102.
234. Prochaska J, Frank B, Nagler M, Lamparter H, Weisser G, Schulz A, et al. Age-related diagnostic value of D-dimer testing and the role of inflammation in patients with suspected deep vein thrombosis. Sci Rep. 2017 Dec 1;Jul 4(7(1)):4591.
235. Robert-Ebadi H, Bertoletti L, Combescure C, Le Gal G, Bounameaux H, Righini M. Effects of impaired renal function on levels and performance of D-dimer in patients with suspected pulmonary embolism. Thromb Haemost. 2014 Sep;112(3):614–20.
236. Haase C, Joergensen M, Ellervik C, Joergensen MK, Bathum L. Age- and sex-dependent reference intervals for D-dimer: evidence for a marked increase by age. Thromb Res. 2013;132(6):676–80.
237. Epstein SE, Hopper K, Mellema MS, Johnson LR. Diagnostic Utility of D-dimer Concentrations in Dogs with Pulmonary Embolism. J Vet Intern Med. 2013;27:1646–9.
238. Sandholm M, Vidovic A, Puotunen-Reinert A, Sankari S, Nyholm K, Rita H. D-Dimer Improves the Prognostic Value of Combined Clinical and Laboratory Data in Equine Gastrointestinal Colic. Acta Vet Scand. 1995;36(2):255–72.
239. Delgado MA, Monreal L, Armengou L, Ríos J, Segura D. Peritoneal D-dimer concentration for assessing peritoneal fibrinolytic activity in horses with colic. J Vet Intern Med. 2009;23(4):882–9.
240. Armengou L, Monreal L, Tarancón I, Navarro M, Ríos J, Segura D. Plasma D-Dimer Concentration in Sick Newborn Foals. J Vet Intern Med. 2008;22:411–7.
241. Monreal L, Anglés A, Espada Y, Monasterio J, Monreal M. Hypercoagulation and hypofibrinolysis in horses with colic and DIC. Equine Vet J Suppl. 2000 Jun;(32):19–25.
242. Monreal L. D-dimer as a new test for the diagnosis of DIC and thromboembolic disease. Vol. 17, Journal of veterinary internal medicine. United States; 2003. p. 757–9.
243. Gara-Boivin C, de Castillo JRE, Dunn ME, Bédard C. Effect of dalteparin administration on thrombin generation kinetics in healthy dogs. Vet Clin Pathol. 2017;46(2):269–77.
244. Wang Z, Li J, Cao Q, Wang L, Shan F, Zhang H. Comparison Between Thromboelastography and Conventional Coagulation Tests in Surgical Patients With Localized Prostate Cancer. Clin Appl Thromb. 2018;24(5):755–63.
245. Thakur M, Ahmed AB. A review of thromboelastography. Int J Perioper Ultrasound Appl Technol. 2012;1(1):25–9.
246. Mallett S V, Cox DJ. Thrombelastography. Br J Anaesth. 1992 Sep;69(3):307–13.

247. Hartert H. Blutgerinnungsstudien mit der Thrombelastographie, einem neuen Untersuchungsverfahren. Klin Wochenschr. 1948;26(37–38):577–83.
248. Karon BS. Why is everyone so excited about thromboelastrography (TEG)? Clin Chim Acta. 2014;436:143–8.
249. Epstein KL, Brainard BM, Lopes MAF, Barton MH, Moore JN. Thrombelastography in 26 healthy horses with and without activation by recombinant human tissue factor. J Vet Emerg Crit Care. 2009;19(1):96–101.
250. Lemon A V., Goddard A, Hooijberg EH. Effects of storage time and temperature on thromboelastographic analysis in dogs and horses. Vet Clin Pathol. 2021;50(1):9–19.
251. Hyldahl Laursen S, Andersen PH, Kjelgaard-Hansen M, Wiinberg B. Comparison of components of biological variation between 3 equine thromboelastography assays. Vet Clin Pathol. 2013;42(4):443–50.
252. Johansson PI, Bochsen L, Andersen S, Viuff D. Investigation of the effect of kaolin and tissue factor-activated citrated whole blood, on clot forming variables, as evaluated by thromboelastography. Transfusion. 2008;48(11):2377–83.
253. Marschner CB, Kristensen AT, Rozanski EA, McEvoy FJ, Kühnel L, Taeymans O, et al. Diagnosis of canine pulmonary thromboembolism by computed tomography and mathematical modelling using haemostatic and inflammatory variables. Vet J. 2017;229:6–12.
254. Goggs R, Brainard B, De Laforcade AM, Flatland B, Hanel R, Mcmichael M, et al. Partnership on Rotational ViscoElastic Test Standardization (PROVETS): Evidence-based guidelines on rotational viscoelastic assays in veterinary medicine. J Vet Emerg Crit Care. 2014;24(1):1–22.
255. Vig S, Chitolie A, Bevan DH, Halliday A, Dormandy J. Thromboelastography: A reliable test? Blood Coagul Fibrinolysis. 2001;12(7):555–61.
256. Wiinberg B, Jensen AL, Rojkjaer R, Johansson P, Kjelgaard-Hansen M, Kristensen AT. Validation of human recombinant tissue factor-activated thromboelastography on citrated whole blood from clinically healthy dogs. Vet Clin Pathol. 2005;34(4):389–93.
257. Fenty RK, Delaforcade AM, Shaw SP, Toole TEO. Identification of hypercoagulability in dogs with primary immune-mediated hemolytic anemia by means of thromboelastography. J Am Vet Med Assoc. 2011;238(4):463–7.
258. Wasowicz M, Srinivas C, Meineri M, Banks B, McCluskey SA, Karkouti K. Technical report: Analysis of citrated blood with thromboelastography: Comparison with fresh blood samples. Can J Anesth. 2008;55(5):284–9.
259. Leclere M, Lavoie JP, Dunn M, Bédard C. Evaluation of a modified thrombelastography assay initiated with recombinant human tissue factor in clinically healthy horses. Vet Clin Pathol. 2009;38(4):462–6.
260. Müller M, J. MC, Vroom M, Juffermans N. Utility of thromboelastography and/or thromboelastometry in adults with sepsis: a systematic review. Crit care. 2014;18(1):30–30.
261. Bolliger D, Seeberger MD, Tanaka KA. Principles and Practice of Thromboelastography in Clinical Coagulation Management and Transfusion Practice. Transfus Med Rev. 2012;26(1):1–13.
262. Hemker HC. Thrornbin generation, an essential step in haemostasis and thrombosis. Hemost Thromb. 1994;3:477–92.
263. Hemker HC, Giesen P, AlDieri R, Regnault V, De Smed E, Wagenvoord R, et al. The Calibrated Automated Thrombogram (CAT): A universal routine test for hyper- and hypocoagulability. Pathophysiol Haemost Thromb. 2002;32(5–6):249–53.
264. Machlus KR, Colby EA, Wu JR, Koch GG, Key NS, Wolberg S. Effects of tissue factor, thrombomodulin and elevated clotting factor levels on thrombin generation in the calibrated automated thrombogram. Thromb Haemost. 2010;102(5):936–44.
265. Hemker HC, Giesen P, Al Dieri R, Regnault V, De Smedt E, Wagenvoord R, et al. Calibrated automated thrombin generation measurement in clotting plasma. Pathophysiol Haemost Thromb. 2003;33(1):4–15.
266. Castoldi E, Rosing J. Thrombin generation tests. Thromb Res. 2011;127(SUPPL. 3):S21–5.

267. Duchemin J, Pan-Petesch B, Arnaud B, Blouch MT, Abgrall JF. Influence of coagulation factors and tissue factor concentration on the thrombin generation test in plasma. Thromb Haemost. 2008;99(4):767–73.
268. Saliba W, Awad K, Ron G, Elias M. The Effect of Vitamin D Supplementation on Thrombin Generation Assessed by the Calibrated Automated Thrombogram. Clin Appl Thromb. 2016;22(4):340–5.
269. Lutsey PL, Folsom AR, Heckbert SR, Cushman M. Peak thrombin generation and subsequent venous thromboembolism: The Longitudinal Investigation of Thromboembolism Etiology (LITE) study. J Thromb Haemost. 2009;7(10):1639–48.
270. Wexels F, Dahl OE, Pripp AH, Seljeflot I. Thrombin Generation in Patients with Suspected Venous Thromboembolism. Clin Appl Thromb. 2017;23(5):416–21.
271. Ten Cate-Hoek AJ, Dielis AWJH, Spronk HMH, Van Oerle R, Hamulyák K, Prins MH, et al. Thrombin generation in patients after acute deep-vein thrombosis. Thromb Haemost. 2008;100(2):240–5.
272. Tripodi A, Martinelli I, Chantarangkul V, Battaglioli T, Clerici M, Mannucci PM. The endogenous thrombin potential and the risk of venous thromboembolism. Thromb Res. 2007;121(3):353–9.
273. Van Hylckama Vlieg A, Christiansen SC, Luddington R, Cannegieter SC, Rosendaal FR, Baglin TP. Elevated endogenous thrombin potential is associated with an increased risk of a first deep venous thrombosis but not with the risk of recurrence. Br J Haematol. 2007;138(6):769–74.
274. Hron G, Kollars M, Binder BR, Eichinger S, Kyrle P a. Identification of patients at low risk for recurrent venous thromboembolism by measuring thrombin generation. JAMA. 2006;296(4):397–402.
275. Hudák R, Székely EG, Kovács KR, Nagy A, Hofgárt G, Berényi E, et al. Low thrombin generation predicts poor prognosis in ischemic stroke patients after thrombolysis. PLoS One. 2017;12(7):1–13.
276. Madsen DE, Nichols TC, Merricks EP, Waters EK, Wiinberg B. Global measurement of coagulation in plasma from normal and haemophilia dogs using a novel modified thrombin generation test - Demonstrated in vitro and ex vivo. PLoS One. 2017;12(4):1–16.
277. Allegret V, Dunn M, Bédard C. Monitoring unfractionated heparin therapy in dogs by measuring thrombin generation. Vet Clin Pathol. 2011;40(1):24–31.
278. Rose LJ, Dunn ME, Allegret V, Bédard C. Effect of prednisone administration on coagulation variables in healthy Beagle dogs. Vet Clin Pathol. 2011;40(4):426–34.
279. Rose L, Dunn ME, Bédard C. The Effect of Canine Hyperadrenocorticism on Coagulation Parameters. J Vet Intern Med. 2013;27:207–11.
280. Mischke R, Teuber M, A. Tiede. Measurements of endogenous thrombin potential using the CAT method in cats: Reference values and influence of the direct factor Xa inhibitor apixaban. Res Vet Sci. 2019;127(August):113–21.
281. Lyons ET, Drudge JH, Tolliver SC. Prevalence of some internal parasites found (1971–1989) in horses born on a farm in central kentucky. J Equine Vet Sci. 1990;10(2):99–107.
282. Adcock DM, Hoefner DM, Kottke-Marchant K, Marlar RA, Szamosi DI, Warunek DJ. Collection, Transport, and Processing of Blood Specimens for Testing Plasma-Based Coagulation Assays and Molecular Hemostasis Assays; Approved Guideline - Fith Edition. CLSI Guidel H21-A5. 2008;28(5):1–47.
283. Jeong JC, Kim JE, Ryu JW, Joo KW, Kim HK. Plasma haemostatic potential of haemodialysis patients assessed by thrombin generation assay: Hypercoagulability in patients with vascular access thrombosis. Thromb Res. 2013;132(5):604–9.
284. Salooja N, Perry DJ. Thrombelastography. Blood Coagul Fibrinolysis. 2001;12(5):327–37.
285. Hemker HC, Giesen PLA, Ramjee M, Wagenvoord R, Béguin S. The thrombogram: Monitoring thrombin generation in platelet rich plasma. Thromb Haemost. 2000;83(4):589–91.
286. Trigg DE, Wood MG, Kouides PA, Kadir RA. Hormonal influences on hemostasis in women. Semin Thromb Hemost. 2011;37(1):77–86.
287. Nagy P, Guillaume D, Daels P. Seasonality in mares. Anim Reprod Sci. 2000;60(61):245–62.
288. Ginther O, Gastal E, Gastal M, Beg M. Seasonal influence on equine follicle dynamics. Anim Reprod. 2004;1:31–44.

289. Southwood LL, Russell G. The use of clinical findings in the identification of equine peritonitis cases that respond favorably to medical therapy: Retrospective Study. J Vet Emerg Crit Care. 2007;17(4):382–90.
290. Tritten L, Gillis-Germitsch N, Kockmann T, Schnyder M. Quantitative proteomics analysis of Angiostrongylus vasorum-induced alterations in dog serum sheds light on the pathogenesis of canine angiostrongylosis. Sci Rep. 2021;11(1):1–11.
291. Butterfield C, Grumpelt B, Kimmel D, Patterson R, Jones K, Scott SL, et al. The Pretransport Management of Stress in Performance Horses. J Equine Vet Sci. 2018;69:145–8.
292. Folsom RW, Littlefield-Chabaud MA, French DD, Pourciau SS, Mistric L, Horohov DW. Exercise alters the immune response to equine influenza virus and increases susceptibility to infection. Equine Vet J. 2001;33(7):664–9.
293. Andersen U V, Reinemeyer CR, Toft N, Olsen SN, Jacobsen S, Nielsen MK. Physiologic and systemic acute phase inflammatory responses in young horses repeatedly infected with cyathostomins and *Strongylus vulgaris*. Vet Parasitol. 2014;201(1–2):67–74.
294. Borges AS, Divers TJ, Stokol T, Mohammed OH. Serum iron and plasma fibrinogen concentrations as indicators of systemic inflammatory diseases in horses. J Vet Intern Med. 2007;21(3):489–94.
295. Lyons ET, Drudge JH, Tolliver SC. Ivermectin: activity against larval *Strongylus vulgaris* and adult Trichostrongylus axei in experimental infections in ponies. Am J Vet Res. 1982 Aug;43(8):1449–50.
296. Monahan CM, Chapman MR, Klei TR, Taylor HW, French DD. Dose titration of moxidectin oral gel against migrating *Strongylus vulgaris* and Parascaris equorum larvae in pony foals. Vet Parasitol. 1995;60(1–2):103–10.
297. Nielsen MK, Scare J, Gravatte HS, Bellaw JL, Prado JC, Reinemeyer CR. Changes in serum *Strongylus vulgaris*-specific antibody concentrations in response to anthelmintic treatment of experimentally infected foals. Front Vet Sci. 2015;2:1–6.
298. Flatland B, Koenigshof AM, Rozanski EA, Goggs R, Wiinberg B. Systematic evaluation of evidence on veterinary viscoelastic testing Part 2: Sample acquisition and handling. J Vet Emerg Crit Care. 2014;24(1):30–6.
299. Lippi G, Franchini M, Montagnana M, Salvagno GL, Poli G, Guidi GC. Quality and reliability of routine coagulation testing: Can we trust that sample? Blood Coagul Fibrinolysis. 2006;17(7):513–9.
300. Adcock DM, Kressin DC, Marlar RA. Effect of 3.2 % vs 3.8 % Sodium Citrate Concentration on Routine Coagulation Testing. Coagul Transfus Med. 1996;107(1):105–10.
301. Morales F, Couto CG, Iazbik MC. Effects of 2 concentrations of sodium citrate on coagulation test results, von Willebrand factor concentration, and platelet function in dogs. J Vet Intern Med. 2007;21(3):472–5.
302. Lippi G, Salvagno GL, Montagnana M, Guidi GC. Influence of two different buffered sodium citrate concentrations on coagulation testing. Blood Coagul Fibrinolysis. 2005;16:381–3.
303. Mackenzie CJ, McGowan CM, Pinchbeck G, Carslake HB. Comparison of two blood sampling techniques for the determination of coagulation parameters in the horse: Jugular venipuncture and indwelling intravenous catheter. Equine Vet J. 2017;1–6.
304. Gosselin RC, Janatpour K, Larkin EC, Lee YP, Owings JT. Comparison of samples obtained from 3.2% sodium citrate glass and two 3.2% sodium citrate plastic blood collection tubes used in coagulation testing. Am J Clin Pathol. 2004;122(6):843–8.
305. Regnault V, Beguin S, Lecompte T. Calibrated automated thrombin generation in frozen-thawed platelet-rich plasma to detect hypercoagulability. Pathophysiol Haemost Thromb. 2003;33(1):23–9.
306. Beguin S, Keulartz I. On the Coagulation of Platelet Rich Plasma. Hemostasis. 1999;29:50–7.
307. Buriko Y, Drobatz K, Silverstein DC. Establishment of normal reference intervals in dogs using a viscoelastic point-of-care coagulation monitor and its comparison with thromboelastography. Vet Clin Pathol. 2020;49(4):567–73.
308. Brearton C, Rushton A, Parker J, Martin H, Hodgson J. Performance Evaluation of a New Point of Care Viscoelastic Coagulation Monitoring System in Major Abdominal, Orthopaedic and Vascular Surgery.

Platelets. 2020;31(8):1052–9.
309. Rosati T, Jandrey KE, Burges JW, Kent MS. Establishment of a reference interval for a novel viscoelastic coagulometer and comparison with thromboelastography in healthy cats. Vet Clin Pathol. 2020;49(4):660–4.
310. Kemper A, Burges J, Bishop R, Wilkins P. Evaluation of a point-of-care viscoelastic coagulation monitor (VCM-VET) with preliminary establishment of healthy adult horse reference intervals. J Vet Emerg Crit care. 2021;30(S1):S18.
311. Hellman S. Generation of equine enteroids and enteroid-derived 2D monolayers that are responsive to microbial mimics. Vet Res. 2021;52(1):108.
312. Hellman S. Immunological insights into equine responses against *Strongylus vulgaris* Ex vivo studies using equine intestinal organoids and blood mononuclear cells. PhD Thesis. 2021;1–148.

Paper I

Investigation of Two Different Human D-dimer Assays in The Horse

Honoré, M.L., Pihl, T.H., Busk-Anderson, T.M., Flintrup, L.L, Nielsen, L.N.

Submitted to BMC Veterinary Research, July 2021.

Investigation of Two Different Human D-dimer Assays in The Horse

Marie Louise Honoré[1], Tina H. Pihl[2], Tanne M. Busk-Anderson[3], Laura L. Flintrup[4], Lise N. Nielsen[5]

Affiliation details:

[1] Marie Louise Honoré Lytzhøft-Olsen, PhD student, Department of Veterinary Clinical Sciences, Faculty of Health and Medical Sciences (SUND), University of Copenhagen, Hoejbakkegaard Allé 5a, 2630 Taastrup, Denmark, mlhj@sund.ku.dk

[2] Tina Holberg Pihl, Associate Professor, Department of Veterinary Clinical Sciences, Faculty of Health and Medical Sciences (SUND), University of Copenhagen, Hoejbakkegaard Allé 5a, 2630 Taastrup, Denmark, thpi@sund.ku.dk

[3] Tanne M. Busk-Anderson, Department of Veterinary Clinical Sciences, Faculty of Health and Medical Sciences (SUND), University of Copenhagen, Hoejbakkegaard Allé 5a, 2630 Taastrup, Denmark, tannebusk94@hotmail.com

[4] Laura L. Flintrup, Department of Veterinary Clinical Sciences, Faculty of Health and Medical Sciences (SUND), University of Copenhagen, Hoejbakkegaard Allé 5a, 2630 Taastrup, Denmark, lauralindf@hotmail.com

[5] Lise Nikolic Nielsen, Professor, Section for Internal Medicine, Oncology and Clinical Pathology, Faculty of Health and Medical Sciences (SUND), University of Copenhagen, Dyrlaegevej 16, 1870 Frederiksberg C, Denmark, lini@sund.ku.dk

Correspondence:

Marie Louise Honoré, Department of Veterinary Clinical Sciences, Faculty of Health and Medical Sciences (SUND), University of Copenhagen, Denmark, mlhj@sund.ku.dk

Abstract

Background

D-dimer has value as a marker of thrombosis in critically ill horses and can provide additional information about prognosis. However, there are currently no equine species-specific d-dimer assays available, nor has there been any formal investigation of the applicability of human d-dimer assays in horses, so it is unknown, which assay performs best in this species. The aim of this study was therefore to evaluate and compare two human d-dimer assays for applicability in horses.

The study included four groups of horses: clinically healthy horses, horses with gastrointestinal (GI) disease and mild systemic inflammation based on low serum amyloid A (SAA) (low SAA), horses with GI disease and strong systemic inflammation based on high SAA (high SAA), and horses with non-strangulating intestinal infarction (NSII) caused by *Strongylus vulgaris*. The assays evaluated were the STAGO STA-Liatest D-di+ (Stago) and NycoCard™ D-dimer (NycoCard). Intra- and inter-coefficients of variation (CV) were assessed on two d-dimer concentrations, and linearity under dilution was evaluated. A group comparison was performed for both assays across the four groups of horses. A Spaghetti plot, Spearman Correlation, Passing Bablok regression and Bland-Altman plot were used to compare methods in terms of agreement.

Results

Ten horses were included in the clinically healthy group, eight in the low SAA group, eight in the high SAA group, and seven in the NSII group. For the Stago assay, intra- and inter-CVs were below the accepted level except for one inter-CV. The NycoCard assay did not meet the accepted level for any of the CVs. The linearity under dilution was acceptable for both the Stago and NycoCard. In the group comparison, both methods detected a significantly higher d-dimer concentration in the high SAA and NSII groups compared to the clinically healthy group. Method agreement showed slightly higher d-dimer concentrations with NycoCard compared to Stago. The overall agreement was stronger for the lower d-dimer concentrations.

Conclusion

Both the Stago and the NycoCard were found to be applicable for use in horses but were not directly comparable.

Keywords

Fibrin degradation products, equine, hemostasis, hypercoagulation, thrombosis, validation

Background

Making a definitive diagnosis of thrombosis in equine medicine is often challenging as there, to the best of the authors knowledge, currently is no established gold standard for use in equine clinical practice. In humans, a definitive diagnosis of thrombosis relies on different imaging modalities such as contrast venography and different ultrasonographic techniques (1–4). It is often not practical to use these techniques in horses in a clinical setting due to the size of the animal and the potential need for general anesthesia.

D-dimer represents a good surrogate biomarker of thrombosis. It is the end product of fibrinolysis and can only be measured in plasma after plasmin degradation of cross-linked fibrin (5,6). It is thus a marker of the fibrinolytic system (7). A low d-dimer concentration is an effective rule-out marker for thrombosis, while a high concentration is indicative of, but cannot confirm thrombosis (8,9). Due to the lack of a species-specific assay, d-dimer analyzes in equine medicine have been performed using different human assays (10–17). To the best of the authors' knowledge, there has not been a full investigation into the applicability of these assays in horses and thus, it is currently uncertain, which assay performs most advantageously in this species (10). Plasma d-dimer has been examined in horses with diseases predisposing for concurrent hemostatic imbalances. In particular, horses with gastrointestinal (GI) disease and a strong systemic inflammatory response may develop hemostatic aberrations (15,16,18). D-dimer measurements at admission and during hospitalization can be used to monitor the improvement in medical and surgical colic patients (16), and function as a prognostic marker of survival (15,18). The NycoCard™ D-dimer assay (NycoCard) has been used in horses with severe GI disease. An increased d-dimer concentration was found in horses with surgical colic (12), and d-dimer was found to be a valuable test for diagnosing disseminated intravascular coagulation (DIC) (10).

The parasite *Strongylus vulgaris* (*S. vulgaris*) migrates in the mesenteric arteries, causing thrombosis (19–22) that can lead to non-strangulating intestinal infarctions (NSII) (13). The STAGO STA-Liatest D-di+ (Stago) assay has been used to show that foals with migrating *S. vulgaris* larvae have an increased d-dimer concentration that correlates with the number of larvae in the cranial mesenteric artery (CMA) (23). However, no studies have evaluated d-dimer concentrations in adult horses with NSII.

Due to the lack of a formal investigation into the applicability of the Stago and NycoCard assays in equine medicine, the aim of this study was to evaluate and compare these two human d-dimer assays in relation to their application in horses.

Results

Horses

The clinically healthy group consisted of ten horses (eight mares and two geldings) with a mean age of 11.2 years (min. 4 – max. 22.8 years) and a mean bodyweight (BW) of 559.5 kg (min. 411 – max. 687 kg). Breed distribution was as follows: seven Standardbreds, two warmbloods and one of unknown breed. The low SAA group included eight horses with GI disease and a mild systemic inflammatory response with SAA between 30-100 mg/L at admission. This group consisted of five mares and three geldings with a mean age of 11.8 (min. 4 – max. 23.2 years) and a mean BW of 404.3 kg (min. 286 – max. 700 kg). Breed distribution was as follows: three Icelandic horses, one Clydesdale, three ponies, and one of unknown breed. The high SAA group included eight horses with GI disease and a strong systemic inflammatory response with SAA > 1,000 mg/L at admission. This group consisted of two mares, five geldings, and one stallion with a mean age of 13.4 years (min. 3.1 – max. 24.1 years) and a mean BW of 529.8 kg (min. 350 – max. 666 kg). Breed distribution was as follows: two Icelandic horses, one pony, four warmbloods, and one of unknown breed. The NSII group included seven horses with NSII: four mares and three geldings with a mean age of 11.8 years (min. 9 – max. 17.4 years) a mean BW of 446.8 kg (min. 361 – max. 540 kg). Breed distribution was four warmbloods and three ponies.

Sample handling

Samples for two out of the seven horses in the NSII group were collected in 3.8% (rather than 3.2%) sodium citrate tubes.

Test evaluation

The intra- and inter-CVs for the Stago assay were below the accepted level of 5%, with the exception of the inter-CV for the low pool, whereas the intra- and inter-CVs for the NycoCard were above the accepted level of 10% for all four CVs (Table 1).

Table 1. Imprecision study for the STAGO STA-Liatest D-di+ (Stago) and NycoCard™ D-dimer (NycoCard) assays. Intra- and inter-coefficients of variation (CV) for the low and medium pool for the Stago and NycoCard were calculated as CV % = (standard deviation / mean) * 100.

	Stago	NycoCard
Intra-CV % Low pool	4.66% (0.23; 0.01)	35.14% (0.15; 0.053)
Inter-CV % Low pool	5.98% (0.21; 0.01)	33.99% (0.13; 0.04)
Intra-CV % Medium pool	1.79% (0.51; 0.01)	11.11% (0.60; 0.07)
Inter-CV % Medium pool	2.63% (0.49; 0.01)	12.33% (0.58; 0.07)

(mean; standard deviation)

The results from the linearity under dilution showed compliance values between the observed and expected values of between 89% and 70% for the Stago and 97% and 65% for the NycoCard assay. The linearity under dilution for NycoCard showed a slope of 1.00 (95% Confidence interval (CI): 0.73-1.37) and Y-intercept of -0.075 (95% CI: -0.58-0.00). For the Stago, a slope of 1.00 (95% CI: 0.75-1.14) and Y-intercept of 0.065 (95% CI: 0.04-0.17) were found (Figure 1).

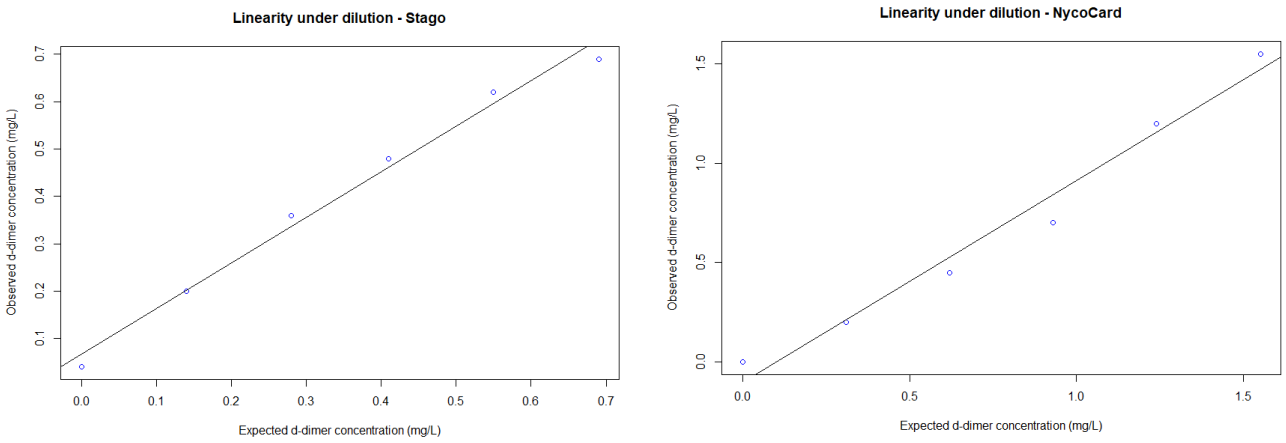


Figure 1. Linearity under dilution for the STAGO STA-Liatest D-di+ (Stago) and NycoCard™ D-dimer (NycoCard) assays using a Passing Bablok regression. A slope of 1.00 (95% confidence interval (CI) 0.75-1.14) and a Y-intercept of 0.065 (95% CI 0.04-0.17) were found for Stago, while a slope of 1.00 (95% CI 0.73-1.37) and Y-intercept of -0.075 (95% CI -0.58-0.00) were found for NycoCard. Note the different axes in the two graphs.

Group comparison

The group comparison showed a statistically significant difference between the groups for both assays ($p = 0.0003$). For the Stago assay, the *post hoc* analysis showed a significantly higher d-dimer concentration in the high SAA group (median 0.3 mg/L, 1st and 3rd quartile (Q_1-Q_3): 0.18-0.38) ($p = 0.002$) and in the NSII group (median 0.21 mg/L, Q_1-Q_3: 0.17-0.55) ($p = 0.001$) compared to the clinically healthy group (median 0.075 mg/L, Q_1-Q_3: 0.07-0.09). For the NycoCard assay, the *post hoc* analysis showed a significantly higher d-dimer concentration in the high SAA group (median 0.4 mg/L, Q_1-Q_3: 0.31-0.55) ($p = 0.003$) and the NSII group (median 0.3 mg/L, Q_1-Q_3: 0.25-1.3) ($p = 0.002$) compared to the clinically healthy group (median < 0.1 mg/L, Q_1-Q_3: < 0.1-< 0.1) (Figure 2).

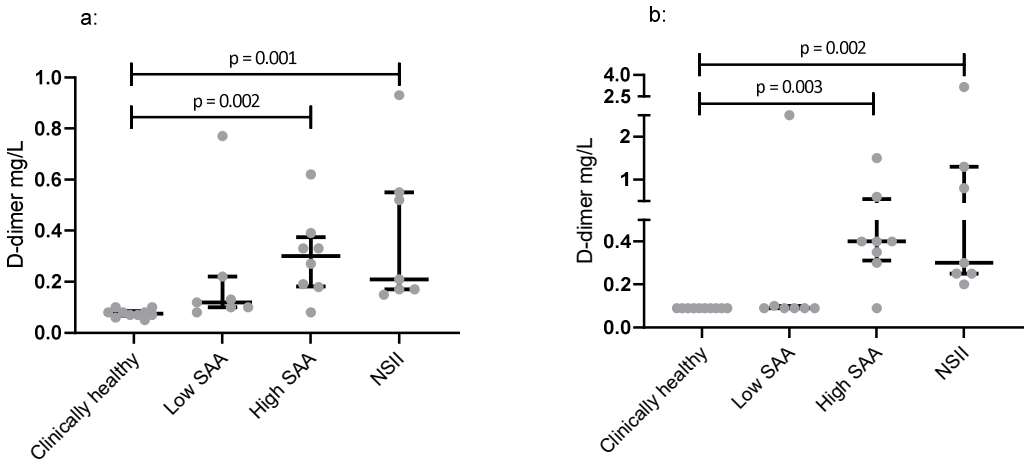


Figure 2. Group comparison across the four groups of horses using the STAGO STA-Liatest D-di+ (Stago) and NycoCard™ D-dimer (NycoCard) assays. a) For the Stago assay, a significant difference was seen between the clinically healthy group, the high serum amyloid A (SAA) group and the non-strangulating intestinal infarctions (NSII) group. b) For the NycoCard assay, a significant difference was seen between the clinically healthy group, the high SAA group and the NSII group. The highest value in the low SAA group for the Stago and the highest value in the low SAA group for the NycoCard are not the same horse. Horizontal lines represent the median and interquartile range. Note the different y-axes in the two graphs.

One horse from the low SAA group was excluded based on Cook's distance. The horse had a d-dimer concentration of 9.38 mg/L as measured by the Stago assay. For completeness, we also included this horse in the group comparison. The only change was that a significant difference was then also seen between the clinically healthy group and the low SAA group ($p = 0.04$).

Method comparison

A spaghetti plot showed that d-dimer measurements from the NycoCard assay were generally higher than Stago measurements, but agreement between the two assays seems to be good, especially for the lower d-dimer concentrations. Three horses had noticeably different measurements across the two assays. Two horses had a markedly higher d-dimer concentration with the NycoCard compared to the Stago assay, whereas one horse had a markedly higher d-dimer concentration with the Stago compared to the NycoCard assay (Figure 3).

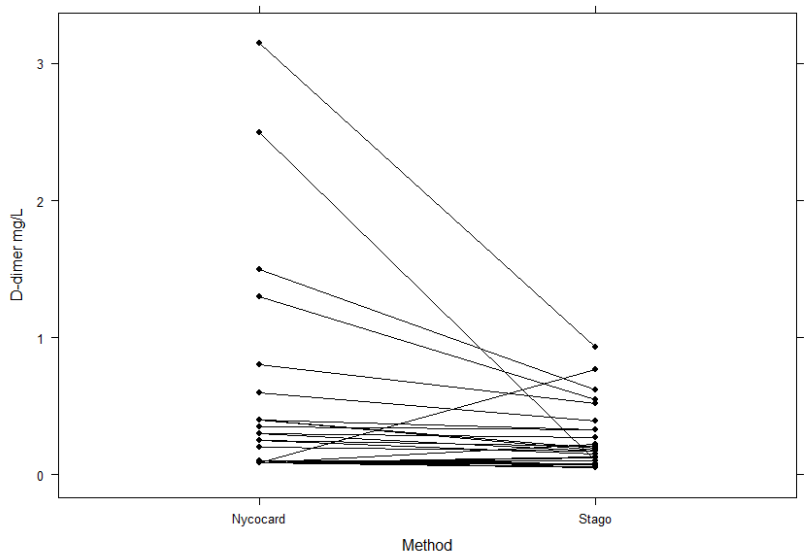


Figure 3. Spaghetti plot displaying the subject-wise trends for the d-dimer (mg/L) concentration for each horse measured with the STAGO STA-Liatest D-di+ (Stago) and NycoCard™ D-dimer (NycoCard) assays. NycoCard measurements were generally higher than the Stago measurements, but there was relatively good agreement between the two assays, especially for the lower concentrations. Three horses had markedly varying results: two were markedly higher with the NycoCard assay, and one was markedly higher with the Stago assay.

A Passing Bablok regression between the Stago and NycoCard measurements gave a slope of 1.61 (95% CI: 1.02-2.61) with an intercept of -0.04 (95% CI: -0.14-0.02). The Spearman correlation between the Stago and the NycoCard assays showed a significantly ($p < 0.0001$) positive correlation, with an r-value of 0.7 (95% CI: 0.46-0.85).

A Bland-Altman plot showed that all but two measurements were within the 95% limits of agreement of -2.9-2.11 mg/L (log2) with a mean of -0.4 mg/L (log2) and a median of -0.3 mg/L (log2) (Figure 4).

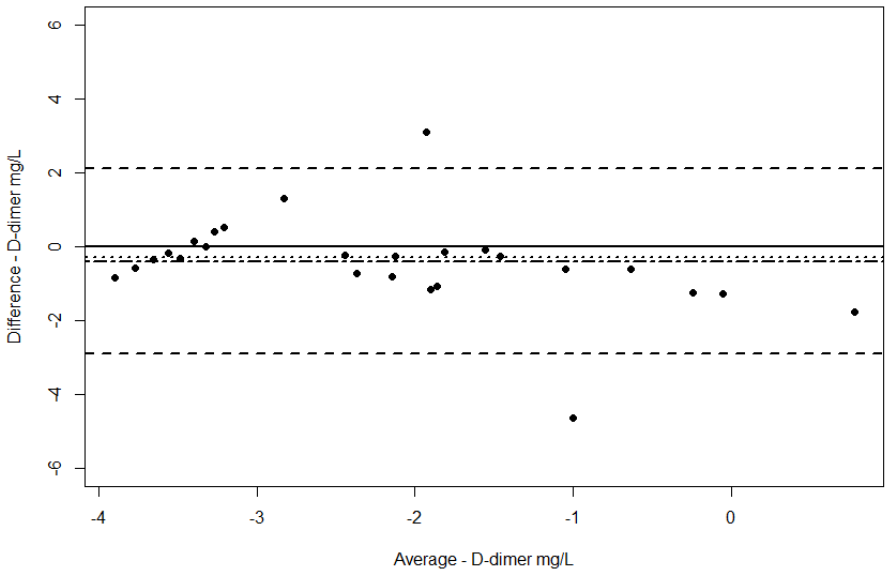


Figure 4. Bland-Altman plot displaying the difference plotted against the average of log2 transformed STAGO STA-Liatest D-di+ (Stago) and NycoCard™ D-dimer (NycoCard) assay measurements including 95% limits of agreement (LOA). Mean -0.4 (dot-dashed line), median -0.3 (dotted line), and 95% LOA -2.9-2.11 (dashed lines).

Discussion

Both assays were able to measure equine d-dimer in PPP and performed acceptably in terms of reliability and validity. They found a significantly higher d-dimer concentration in the high SAA and NSII groups compared to the clinically healthy horses and showed good agreement in terms of classifying horses as having a low or high d-dimer concentration.

The NycoCard assay did not meet the accepted intra- and inter-CV limits of 10% for manual assays (24). The deviation was most substantial in the low pool (Table 1). One explanation for this could be that the assay is relatively crude when displaying results showing only a single decimal. The d-dimer concentrations in the low pool varied from 0.1 to 0.2 mg/L, which is a 100% increase, thereby resulting in a large variability between measurements. In the medium pool, the d-dimer concentrations varied from 0.5 to 0.7 mg/L, which is a proportionally smaller increase, thus resulting in CVs that were much closer to the accepted limit. In addition, the low d-dimer concentrations of 0.1 and 0.2 mg/L are likely to be of minimal clinical relevance as they historically are considered within normal reference intervals when measured using alternative d-dimer assays (15). The Stago assay met the accepted limit of 5% for intra- and inter-CVs (24), with the exception of the low pool, which had an inter-CV of 5.98%. This is a small deviation from the intended precision level and perhaps repeating measurements more than ten times would have given an inter-CV below 5% (24). Furthermore, as with the NycoCard assay, results regarding the low pool are of less clinical relevance, so a slightly larger variation can be tolerated (15). Regardless of the inter- and intra-CV values, both assays were still considered to be of clinical relevance as often the most important thing in a clinical setting is to know that horses are classified correctly in terms of having a low or high d-dimer concentration, and not necessarily, what the exact concentration is.

The agreement between the expected and obtained values for the Stago assay varied between 89% and 70%, with the highest agreement in the 80% pool and the lowest agreement in the 20% pool. For the NycoCard assay, the agreement varied between 97% and 65% for the same pools. Agreement should ideally be 100% but is usually between 80% and 120% (24). For the Stago assay, the low agreement seen in the pool with the low d-dimer concentration could possibly be explained by the d-dimer concentration being below the preset detection limit for the assay, which increases the inaccuracy of the measurements. For the NycoCard assay, the similar low agreement in the 20% pool could be explained by the measuring increments of 0.1 mg/L, which has a substantially larger influence in low d-dimer concentrations. The Passing Bablok regression showed good coherence between the obtained and expected d-dimer concentrations for both assays. For the Stago assay, the 95% CI for the slope contained 1 and the CI for the Y-intercept was very close to containing 0. For the NycoCard assay, the 95% CI for the slope contained 1 and for the Y-intercept contained 0, which are considered optimal values for a 100% agreement between expected and observed values (24).

Due to the lack of an equine species-specific recombinant d-dimer antibody, the linearity under dilution was investigated using a heterologous d-dimer antibody. A previous study (25) found that the fibrinogen molecule is highly conserved between species and that there is extensive amino acid homology between specific human and equine fibrinogen sequences. However, whether the

breakdown products of cross-linked fibrin are identical in horses and humans remains unknown. The analyzed assays are heterologous, and we do not have any available equine d-dimer that can be added and assessed in terms of recovery. As the human assays resulted in d-dimer concentrations within the expected range for the four different groups of horses, we choose to interpret that the assays could in fact measure equine d-dimer.

The linearity under dilution was investigated on a d-dimer pool with a d-dimer concentration of 0.72 mg/L. This was the highest pool it was possible to create in this study. It could be argued that a pool with even higher values, as found in the literature (15), would provide a better view of how precisely the two assays can measure the higher d-dimer concentrations.

In the group comparison, both assays measured a significantly increased d-dimer concentration in the high SAA and NSII groups compared to the clinically healthy group. However, no significant difference was seen across the three different disease groups. This indicates that d-dimer increases in horses with GI disease and systemic inflammation, as well as in horses with GI disease and thrombosis. It was not possible to differentiate between horses with inflammation and NSII in this study. In comparison, increased d-dimer concentrations have been found in dogs with systemic inflammation, rendering d-dimer a good rule-out marker for thrombosis, though not necessarily a good rule-in marker (26,27).

One horse in the low SAA group had a markedly increased d-dimer concentration measured by the Stago assay (9.38 mg/L), while it had a d-dimer concentration of < 0.1 mg/L when measured by the NycoCard assay. The sample from this horse was not collected or handled any differently than the samples from the other horses. The measured fibrinogen concentration was within normal limits. The horse was intraoperatively diagnosed with peritonitis of unknown origin, which might explain why it would have an elevated d-dimer concentration compared to clinically healthy horses. However a d-dimer concentration of around 9 mg/L seems improbably high compared to previous studies (15,18), and none of the other horses in the study – even those that were more severely ill – had d-dimer concentrations that came close to this concentration. The d-dimer concentration in this horse was measured several times using both assays with similar results, and even dilution curves confirmed the same findings. The reason for this one high value on the Stago assay is not known. However, it seems most likely that in this case the assay was measuring something other than d-dimer. As the assay was developed for humans and built on murine antibodies, it is feasible that there was a form of cross-reaction with another type of matrix antibody present in this horse, potentially due to its clinical condition. We do not know the cause of this finding, which was only seen in one horse, but it highlights the need to combine laboratory results with the individual patient's clinical manifestations. This horse was excluded from this study based on Cook's distance. The group comparison was performed with and without the outlier. When excluding the outlier, the statistical difference between healthy horses and horses with low SAA disappeared. Both assays were therefore still able to distinguish clinically healthy horses from the high SAA and the NSII groups, but were not able to distinguish the different disease groups. It therefore had no effect on the applicability of the two assays.

It is relevant to know whether d-dimer measurements from the Stago and NycoCard assays can be compared and whether they classify horses in the same way. The Spaghetti plot shows a relatively good agreement between the two methods, particularly for the lower d-dimer concentrations. However, it seems that d-dimer measurements from the NycoCard assay are consistently higher than for the Stago assay, especially for the higher d-dimer concentrations. This implies that d-dimer measurements from the same horse measured with both assays cannot be compared directly. It does, however, seem that there is good agreement in terms of classifying horses as having a low or high d-dimer concentration, with the exception of three horses with noticeably different results across the two assays. A Passing Bablok regression between the two assays showed that the lower 95% CI for the slope came close to, but did not contain, 1. However, the 95% CI for the intercept did contain 0 (24). This combined with a moderately good (28) positive Spearman correlation indicates acceptable coherence between the two assays. A Bland-Altman plot is one of the most commonly used methods to quantify the agreement between two quantitative measurements (29). The Bland-Altman plot in this study showed a negative bias, which may be the result of average measurements above -2 mg/L (log2). This means that the difference between the two assays is greatest for the higher d-dimer concentrations and that the NycoCard assay measures higher d-dimer concentrations than the Stago assay, as also seen in the spaghetti plot. The best agreement between the two assays is seen for the low d-dimer concentrations. Only two of the measurements are outside the 95% LOA, which is acceptable. Again, this indicates a good agreement between the two assays when it comes to classifying horses with a low or high d-dimer concentration and supports the idea that measurements from the two assays cannot be compared directly.

It has previously been shown that the final concentration of anticoagulant in the blood tube can affect the results of hemostatic markers such as prothrombin time (PT) and activated partial thromboplastin time (aPTT) due to a dilution effect (30–32). The d-dimer concentration in the two samples from the NSII group that were collected in 3.8% as opposed to 3.2% sodium citrate tubes could thus potentially be diluted. However, it has been shown that d-dimer concentration is unaffected by the final anticoagulant concentration in the sampling tube (33). The d-dimer concentration for these two samples did not vary markedly from the remaining measurements in the NSII group and it seems likely that the sodium citrate concentration did not significantly influence the d-dimer measurements.

The two assays vary in their ease of use. The Stago machine is an automated assay that can run up to 20 samples at a time. It requires substantial knowledge regarding the setup and handling of the machine, and trained personnel must be present when samples are analyzed. This limits its usefulness in out-of-hours situations. However, once the samples are loaded and running, the machine does the remaining work, which means that a large quantity of samples can easily be analyzed in a short amount of time. Therefore, this assay might be useful in a hospital setting where a large number of samples are run each week or for research purposes. In contrast, the NycoCard assay is an easy-to-use manual tabletop assay. Each test disc is packed separately, and it is thus highly applicable for testing individual animals, even in emergency situations.

In this study, horses were grouped according to being clinically healthy, the degree of systemic inflammation based on the SAA concentration at admission to the hospital, or a confirmed diagnosis

of NSII caused by *S. vulgaris*. Some of the horses in the low SAA group were diagnosed with an inflammatory disease process after a few days at the hospital, and the low SAA group and high SAA group therefore both included similar types of disease processes but of different duration or severity.

A limitation of the study is the relatively small number of horses in each group for the group comparison. However, it was still possible to observe a significant difference between the clinically healthy horses and the high SAA and NSII groups. Identifying differences between the different disease groups would require around 200–300 horses, which would not be realistic or ethically justifiable when it might not be relevant to daily clinical work. In addition, the investigated assays should ideally be compared to an established gold standard to calculate a cut off value for thrombosis and assess the true diagnostic sensitivity and specificity for both assays. However, as no gold standard is available in equine medicine, and as it was not possible to perform a postmortem examination of all horses, this was not possible.

Conclusion

The Stago assay was found to be reliable for measuring d-dimer in equine PPP based on intra- and inter-CVs and linearity under dilution. It was able to distinguish horses in the high SAA group and the NSII group from clinically healthy horses. The assay is considered only moderately user friendly and may not be an appropriate stall-side test, but it can be used for research purposes and in larger clinics or hospital facilities.

The NycoCard assay was close to the accepted limit with regard to the medium pool for the intra- and inter-CVs. The assay was further from the accepted limit for the low pool. However, as these values are considered to be below the clinically relevant concentration, the assay is still clinically valuable. The assay was deemed to be reliable based on the linearity under dilution, and it was able to distinguish horses in the high SAA group and NSII group from clinically healthy horses. The assay is quick and easy to use and thus might prove to be a useful stall-side test for detecting critically ill horses in the early stages of disease.

Although the d-dimer measurements from the two assays cannot be compared directly, there does seem to be a good agreement between the two assays in terms of classifying horses as having a low or high d-dimer concentration. The agreement seems to be best for the lower d-dimer concentrations. The two assays can thus both be used in a clinical setting to evaluate whether a horse has a low or high d-dimer concentration, but in cases where serial measurements are required, the same assay should be used throughout.

Methods

This study was approved by the ethical review board of the Department of Clinical Veterinary Science at the University of Copenhagen, and relevant guidelines and regulations were followed. All samples were obtained from December 2017 through to December 2018 and analyzed at the laboratory at the Large Animal Teaching Hospital and at the Veterinary Diagnostic Laboratory of the University of Copenhagen, Denmark. Blood samples were collected from clinically healthy horses owned by the University for teaching purposes and following the experimental animal licensing given for the horses. Blood samples from diseased horses were collected from client-owned horses and were collected as part of the diagnostic work-up and with the owner's consent. All procedures were carried out in accordance with the ARRIVE guidelines.

Horses

Four groups of horses were included in the study: *1)* Clinically healthy horses from The Large Animal Teaching Hospital at The University of Copenhagen (clinically healthy group). This group included adult horses (> one year of age) deemed healthy based on clinical examination (heart rate, respiration rate, rectal temperature, mucous membrane color, and borborygmia), complete blood count (CBC), and serum biochemistry profiles (including lactate and acute phase proteins serum amyloid A (SAA) and fibrinogen concentrations) within normal reference intervals. *2)* Horses with GI disease and a mild systemic inflammatory response with a SAA between 30-100 mg/L at admission (low SAA group). *3) H*orses with GI disease and a strong systemic inflammatory response with a SAA > 1000 mg/L at admission (high SAA group). Groups two and three included adult horses (> one year of age). *4)* Horses with NSII caused by *S. vulgaris* (NSII group). This group included adult horses (> one year of age) with NSII confirmed at surgery or necropsy, with an area of localized intestinal infarction and without signs of strangulation or enterocolitis. In addition, signs of migrating *S. vulgaris* larvae, seen as thrombosis and arteritis were observed in the cranial mesenteric artery and/or its branches at post-mortem examination or on histology of the resected intestine from surviving horses as has been described previously (34). The last three groups were all patients referred to The Large Animal Teaching Hospital at The University of Copenhagen. Citrated platelet poor plasma (PPP) from the low and high SAA group were used in the intra- and inter- assay coefficients of variation (CV) studies and the linearity under dilution. Citrated PPP from the low and high SAA group and the NSII group were used in the group comparison and for the method agreement.

Samples

As previously described (35) blood samples were collected by clean venipuncture of the jugular vein using a vacutainer system with a 21 G needle on admission to the hospital, at the same time as routine blood samples (including samples for SAA measurement). Four 3.2% sodium citrate tubes (BD, Franklin Lakes, NJ) were filled according to the manufacturer's recommendation. Samples from two of the horses in the NSII group were drawn in 3.8% rather than 3.2% sodium citrate tubes, but were otherwise handled in the same way. The first tube was discarded. The remaining samples were inverted 4-6 times and then centrifuged within a maximum of four hours at 2,000 *g* for 15 minutes at room temperature (36). Citrated PPP was then immediately aliquoted into cryotubes and stored for a

maximum of 15 months at -80°C until further analysis. Before analysis, all samples were thawed for approximately 4 min. at 37°C in a water bath and divided into additional aliquots of low, medium, and high pools and then frozen again at -80°C. When ready for analysis, these aliquots were thawed in the same manner as mentioned above and then vortexed. All samples therefore underwent two identical freeze-thaw cycles, which should not significantly influence the d-dimer concentration (37).

Pools with low, medium, and high d-dimer concentrations were created based on prior analysis carried out on the Stago assay on the horses from the above-mentioned groups. Horses were categorized in such a way as to achieve the desired d-dimer concentrations: A) the low d-dimer pool (n=4 horses) with an average final d-dimer concentration of 0.23 mg/L, B) the medium d-dimer pool (n=3 horses) with an average final d-dimer concentration of 0.52 mg/L, and C) the high d-dimer pool (n=3 horses) with an average final d-dimer concentration of 0.72 mg/L.

Stago assay

The STAGO STA-Liatest D-di+ (Stago) assay (Trioloab, Brøndby, Denmark) is a fully automated humane immunoturbidimetric assay. It performs a photometric measurement of the change in turbidity in a suspension of sample material and reagent containing micro particles. These micro latex particles are covered with two murine monoclonal antibodies with a high specificity for human d-dimer. The binding of antibodies with d-dimer leads to an agglutination of the micro latex particles and thus a change in turbidity depending on the d-dimer concentration in the sample. The assay provides a quantitative determination of d-dimer in mg/L fibrinogen equivalent units (FEU), the true mg/L d-dimer concentration is considered to be approximately half the FEU value. The assay has a preset measuring range of 0.27-20 mg/L FEU (0.13-10 mg/L d-dimer) based on human d-dimer samples, and the machine is set to automatically dilute samples above 4.0 mg/L FEU. The measuring interval is 0.01 mg/L (38).

NycoCard assay

The NycoCard™ D-dimer (NycoCard) assay (Abbott Laboratories, Copenhagen, Denmark) (39), is based on an immunometric flow through principle, with plasma d-dimer molecules being trapped on a surface membrane with d-dimer specific monoclonal antibodies. The addition of a secondary labelled antibody generates a subsequent color development, with the color intensity being proportional to the d-dimer concentration. The assay has a measuring range of 0.1-20 mg/L, a calibrated human assay range of 0.1-10.0 mg/L and a measuring interval of 0.1mg/L (NycoCardTM D-dimer, technical support, Abbott Laboratories, Copenhagen, Denmark) (40). Measurements displayed by the machine as < 0.1 mg/L in this study were converted to 0.09 mg/L for statistical purposes.

Test evaluation

Imprecision was evaluated in both tests by routine descriptive analytical procedures. Intra- and inter-CVs were examined by running the low and medium d-dimer pools in duplicate ten times in one day

and then once daily for 10 consecutive days with an imprecision performance acceptance level of 5% for automated assays (Stago) and 10% for manual assays (NycoCard) (24).

Linearity under dilution was used to determine inaccuracy for both assays by analyzing the high d-dimer pool both undiluted and diluted with 0.9% saline to obtain the following concentrations of d-dimer: 100%, 80%, 60%, 40%, 20% and 0%. A Passing Bablok regression was used for both tests. Linearity performance was considered acceptable if the confidence interval of the slope and Y-intercept included 1 and 0, respectively.

Group comparisons of the assays were evaluated by comparing the median and interquartile range across the four groups of horses (clinically healthy, low SAA, high SAA and NSII) for 32 horses. Data did not follow a normal distribution and applying various log transformations did not improve this, so a Kruskal-Wallis test was used. A Dunn's multiple comparisons test was used for *post hoc* analysis.

Method comparison

A method comparison was performed by examining d-dimer concentrations using the Stago and NycoCard assays in parallel across the four groups of horses. The agreement between the two assays was illustrated in a spaghetti plot, with a Passing Bablok regression, a Spearman correlation test, and a Bland-Altman plot on log2 transformed data, which brought the difference between the two assays closer to a normal distribution than with the original data. The Spearman correlation was considered poor if the r-squared value was $\leq \pm 0.2$, fair with a value $> \pm 0.2$ to $\leq \pm 0.5$, moderate with a value $> \pm 0.5$ to $\leq \pm 0.7$, and strong with a value $\geq \pm 0.8$ (28).

Statistical analyses were performed using Microsoft Excel 2016 (Washington, USA), R Studio (Boston, MA, USA) and GraphPad Prism 8.1.0 (San Diego, USA). Data were assessed for normality using graphic evaluation. A significance level of $< 0.05\%$ was set for all analyses.

The complete dataset was examined for extreme measurements using graphic representation and Cook's distance (= 4/n). If a variable was found to be an extreme outlier with a strong influence on the statistical analysis, it was excluded. Any exclusions were accounted for in the results paragraph.

Abbreviations

aPTT	Activated partial thromboplastin time
BW	Body weight
CBC	Complete blood count
CI	Confidence interval
CV	Coefficients of variation
GI	Gastrointestinal
LOA	Limits of agreement
NSII	Non-strangulating intestinal infarction
NycoCard	NycoCard™ D-dimer assay
PPP	Platelet poor plasma
PT	Prothrombin time
Q_1	1st quartile
Q_3	3rd quartile
SAA	Serum amyloid A
Stago	STAGO STA-Liatest D-di+ assay
S. vulgaris	*Strongylus vulgaris*

Declarations

Ethics approval and consent to participate

All samples were collected at The Large Animal Teaching Hospital at The University of Copenhagen, Denmark, in 2017 and 2018. Approval was obtained from the ethical board of the Department of Veterinary Clinical Sciences, University of Copenhagen, and relevant guidelines and regulations was followed. Written consent from the owners of the horses was obtained. All procedures were carried out in accordance with the ARRIVE guidelines.

Consent for publication

Not applicable

Availability of data and materials

If deemed relevant or is of interest raw data can be submitted.

Competing interests

The authors declare that they have no competing interests

Funding

The Danish Horse Levy Foundation funded the project but played no role in the design, analysis and reporting of the study.

Authors' contributions

All authors have provided substantial contribution to the manuscript. MLH has acquired the sample material, and owner consent, conducted and assisted in data analysis, interpreted results, and have drafted the work and continuously revised it. TMBA and LLF has conducted and assisted in data analysis, interpreted results and has assisted in drafting the work and revised it. LNN has contributed to the design of the work and data interpretation and has revised the work. THP has contributed to the acquisition and interpretation of data and has revised the work. All authors have read and approved the final manuscript.

Acknowledgements

The authors are grateful to medical laboratory technologist Louise Bochsen, to laboratory technician Tina Roust and to technician trainee Peter Trappaud Rønne from the Department of Veterinary Clinical Sciences, Faculty of Health and Medical Sciences (SUND), University of Copenhagen, Denmark for their laboratory assistance during this study and to the horse owners who kindly enrolled their horses in the study. The authors would also like to thank the Danish Horse Levy Foundation for funding the project.

References

1. Tovey C, Wyatt S. Diagnosis, investigation, and management of deep vein thrombosis. Br Med J. 2003;326(May):1180–4.
2. Philbrick JT, Heim S. The d-dimer test for deep venous thrombosis: Gold standards and bias in negative predictive value. Clin Chem. 2003;49(4):570–4.
3. Kainz B, Heinrich MP, Makropoulos A, Oppenheimer J, Mandegaran R, Sankar S, et al. Non-invasive diagnosis of deep vein thrombosis from ultrasound imaging with machine learning. npj Digit Med. 2021;4(1):1–18.
4. Carpenter JP, Holland GA, Baum RA, Owen RS, Carpenter JT, Cope C. Magnetic resonance venography for the detection of deep venous thrombosis: Comparison with contrast venography and duplex Doppler ultrasonography. J Vasc Surg. 1993;18(5):734–41.
5. Adam SS, Key NS, Greenberg CS. D-dimer antigen: current concepts and future prospects. Am Soc Hematol. 2009;113(13):2878–87.
6. Gaffney PJ, Joe F. The lysis of crosslinked human fibrin by plasmin yields initially a single molecular complex, D dimer-E. Thromb Res. 1979;15(5):673–87.
7. Weitz JI, Fredenburgh JC, Eikelboom JW. A Test in Context: D-Dimer. J Am Coll Cardiol. 2017;70(19):2411–20.
8. Epstein SE, Hopper K, Mellema MS, Johnson LR. Diagnostic Utility of D-dimer Concentrations in Dogs with Pulmonary Embolism. J Vet Intern Med. 2013;27:1646–9.
9. Johnson ED, Schell JC, Rodgers GM. The D-dimer assay. Am J Hematol. 2019;94(7):833–9.
10. Stokol T, Erb HN, De Wilde L, Tornquist SJ, Brooks M. Evaluation of latex agglutination kits for detection of fibrin(ogen) degradation products and D-dimer in healthy horses and horses with severe colic. Vet Clin Pathol. 2005;34(4):375–82.
11. Dallap BL, Dolente B, Boston R. Coagulation profiles in 27 horses with large colon volvulus. J Vet Emerg Crit Care. 2003;13(4):215–25.
12. Feige K, Schwarzwald CC, Bombeli T. Comparison of unfractioned and low molecular weight heparin for prophylaxis of coagulopathies in 52 horses with colic: a randomised double-blind clinical trial. EQUINE Vet J Equine vet J. 2003;35(5):506–13.
13. Pihl TH, Nielsen MK, Jacobsen S. Changes in Hemostatic Indices in Foals Naturally Infected With *Strongylus vulgaris*. J Equine Vet Sci. 2017;54:1–7.
14. Dunkel B, Chan DL, Boston R, Monreal L. Association between Hypercoagulability and Decreased Survival in Horses with Ischemic or Inflammatory Gastrointestinal Disease. J Vet Intern Med. 2010;24:1467–74.
15. Cesarini C, Monreal L, Armengou L, Delgado MÁ, Ríos J, Jose-Cunilleras E. Association of Admission Plasma D-dimer Concentration with Diagnosis and Outcome in Horses with Colic. J Vet Intern Med. 2010;24:1490–7.
16. Cesarini C, Monreal L, Armengou L, Delgado MÁ, Ríos J, Jose-Cunilleras E. Progression of plasma D-dimer concentration and coagulopathies during hospitalization in horses with colic. J Vet Emerg Crit Care. 2014;24(6):672–80.
17. Armengou L, Monreal L, Tarancón I, Navarro M, Ríos J, Segura D. Plasma D-Dimer Concentration in Sick Newborn Foals. J Vet Intern Med. 2008;22:411–7.
18. Nikvand AA, Jalali SM, Ghadrdan Mashhadi A, Razi Jalali M, Hassanpour Amirabadi S. Clinical, hematologic, hemostatic, and serum biochemical findings related to survival in Arabian horses with colic. Vet Clin Pathol. 2019;48(3):441–8.
19. Enigk K. On the Development of *Strongylus vulgaris* (Nematoda) in the Host Animal. Zeitschrift für Tropenmedizin und Parasitol und Parasitol. 1950;2(2):223–46.
20. Enigk K. Further Investigations on the Biology of *Strongylus Vulgaris* (Nematoda) in the Host

Animal. Zeitschrift fiir Tropenmedizin und Parasitol. 1951;2(4):247–63.
21. White NA. Intestinal infarction associated with mesenteric vascular thrombotic disease in the horse. J Am Vet Med Assoc. 1981;178(3):259–62.
22. Duncan RB, Duncan JL, Pirie HM. The pathogenesis of single experimental infections with *Strongylus vulgaris* in foals. Res Vet Sci. 1975;18(1):82–93.
23. Pihl TH, Nielsen MK, Jacobsen S. Changes in Hemostatic Indices in Foals Naturally Infected With *Strongylus vulgaris*. J Equine Vet Sci. 2017;54:1–7.
24. Jensen AL, Kjelgaard-hansen M. Diagnostic Test Validation. Schalm's Vet Hematol. 2010;6:1027–33.
25. Sobel JH, Thibodeau CA, Kolks MAG, Canfield RE. Isolation and Partial Structural Characterization of an Equine Fibrinogen CNBr: Fragment That Exhibits Immunologic Cross-Reactivity with an Aα-Chain Cross-Linking Region of Human Fibrinogen. Biochemistry. 1990;29(38):8907–16.
26. Dewhurst E, Cue S, Crawford E, Papasouliotis K. A retrospective study of canine D-dimer concentrations measured using an immunometric "Point-of-Care" test. J Small Anim Pract. 2008 Jul 1;49(7):344–8.
27. Marschner CB, Kristensen AT, Rozanski EA, McEvoy FJ, Kühnel L, Taeymans O, et al. Diagnosis of canine pulmonary thromboembolism by computed tomography and mathematical modelling using haemostatic and inflammatory variables. Vet J. 2017;229:6–12.
28. Akoglu H. User's guide to correlation coefficients. Turkish J Emerg Med. 2018;18(3):91–3.
29. Giavarina D. Understanding Bland Altman Analysis. Biochem medica. 2018;25(2):141–51.
30. Adcock DM, Kressin DC, Marlar RA. Effect of 3.2 % vs 3.8 % Sodium Citrate Concentration on Routine Coagulation Testing. Coagul Transfus Med. 1996;107(1):105–10.
31. Adcock DM, Duff S. Enhanced standardization of the International Normalized Ratio through the use of plasma calibrants: a concise review. Blood Coagul Fibrinolysis. 2000;11(7).
32. van den Besselaar AM, Chantarangkul V, Tripodi A. A comparison of two sodium citrate concentrations in two evacuated blood collection systems for prothrombin time and ISI determination. Thromb Haemost. 2000 Oct;84(4):664–7.
33. Lippi G, Salvagno GL, Montagnana M, Guidi GC. Influence of two different buffered sodium citrate concentrations on coagulation testing. Blood Coagul Fibrinolysis. 2005;16:381–3.
34. Pihl TH, Nielsen MK, Olsen SN, Leifsson PS, Jacobsen S. Nonstrangulating intestinal infarctions associated with *Strongylus vulgaris*: Clinical presentation and treatment outcomes of 30 horses (2008-2016). Equine Vet J. 2017;1–7.
35. Honoré ML, Pihl TH, Nielsen LN. A pilot study evaluating the Calibrated Automated Thrombogram assay and application of plasma-thromboelastography for detection of hemostatic aberrations in horses with gastrointestinal disease. BMC Vet Res. 2021;17(1):1–11.
36. Adcock DM, Hoefner DM, Kottke-Marchant K, Marlar RA, Szamosi DI, Warunek DJ. Collection , Transport, and Processing of Blood Specimens for Testing Plasma-Based Coagulation Assays and Molecular Hemostasis Assays; CLSI Approved Guideline-Fifth Edition. H21-A5. Clin Lab Stand Inst. 2008;28(5):H21-A5.
37. Gosselin RC, Honeychurch K, Kang HJ, Dwyre DM. Effects of storage and thawing conditions on coagulation testing. Int J Lab Hematol. 2015;37(4):551–9.
38. Diagnostica-Stago. STA-Liatest D-DI plus Immunturbidimetrisk bestemmelse af D-dimer (REF 00662). Prod Inf. 2018;
39. Abbott Diagnostics Technologies AS. NycoCard Reader II User Manual (Multilingual). 2019. p. 1–92.
40. AXIS-SHIELD PoC AS. NycoCard D-dimer Single Test Product Description. 2007. p. 1–8.

Paper II

A pilot study evaluating the Calibrated Automated Thrombogram assay and application of plasma-thromboelastography for detection of hemostatic aberrations in horses with gastrointestinal disease

Honoré, M.L., Pihl, T.H., Nielsen, L.N.

BMC Veterinary Research, 2021, 17(1), p. 1-11.

BMC Veterinary Research

RESEARCH

Open Access

A pilot study evaluating the Calibrated Automated Thrombogram assay and application of plasma-thromboelastography for detection of hemostatic aberrations in horses with gastrointestinal disease

Marie Louise Honoré[1*], Tina Holberg Pihl[1] and Lise Nikolic Nielsen[2]

Abstract

Background: Critically ill horses, such as horses with gastrointestinal (GI) disease, often suffer from hemostatic aberrations. Global hemostatic tests examining the initiation of coagulation, clot strength and fibrinolysis, such as the Calibrated Automated Thrombogram (CAT) and plasma-thromboelastography (TEG) have not been evaluated in horses. This study aimed to evaluate CAT and apply plasma-TEG in horses.

Test performance of CAT was evaluated on equine platelet poor plasma with intra- and inter-assay variability (CV) and a heparin dilution curve. To examine clinical performance of both tests, group comparisons were assessed comparing healthy horses, horses with mild and severe GI disease with both CAT and plasma-TEG.

Results: For CAT, intra- and inter-assay CVs were established for lag-time (1.7, 4.7%), endogenous thrombin potential (1.6, 4.6%), peak (2.6, 3.9%) and time to peak (ttPeak) (1.9, 3.4%). Increasing heparin concentrations led to the expected decrease in thrombin generation. In the group comparison analysis, CAT showed significant higher peak ($p = 0.04$) and ttPeak ($p = 0.008$) in the severe GI disease group compared to horses with mild GI disease and healthy horses, respectively. Plasma-TEG showed an increased angle ($p = 0.032$), maximum amplitude ($p = 0.017$) and shear elastic force (G) ($p = 0.017$) in the severe GI disease group compared to healthy horses.

Conclusions: CAT performed well in horses. Both CAT and plasma-TEG identified hemostatic aberrations in horses with severe GI disease compared to healthy horses. Further studies including more horses, are needed to fully appreciate the use of CAT and plasma-TEG in this species.

Keywords: Equine, Global hemostatic tests, Hypercoagulation, Tissue factor, Platelet poor plasma

Background

Critically ill horses, such as horses with ischemic or inflammatory gastrointestinal (GI) disease, often suffer from marked hemostatic aberrations [1–5]. The most frequent hemostatic aberration is a hypercoagulable state with a decrease in platelet (PLT) count, an increase in thrombin–antithrombin (TAT) complexes, a prolonged prothrombin (PT) and activated partial

*Correspondence: mlhj@sund.ku.dk
[1] Department of Veterinary Clinical Sciences, Faculty of Health and Medical Sciences (SUND), University of Copenhagen, Hoejbakkegaard Allé 5a, 2630 Taastrup, Denmark
Full list of author information is available at the end of the article

thromboplastin (aPTT) time, decreased anti-thrombin (AT) and increased D-dimer [1, 2, 4–6]. When this consumption coagulopathy overwhelms the inhibitory system, the coagulopathy is then said to be uncompensated, and an imbalance arises, that can lead to disseminated intravascular coagulation (DIC) and thrombus formation. This hypercoagulable state can however also progress into a hypocoagulable state in the later stages of the disease process due to the depletion of procoagulant factors [3]. This proves that coagulopathies are dynamic and complex pathophysiologic processes. This might explain why one study looking at TEG and conventional hemostatic markers found horses with severe GI disease (ischemic and inflammatory lesions) to be hypocoagulable [3], whereas another study looking at TEG and conventional hemostatic markers suggested horses with severe GI disease (ischemic and inflammatory lesions) to be hypercoagulable [2]. There has however only been moderate progression in the use of more advanced diagnostic global hemostatic tests in horses [2, 3, 7–14], with more conventional hemostatic markers such as PLT count, aPTT, PT, AT, fibrinogen, and D-dimer concentration still being the most commonly reported parameters [15, 16].

These hemostatic parameters, however, have some limitations since they are snapshots of different aspects of the hemostatic process. Global hemostatic tests such as thrombin generation time or thromboelastography (TEG) on the other hand are dynamic and cover the entire hemostatic process from initiation to clot development and fibrinolysis [17, 18]. Whole blood-TEG has been evaluated in equine medicine in horses suffering from non-strangulating and non-inflammatory GI disorders such as large colon impactions, inflammatory GI disorders such as enterocolitis, and ischemic GI lesions such as intestinal volvulus [2, 3, 7, 19]. One drawback for this method is that TEG should be analyzed with a fixed storage time of ideally 30 min post-sampling in horses to avoid unnecessary time-related aberrations in the TEG parameters [8]. This shortness in time may defer or reduce the applicability of whole blood-TEG in clinical practice as horses are required to be in a hospital setting for the analysis to be performed. If plasma-based global hemostatic tests could replace the whole blood-TEG, this might improve the applicability of the tests in a clinical setting in equine medicine. TEG analyzed with citrated plasma (plasma-TEG) has been investigated in a couple of species. Plasma-TEG was successfully applied in dogs looking at biological variation [20], while in septic pigs plasma-TEG was shown to display a similar increased hemostatic response as identified with whole blood-TEG [21]. In human medicine, plasma-TEG has been applied in studies examining the TEG device, but also in experimental human endotoxemia [22, 23]. Although plasma-TEG is devoid of the cellular components of hemostasis, the coagulation factors and fibrinogen concentration are still present, and changes in test parameters may still be of interest in patients at risk of hemostatic imbalance.

The plasma based thrombin generation time using the Calibrated Automated Thrombogram (CAT) assay, has previously been investigated in both humans [10, 24, 25] and dogs [13, 26]. CAT permits the direct measurement of thrombin generation in a more physiological setting than conventional clotting assays. In humans, it is considered valuable for the study of hypo- and hypercoagulation [10, 27, 28] and has been recommended for assessing patients with venous thromboembolisms [24], evaluating the prognosis in patients with myocardial infarcts [29], and monitoring anticoagulant therapy [10, 13]. An extensive search through the current literature did not identify the use of the CAT assay in horses.

The aim of the present study was, therefore, to evaluate the performance of the CAT assay in equine citrated platelet poor plasma (PPP) and to apply plasma-TEG as an alternative to whole blood-TEG.

Our hypotheses were that the CAT assay would show a high degree of validity and reliability, that both assays would be applicable in equine PPP, and that hemostatic aberrations would be detectable in horses with GI disease compared to clinically healthy horses.

Results

Demographic data regarding the included horses in the three groups are displayed in Table 1.

It was possible to use the CAT assay in the horses. Intra-assay coefficient of variation ranged from 1.6–2.6% and inter-assay coefficient of variability from 3.4–4.7% for lag-time, ETP, peak, and ttPeak, respectively (Table 2).

Increasing concentrations of unfractionated heparin resulted in decreasing thrombin generation except for the two concentrations 0.01125 U/ml and 0.0225 U/ml where there was a slight increase in peak and ETP compared to the trace with a heparin concentration of 0.0 U/mL (Fig. 1, supporting information Table 1 s). Heparin concentrations higher than 0.18 U/ml completely inhibited thrombin generation.

Comparing the CAT assay results for the three groups of horses, significant differences were found in peak ($p = 0.04$) and ttPeak ($p = 0.01$) (Table 3).

Post hoc analyses identified a significantly higher peak in horses with severe GI disease compared to horses with mild GI disease ($p = 0.04$). A significantly lower ttPeak was seen in horses with severe GI disease compared to the clinically healthy horses ($p = 0.008$) (Fig. 2).

Table 1 An overview of the demographic data regarding the horses included in the study in the three different groups (clinically healthy, horses with mild gastrointestinal (GI) disease, and severe GI disease). The included parameters are; number of horses, age in years, bodyweight (BW) in kilograms (kg), sex, breed, and diagnosis. [a] Values are displayed as mean (minimum-maximum)

	Clinically healthy	Mild GI disease	Severe GI disease
Number of horses	10	9	15
Age [a] (years)	11.2 (4–22.8)	8.4 (5.9–12.9)	12.7 (4.8–24)
BW [a] (kg)	559.5 (411–687)	492.9 (311–606)	492.5 (139–700)
Sex	8 mares 2 geldings	5 mares 4 geldings	7 mares 8 geldings
Breed	Standardbreds ($n=7$) Warm bloods ($n=2$) Unknown ($n=1$)	Warm bloods ($n=5$) Icelandic horses ($n=2$) Pony ($n=1$) Unknown ($n=1$)	Warm bloods ($n=7$) Frisian ($n=1$) Icelandic horses ($n=2$) Pony ($n=2$) Cold bloods ($n=2$)
Diagnosis	NA	Large intestinal impactions ($n=6$) Large intestinal non-strangulating displacements ($n=3$)	Peritonitis associated to the GI-tract ($n=2$) Acute colitis ($n=4$) Strangulated small intestine ($n=5$) Colon torsion ($n=1$) Severe gastric ulcers ($n=1$) Ruptured intestine ($n=2$)

Table 2 Imprecision study of the calibrated automated thrombogram (CAT). Intra- and inter- assay coefficients of variation (CV) in percentages established for lag-time, endogenous thrombin potential (ETP), peak and time to peak (ttPeak) are displayed

	Lag-time	ETP	Peak	ttPeak
Intra- assay CV %	1.7	1.6	2.6	1.9
Inter-assay CV %	4.7	4.6	3.9	3.4

The plasma-TEG ran successfully in all horses with the exception of not reaching K-values in 12/34 horses. Significant differences were seen for α ($p=0.016$), MA ($p=0.023$) and G ($p=0.022$) between the three groups of horses, with post hoc analysis identifying the differences between horses with severe GI disease and clinically healthy horses (α: $p=0.032$; MA: $p=0.017$; G: $p=0.017$) (Fig. 3 and Table 3).

No significant differences in whole blood-TEG parameters were detected between the three groups of horses (Table 4). When comparing whole blood-TEG and plasma-TEG a significantly lower α ($p=0.003$), MA ($p<0.0001$) and G ($p<0.0001$) were found for plasma-TEG.

There were no differences identified between the 3 groups of horses for fibrinogen concentration, aPTT, PT, or AT. Whereas for D-dimer, a significant difference was found between groups ($p<0.0001$), with post hoc analysis identifying a difference both between horses with severe

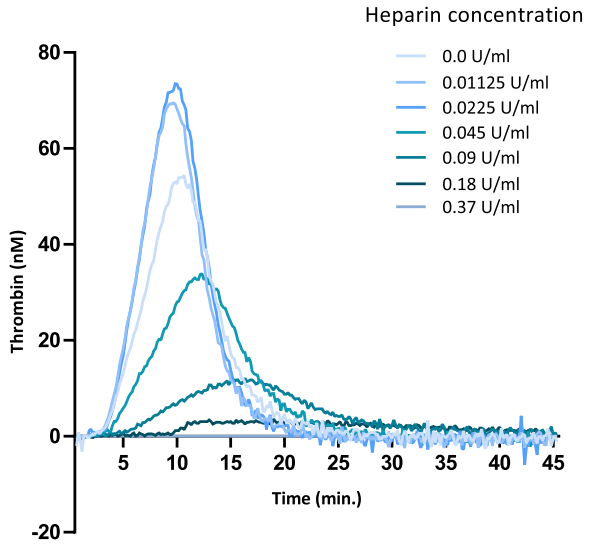


Fig. 1 Thrombin generation curves obtained by the calibrated automated thrombogram (CAT) assay for serial dilutions of unfractionated heparin in pooled equine citrated plasma. Thrombin generation decreased with increasing concentrations of unfractionated heparin. Heparin concentrations above 0.18 U/mL completely inhibited thrombin generation and produced a flat-line (0 nM thrombin) and 0.37 U/mL, 0.75 U/mL, 1.5 U/mL and 3 U/mL are therefore not visible on the graph. A heparin concentration of 0.01125 U/ml and 0.0225 U/ml produced a slightly higher peak and endogenous thrombin potential than heparin concentration of 0.0 U/mL

Table 3 Analysis of the calibrated automated thrombogram (CAT) and plasma-thromboelastography (plasma-TEG) between clinically healthy, mild gastrointestinal (GI) and severe GI disease horse groups. Median and range for plasma-TEG and thrombin generation parameters lag-time, time to peak (ttPeak), peak and endogenous thrombin potential (ETP). *P*-value column: one-way ANOVA or a Kruskal-Wallis test depending on normality of data across all three groups with a significance level of < 0.05. [a] Post hoc statistical significant difference between healthy and severe GI disease, [b] Post hoc statistical significant difference between mild and severe GI disease. SP: split point, R: reaction time, K: clot formation time, α: alpha angle, MA: maximum amplitude, G: shear elastic force, LY30: lysis 30 min, LY60: lysis 60 min (min.)

		Clinically healthy	Mild GI disease	Severe GI disease	*P*-value
Plasma-TEG	SP (min.)	11.55 (8.6–16)	11.5 (7.8–16.8)	9.2 (4.2–20.1)	0.53
	R (min.)	13.6 (10.9–20.4)	14.6 (9.3–21.3)	11.3 (4.8–24.5)	0.38
	α (degrees)	18.1 (8.4–25.4)	17.1 (9.5–32.6)	27 (10.2–68.5)	0.016 0.032[a]
	K (min.)	11.85 (9.5–17.9)	10.85 (7.9–19.2)	8.45 (1.4–16.9)	0.14
	MA (mm)	18.45 (11.7–25.2)	22.3 (17–26)	22.1 (13.4–34.4)	0.023 0.017[a]
	G (dynes/cm^2)	1.1 (0.7–1.7)	1.4 (1–1.8)	1.4 (0.8–2.6)	0.022 0.017[a]
	Ly30 (%)	0 (0–0)	0 (0–0)	0 (0–2.3)	0.53
	Ly 60 (%)	0 (0–0)	0 (0–0)	0 (0–1.1)	0.53
CAT	Lag-time (min.)	4.05 (3.4–5.33)	3.72 (2.17–5.67)	3.31 (2.7–7.22)	0.20
	Peak (nM)	32.69 (19.49–47.57)	29.2 (19.63–45.49)	51.46 (15.35–112.4)	0.040 0.04[b]
	ttPeak (min.)	11.87 (10.08–13)	10.28 (8.75–13.89)	9.33 (6.5–13.01)	0.01 0.008[a]
	ETP (nM/min.)	333.1 (270.3–422.1)	292 (179.7–350.8)	346.8 (166.8–468.1)	0.11

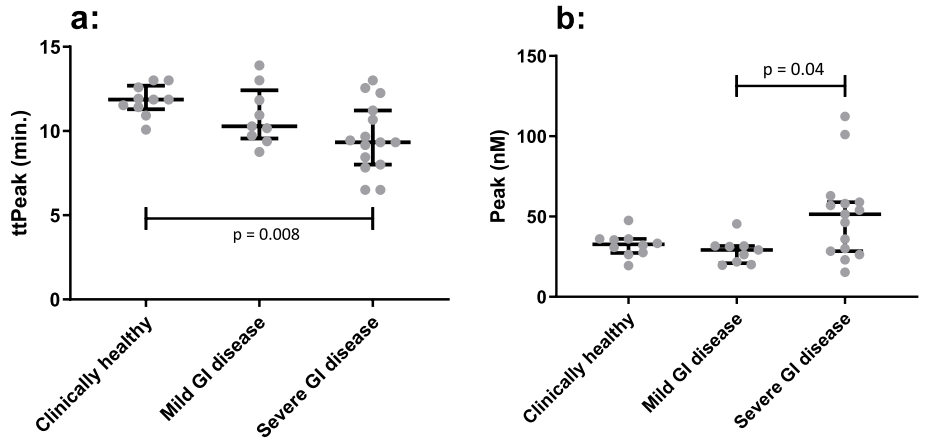


Fig. 2 Group comparison between the three groups of horses (clinically healthy, mild and severe gastrointestinal (GI) disease) for the calibrated automated thrombogram (CAT) assay showing a significantly lower time to peak (ttPeak) ($p = 0.008$) in the severe GI group compared to the clinically healthy horses (**a**). Likewise a significantly higher Peak ($p = 0.04$) was found in the severe GI group when compared to the mild GI group (**b**). The horizontal bars are displaying median, 1st, and 3rd quartile

and mild GI disease ($p = 0.0084$) and between severe GI disease and clinically healthy horses ($p < 0.0001$) (Table 4).

Correlation analyses between plasma-TEG MA and fibrinogen and between plasma-TEG G and fibrinogen both revealed a moderate and significant Spearman's correlation coefficient (r) of 0.61 (95% CI: 0.33–0.79, $p = 0.0001$).

Discussion

CAT ran successfully on equine citrated PPP with satisfactory repeatability based on intra- and inter-assay coefficient of variations for both lag-time, ETP, peak, and ttPeak all below the accepted limit of 5% [30].

In the CAT assay the PPP trigger reagent with 5 pM tissue factor was applied to initiate the thrombin generation

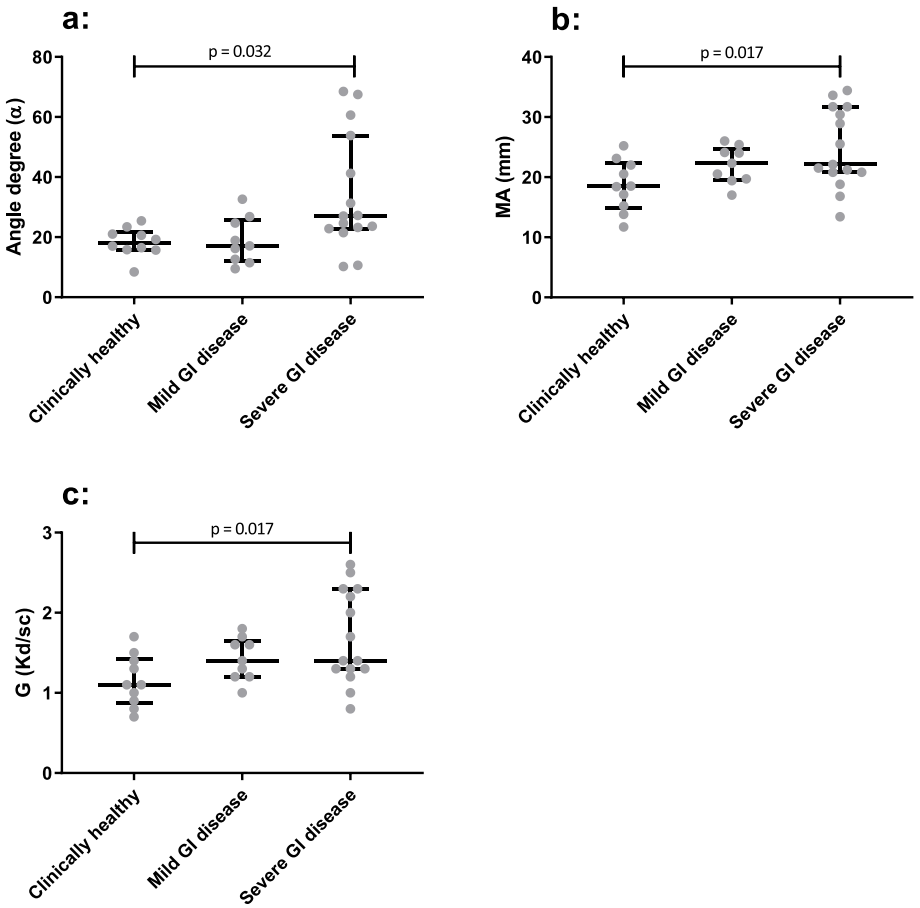


Fig. 3 Group comparison between the three groups of horses (clinically healthy, mild and severe gastrointestinal (GI) disease) for plasma-thromboelastography (plasma-TEG) showing a significantly higher angle degree (α) ($p = 0.032$) (**a**), maximum amplitude (MA) ($p = 0.017$) (**b**) and shear elastic modulus strength (G) ($p = 0.017$) (**c**) in the severe GI group compared to the clinically healthy horses. Plots are displaying median, 1st, and 3rd quartile

process, similarly to what has previously been applied in humans [31] and dogs [32]. The manufacturer provides three different triggering reagents with either 1 pM (PPP low), 5pM (PPP) or 20 pM (PPP high) tissue factor [33]. In human research, the PPP low and high are used in hemophilic patients or patients treated with anticoagulant therapy, respectively. In a recent study in cats [34], all three PPP trigger reagents were tested in depth. Based on performance of the three triggering reagents, PPP low was selected as the most appropriate for the feline species. It is possible that more subtle hemostatic aberrations between healthy horses and horses with severe GI disease could have been detected, if a similar set up had been applied in our study. This needs to be investigated further in future studies.

Endogenous thrombin potential did not differ between horses with severe GI disease and healthy horses. While some consider ETP a more consistently predictive parameter for hemostatic aberrations compared to the remaining CAT parameters, others report an equal importance of lag-time, peak, and ttPeak in the CAT assay [26, 32, 35]. In humans an increased peak and ttPeak has been found as a sign of hypercoagulability in patients with both inherited and acquired hemostatic disorders [36, 37]. We did not expect horses with mild GI disease to have hemostatic aberrations when looking at group comparison, but, surprisingly, it was not possible to distinguish horses with severe and mild GI disease with the global hemostatic tests, apart from peak in the CAT assay. This might have been the result of our two groups with GI disease having disease processes that were too similar concerning their pathophysiology even though we intended to include horses with different severity of inflammation and ischemia. Nevertheless, several parameters were significantly different in both the CAT assay and the plasma-TEG between healthy horses and horses with severe GI disease without being mirrored in the whole blood-TEG. Furthermore, the defrosting and

Table 4 Analysis of whole blood-thromboelastography (WB-TEG) and routine plasma based coagulation tests between clinically healthy, mild gastrointestinal (GI) and severe GI disease horse groups. Median and range for WB-TEG and routine coagulation parameters fibrinogen, activated partial thromboplastin time (aPTT), prothrombin time (PT), anti-thrombin (AT) and d-dimer in healthy horses (healthy control), and horses with mild or severe GI diseases. *P*-value column: one-way ANOVA or a Kruskal-Wallis test depending on normality of data across all three groups with a significant level of < 0.05. [a] Post hoc statistical difference between healthy and severe GI disease, [b] Post hoc statistical difference between mild and severe GI disease. SP: split point, R: reaction time, K: clot formation time, α: alpha angle, MA: maximum amplitude, G: shear elastic force, LY30: lysis 30 min, LY60: lysis 60 min (min.)

		Clinically healthy	Mild GI disease	Severe GI disease	*P*-value
WB-TEG	SP (min.)	12.7 (8.2–17.1)	9.8 (4.4–22.6)	10.8 (5.8–15.9)	0.23
	R (min.)	16.6 (10.5–20.6)	11.6 (5.2–29.5)	13.5 (7.2–19.1)	0.29
	α (degrees)	26.35 (17.2–41.7)	39.3 (13.3–65.4)	35.6 (18.5–54.5)	0.26
	K (min.)	7.15 (4.2–13)	4.9 (2–18.4)	5.4 (3.4–11.8)	0.13
	MA (mm)	51.55 (35–60.1)	56.4 (49.5–73.6)	52.2 (40.9–77.6)	0.18
	G (dynes/cm^2)	5.3 (2.7–7.5)	7 (4.9–13.9)	5.5 (3.5–17.4)	0.15
	Ly30 (%)	0 (0–1.5)	0.1 (0–0.6)	0 (0–0.9)	0.9
	Ly60 (%)	2.35 (0.2–6.1)	2.9 (0–4.4)	1.3 (0.1–4.4)	0.32
Conventional haemostatic markers	Fibrinogen (g/L)	3.17 (2.87–3.53)	3.72 (2.17–5.67)	3.17 (2.57–7.01)	0.14
	aPTT (sec)	47.15 (44.30–57.90)	48.80 (39.30–51.4)	48.00 (40.60–56.30)	0.62
	PT (sec)	13.85 (13.10–14.00)	13.60 (12.70–14.10)	14.10 (12.50–17.30)	0.15
	AT (%)	210.5 (189.00–239.00)	223.00 (195.00–246.00)	197.00 (158.00–260.00)	0.14
	D-dimer	0.08 (0.05–0.1)	0.09 (0.05–0.18)	0.25 (0.1–6.41)	< 0.0001 < 0.0001[a] 0.0084[b]

centrifugation process in the CAT assay and plasma-TEG could activate the low number of existing platelets as well as procoagulant platelet micro-particles [38]. Potentially, this could contribute to the initiation phase of plasma-based tests and perhaps affect all parameters in the CAT assay as well as α, K, MA, and G in the plasma-TEG. In people, platelet microparticles are known to contribute to a prothrombotic state in different types of GI diseases [39, 40]. Microparticles were not measured in this study, but it could be speculated that horses with severe GI disease would have a higher concentration of platelet microparticles and thus be more procoagulant than healthy horses. In humans a shorter R and K value and an increased angle degree and MA has been found as a sign of hypercoagulability [41, 42].

Although the plasma-TEG was analyzed in all horses, the K parameter did not produce a reading in 12 of these. This is not a problem of clinical relevance, as it is caused by the fact that the pre-set standard value for K in the TEG machine is defined in the software as the time to clot strength at 20 mm based on human whole blood measurements and due to the narrower tracing of the plasma-TEG, this was not achieved in all cases. In general, the plasma-TEG readings had a lower α, MA, and G compared to whole blood TEG, which likely is due to the different composition of PPP compared to whole blood, probably most importantly the lack of platelets.

Contrary to what have been found in previous studies [2, 3, 43], no differences were identified between the three groups of horses when analyzed with whole blood-TEG. The reason for this is not entirely clear. It is most likely due to the complexity of dealing with coagulopathies [3].

In TEG, MA and G are the comprehensive assessment of the fibrinogen function and concentration combined with the platelet count [44]. When analyzing plasma-TEG using PPP, it could thus be claimed that MA and G of such an assay are no more than an elaborate fibrinogen analysis. In the present study, there was a positive correlation between fibrinogen, MA and G. However, as opposed to plasma-TEG MA and G measured by plasma-TEG, the fibrinogen concentration alone was not able to detect a difference between the three groups of horses. Plasma-TEG has still not been validated extensively in veterinary medicine and whether the plasma-TEG is a more sensitive assay than measuring the fibrinogen concentration on its own remains to be fully elucidated.

The decreased ttPeak and elevated peak for the CAT assay and an elevated angle degree, MA and G for the plasma-TEG in the severe GI disease group corresponds well. Both support a hypercoagulable state which was what we expected looking at the existing literature [2, 6]. For the routine coagulation markers an increased D-dimer was additionally seen in the severe GI disease group, which also supports the finding of a hypercoagulable state [4, 5]. It thus seems that horses with severe GI disease suffers from a hypercoagulable state which in a

clinical setting can be evaluated with the use of the CAT assay and plasma-TEG.

The main limitation of the present study is the low number of horses in each group and the variability of the disease processes within each group. The low number of horses was a result of the study being conducted as a pilot study. However, the presented preliminary results do suggest that these global hemostatic tests could be of value in horses and it would be of interest to explore these assays in more detail. Studies could focus on applying the tests to larger groups of horses, horses with visible thromboembolic disease or bleeding disorders to fully examine the potential of both the CAT assay and plasma-TEG in equine medicine.

Conclusion

The CAT assay performed well in horses with intra- and inter-assay coefficients of variation below the accepted limits and are thus reliable. Additionally the heparin dilution curve showed that the CAT assay is valid in horses. The CAT assay and plasma-TEG were applicable for use in equine citrated PPP and both assays showed hemostatic aberrations in horses with GI disease. Except for ttPeak in the CAT assay, the assays could not distinguish mild from severe GI disease.

Methods

This study was conducted as a pilot study. All samples were collected at The Large Animal Teaching Hospital at The University of Copenhagen, Denmark, in 2018. Approval was obtained from the ethical board of the Department of Veterinary Clinical Sciences, University of Copenhagen, as was written consent from the owners of the horses.

Study design

For the evaluation of the CAT assay, intra- and inter-assay coefficients of variation were established in order to establish the reliability of the test in equine PPP. This was performed based on replicate measurements on pooled plasma from 10 clinically healthy horses analyzed 10 times on the same day and once daily for 10 consecutive days. To confirm the capacity of the human CAT assay to measure equine thrombin in PPP and thus establish the validity of the test, unfractionated heparin was added in decreasing concentrations to aliquots of the plasma pool: 0.0, 0.01125, 0.0225, 0.045, 0.09, 0.18, 0.37, 0.75, 1.5 and 3.0 U/mL [45–47].

To examine if these global hemostatic tests deviated in horses with GI disease, group comparisons of both the CAT assay, plasma-TEG, and whole blood-TEG were performed, comparing horses with severe and mild GI disease to healthy horses. The purpose of a group comparison is to detect differences between healthy and clearly sick individuals and is defined as a phase II of test validation [48]. This is performed before proceeding to the more complex phase III involving several different disease and severity groups. We would expect horses with severe GI disease to be hypercoagulable in comparison to clinically healthy horses and horses with mild GI disease.

Whole blood-TEG and the individual hemostatic parameters; D-dimer, fibrinogen, aPTT, PT, and AT were analyzed in the 3 groups of horses in order to compare them to the new global hemostatic tests.

Horses

Three groups of horses were included in the study: Clinically healthy, mild GI disease, and severe GI disease. The clinically healthy group included adult horses (> 1 year of age) deemed healthy based on clinical examination, complete blood count (CBC) and serum biochemistry profiles, including lactate and the acute phase proteins serum amyloid A (SAA) and fibrinogen concentration being within normal reference intervals. All horses in this group were owned and stabled by The Large Animal Teaching Hospital at The University of Copenhagen. Blood from horses in this group were used in both the intra- and inter-CV studies, the heparin dilution curve and the group comparison. Horses included in the two GI disease groups consisted of adult horses (> 1 year of age) admitted to The Large Animal Teaching Hospital with acute abdominal pain in the period March through June 2018. All samples from hospital patients were collected at admission as part of the initial diagnostic work-up, before placement of intravenous-catheters or administration of any treatments. Horses were assigned to the mild GI disease group if the horses had non-strangulating intestinal obstructions or displacements without signs of secondary inflammation or ischemia of the intestines. Conversely, horses were placed in the severe GI disease group if the horses had inflammatory or strangulating intestinal diseases with ischemia of the intestine. Grouping of the horses was based on clinical signs (heart rate, respiratory rate, temperature and mucous membrane color), CBC, serum biochemistry including SAA, fibrinogen, rectal palpation, naso-gastric intubation, and, where appropriate, abdominocentesis, transabdominal ultrasonography, surgery and post-mortem findings. Horses were considered to have systemic inflammation when there was both clinical or post mortem signs of inflammation like fever or edema in the intestinal wall and inflammatory changes in the blood samples such as leukopenia and increased SAA. Horses were considered to have intestinal ischemia when a segment of the intestine was found to be

strangulated and with circulatory changes at surgery or post mortem. Mares included in the study all were reproductively inactive at the sampling point.

Blood sample handling and routine blood analysis

Blood samples were collected by jugular venipuncture using a 21 g needle and a vacutainer system (BD, Franklin Lakes, NJ). Blood tubes (BD, Franklin Lakes, NJ) were collected in the recommended order [49] starting with the 3.2% 0.109 M sodium citrate tubes in a 1:9 ratio citrate/blood, then serum separator tubes, and finally EDTA tubes. A total of 4 sodium citrate, 1 serum and 1 EDTA tubes were obtained in that order, with the first sodium citrate tube being discarded [49]. The remaining three sodium citrate blood tubes were used for hemostatic assays, while the serum and EDTA tubes were used for the routine biochemistry analysis and CBC (ADVIA 2120i, Siemens Healthcare A/S, Ballerup, Denmark) including a blood smear evaluation, at the hospital's Diagnostic Laboratorium. The serum and EDTA samples were stored at 4 °C until analysis.

Whole blood TEG with diluted tissue factor (TF) was measured 30 min after sampling in all horses. Platelet poor plasma (PPP) was created within a maximum of 1 h by centrifugation of the sodium citrate tubes at room temperature at 2000 *g* for 15 min [13].. The PPP was stored at -80 °C until batch analysis of plasma-TEG, CAT, d-dimer, fibrinogen, aPTT, PT, and AT. Samples were thawed in a water bath for 4 min at 37 °C and then thoroughly mixed [49]. A platelet count below 10×10^9/L [50] in the PPP was ensured by platelet measurement prior to hemostatic analysis (ADVIA 2120i, Siemens Healthcare A/S, Ballerup, Denmark).

Hemostatic assays

Thrombin generation was measured with the CAT assay. The following four parameters were evaluated: Lag-time (min), which is the time until 1/6 of the total thrombin concentration is reached; the endogenous thrombin potential (ETP) (nM/min), which represents the total amount of thrombin generated; peak (nM), which is the maximal thrombin concentration, and time to peak (ttPeak) (min) (Thrombinoscope BV, Maastricht, The Netherlands). The Thrombinoscope software is for now only intended for research purposes.

The assay was activated by a manufacturer-supplied trigger solution (PPP reagent) (Triolab AS, Denmark). The measuring interval was set to 20 s, the temperature to 37.5 °C, and the measured time to 45 min. All analyses were performed in triplicates. In each well 80 µl of citrated plasma was added to 20 µl of either the activator solution or thrombin calibrator solution as per the manufacturers recommendation and described previously [13]. The activator solution used contained 5 pM TF and 4 µM phospholipids. Thrombin generation was activated as per the manufacturers (Thrombinoscope BV, Maastricht, The Netherlands) instruction by adding 20 µl of FluCa consisting of Fluo-Buffer containing $CaCl_2$ and a fluorescent, and read by an automated highly specific plate reader.

Both plasma-TEG and whole blood-TEG analyses were performed using a computerized thromboelastograph (TEG 5000 Haemostasis Analyzer, Haemoscope Corporation, Illinois, USA). The TEG machines were evaluated prior to daily use with the E-test, which is an electronic quality control. The following TEG parameters were recorded: Split point (SP) which is time to initial conversion of fibrinogen to fibrin; reaction time (R) which is time to initial fibrin clot formation; Clotting time (K) which is the time from initial clot formation until reaching a predetermined level of clot strength (20 mm); Angle (α) which represents the speed of fibrin build up and cross-linking; Maximum amplitude (MA) which represents the maximum clot strength; Shear elastic modulus strength (G) which is a linear function of the MA and considered a measure of global clot strength [51] and degree of fibrinolysis at 30 min (LY30%) and 60 min (LY60%) after MA. The analyses were run as per the manufacturer's protocol for 2 h. Hypercoagulability for the CAT assay was defined as a shortened lagtime, ttPeak and a higher ETP and Peak compared to the clinically healthy group with one or more altered variable [52]. For TEG hypercoagulability was defined as a shorter R and K value and an increased angle degree, MA, and G (with any $\geq$1 index being abnormal) compared to the clinically healthy group. For both tests, the opposite situation was defined as hypocoagulability [3, 53–55].

Fibrinogen was measured with a PT-based assay using HemosIL RecombiPlastin on an ACL Top 500 (ILS Danmark, Allerød, Denmark). D-dimer, aPTT, PT, and AT were measured on the automated machine STAGO STA Satellite coagulation analyzer (Triolab, Brøndby, Denmark) with different assays for each parameter according to the manufacturers recommendations. D-dimer was analyzed with STA-Liatest D-Di + which is a photometric antibody-antigen assay based on murine D-dimer antibodies [16, 56], aPTT with STA-Cephascreen using kefalin as an activator which together with PT is a coagulometric assay, PT with STA-NeoPTimal using rabbit thromboplastin, and AT with STA-Stachrom ATIII using bovine thrombin which is a chromogenic assay [16].

Statistical analysis

Intra- and inter-assay coefficient of variation levels were calculated using Microsoft Excel 2016 (Washington,

USA), and levels below 5% were considered acceptable [30]. Remaining analyses were performed using GraphPad Prism 8.3.0 (San Diego, California, USA).

Normality was assessed by the D'Agostino-Pearsons K-squared test. Comparisons between the three groups of horses were performed using a one-way ANOVA or a Kruskal-Wallis test depending on normality of data. As a post hoc test, either Tukey's multiple comparisons test or Dunn's multiple comparisons test was applied. Correlation tests (Spearman) between plasma-TEG MA, plasma-TEG G, and fibrinogen concentration were performed in order to evaluate whether these values were connected. The probability of significance was set at a level of < 0.05.

Abbreviations

α: Angle degree (TEG); aPTT: Activated partial thromboplastin time; AT: Anti-thrombin; BW: Body weight; CAT: Calibrated Automated Thrombogram; CBC: Complete blood count; CV: Coefficients of variation; DIC: Disseminated intravascular coagulation; ETP: Endogenous thrombin potential (CAT); G: Shear elastic force (TEG); GI: Gastrointestinal; K: Clotting time (TEG); LY30%: Fibrinolysis at 30 min after MA (TEG); LY60% : Fibrinolysis at 60 min after MA (TEG); MA: Maximum amplitude (TEG); PLT: Platelet; PPP: Platelet poor plasma; PT: Prothrombin time; R: Reaction time (TEG); SAA: Serum amyloid A; SP: Split point (TEG); TAT: Thrombin–antithrombin; TEG: Thromboelastography; TF: Tissue factor; ttPeak: Time to peak (CAT); WB-TEG: Whole blood-thromboelastography.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12917-021-03058-7.

Additional file 1: Table 1s. Heparin dilution curve performed in the calibrated automated thrombin assay. Lag-time, time to peak (ttPeak), peak and endogenous thrombin potential (ETP) are listed. Note that the thrombin potential is completely inhibited at a heparin concentration of 0.037 U/mL. At the lowest concentration of heparin (0.01125 and 0.0225 U/mL), the lag-time and ttPeak are almost identical to 0.0 U/mL, but with a slight 15–20% increase in both Peak and ETP.

Acknowledgements

The authors are grateful to medical laboratory technologist Louise Bochsen and to laboratory technician Tina Roust from the Department of Veterinary Clinical Sciences, Faculty of Health and Medical Sciences (SUND), University of Copenhagen, Denmark for their laboratory assistance during this study and to the horse owners who kindly enrolled their horses in the study.

The authors would also like to thank Associate professor Dr. Rebecca Langhorn, University of Copenhagen, Denmark for language editing and the Danish Horse Levy Foundation for funding the project.

Authors' contributions

All authors have provided substantial contribution to the manuscript. MLH has acquired the sample material, and owner consent, conducted and assisted in data analysis, interpreted results, and have drafted the work and continuously revised it. LNN has made contributions to the design of the work and data interpretation and has revised the work. THP has made contributions to the acquisition and interpretation of data and has revised the work. All authors have read and approved the final manuscript.

Funding

The Danish Horse Levy Foundation funded the project but played no role in the design, analysis and reporting of the study.

Availability of data and materials

If deemed relevant or is of interest raw data can be submitted.

Declarations

Ethics approval and consent to participate

All samples were collected at The Large Animal Teaching Hospital at The University of Copenhagen, Denmark, in 2018. Approval was obtained from the ethical board of the Department of Veterinary Clinical Sciences, University of Copenhagen, as was written consent from the owners of the horses. All procedures were carried out in accordance with the ARRIVE guidelines.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

[1]Department of Veterinary Clinical Sciences, Faculty of Health and Medical Sciences (SUND), University of Copenhagen, Hoejbakkegaard Allé 5a, 2630 Taastrup, Denmark. [2]Section for Internal Medicine, Oncology and Clinical Pathology, Faculty of Health and Medical Sciences (SUND), University of Copenhagen, Dyrlaegevej 16, 1870 Frederiksberg C, Denmark.

Received: 18 June 2021 Accepted: 19 October 2021
Published online: 08 November 2021

References

1. Dallap BL, Dolente B, Boston R. Coagulation profiles in 27 horses with large colon volvulus. J Vet Emerg Crit Care. 2003;13(4):215–25.
2. Dunkel B, Chan DL, Boston R, Monreal L. Association between hypercoagulability and decreased survival in horses with ischemic or inflammatory gastrointestinal disease. J Vet Emerg Crit Care. 2010;24:1467–74.
3. Epstein KL, Brainard BM, Gomez-Ibanez SE, Lopes MAF, Barton MH, Moore JN. Thrombelastography in horses with acute gastrointestinal disease. J Vet Intern Med. 2011;25:307–14.
4. Cesarini C, Monreal L, Armengou L, Delgado MÁ, Ríos J, Jose-Cunilleras E. Progression of plasma D-dimer concentration and coagulopathies during hospitalization in horses with colic. J Vet Emerg Crit Care. 2014;24(6):672–80.
5. Cesarini C, Monreal L, Armengou L, Delgado MÁ, Ríos J, Jose-Cunilleras E. Association of admission plasma D-dimer concentration with diagnosis and outcome in horses with colic. J Vet Intern Med. 2010;24:1490–7.
6. Monreal L, Cesarini C. Coagulopathies in horses with colic. Vet Clin North Am - Equine Prac. 2009;25(2):247–58. Available from: https://doi.org/10.1016/j.cveq.2009.04.001.
7. Hyldahl Laursen S, Andersen PH, Kjelgaard-Hansen M, Wiinberg B. Comparison of components of biological variation between 3 equine thromboelastography assays. Vet Clin Pathol. 2013;42(4):443–50.
8. Leclere M, Lavoie JP, Dunn M, Bédard C. Evaluation of a modified thrombelastography assay initiated with recombinant human tissue factor in clinically healthy horses. Vet Clin Pathol. 2009;38(4):462–6.
9. Hron G, Kollars M, Binder BR, Eichinger S, Kyrle PA. Identification of patients at low risk for recurrent venous thromboembolism by measuring thrombin generation. JAMA. 2006;296(4):397–402.
10. Hemker HC, Giesen P, AlDieri R, Regnault V, De Smed E, Wagenvoord R, et al. The Calibrated Automated Thrombogram (CAT): a universal routine test for hyper- and hypocoagulability. Pathophysiol Haemost Thromb. 2002;32(5–6):249–53.
11. Deppe AC, Weber C, Zimmermann J, Kuhn EW, Slottosch I, Liakopoulos OJ, et al. Point-of-care thromboelastography/thromboelastometry-based coagulation management in cardiac surgery: a meta-analysis of 8332 patients. J Surg Res [Internet]. 2016;203(2):424–33. Available from: https://doi.org/10.1016/j.jss.2016.03.008.
12. Fenty RK, Delaforcade AM, Shaw SP, Toole TEO. Identification of hypercoagulability in dogs with primary immune-mediated hemolytic anemia by means of thromboelastography. J Am Vet Med Assoc. 2011;238(4):463–7.

13. Allegret V, Dunn M, Bédard C. Monitoring unfractionated heparin therapy in dogs by measuring thrombin generation. Vet Clin Pathol. 2011;40(1):24–31.
14. Scruggs JL, Flatland B, Mccormick KA, Reed A. Biological variation of thromboelastrography variables in 10 clinically healthy horses. J Vet Emerg Crit Care. 2016;26(1):80–4.
15. Dallap BL. Coagulopathy in the equine critical care patient. Vet Clin North Am - Equine Pract. 2004;20(1):231–51.
16. Pihl TH, Nielsen MK, Jacobsen S. Changes in hemostatic indices in foals naturally infected with strongylus vulgaris. J Equine Vet Sci. 2017;54:1–7. Available from: https://doi.org/10.1016/j.jevs.2017.02.002.
17. Lancé MD. A general review of major global coagulation assays: thromboelastography, thrombin generation test and clot waveform analysis. Thromb J. 2015;13(1):1–6.
18. Spahn DR, Bouillon B, Cerny V, Coats TJ, Duranteau J, Fernández-mondéjar E, et al. Management of bleeding and coagulopathy following major trauma: an updated European guideline. Crit Care. 2013;17(2):R76. https://doi.org/10.1186/cc12685.
19. Mendez-angulo JL, Mudge MC, Vilar P, Stingle N, Couto CG. Thromboelastography in healthy horses and horses with inflammatory gastrointestinal disorders and suspected coagulopathies. J Vet Emerg Crit Care. 2010;20(5):488–93.
20. Wiinberg B, Jensen AL, Kjelgaard-Hansen M, Rojkjaer R, Johansson PI, Gade LP, et al. Study on biological variation of haemostatic parameters in clinically healthy dogs. Vet J. 2007;174(1):62–8.
21. Krogh AK, Brunse A, Thymann T, Kristensen AT, Nielsen LN. Plasma thromboelastography in septic preterm pigs and full-term pigs at birth and 11 days post-partum. In: Joint Congress of Veterinary Pathology and Veterinary Clinical Pathology 25–28 September 2019 | Arnhem, the Netherlands; 2019. p. 287.
22. Contreras-García A, Merhi Y, Ruiz JC, Wertheimer MR, Hoemann CD. Thromboelastography (TEG) cups and pins with different PECVD coatings: effect on the coagulation cascade in platelet-poor blood plasma. Plasma Process Polym. 2013;10(9):817–28.
23. Ostrowski SR, Berg RMG, Windeløv NA, Meyer MAS, Plovsing RR, Møller K, et al. Discrepant fibrinolytic response in plasma and whole blood during experimental endotoxemia in healthy volunteers. PLoS One. 2013;8(3)1-9. https://doi.org/10.1371/journal.pone.0059368.
24. Wexels F, Dahl OE, Pripp AH, Seljeflot I. Thrombin generation in patients with suspected venous thromboembolism. Clin Appl Thromb. 2017;23(5):416–21.
25. Dargaud Y, Wolberg AS, Luddington R, Regnault V, Spronk H, Baglin T, et al. Evaluation of a standardized protocol for thrombin generation measurement using the calibrated automated thrombogram: an international multicentre study. Thromb Res. 2012;130(6):929–34. Available from: https://doi.org/10.1016/j.thromres.2012.07.017.
26. Rose LJ, Dunn ME, Allegret V, Bédard C. Effect of prednisone administration on coagulation variables in healthy beagle dogs. Vet Clin Pathol. 2011;40(4):426–34.
27. Machlus KR, Colby EA, Wu JR, Koch GG, Key NS, Wolberg S. Effects of tissue factor, thrombomodulin and elevated clotting factor levels on thrombin generation in the calibrated automated thrombogram. Thromb Haemost. 2010;102(5):936–44.
28. Hemker HC, Giesen P, Al Dieri R, Regnault V, De Smedt E, Wagenvoord R, et al. Calibrated automated thrombin generation measurement in clotting plasma. Pathophysiol Haemost Thromb. 2003;33(1):4–15.
29. Hudák R, Székely EG, Kovács KR, Nagy A, Hofgárt G, Berényi E, et al. Low thrombin generation predicts poor prognosis in ischemic stroke patients after thrombolysis. PLoS One. 2017;12(7):1–13.
30. Jensen AL, Kjelgaard-hansen M. Diagnostic Test Validation. Schalm's Vet Hematol. 2010;6:1027–33.
31. Jeong JC, Kim JE, Ryu JW, Joo KW, Kim HK. Plasma haemostatic potential of haemodialysis patients assessed by thrombin generation assay: hypercoagulability in patients with vascular access thrombosis. Thromb Res. 2013;132(5):604–9. Available from: https://doi.org/10.1016/j.thromres.2013.09.002.
32. Rose L, Dunn ME, Bédard C. The effect of canine hyperadrenocorticism on coagulation parameters. J Vet Intern Med. 2013;27:207–11.
33. Thrombinoscope.com. Thrombinoscope: methods and products. Accessed 13 Mar 2020.
34. Mischke R, Teuber M, Tiede A. Research in veterinary science measurements of endogenous thrombin potential using the CAT method in cats : reference values and in fl uence of the direct factor Xa inhibitor apixaban. Res Vet Sci. 2019;127(August):113–21. Available from: https://doi.org/10.1016/j.rvsc.2019.08.030.
35. Mcmichael MA, Brien MO, Smith SA. Hypercoagulability in dogs with blastomycosis. J Vet Intern Med. 2015;29(2):499–504.
36. Tournier A, Wahl D, Chaouat A, Max JP, Regnault V, Lecompte T, et al. Calibrated automated thrombography demonstrates hypercoagulability in patients with idiopathic pulmonary arterial hypertension. Thromb Res. 2010;126(6):e418–22. Available from: https://doi.org/10.1016/j.thromres.2010.08.020.
37. Regnault V, Hemker HC, Wahl D, Lecompte T. Phenotyping the haemostatic system by thrombography - potential for the estimation of thrombotic risk. Thromb Res. 2004;114(5–6 SPEC. ISS):539–45.
38. Stepien E, Gruszczynski K, Kapusta P, Kowalik A, Wybranska I. Plasma centrifugation does not influence thrombin-antithrombin and plasmin-antiplasmin levels but determines platelet microparticles count. Biochem Med. 2015;25(2):222–9.
39. Chamouard P, Desprez D, Hugel B, Kunzelmann C, Gidon-Jeangirard C, Lessard M, et al. Circulating cell-derived microparticles in Crohn's disease. Dig Dis Sci. 2005;50(3):574–80.
40. Pamuk GE, Vural Ö, Turgut B, Demir M, Ümit H, Tezel A. Increased circulating platelet – neutrophil , platelet – monocyte complexes , and platelet activation in patients with ulcerative colitis: a comparative study. Am J Hematol. 2006;81(10):753–9.
41. Donahue SM, Otto CM. Thromboelastography: a tool for measuring hypercoagulability, hypocoagulability, and fibrinolysis. J Vet Emerg Crit Care. 2005;15(1):9–16.
42. Kupcinskiene K, Trepenaitis D, Petereit R, Kupcinskas J, Gudaityte R, Maleckas A, et al. Monitoring of hypercoagulability by thromboelastography in bariatric surgery. Med Sci Monit. 2017;23:1819–26.
43. Müller MC, Meijers JCM, Vroom MB, Juffermans NP. Utility of thromboelastography and/or thromboelastometry in adults with sepsis: a systematic review. Crit Care. 2014;18(1):30.
44. Salooja N, Perry DJ. Thrombelastography. Blood Coagul Fibrinolysis. 2001;12(5):327–37.
45. Epstein KL, Brainard BM, Giguere S, Vrono Z, Moore JN. Serial viscoelastic and traditional coagulation testing in horses with gastrointestinal disease. J Vet Emerg Crit Care. 2013;23(5):504–16.
46. Moore BR, Hinchcliff KW. Heparin: a review of its pharmacology and therapeutic use in horses. J Vet Intern Med. 1994;8(1):26–35.
47. Duncan SG, Reed SM. Heparin anticoagulant therapy in equine colic. Mod Vet Pract. 1984;65:601–5.
48. Kjelgaard-Hansen M, Jacobsen S. Assay validation and diagnostic applications of major acute-phase protein testing in companion animals. Clin Lab Med. 2011;31(1):51–70. Available from: https://doi.org/10.1016/j.cll.2010.10.002.
49. Adcock DM, Hoefner DM, Kottke-Marchant K, Marlar RA, Szamosi DI, Warunek DJ. Collection , transport, and processing of blood specimens for testing plasma-based coagulation assays and molecular hemostasis assays; CLSI approved guideline-fifth edition. H21-A5. Clin Lab Stand Inst. 2008;28(5):H21–A5.
50. Nielsen LN, Wiinberg B, Kristensen AT. The presence of antiphospholipid antibodies in healthy Bernese Mountain dogs. J Vet Intern Med. 2011;25(6):1258–63.
51. Hanel RM, Chan DL, Conner B, Gauthier V, Istvan S, Walker JM, et al. Systematic evaluation of evidence on veterinary viscoelastic testing part 4 : definitions and data reporting. J Vet Emerg Crit Care. 2014;24(1):47–56.
52. Saliba W, Awad K, Ron G, Elias M. The effect of vitamin D supplementation on thrombin generation assessed by the calibrated automated thrombogram. Clin Appl Thromb. 2016;22(4):340–5.
53. Wang Z, Li J, Cao Q, Wang L, Shan F, Zhang H. Comparison between thromboelastography and conventional coagulation tests in surgical patients with localized prostate cancer. Clin Appl Thromb.

2018;24(5):755–63. Available from: https://doi.org/10.1177/1076029617724229.
54. Lemon AV, Goddard A, Hooijberg EH. Effects of storage time and temperature on thromboelastographic analysis in dogs and horses. Vet Clin Pathol. 2021;50(1):9–19.
55. Thakur M, Ahmed AB. A review of thromboelastography. Int J Perioper Ultrasound Appl Technol. 2012;1(1):25–9.
56. Armengou L, Monreal L, Tarancón I, Navarro M, Ríos J, Segura D. Plasma D-dimer concentration in sick newborn foals. J Vet Intern Med. 2008;22:411–7.

Publisher's Note

Paper III

Hemostatic changes identified in horses with migrating *Strongylus vulgaris* using plasma-thromboelastography and the Calibrated Automated Thrombogram assay

Honoré, M.L., Nielsen, L. N., Stærk-Østergaard, J., Týden, E., Hedberg-Alm, Y., Riihimäki, M., Anlén, K. G., Nielsen, M. K., Pihl, T. H.

Manuscript in preparation for submission to BMC Veterinary Research.

Hemostatic changes identified in horses with migrating *Strongylus vulgaris* using plasma-thromboelastography and the Calibrated Automated Thrombogram assay

Marie Louise Honoré[1], Lise Nikolic Nielsen[2], Jacob Stærk-Østergaard[3], Eva Týden[4], Ylva Hedberg-Alm[5], Miia Riihimäki[6], Karin Anlén[7], Martin Krarup Nielsen[8], Tina Holberg Pihl[9]

Affiliation details:

[1] Marie Louise Honoré Lytzhøft-Olsen, PhD-student, Department of Veterinary Clinical Sciences, Faculty of Health and Medical Sciences (SUND), University of Copenhagen, Hoejbakkegaard Allé 5a, 2630 Taastrup, Denmark, mlhj@sund.ku.dk

[2] Lise Nikolic Nielsen, Professor, Section for Internal Medicine, Oncology and Clinical Pathology, Faculty of Health and Medical Sciences (SUND), University of Copenhagen, Dyrlaegevej 16, 1870 Frederiksberg C, Denmark, lini@sund.ku.dk

[3] Jacob Stærk-Østergaard, Assistant professor, Department of Veterinary and Animal Sciences, Faculty of Health and Medical Sciences (SUND), University of Copenhagen, Groennegaardsvej 8, 1870 Frederiksberg C, Denmark, ostergaard@sund.ku.dk

[4] Eva Týden, Associate professor, Swedish University of Agricultural Sciences, Department of Biomedical Science and Veterinary Public Health, Parasitology Unit, Ulls Väg 26, Uppsala, Sweden, eva.tyden@slu.se

[5] Ylva Hedberg-Alm, DVM, University Animal Hospital, Swedish University of Agricultural Sciences, Ultunaallén 3C, Uppsala, Sweden, ylva.hedberg.alm@slu.se

[6] Miia Riihimaki, Assistant professor, University Animal Hospital, Swedish University of Agricultural Sciences, Ultunaallén 3C, Uppsala, Sweden, miia.riihimaki@slu.se

[7] Karin Anlén, DVM, Evidensia Helsingborg Equine Referral Hospital, Bergavägen 3, 254 66 Helsingborg, Sweden, karin.anlen@evidensia.se

[8] Martin Krarup Nielsen, Professor, 343, Gluck Equine Research Center, University of Kentucky, Lexington, KY 40546-0099, USA, martin.nielsen@uky.edu

[9] Tina Holberg Pihl, Associate Professor, Department of Veterinary Clinical Sciences, Faculty of Health and Medical Sciences (SUND), University of Copenhagen, Hoejbakkegaard Allé 5a, 2630 Taastrup, Denmark, thpi@sund.ku.dk

Correspondence:

Marie Louise Honoré, Department of Veterinary Clinical Sciences, Faculty of Health and Medical Sciences (SUND), University of Copenhagen, Denmark, mlhj@sund.ku.dk

Abstract

Background

Strongylus vulgaris causes thrombosis in the cranial mesenteric artery of the horse, which in some leads to non-strangulating intestinal infarctions (NSII). Diagnosing migrating *S. vulgaris* and NSII is challenging, and improved pathophysiological understanding and novel diagnostic markers are needed.

The objectives of this study were to investigate whether horses with migrating *S. vulgaris* with and without NSII had hemostatic changes measurable with plasma-thromboelastography (TEG) and the Calibrated Automated Thrombogram (CAT), and whether they were distinguishable from clinically healthy horses (without *S. vulgaris*) and horses with severe GI diseases mimicking NSII.

Horses underwent clinical examination and hematological and biochemical (including inflammatory makers) analysis including measurement of *S. vulgaris* antibodies. Citrated plasma samples were analyzed using d-dimer, plasma-TEG and CAT. Groups were compared using ANOVA and Tukey's *post hoc* test. A logistic regression, receiver operating curves, and multinomial regression were used to differentiate the *S. vulgaris* NSII positive group.

Results

Clinically healthy horses (17), *S. vulgaris* NSII negative (18), *S. vulgaris* NSII positive (16), idiopathic peritonitis (20), enterocolitis (26), and strangulating lesions (26) were included.

Plasma-TEG and CAT identified significant hemostatic changes in the *S. vulgaris* NSII negative and positive groups compared to the other groups. The *S. vulgaris* NSII positive group had a longer reaction time (TEG) than horses with strangulating lesions, as well as a higher maximum amplitude (MA, TEG), longer lag time (CAT) and time to peak (CAT) compared to horses with enterocolitis and strangulating lesions. The *S. vulgaris* NSII negative group had a higher angle (TEG) and MA as well as a longer lag time than the clinically healthy horses.

A model including iron, serum amyloid A, angle, and endogenous thrombin potential (CAT) distinguished the *S. vulgaris* NSII positive group from the remaining groups to some extent.

Conclusions

Plasma-TEG and CAT showed hemostatic changes in the *S. vulgaris* NSII negative and positive groups. An overlap with the other groups made them suboptimal as diagnostic markers, yet they provided valuable information on hemostasis. A model combining inflammatory and hemostatic markers seemed to differentiate the *S. vulgaris* NSII positive group from the remaining groups.

Keywords

Equine, global hemostatic tests, hypercoagulability, non-strangulating intestinal infarctions, diagnostic biomarkers

Background

Strongylus vulgaris is considered the most pathogenic intestinal parasite in the horse (1,2). It has a nearly six-month long prepatent phase during which it migrates in the cranial mesenteric artery and its branches. This migratory phase disrupts the endothelium of the arterial wall, which leads to verminous arteritis, thrombosis, and ultimately non-strangulating intestinal infarctions (NSII) in some horses (3). It is challenging to differentiate horses with idiopathic peritonitis from horses with NSII, as both are dominated by peritoneal inflammation (4,5). Both groups often present with mild intermittent colic, pyrexia, and peritonitis (4–6). Horses with idiopathic peritonitis most often require medical treatment, in contrast to horses with NSII that need immediate surgery for intestinal resection in order to survive (3–5). It is currently unknown why only some horses develop NSII (7,8), and it could be speculated that the development of NSII is related to horses experiencing a concomitant systemic inflammation that tips the hemostatic balance. An increase in the prevalence of *S. vulgaris* has been identified in Denmark (3,6,9), Sweden (10), and Italy (11) in recent decades. In the Nordic countries, it has been suggested that this is the result of a selective treatment strategy (9,10) combined with the difficulties in diagnosing *S. vulgaris* during the migratory larval stages. The only current definitive diagnostic method for NSII is an exploratory laparotomy or postmortem examination (6). Neither clinical nor standard laboratory variables are able to differentiate horses with NSII from horses with e.g. idiopathic peritonitis or enterocolitis.

Pathophysiological alterations in the vasculature caused by the migrating stages of *S. vulgaris* affect the hemostasis of the host (2,12,13). In equine medicine, conventional hemostatic markers such as activated partial thromboplastin time (aPTT), prothrombin time (PT), antithrombin (AT), and d-dimer concentration have most commonly been used to evaluate hemostatic changes (14,15). However, these are static snapshots and only provide information about isolated aspects of the hemostatic process (16–19). Global hemostatic tests evaluate the entire hemostatic process from initiation to clot formation and fibrinolysis (20,21). Whole blood thromboelastography (TEG) has previously been used in horses (14,22–25). This method requires blood samples to be analyzed 30 min. after sampling to avoid unreliable results (24), which renders this test inaccessible for clinics without a TEG machine. Studies in dogs (26), pigs (27), and a recent study in horses (28) have examined TEG analyzed on citrated plasma. Despite the lack of cellular components, plasma-TEG has the advantage that samples can be stored for longer than whole blood prior to analysis, making TEG more available within a clinical setting. The Calibrated Automated Thrombogram (CAT) assay is used in humans (29–31) and dogs (32) and has recently been evaluated for use with equine citrated plasma (28). The CAT assay is a global hemostatic test, which allows for the direct measurement of thrombin generation. In human medicine, it has been used to evaluate both hypo- and hypercoagulable conditions (29,33,34). Both plasma-TEG and the CAT assay have recently been shown to detect hypercoagulability in horses with severe gastrointestinal (GI) disease (28), and it would therefore be of interest to examine their potential as diagnostic markers in horses with *S. vulgaris* both with and without clinical disease (NSII) in combination with systemic inflammatory markers. The objectives of this study were to investigate whether horses with migrating *S. vulgaris* with and without NSII had hemostatic changes measurable with plasma-TEG and CAT, and whether they were distinguishable

from clinically healthy horses (without *S. vulgaris*) and horses with severe GI diseases mimicking NSII.

Results

Horses

The inclusion and exclusion of samples collected at the four facilities can be found in Fig. 1. Demographics for the six groups can be found in Table 1s (supporting information). There were no significant differences among groups for age or body weight (BW). A significant ($p < 0.05$) variation was seen in sex and breed distribution.

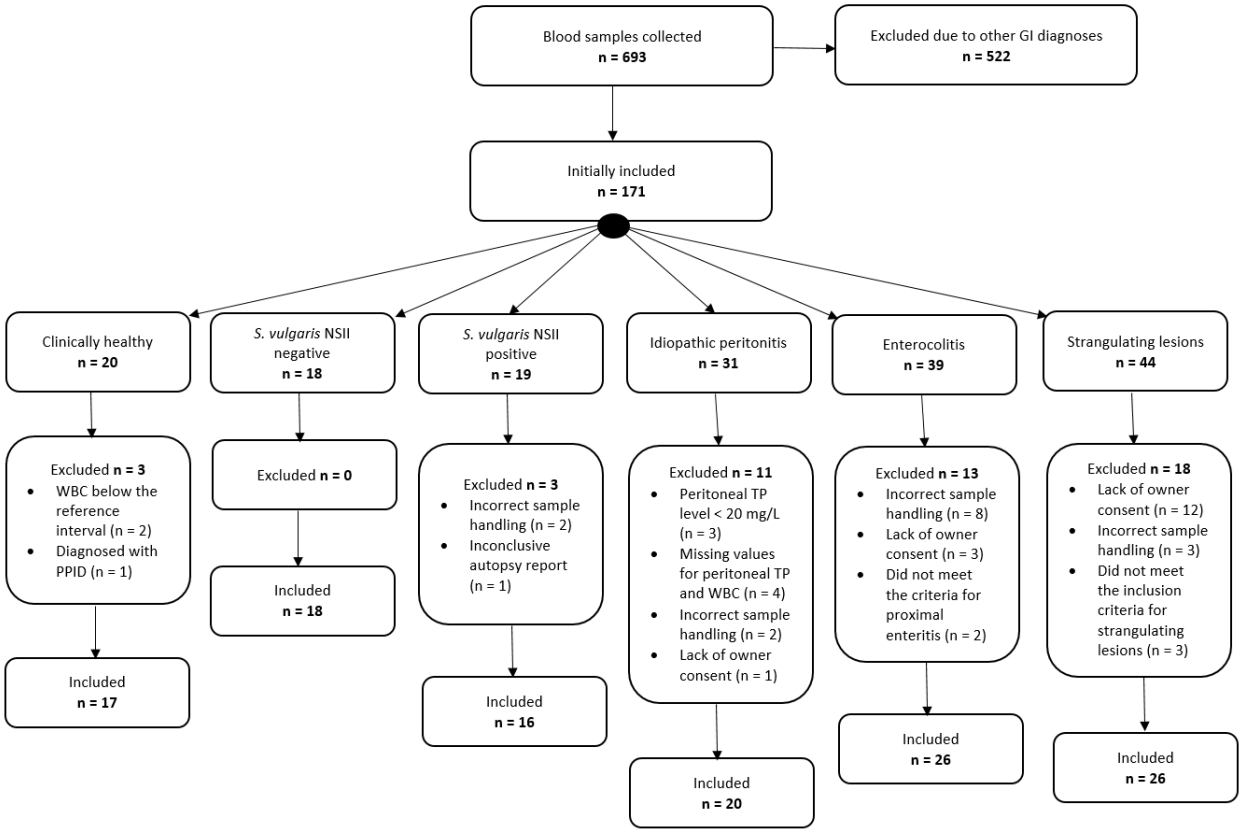


Figure 1. An overview of the total number of sampled horses and inclusion and exclusion from each of the six groups: clinically healthy horses without migrating *Strongylus vulgaris*, horses with migrating *S. vulgaris* without clinical disease (*S. vulgaris* non-strangulating intestinal infarction (NSII) negative group), horses with migrating *S. vulgaris* with clinical disease (*S. vulgaris* NSII positive group), horses with idiopathic peritonitis, horses with enterocolitis, and horses with strangulating lesions. GI: gastrointestinal, PPID: Pituitary Pars Intermedia Dysfunction, TP: total protein, WBC: white blood cell count.

From the *S. vulgaris* NSII positive group 8/16 horses originated from one of the two Swedish sampling centers (the University Animal Hospital at the Swedish University of Agricultural Sciences, or Evidensia Helsingborg Equine Referral Hospital), with it being so for 17/20 horses with idiopathic peritonitis.

Group comparison (plasma-thromboelastography)

A group comparison of the investigated plasma-TEG variables can be found in Fig. 2 and Table 2s (supporting information). The *S. vulgaris* NSII negative group had a significantly increased angle ($p = 0.0004$), maximum amplitude (MA) ($p = 0.01$), and shear elastic force (G) ($p = 0.003$) compared to the clinically healthy group. The *S. vulgaris* NSII positive group had a significantly longer reaction time (R) ($p = 0.04$) than the horses with strangulating lesions. In addition the *S. vulgaris* NSII positive group had a significantly higher MA than the enterocolitis ($p = 0.02$) and strangulating lesions ($p = 0.02$) groups, as well as a significantly higher G than the enterocolitis ($p = 0.03$) and strangulating lesions ($p = 0.03$) groups.

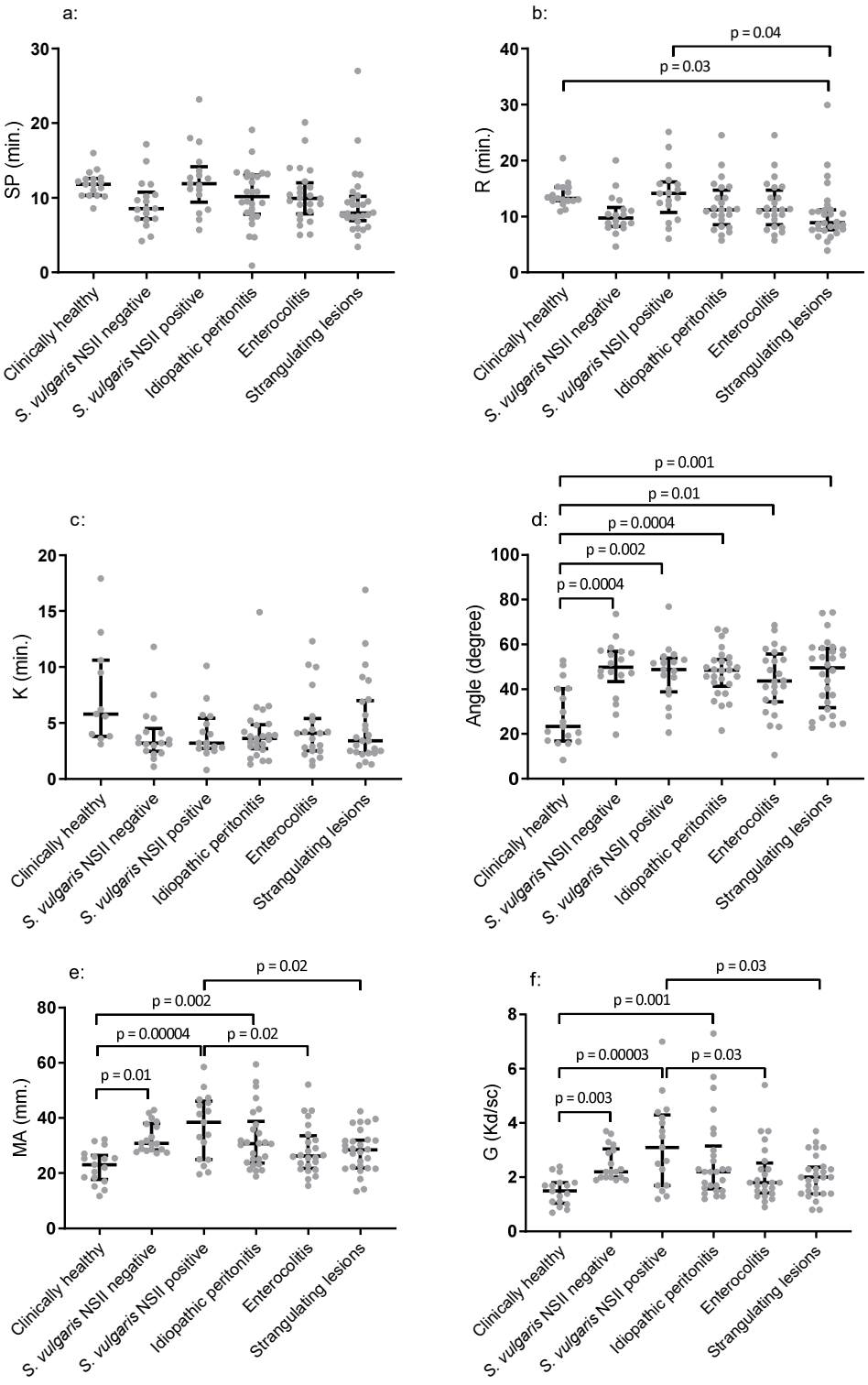


Figure 2. Group comparison for plasma-thromboelastography (TEG) across the six groups of horses: clinically healthy horses without migrating *Strongylus vulgaris*, horses with migrating *S. vulgaris* without clinical signs (*S. vulgaris* non-strangulating intestinal infarction (NSII) negative group), horses with migrating *S. vulgaris* with clinical disease (*S. vulgaris* NSII positive group), horses with idiopathic peritonitis, horses with enterocolitis, and horses with strangulating lesions. Lines show the median and interquartile range. SP: split point, MA: maximum amplitude, G: shear elastic force.

Group comparison (Calibrated Automated Thrombogram)

A group comparison of the investigated CAT variables can be found in Fig. 3 and Table 3s (supporting information). The *S. vulgaris* NSII negative group had a significantly longer lag time (p = 0.02) than the clinically healthy group. The *S. vulgaris* NSII positive group had a significantly longer lag time than the horses with enterocolitis (p = 0.01) and strangulating lesions (p = 0.001). In addition, the *S. vulgaris* NSII positive group had a significantly longer time to peak (ttPeak) than the enterocolitis group (p = 0.002) and the horses with strangulating lesions (p = 0.002).

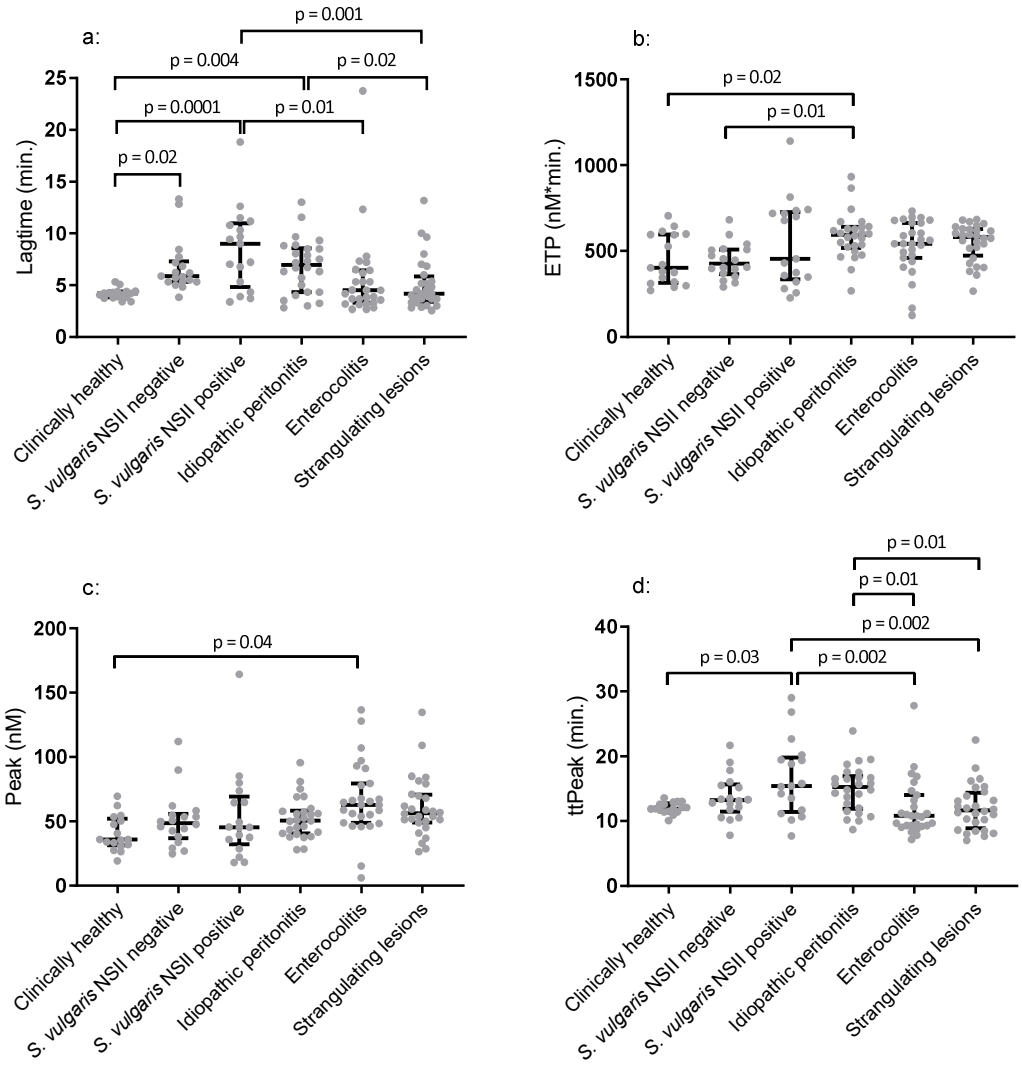


Figure 3. Group comparison for the Calibrated Automated Thrombogram (CAT) across the six groups of horses: clinically healthy horses without migrating *Strongylus vulgaris*, horses with migrating *S. vulgaris* without clinical disease (*S. vulgaris* non-strangulating intestinal infarction (NSII) negative group), horses with migrating *S. vulgaris* with clinical disease (*S. vulgaris* NSII positive group), horses with idiopathic peritonitis, horses with enterocolitis, and horses with strangulating lesions. Lines show the median and interquartile range. ETP: endogenous thrombin potential, ttPeak: time to peak.

Group comparison (clinical and laboratory variables)

A comparison of the investigated clinical variables for each group can be found in Table 4s (supporting information). Clinical variables were not available for the *S. vulgaris* NSII negative group due to their semi-feral nature. There were no significant differences in heart rate (HR) among the groups with GI disease. Horses with idiopathic peritonitis had a significantly lower respiration rate (RR) than horses with strangulating lesions ($p = 0.001$). Rectal temperature was significantly higher in the *S. vulgaris* NSII positive group ($p = 0.02$) and idiopathic peritonitis group ($p < 0.0001$) than in the strangulating lesions group, as well as in the idiopathic peritonitis group ($p = 0.0002$) compared to the enterocolitis group.

A comparison of the investigated laboratory variables for each group can be found in Fig. 4 and Table 5s (supporting information). The *S. vulgaris* NSII positive group had significantly lower hematocrit (HCT) ($p = 0.04$) and iron ($p = 0.01$) than the enterocolitis group, and the iron concentration was also significantly lower than for the strangulating lesions group ($p < 0.0001$). Furthermore, the *S. vulgaris* NSII positive group had a significantly higher serum amyloid A (SAA) concentration than the enterocolitis ($p = 0.0003$) and strangulating lesions groups ($p < 0.0001$). The fibrinogen concentration was also significantly higher for the *S. vulgaris* NSII positive group than for the idiopathic peritonitis ($p = 0.03$) and strangulating lesions groups ($p = 0.003$). The strangulating lesions group had a significantly lower S. vulgaris antibody concentration ($p = 0.04$) than the *S. vulgaris* NSII positive group.

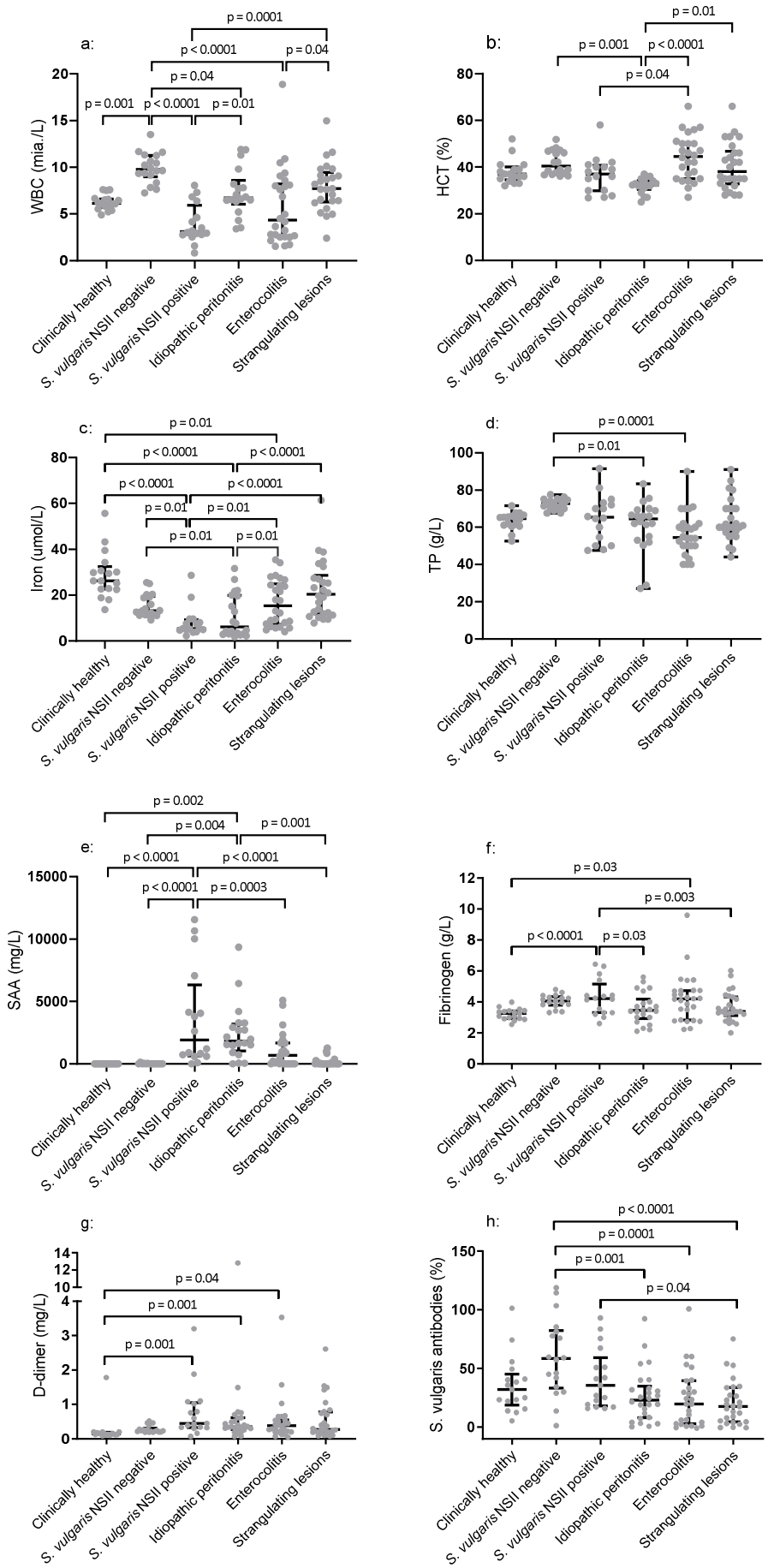

a:
WBC (mia./L)
p = 0.0001
p < 0.0001
p = 0.04
p = 0.04
p = 0.001
p < 0.0001
p = 0.01
b:
HCT (%)
p = 0.01
p = 0.001
p < 0.0001
p = 0.04
c:
Iron (umol/L)
p = 0.01
p < 0.0001
p < 0.0001
p < 0.0001
p < 0.0001
p = 0.01
p = 0.01
p = 0.01
p = 0.01
d:
TP (g/L)
p = 0.0001
p = 0.01
e:
SAA (mg/L)
p = 0.002
p = 0.004
p = 0.001
p < 0.0001
p < 0.0001
p < 0.0001
p = 0.0003
f:
Fibrinogen (g/L)
p = 0.03
p = 0.003
p < 0.0001
p = 0.03
g:
D-dimer (mg/L)
p = 0.04
p = 0.001
p = 0.001
h:
S. vulgaris antibodies (%)
p < 0.0001
p = 0.0001
p = 0.001
p = 0.04
Clinically healthy
S. vulgaris NSII negative
S. vulgaris NSII positive
Idiopathic peritonitis
Enterocolitis
Strangulating lesions

Figure 4. Group comparison for laboratory parameters across the six groups of horses: clinically healthy horses without migrating *Strongylus vulgaris*, horses with migrating *S. vulgaris* without clinical disease (*S. vulgaris* non-strangulating intestinal infarction (NSII) negative group), horses with migrating *S. vulgaris* with clinical disease (*S. vulgaris* NSII positive group), horses with idiopathic peritonitis, horses with enterocolitis, and horses with strangulating lesions. Lines show the median and interquartile range. WBC: white blood cell count, HCT: hematocrit, SAA: serum amyloid A.

Differentiating horses with non-strangulating intestinal infarctions

With the use of logistic regression, a model including iron, SAA, angle (plasma-TEG), and endogenous thrombin potential (ETP) (CAT) was able to classify whether or not horses belonged to the *S. vulgaris* NSII positive group (Table 1, Fig. 5, Fig. 6).

Table 1. The model based on logistic regression included iron, serum amyloid A (SAA), angle (plasma-thromboelastography), and endogenous thrombin potential (ETP) (Calibrated Automated Thrombogram) and was able to classify whether or not horses belonged to the *Strongylus vulgaris* non-strangulating intestinal infarction (NSII) positive group.

	Estimate	**Standard Error**	**P-value**
Intercept	1.74	1.58	0.27
Iron (mmol/L)	-0.14	0.06	0.01
SAA (mg/L)	0.0008	0.0002	0.0002
Angle (degree)	0.05	0.03	0.14
ETP (nM*min.)	-0.01	0.004	0.002

A decreased iron concentration, increased SAA concentration, increased angle (plasma-TEG), and decreased ETP (CAT) were indicative of belonging to the *S. vulgaris* NSII positive group (Table 2).

Table 2. The odds including 95% confidence interval (CI) for iron, serum amyloid A (SAA), angle, and endogenous thrombin potential (ETP) of being classified in the *Strongylus vulgaris* non-strangulating intestinal infarction (NSII) positive group. The considered change for SAA is +/- 1,000. For the remaining variables, it is +/- 1.

	Odds	**95% CI**
Iron (mmol/L)	0.87	0.78 - 0.97
SAA (mg/L)	2.31	1.48 - 3.6
Angle (degree)	1.05	0.99 - 1.11
ETP (nM*min.)	0.99	0.98 - 0.99

A probability set to 0.4 correctly classified 10/16 horses in the *S. vulgaris* NSII positive group, with 6/16 horses as false negatives, and 5/102 horses as false positives (Fig. 5).

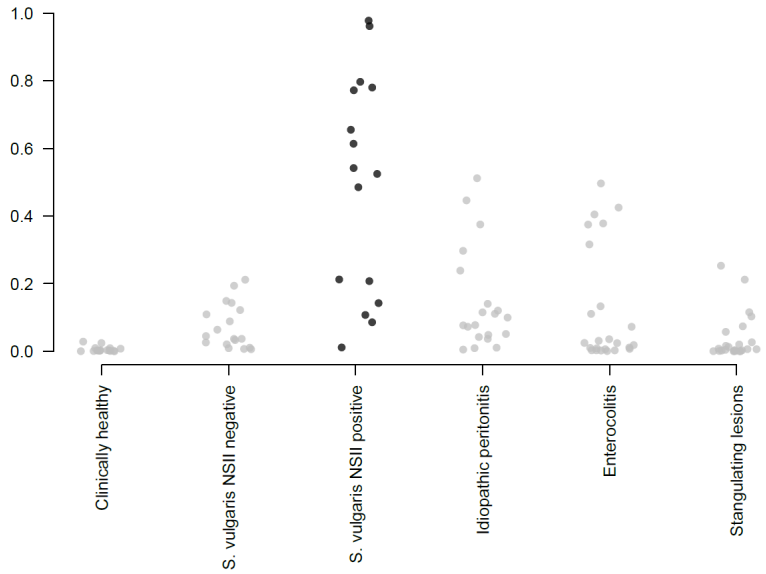


Figure 5. The estimated probability of being classified in the *Strongylus vulgaris* non-strangulating intestinal infarctions (NSII) positive group based on a model including iron, serum amyloid A (SAA), angle (plasma-thromboelastography), and endogenous thrombin potential (ETP) (Calibrated Automated Thrombogram).

The cross-validated receiver operating curve (ROC) curve had an area under the curve (AUC) of 0.83 and a cut-off set to 51.2% with an accuracy of 90.0%, which resulted in 2/103 being classified as false positives and 10/16 as false negatives (Fig. 6).

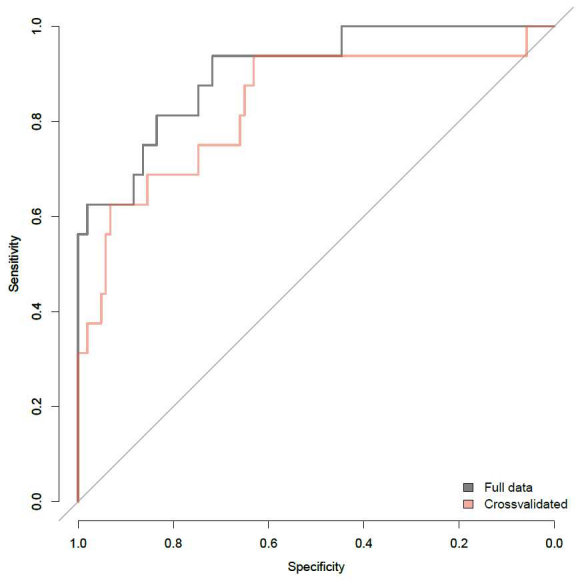


Figure 6. Receiver operating curves (ROC) for the full dataset (black line) and the cross-validation (red line) for the logistic regression model including iron, serum amyloid A (SAA), angle (plasma-thromboelastography), and endogenous thrombin potential (ETP) (Calibrated Automated Thrombogram).

Using a multinomial regression, the clinically healthy horses without *S. vulgaris* were classified with 100% accuracy by the model without any overlap from the remaining groups. The *S. vulgaris* NSII negative group was also accurately classified. The four groups with severe GI disease showed a more substantial overlap in their predicted classification probabilities. The *S. vulgaris* NSII positive group showed a particularly marked overlap with the idiopathic peritonitis group, while the enterocolitis group showed a marked overlap with the strangulating lesions group (Fig. 7).

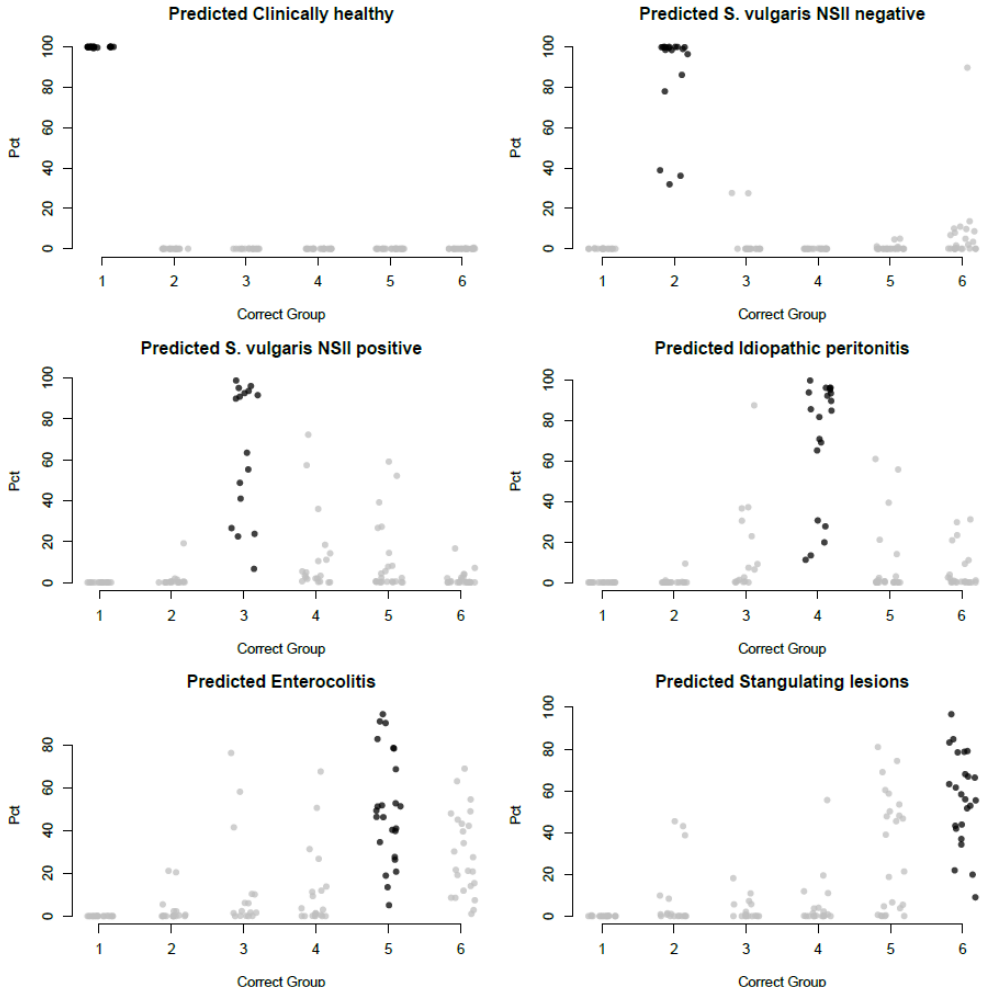


Figure 7. A dot plot based on a multinomial regression showing the predicted probability of being classified in the six different groups of horses (clinically healthy horses without migrating *Strongylus vulgaris*, horses with migrating *S. vulgaris* without clinical disease (*S. vulgaris* non-strangulating intestinal infarction (NSII) negative group), horses with migrating *S. vulgaris* with clinical disease (*S. vulgaris* NSII positive group), horses with idiopathic peritonitis, horses with enterocolitis, and horses with strangulating lesions) based on a model including iron, serum amyloid A (SAA), angle (plasma-thromboelastography), and endogenous thrombin potential (ETP) (Calibrated Automated Thrombogram).

Discussion

Plasma-TEG and CAT showed significant hemostatic changes in the *S. vulgaris* NSII negative and positive groups compared to clinically healthy horses without *S. vulgaris* and the remaining groups with severe GI disease (idiopathic peritonitis, enterocolitis, and strangulating lesions) (Fig. 2 and Fig. 3).

The inclusion of the *S. vulgaris* NSII negative group is unique and provides valuable information concerning subclinical changes in hemostatic variables within this population. This may support our understanding of the hemostatic pathophysiological changes caused by the parasite without the presence of a simultaneous systemic inflammation. In this study, both the *S. vulgaris* NSII negative group and the *S. vulgaris* NSII positive group had mixed hemostatic changes, as seen in Fig. 2 and Fig. 3. While the *S. vulgaris* NSII negative group were found to be hypercoagulable based on the plasma-TEG variables (23,35–37) when compared to clinically healthy horses (without *S. vulgaris*) (Fig. 2), a longer lag time in the CAT assay (Fig. 3) could be interpreted as a sign of hypocoagulability (38). For the *S. vulgaris* NSII positive group, plasma-TEG demonstrated a prolonged R time (Fig. 2), and CAT revealed a prolonged lag time and ttPeak (Fig. 3), which could be interpreted as hypocoagulability (23,35–38). In contrast, the *S. vulgaris* NSII positive group also had an increased MA and G for plasma-TEG (Fig. 2), which could be interpreted as hypercoagulability (23,35–37). It therefore seems that there was a prolonged initiation of clot formation, but an increased clot strength once the clot had formed. There is currently no precise explanation for this, but it could be related to the different sensitivities of the two assays in detecting hemostatic changes and the choice of activator in each assay. Both plasma-TEG and CAT have only recently been used in equine medicine (28) and there is a substantial overlap for the investigated variables among the groups (Fig. 2 and 3). These findings should be explored further in future studies. However, for the *S. vulgaris* NSII positive group, it could be speculated that a delay in admission to a hospital facility in some of these cases might occur as they often initially present with pyrexia and none too mild colic signs (6). In addition, as horses with NSII caused by *S. vulgaris* mimic horses with idiopathic peritonitis and enterocolitis requiring medical treatment (4,39,40), the timespan from admission to surgery might also be prolonged. The long initiation phase in the assays could therefore be explained by a consumption of procoagulant factors, insufficient coagulation factor activity, or an imbalance in the regulatory and inhibitory hemostatic system. The increased clot strength could be explained by the presence of microparticles from activated endothelial cells (41) or an imbalance in the fibrinolytic system. These hypotheses should be investigated further. Nonetheless, global hemostatic markers still proved valuable in assessing the hemostatic balance of the horse.

The identified model showed that a combination of inflammatory markers (decreased iron concentration and increased SAA concentration) and hemostatic parameters (increased angle and decreased ETP) seemed to be able to distinguish the *S. vulgaris* NSII positive group from the remaining groups (Table 2). However, using this model as the sole diagnostic tool would mean that some horses in the *S. vulgaris* NSII positive group would go unnoticed (Fig. 5), resulting in a fatal outcome. This model can therefore not stand alone in diagnosing NSII caused by *S. vulgaris*. It may be that combining this model with clinical findings indicative of NSII might add to the diagnostic

sensitivity of the model. As a consequence, it appears that the safest protocol in areas with a high prevalence of *S. vulgaris* in horses with a history of insufficient deworming presenting with a rectal mass, septic peritonitis, and during winter (5,6,42) would be to recommend an explorative laparotomy to confirm or refute NSII (5,42). This might be unrealistic in countries with a high prevalence of idiopathic peritonitis (42), thus underlining the need for a specific diagnostic tool for NSII.

It would be interesting to explore why the *S. vulgaris* NSII negative group with large numbers of *S. vulgaris* and clear hemostatic changes do not develop clinical disease. One likely explanation is that this herd is subject to minimal external stressors. They are not exercised, transported, or moved, and remain within the same herd under the same management routines. Stress has been shown to negatively influence the immune response (43,44) and it is possible that horses can cope with a *S. vulgaris* infection as long as they do not experience considerable stress and are not affected by concurrent disease causing systemic inflammation. This is supported by the established model, which includes both inflammatory markers and hemostatic parameters in order to distinguish the *S. vulgaris* NSII positive group.

As shown in Fig. 7, there was a marked overlap between the *S. vulgaris* NSII positive group, the idiopathic peritonitis group, and the enterocolitis group when the model was used to predict the *S. vulgaris* NSII positive group. It is challenging to differentiate horses with idiopathic peritonitis (4–6) and peracute enterocolitis (5,39,40) from horses with NSII (4–6), though horses with enterocolitis are often more systemically affected, as reflected by the higher HCT (Fig. 4). In this study, we could not be certain that the horses with idiopathic peritonitis, enterocolitis, and strangulating lesions were free of migrating *S. vulgaris* larvae, but none of them suffered from NSII. It could therefore be speculated that some of the horses in these disease groups, both in the study and in general, could also have lesions caused by migrating *S. vulgaris* larvae.

Only horses with strangulating lesions had a *S. vulgaris* antibody concentration that differed from the *S. vulgaris* NSII positive group. In addition, the clinically healthy horses without *S. vulgaris* had antibody concentrations that would be considered positive for *S. vulgaris* (5,45). However, the finding of seropositive horses in the clinically healthy group is not surprising as *S. vulgaris* is enzootic in Denmark. The clinically healthy horses were treated with an anthelmintic product containing either ivermectin or moxidectin two weeks prior to being sampled. It has been shown that horses treated with ivermectin can have a significant increase in *S. vulgaris* antibody concentration for up to 53 days post treatment, and that it can take up to five months for concentrations to decrease back to normal (46). The finding therefore indicates that the clinically healthy horses had a current or previous infection with *S. vulgaris* at the time of anthelmintic treatment, and paired antibody measurements could potentially help to clarify this. Furthermore, there could also be a level of cross-reactivity with other large strongyles, although this seems less likely from the literature (45). The antibody concentration therefore does not seem applicable as a diagnostic marker for acute disease related to *S. vulgaris* in a clinical setting.

This study should be considered as an exploratory study laying the groundwork for further research. Even though the total number of horses included in this study was relatively large, assigning them to

six different groups rendered the group sizes relatively small. *A priori* power calculations indicated that a group size of around 20 horses per group should be sufficient, but a larger population of horses was required for some variables due to a large overlap between groups.

Conclusion

Plasma-TEG and CAT showed significant hemostatic changes in the *S. vulgaris* NSII negative and positive horses. Both groups had a mixture of hemostatic changes and could be considered both hypo- and hypercoagulable compared to the clinically healthy horses without *S. vulgaris* and horses with other severe GI diseases (idiopathic peritonitis, enterocolitis, and strangulating lesions). A model combining serum iron, SAA, angle, and ETP was able to distinguish the *S. vulgaris* NSII positive group from the remaining groups to a certain extent. Global hemostatic tests appear to be useful in describing the horse's hemostatic balance and can aid in assessing the risk of NSII.

Methods

This was an explorative international multicenter study. Samples were collected at ***a)*** the Large Animal Teaching Hospital at the University of Copenhagen, Denmark from December 2017 through March 2021; ***b)*** the University Animal Hospital at the Swedish University of Agricultural Sciences, Sweden, and ***c)*** Evidensia Helsingborg Equine Referral Hospital, Sweden, from January 2018 through March 2021. In addition, samples were collected at ***d)*** Gluck Equine Research Center at the University of Kentucky, USA, in October and November 2018. Approval was obtained from the ethical boards at each facility and written consent was acquired from the owners of the horses.

Horses

Six groups of horses were included in the study: *i)* clinically healthy horses without *S. vulgaris*, *ii)* horses with migrating *S. vulgaris* larvae without clinically overt disease (*S. vulgaris* NSII negative), *iii)* horses with migrating *S. vulgaris* larvae with clinically overt disease (*S. vulgaris* NSII positive), *iv)* horses with idiopathic peritonitis, *v)* horses with enterocolitis, and *vi)* horses with strangulating intestinal lesions. Horses were excluded from the study if they were younger than one year. For specific inclusion criteria for each group, see Table 3.

Table 3. Inclusion criteria for the six groups of horses.

Group	Sample facility	Inclusion criteria
i) Clinically healthy	*a*	- Horses with a clinical examination (heart rate, respiration rate, rectal temperature, borborygmus), complete blood count (CBC), and serum biochemistry profiles including serum amyloid A (SAA) and fibrinogen concentration within normal limits. - No recent history or current signs of colic. - No clinical or laboratory signs of acute or chronic systemic inflammation. - Treated with an anthelmintic treatment containing either ivermectin or moxidectin between one and two weeks prior to being examined and samples being collected.
ii) S. vulgaris NSII negative	*d*	- Horses with migrating *S. vulgaris* larvae without clinically overt disease (NSII), i.e. no recent history or current signs of colic. - A well-described herd with no anthelmintic intervention since 1979 (47), in which large strongyles including *S. vulgaris* are highly prevalent (7,8). - Presence of *S. vulgaris* confirmed via coproculture as previously described (48) and a positive *S. vulgaris* antibody titer (> 13.47%) (45). - The antibody titer was established with an indirect antibody enzyme-linked immunosorbent assay (ELISA) using recombinant SvSXP protein as antigen as previously described (3,45). Results were reported as the normalized value, percentage of a positive control (PP) (45). - Horses were semi-feral and of mixed light breed.

iii) S. vulgaris NSII positive	*a, b, c*	- Horses with migrating *S. vulgaris* larvae with clinically overt disease (NSII)
		- Confirmed at surgery or postmortem examination.
		- Criteria for NSII were as previously described (6):
		  - One or more areas of localized intestinal infarction identified at surgery or postmortem examination without signs of strangulation such as a clear volvulus or signs of diffuse enterocolitis.
		  - Additionally, in cases of euthanasia, horses in this group were found to have signs of migrating *S. vulgaris* larvae, seen as thrombosis and arteritis, in the cranial mesenteric artery and/or its branches, present at postmortem examination.
		  - Horses that survived had signs of arteritis and/or larvae present on histology of the resected intestinal segment.

iv) Idiopathic peritonitis	*a, b, c*	- Horses with a peritoneal fluid sample with a white blood cell count (WBC) > $10x10^9$ cells/L and a peritoneal total protein > 20 g/L. - Horses survived and recovered solely with medical treatment or had no apparent cause for the peritonitis identified at surgery or postmortem (49).
v) Enterocolitis	*a*	Horses with peracute and acute colitis: - Horses referred for evaluation of acute abdominal pain or diarrhea of maximum 48 hours duration prior to admission, - or identified with diarrhea within 48 of admission, - or horses without diarrhea but a very short, intense course of disease and a postmortem diagnosis of enterocolitis or typhlocolitis.

		- Postmortem diagnosis was based on macroscopic signs of generalized intestinal inflammation (hyperemia, necrosis and edema) with no signs of strangulation. Horses with duodenitis-proximal jejunitis: - Horses with excessive gastric reflux (> 20 L) of > 24 hours duration that responded to medical treatment or where no concomitant mechanical obstruction was identified at surgery or postmortem (50).
v) Strangulating lesions	*a*	- One or more strangulating intestinal lesions in the ileum, jejunum, and/or ascending colon confirmed at either surgery or postmortem.

Power calculations based on ETP, R, angle, and MA values from a recent study looking at plasma-TEG and CAT in horses with GI disease (28) indicated that around 16-35 horses in each group would be sufficient to detect a potential difference.

Blood sample handling and blood analysis

As described previously (28) blood samples were taken by jugular venipuncture using a 21 g needle and a vacutainer system. Blood tubes were collected starting with the 3.2% 0.109M sodium citrate tubes with a 1:9 citrate/blood ratio, then serum separator tubes, and lastly EDTA tubes, which is the recommended order (51). The first sodium citrate tube was discarded. The remaining sodium citrate blood tubes were used for hemostatic analysis (fibrinogen, d-dimer, plasma-TEG, and CAT). The serum tubes were used for routine biochemical analysis including iron and SAA, and the EDTA tubes were used for complete blood cell count (CBC).

Samples collected at the Large Animal Teaching Hospital at the University of Copenhagen were analyzed according to in-house protocols for CBC (ADVIA 2120i, Siemens Healthcare A/S, Ballerup, Denmark) and biochemistry (ADVIA 1800, Siemens Healthcare A/S, Ballerup, Denmark). The latter was also used for samples from the *S. vulgaris* NSII negative group and samples collected at the University Animal Hospital at the Swedish University of Agricultural Science and Evidensia Helsingborg Equine Referral Hospital. For the *S. vulgaris* NSII negative group, CBC was analyzed according to in-house protocols at the University of Kentucky Vet Diagnostic Laboratory (Genesis Hematology Analyzer, Oxford Science, Oxford, Connecticut, USA). For horses from Sweden, CBC was analyzed according to in-house protocols at the University Animal Hospital at the Swedish University of Agricultural Science (ADVIA 2120i, Siemens, Erlangen, Germany) or Evidensia Helsingborg Equine Referral Hospital (Idexx Procyte Dx, Idexx Europe, Netherlands). The serum and EDTA samples were stored at 4°C until analysis. The sodium citrate tubes were centrifuged at room temperature at 2,000 *g* for 15 min. within a maximum of four hours from sample collection (32). The citrated plasma was stored at -80°C until batch analysis (plasma TEG, CAT, d-dimer, and fibrinogen concentration). Samples were thawed in a water bath for 4 minutes at 37°C and thoroughly mixed prior to analysis (51).

Hemostatic assays

Plasma-TEG was performed as previously described (26,28,52). Diluted tissue factor (TF) was applied as the activator using a computerized thromboelastograph (TEG 5000 Hemostasis Analyzer, Haemoscope Corporation, Illinois, USA). The TEG machines were prior to daily use evaluated with an E-test, which is an electronic quality control, and analyses were run according to the manufacturer's protocol. The recorded plasma-TEG variables are described in Table 4.

Table 4. Variables recorded for plasma-thromboelastography (TEG).

Variable	Unit	Description
Split point (SP)	min.	Time to initial conversion of fibrinogen to fibrin.
Reaction time (R)	min.	Time to initial fibrin clot formation.
Clotting time (K)	min.	Time from initial clot formation until a preset level of clot strength is reached (20 mm).
Angle	Degrees	Speed of fibrin build up and cross-linking.
Maximum amplitude (MA)	mm.	Maximum clot strength.
Shear elastic force (G)	(dynes/cm^2)	A linear function of the MA and a measure of global clot strength.
Lysis 30 min. (Ly30)	%	Degree of fibrinolysis 30 minutes after MA.
Lysis 60 min. (Ly60)	%	Degree of fibrinolysis 60 minutes after MA.

Thrombin generation was measured using the CAT assay (Thrombinoscope BV, Maastricht, The Netherlands) (28,32). The recorded CAT variables are described in Table 5.

Table 5. Variables recorded for the Calibrated Automated Thrombogram (CAT) assay.

Variable	Unit	Description
Lag time	min.	Time until 1/6 of the total thrombin concentration is reached.
Endogenous Thrombin potential (ETP)	nM*min.	The total amount of thrombin generated.
Peak	nM	Maximal thrombin concentration.
Time to peak (ttPeak)	min.	Time to maximal thrombin concentration.

As previously described (28), the assay was activated by a trigger solution (PPP reagent) (Triolab AS, Denmark). The temperature was set to 37.5°C, with a measuring interval of 20 seconds, and the measured time was set to 45 minutes. All analyses were performed in duplicate. In each well, 80 µl of citrated plasma was added to 20 µl of either the activator solution or thrombin calibrator solution as per the manufacturer's recommendation and as previously described (28,32). The activator solution used contained 5 pM TF and 4 µM phospholipids. Thrombin generation was activated by adding 20

µl of FluCa, consisting of Fluo-Buffer containing $CaCl_2$ and a fluorescent reagent, and was read by an automated plate reader.

For plasma-TEG, hypercoagulability was defined as a shorter R and/or K time and/or an increased angle, MA, and G, with one or more altered variables compared to the clinically healthy group (23,35–37). Hypercoagulability for CAT was defined as a shortened lag time and/or ttPeak and/or a higher ETP and peak with one or more altered variables compared to the clinically healthy group (38). For both tests, the opposite finding was defined as hypocoagulability.

The fibrinogen concentration was measured on an ACL Top 500 with a PT-based assay using HemosIL RecombiPlastin (ILS Danmark, Allerød, Denmark) as previously described (28). D-dimer was measured on the automated machine STAGO STA Satellite coagulation analyzer (Triolab, Brøndby, Denmark). D-dimer was analyzed using STA-Liatest D-Di+, which is a photometric antibody-antigen assay based on murine D-dimer antibodies (15,16).

Additional variables

All samples were analyzed for *S. vulgaris*-specific antibodies expressed as a percentage of a positive control (PP) using a validated ELISA at the University of Kentucky, as previously described (3,45) (Table 3).

Statistical analysis

Analyses were performed using R (R Core Team (2020) R: A Language and Environment for Statistical Computing, Addison R Foundation for Statistical Computing, Vienna, Austria) and GraphPad Prism 8.3.0 (San Diego, California, USA). The probability of significance was set to 5%. Normality was assessed visually and using Shapiro-Wilk's test. Where necessary, data were log or log2 transformed in order to achieve normality. ANOVA was used to compare groups with regard to: HR, RR, temperature, white blood cell count (WBC), HCT, platelet count (PLT), total protein (TP), iron, SAA, fibrinogen, mean platelet volume (MPV), mean platelet component (MPC), d-dimer, *S. vulgaris* antibodies, for plasma TEG: SP, R, K, angle, MA, G, Ly30, Ly60, and for CAT: lag time, ETP, peak and ttPeak. *Post hoc* analyses were carried out with Tukey's multiple comparisons test. A logistic regression analysis was performed to assess potential differences in chosen diagnostic variables (iron, SAA, fibrinogen, *S. vulgaris* antibodies, d-dimer; plasma-TEG: R, K, angle, MA; CAT: lag time, ETP, peak, and ttPeak) linked to the six groups of horses. Initially, data imputation was performed to address a number of missing values (for details, see the Statistical appendix in the supporting information), then a logistic regression model was used to analyze differences between the *S. vulgaris* NSII positive group (y=1) and the other groups (y=0). For details on model selection and diagnostics, please refer to the Statistical appendix (supporting information). Two ROC curves were generated from the model, one with all data included when fitting the model and the other using a jackknife, i.e. leave-one-out, cross-validation. As an extension to this analysis, a multinomial regression model was used to investigate differences across all six groups.

Abbreviations

aPTT	Activated partial thromboplastin time
AT	Antithrombin
AUC	Area under the curve
BW	Body weight
CAT	Calibrated Automated Thrombogram
CBC	Complete blood count
CI	confidence interval
ELISA	Enzyme-linked immunosorbent assay
ETP	Endogenous thrombin potential (CAT)
G	Shear elastic force (TEG)
GI	Gastrointestinal
HCT	Hematocrit
HR	Heart rate
K	Clotting time (TEG)
LY30	Fibrinolysis 30 min after MA (TEG)
LY60	Fibrinolysis 60 min after MA (TEG)
MA	Maximum amplitude (TEG)
MPC	Mean platelet component
MPV	Mean platelet volume
NSII	Non-strangulating intestinal infarction
PLT	Platelet
PT	Prothrombin time
R	Reaction time (TEG)
ROC	Receiver operating curve
RR	Respiration rate
SAA	Serum amyloid A
SP	Split point (TEG)
TEG	Thromboelastography
TF	Tissue factor
TP	Total protein
ttPeak	Time to peak (CAT)
WBC	White blood cell count

Declarations

Ethics approval and consent to participate

Samples were collected at the Large Animal Teaching Hospital at the University of Copenhagen, Denmark from December 2017 through March 2021; the University Animal Hospital at the Swedish University of Agricultural Sciences, Sweden, and Evidensia Helsingborg Equine Referral Hospital, Sweden, from January 2018 through March 2021. In addition, samples were collected at Gluck Equine Research Center at the University of Kentucky, USA, in October and November 2018. Approval was obtained from the ethical boards at each facility and relevant guidelines and regulations were followed. Written consent from the owners of the horses was obtained. All procedures were carried out in accordance with the ARRIVE guidelines.

Consent for publication

Not applicable

Availability of data and materials

If deemed relevant or is of interest raw data can be submitted.

Competing interests

The authors declare that they have no competing interests

Funding

The Danish Horse Levy Foundation, the Swedish-Norwegian Foundation for Equine Research, and Sveland funded the project but played no role in the design, analysis and reporting of the study.

Authors' contributions

All authors have provided substantial contribution to the manuscript. MLH has contributed to the design of the work, in partial acquired the sample material, and owner consent. MLH has conducted and assisted in data analysis, interpreted results, and have drafted the manuscript and continuously revised it. JSØ has assisted with statistical analyses and have revised the manuscript. LNN has contributed with data interpretation and has revised the manuscript. ET, YHA, MR, KA have in partial acquired the sample material, and owner consent; and they have revised the manuscript. MKN has in partial acquired the sample material and have revised the manuscript. THP has contributed to the design of the work, the acquisition and interpretation of data and has revised the manuscript. All authors have read and approved the final manuscript.

Acknowledgements

The authors are grateful to medical laboratory technologist Louise Bochsen, laboratory technicians Birgitte Klitgaard and Tina Roust from the Department of Veterinary Clinical Sciences, Faculty of

Health and Medical Sciences (SUND), University of Copenhagen, Denmark for their laboratory assistance during this study.

Thank you to Professor Søren Saxmose Nielsen for assistance with statistical analysis.

The authors would also like to extend their gratitude to the clinical staff at the Large Animal Teaching Hospital at the University of Copenhagen; at the University Animal Hospital at the Swedish University of Agricultural Sciences, and at Evidensia Helsingborg Equine Referral Hospital for assisting with sample collection.

In addition, the authors would like to thank the farm crew at the Barn 10 research herd at the University of Kentucky's Maine Chance Farm. A big thank you also goes to undergraduate and graduate students at Martin K. Nielsen's laboratory, at the Gluck Equine Research Center, and at the University of Kentucky for assisting with sample collection.

We are grateful to the horse owners in Denmark and Sweden who kindly enrolled their horses in the study.

Lastly, the authors would like to acknowledge the Danish Horse Levy Foundation, Stiftelsen Hästforskning, and Sveland for funding the project.

References

1. Duncan JL, Pirie HM. The life cycle of *Strongylus vulgaris* in the horse. Res Vet Sci. 1972;13(4):374–9.
2. Duncan RB, Duncan JL, Pirie HM. The pathogenesis of single experimental infections with *Strongylus vulgaris* in foals. Res Vet Sci. 1975;18(1):82–93.
3. Nielsen MK, Jacobsen S, Olsen SN, Bousquet E, Pihl T. Nonstrangulating intestinal infarction associated with *Strongylus vulgaris* in referred Danish equine cases. Spec Focus Parasitol. 2016;48(3):376–9.
4. Odelros E, Kendall A, Hedberg-Alm Y, Pringle J. Idiopathic peritonitis in horses: A retrospective study of 130 cases in Sweden (2002-2017). Acta Vet Scand. 2019;61(1):1–8.
5. Poulsen L, Henriksson FV, Dahl M, Honoré ML, Olsen SN, Leifsson PS, et al. Differentiating *Strongylus vulgaris*-associated, non-strangulating intestinal infarctions from idiopathic peritonitis and acute colitis. Equine Vet J. 2021;(In review).
6. Pihl TH, Nielsen MK, Olsen SN, Leifsson PS, Jacobsen S. Nonstrangulating intestinal infarctions associated with *Strongylus vulgaris*: Clinical presentation and treatment outcomes of 30 horses (2008-2016). Equine Vet J. 2017;1–7.
7. Lyons ET, Tolliver SC, Stamper S, Drudge JH, Granstrom DE, Collins SS. Transmission of some species of internal parasites in horses born in 1990, 1991, and 1992 in the same pasture on a farm in central Kentucky. Vet Parasitol. 1994;52(3–4):257–69.
8. Lyons ET, Tolliver SC, Collins SS, Drudge JH, Granstrom DE. Transmission of some species of internal parasites in horses born in 1993, 1994, and 1995 on the same pasture on a farm in central Kentucky. Vet Parasitol. 1997;70:225–40.
9. Nielsen MK, Vidyashankar AN, Olsen SN, Monrad J, Thamsborg SM. *Strongylus vulgaris* associated with usage of selective therapy on Danish horse farms-Is it reemerging? Vet Parasitol. 2012;189(2–4):260–6.
10. Tydén E, Enemark HL, Franko MA, Höglund J, Osterman-Lind E. Prevalence of *Strongylus vulgaris* in horses after ten years of prescription usage of anthelmintics in Sweden. Vet Parasitol. 2019;2(January):1–7.
11. Scala A, Tamponi C, Sanna G, Predieri G, Dessì G, Sedda G, et al. Gastrointestinal strongyles egg excretion in relation to age, gender, and management of horses in Italy. Animals. 2020;10(12):1–12.
12. Duncan JL. *Strongylus vulgaris* infection in the horse. Vet Rec. 1974;95(2):34–7.
13. White NA, Moore JN, Douglas M. SEM study of *Strongylus vulgaris* larva-induced arteritis in the pony. Equine Vet J. 1983;15(4):349–53.
14. Dallap BL. Coagulopathy in the equine critical care patient. Vet Clin North Am - Equine Pract. 2004;20(1):231–51.
15. Pihl TH, Nielsen MK, Jacobsen S. Changes in Hemostatic Indices in Foals Naturally Infected With *Strongylus vulgaris*. J Equine Vet Sci. 2017;54:1–7.
16. Armengou L, Monreal L, Tarancón I, Navarro M, Ríos J, Segura D. Plasma D-Dimer Concentration in Sick Newborn Foals. J Vet Intern Med. 2008;22:411–7.
17. Cesarini C, Monreal L, Armengou L, Delgado MÁ, Ríos J, Jose-Cunilleras E. Association of Admission Plasma D-dimer Concentration with Diagnosis and Outcome in Horses with Colic. J Vet Intern Med. 2010;24:1490–7.
18. Cesarini C, Monreal L, Armengou L, Delgado MÁ, Ríos J, Jose-Cunilleras E. Progression of plasma D-dimer concentration and coagulopathies during hospitalization in horses with colic. J Vet Emerg Crit Care. 2014;24(6):672–80.
19. Dallap BL, Dolente B, Boston R. Coagulation profiles in 27 horses with large colon volvulus.

J Vet Emerg Crit Care. 2003;13(4):215–25.
20. Lancé MD. A general review of major global coagulation assays: Thrombelastography, thrombin generation test and clot waveform analysis. Thromb J. 2015;13(1):1–6.
21. Spahn DR, Bouillon B, Cerny V, Coats TJ, Duranteau J, Fernández-mondéjar E, et al. Management of bleeding and coagulopathy following major trauma: an updated European guideline. Critcal care. 2013;17:1–45.
22. Hyldahl Laursen S, Andersen PH, Kjelgaard-Hansen M, Wiinberg B. Comparison of components of biological variation between 3 equine thromboelastography assays. Vet Clin Pathol. 2013;42(4):443–50.
23. Epstein KL, Brainard BM, Barton MH, Moore JN, Gomez-Ibanez SE, Lopes MAF, et al. Thrombelastography in Horses with Acute Gastrointestinal Disease. J Vet Intern Med. 2011;25(2):307–14.
24. Leclere M, Lavoie JP, Dunn M, Bédard C. Evaluation of a modified thrombelastography assay initiated with recombinant human tissue factor in clinically healthy horses. Vet Clin Pathol. 2009;38(4):462–6.
25. Dunkel B, Chan DL, Boston R, Monreal L. Association between Hypercoagulability and Decreased Survival in Horses with Ischemic or Inflammatory Gastrointestinal Disease. J Vet Intern Med. 2010;24:1467–74.
26. Wiinberg B, Jensen AL, Kjelgaard-Hansen M, Rojkjaer R, Johansson PI, Gade LP, et al. Study on biological variation of haemostatic parameters in clinically healthy dogs. Vet J. 2007;174(1):62–8.
27. Krogh AK, Brunse A, Thymann T, Kristensen AT, Nielsen LN. Plasma Thromboelastography in Septic Preterm Pigs and Full-Term Pigs at Birth and 11 Days Post-Partum. In: Joint Congress of Veterinary Pathology and Veterinary Clinical Pathology 25-28 September 2019 | Arnhem, The Netherlands. 2019. p. 287.
28. Honoré ML, Pihl TH, Nielsen LN. A pilot study evaluating the Calibrated Automated Thrombogram assay and application of plasma-thromboelastography for detection of hemostatic aberrations in horses with gastrointestinal disease. BMC Vet Res. 2021;17(1):1–11.
29. Hemker HC, Giesen P, AlDieri R, Regnault V, De Smed E, Wagenvoord R, et al. The Calibrated Automated Thrombogram (CAT): A universal routine test for hyper- and hypocoagulability. Pathophysiol Haemost Thromb. 2002;32(5–6):249–53.
30. Wexels F, Dahl OE, Pripp AH, Seljeflot I. Thrombin Generation in Patients with Suspected Venous Thromboembolism. Clin Appl Thromb. 2017;23(5):416–21.
31. Hudák R, Székely EG, Kovács KR, Nagy A, Hofgárt G, Berényi E, et al. Low thrombin generation predicts poor prognosis in ischemic stroke patients after thrombolysis. PLoS One. 2017;12(7):1–13.
32. Allegret V, Dunn M, Bédard C. Monitoring unfractionated heparin therapy in dogs by measuring thrombin generation. Vet Clin Pathol. 2011;40(1):24–31.
33. Hemker HC, Giesen P, Al Dieri R, Regnault V, De Smedt E, Wagenvoord R, et al. Calibrated automated thrombin generation measurement in clotting plasma. Pathophysiol Haemost Thromb. 2003;33(1):4–15.
34. Machlus KR, Colby EA, Wu JR, Koch GG, Key NS, Wolberg S. Effects of tissue factor, thrombomodulin and elevated clotting factor levels on thrombin generation in the calibrated automated thrombogram. Thromb Haemost. 2010;102(5):936–44.
35. Wang Z, Li J, Cao Q, Wang L, Shan F, Zhang H. Comparison Between Thromboelastography and Conventional Coagulation Tests in Surgical Patients With Localized Prostate Cancer. Clin Appl Thromb. 2018;24(5):755–63.

36. Lemon A V., Goddard A, Hooijberg EH. Effects of storage time and temperature on thromboelastographic analysis in dogs and horses. Vet Clin Pathol. 2021;50(1):9–19.
37. Thakur M, Ahmed AB. A review of thromboelastography. Int J Perioper Ultrasound Appl Technol. 2012;1(1):25–9.
38. Saliba W, Awad K, Ron G, Elias M. The Effect of Vitamin D Supplementation on Thrombin Generation Assessed by the Calibrated Automated Thrombogram. Clin Appl Thromb. 2016;22(4):340–5.
39. Staempfli HR, Townsend HG, Prescott JF. Prognostic features and clinical presentation of acute idiopathic enterocolitis in horses. Can Vet J. 1991;32(4):232–7.
40. Saville WJ, Hinchcliff KW, Moore BR, Kohn CW, Reed SM, Mitten LA, et al. Necrotizing enterocolitis in horses: a retrospective study. J Vet Intern Med. 1996;10(4):265–70.
41. Epstein KL, Brainard BM, Lopes MAF, Barton MH, Moore JN. Thrombelastography in 26 healthy horses with and without activation by recombinant human tissue factor. J Vet Emerg Crit Care. 2009;19(1):96–101.
42. Hedberg-Alm Y, Tydén E, Tamminen L-M, Linström L, Anlén K, Svensson M, et al. Clinical Features and Treatment Response to Differentiate Idiopathic Peritonitis From Non-strangulating Intestinal Infarction Associated With *Strongylus Vulgaris* Infection in the Horse. BMC Vet Res. 2021;In review:1–23.
43. Butterfield C, Grumpelt B, Kimmel D, Patterson R, Jones K, Scott SL, et al. The Pretransport Management of Stress in Performance Horses. J Equine Vet Sci. 2018;69:145–8.
44. Folsom RW, Littlefield-Chabaud MA, French DD, Pourciau SS, Mistric L, Horohov DW. Exercise alters the immune response to equine influenza virus and increases susceptibility to infection. Equine Vet J. 2001;33(7):664–9.
45. Andersen U V., Howe DK, Dangoudoubiyam S, Toft N, Reinemeyer CR, Lyons ET, et al. SvSXP: A *Strongylus vulgaris* antigen with potential for prepatent diagnosis. Parasites and Vectors. 2013;6(1):1–13.
46. Nielsen MK, Vidyashankar AN, Bellaw J, Gravatte HS, Cao X, Rubinson EF, et al. Serum *Strongylus vulgaris*-specific antibody responses to anthelmintic treatment in naturally infected horses. Parasitol Res. 2014;114(2):445–51.
47. Lyons ET, Drudge JH, Tolliver SC. Prevalence of some internal parasites found (1971–1989) in horses born on a farm in central kentucky. J Equine Vet Sci. 1990;10(2):99–107.
48. Nielsen MK, Olsen SN, Lyons ET, Monrad J, Thamsborg SM. Real-time PCR evaluation of *Strongylus vulgaris* in horses on farms in Denmark and Central Kentucky. Vet Parasitol. 2012;190(3–4):461–6.
49. Pihl TH, Scheepers E, Sanz M, Goddard A, Page P, Toft N, et al. Influence of Disease Process and Duration on Acute Phase Proteins in Serum and Peritoneal Fluid of Horses with Colic. J Vet Intern Med. 2015;29:651–8.
50. Underwood C, Southwood LL, McKeown KP, Knight D. Complications and survival associated with surgical compared with medical management of horses with duodenitis-proximal jejunitis. Equine Vet J. 2008;40(4):373–8.
51. Adcock DM, Hoefner DM, Kottke-Marchant K, Marlar RA, Szamosi DI, Warunek DJ. Collection, Transport, and Processing of Blood Specimens for Testing Plasma-Based Coagulation Assays and Molecular Hemostasis Assays; CLSI Approved Guideline-Fifth Edition. H21-A5. Clin Lab Stand Inst. 2008;28(5):H21-A5.
52. Wiinberg B, Jensen AL, Rojkjaer R, Johansson P, Kjelgaard-Hansen M, Kristensen AT. Validation of human recombinant tissue factor-activated thromboelastography on citrated whole blood from clinically healthy dogs. Vet Clin Pathol. 2005;34(4):389–93.

Supporting information

Table 1s. Demographic data from the six groups of horses: clinically healthy horses without migrating *Strongylus vulgaris*, horses with migrating *S. vulgaris* without clinical disease (*S. vulgaris* non-strangulating intestinal infarction (NSII) negative group), horses with migrating *S. vulgaris* with clinical disease (*S. vulgaris* NSII positive group), horses with idiopathic peritonitis, horses with enterocolitis, and horses with strangulating lesions. Age and body weight (BW) are displayed as the mean with minimum and maximum values.

	Clinically healthy horses		**Horses with severe gastrointestinal disease**				
	Clinically healthy (n = 17)	*S. vulgaris* NSII negative (n = 18)	*S. vulgaris* NSII positive (n = 16)	Idiopathic peritonitis (n = 20)	Entero-colitis (n = 26)	Strangulating lesions (n = 26)	P-value
Sex							
Stallions		1 (6%)		1 (5%)	2 (8%)		
Geldings	2 (12%)		7 (44%)	11 (55%)	15 (58%)	15 (58%)	0.001
Mares	15 (88%)	17 (94%)	9 (56%)	8 (40%)	9 (34%)	11 (42%)	
Breed							
Warm-bloods	16 (94%)		4 (25%)	12 (60%)	12 (46%)	14 (54%)	
Cold-bloods			5 (31%)	8 (40%)	14 (54%)	7 (27%)	< 0.0001
Unknown	1 (6%)	18 (100%)	7 (44%)			5 (19%)	
Age (years)	13.1 (5.9-25.6)	11.6 (6-17)	10.2 (3.5-21)	12 (2-22)	11.8 (2.8-27)	11.9 (1.4-26)	0.7
BW (kg)	564.4 (411-695)	NA [I]	502.9 (387-632)	503.7 (356-600)	478.4 (251-660)	484.8 (338-700)	0.1

[I] Not recorded.

Table 2s. Analysis of plasma-thromboelastography (TEG) for clinically healthy horses without migrating *Strongylus vulgaris*, horses with migrating *S. vulgaris* without clinical signs (*S. vulgaris* non-strangulating intestinal infarction (NSII) negative group), horses with migrating *S. vulgaris* with clinical disease (*S. vulgaris* NSII positive group), horses with idiopathic peritonitis, horses with enterocolitis, and horses with strangulating lesions. Values are displayed as the median and 1st–3rd quartiles.

	Clinically healthy horses		**Horses with severe gastrointestinal disease**				
	Clinically healthy	*S. vulgaris* NSII negative	*S. vulgaris* NSII positive	Idiopathic peritonitis	Entero-colitis	Strangu-lating lesions	P-value
SP (min.)	11.8 (10.3-12.6)	8.6 (7.2-10.8)	12.3 (10.6-14.5)	9.5 (7.9-12.6)	10.0 (7.8-12.0)	9.1 (7.4-10.6)	0.03 *
R (min.)	13.2 (12.8-15.2)	9.7 (8.2-11.6)	14.2 (12.1-16.3)	11.4 (9.1-13.7)	11.2 (8.5-14.7)	10.3 (7.8-11.6)	0.01
K (min.)	5.8 (3.8-10.6)	3.2 (2.5-4.5)	3.2 (2.7-5.6)	3.3 (2.1-3.9)	4.1 (2.5-5.4)	3.4 (2.3-7.1)	0.1
Angle (degree)	23.4 (16.8-40.2)	49.9 (43.4-57.0)	50.2 (38.3-54.2)	48.7 (39.8-55.1)	43.6 (34.4-55.7)	48.4 (29.5-57.3)	0.0001
MA (mm)	23.1 (17.8-26.5)	30.8 (28.5-38.0)	39.9 (26.6-46.4)	31.2 (24.2-42.9)	26.3 (21.8-33.6)	28.6 (22.5-33.8)	< 0.0001
G (dynes/cm^2)	1.5 (1.1-1.8)	2.2 (2.0-3.1)	3.3 (1.9-4.4)	2.3 (1.6-3.8)	1.8 (1.4-2.5)	2.0 (1.5-2.6)	< 0.0001

SP: split point, R: reaction time, K: clot formation time, MA: maximum amplitude, G: shear elastic force, LY30: lysis 30 minutes, LY60: lysis 60 minutes (min.).

* No significant difference between groups on *post hoc* analysis.

Table 3s. Analysis of the Calibrated Automated Thrombogram (CAT) assay for clinically healthy horses without migrating *Strongylus vulgaris*, horses with migrating *S. vulgaris* without clinical signs (*S. vulgaris* non-strangulating intestinal infarction (NSII) negative group), horses with migrating *S. vulgaris* with clinical disease (*S. vulgaris* NSII positive group), horses with idiopathic peritonitis, horses with enterocolitis, and horses with strangulating lesions. Values are displayed as the median and 1st–3rd quartiles.

	Clinically healthy horses		**Horses with severe gastrointestinal disease**				
	Clinically healthy	*S. vulgaris* NSII negative	*S. vulgaris* NSII positive	Idiopathic peritonitis	Entero-colitis	Strangu-lating lesions	P-value
Lag time (min.)	4.2 (3.8-4.4)	5.9 (5.3-7.3)	9.2 (4.9-11.1)	7.3 (5.2-8.9)	4.5 (3.5-6.5)	4.2 (3.5-5.3)	< 0.0001
ETP (nM*min.)	402.1 (315.9-597.2)	427.0 (365.4-509.7)	443.3 (327.7-719.0)	611.3 (556.3-663.8)	542.9 (455.3-668.1)	589.7 (444.3-635.5)	0.01
Peak (nM)	36.0 (31.4-52.0)	48.8 (37.2-55.8)	45.3 (30.5-64.8)	53.9 (42.1-66.9)	62.9 (48.8-82.5)	57.9 (50.8-75.2)	0.03
ttPeak (min.)	11.9 (11.5-12.8)	13.2 (11.5-15.6)	15.6 (12.1-20.0)	15.7 (12.9-17.3)	10.7 (9.3-14.2)	11.5 (8.6-13.5)	< 0.0001

ttPeak: time to peak, ETP: endogenous thrombin potential.

Table 4s. Clinical data from the six groups of horses: clinically healthy horses without migrating *Strongylus vulgaris*, horses with migrating *S. vulgaris* without clinical disease (*S. vulgaris* non-strangulating intestinal infarction (NSII) negative), horses with migrating *S. vulgaris* with clinical disease (*S. vulgaris* NSII positive), horses with idiopathic peritonitis, horses with enterocolitis, and horses with strangulating lesions. Values are displayed as the median with minimum and maximum values.

	Clinically healthy horses		**Horses with severe gastrointestinal disease**				
	Clinically healthy [I] (n = 17)	*S. vulgaris* NSII negative [II] (n = 18)	*S. vulgaris* NSII positive (n = 16)	Idiopathic peritonitis (n = 20)	Entero-colitis (n = 26)	Strangu-lating lesions (n = 26)	P-value
HR (beats/min.)	36-40	NA	56 (40-96)	50 (36-72)	56 (36-84)	55 (36-120)	< 0.0001
RR (breaths/min.)	10-18	NA	24 (10-40)	12 (10-25)	18 (10-80)	24 (8-52)	0.003
Temperature (degrees)	37.5-38.0	NA	38.1 (37.3-39.4)	38.5 (37.4-40.0)	37.6 (35.8-40.3)	37.5 (35.7-38.7)	< 0.0001

[I] Clinical variables were within the indicated intervals.
[II] Heart rate (HR), respiration rate (RR), and temperature not recorded.

Table 5s. Laboratory variables from the six groups of horses: clinically healthy horses without migrating *Strongylus vulgaris*, horses with migrating *S. vulgaris* without clinical disease (*S. vulgaris* non-strangulating intestinal infarction (NSII) negative), horses with migrating *S. vulgaris* with clinical disease (*S. vulgaris* NSII positive), horses with idiopathic peritonitis, horses with enterocolitis, and horses with strangulating lesions. Values are displayed as the median with 1st–3rd quartiles.

	Clinically healthy horses		**Horses with severe gastrointestinal disease**				
	Clinically healthy (n = 17)	*S. vulgaris* NSII negative (n = 18)	*S. vulgaris* NSII positive (n = 16)	Idiopathic peritonitis (n = 20)	Entero-colitis (n = 26)	Strangu-lating lesions (n = 26)	P-value
WBC (mia./L)	6.1 (5.7-6.6)	9.8 (8.9-11.3)	3.1 (2.8-5.9)	6.7 (6.0-8.6)	4.3 (2.5-8.2)	7.7 (6.3-9.5)	< 0.0001
HCT (%)	37.0 (34.5-40.0)	40.5 (37.2-46.9)	37.0 (29.9-40.8)	32.2 (30.5-34.3)	44.5 (35.0-50.3)	38.0 (32.8-46.8)	< 0.0001
PLT (mia./L)	128.0 (116-140)	NA	113.0 (90-151.5)	129.0 (118-176.5)	129.0 (90.8-172)	139.0 (110-185)	0.4
MPV (fl)	7.8 (6.9-8.8)	NA	6.8 (6.5-7.0)	6.8 (6.7-6.9)	6.7 (6.2-7.4)	6.8 (6.5-7.5)	0.02
MPC (g/L)	228.0 (219-244)	NA	259.5 (192-286.5)	251.0 (248-262)	240.0 (213-262.5)	253.0 (229-265)	0.8
SAA (mg/L)	0.0 (0.0-0.4)	0.95 (0.0-14.2)	1912.0 (641.8-6336)	1828.0 (1049-3184)	682.1 (1.4-1663)	2.9 (0.0-51.2)	< 0.0001
Fibrinogen (g/L)	3.3 (2.9-3.4)	4.1 (3.8-4.4)	4.4 (4.1-7.5)	3.9 (3.2-4.9)	4.2 (3.3-4.7)	3.4 (3.2-4.3)	0.0001
TP (g/L)	64.7 (60.9-66.3)	72.8 (70.1-74.9)	65.4 (51.2-72.8)	64.5 (53.5-69.3)	54.5 (50-62)	60.5 (56.9-70)	0.001
Iron (umol/L)	26.2 (22.6-32.5)	13.3 (11.5-19.5)	5.5 (4.8-9.3)	6.2 (3.0-19.9)	15.4 (7.2-24.9)	20.4 (11.8-28.7)	< 0.0001
D-dimer (mg/L)	0.14 (0.13-0.19)	0.24 (0.2-0.31)	0.5 (0.3-1.1)	0.4 (0.3-0.7)	0.4 (0.2-0.6)	0.3 (0.18-0.8)	0.0004
S. vulgaris antibody titer (%)	32.2 (18.9-45.3)	58.4 (33.2-82.4)	38.5 (20.2-63.3)	23.1 (4.8-37.8)	19.8 (2.8-35.0)	15.6 (4.2-29.5)	< 0.0001

WBC: white blood cell count, HCT: hematocrit, PLT: platelet count, MPV: mean platelet volume, MPC: mean platelet component, SAA: serum amyloid A, TP: total protein.

Statistical appendix

Data imputation

A total of 123 horses were included in the study. Among the 15 variables, there were a number of missing values (NAs). Tables 6s and 7s presents the observations per group and the amount of missing information per group. It was noticeable that the clotting time (K) from the plasma-thromboelastography (TEG) were missing in six horses in group 1 (clinically healthy horses) and serum amyloid A (SAA) were missing in four horses in group 2 (*Strongylus vulgaris* non-strangulating intestinal infarction (NSII) negative). In addition, in groups 5 (enterocolitis) and 6 (strangulating lesions) multiple variables had missing values. However, it was found that out of the 123 horses only four (ID no = 18, 19, 319, 460) were missing two or more observations. These horses were all from groups 5 (enterocolitis) and 6 (strangulating lesions) (two in each) which had the most observations, see Table 6s. Hence, we excluded these four horses entirely from the analysis.

Table 6s. Number of observations in each group.

Group	**Observations (n)**
1: Clinically healthy	17
2: *S. vulgaris* NSII negative	18
3: *S. vulgaris* NSII positive	16
4: Idiopathic peritonitis	20
5: Enterocolitis	26
6: Strangulating lesions	26
Total	123

Table 7s. Missing values for each group (1: clinically healthy horses without *Strongylus vulgaris*, 2: *S. vulgaris* non-strangulating intestinal infarction (NSII) negative, 3: *S. vulgaris* NSII positive, 4: idiopathic peritonitis, 5: enterocolitis, and 6: strangulating lesions). TEG: thromboelastography, CAT: Calibrated Automated Thrombogram, SAA: serum amyloid A, R: reaction time, K: clotting time, MA: maximum amplitude, ETP: endogenous thrombin potential, ttPeak: time to peak.

						Plasma-TEG				**CAT**			
Group	Iron	SAA	Fibrin-ogen	D-dimer	*S. vulgaris* antibody titer	R	K	Angle	MA	Lag time	ETP	Peak	ttPeak
1	0	0	0	0	0	0	6	0	0	0	0	0	0
2	0	4	1	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	1	0	0	0	0	0	0
4	0	0	0	0	0	0	1	0	0	0	0	0	0
5	0	0	0	0	0	2	5	2	2	0	0	0	0
6	0	0	1	1	1	1	3	1	1	1	1	1	1

Among the remaining variables, K was missing from a substantial number of horses. This was due to the plasma samples not reaching a preset level of clot strength as this has been established for human whole blood samples. We therefore excluded the K variable.

This left four missing values in SAA and one in fibrinogen among the horses in group 2 (*S. vulgaris* NSII negative). Figure 1s shows boxplots of the observed fibrinogen values for each group. The values in group 2 were distributed with a small dispersion compared to the other groups. From this we concluded that it was sound to impute the missing values within group 2 using the median (fibrinogen (g/L) = 4.06) of the remaining fibrinogen values in the group without skewing the results.

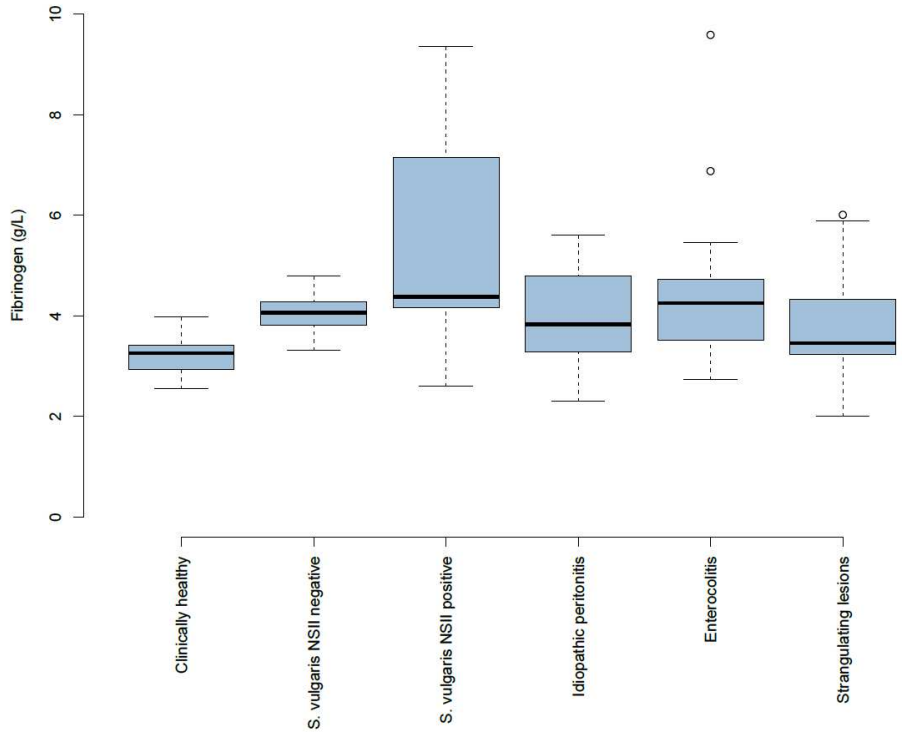


Figure 1s. Boxplot of fibrinogen values for each group. The missing values was in group 2 (*Strongylus vulgaris* non-strangulating intestinal infarction (NSII) negative) in which values exhibited a small dispersion in comparison with the other groups.

For the four missing SAA values, we opted for a random forest imputation, using the R package missForest [1]. Figure 2s highlights the correlation structure of the variables. Evidently, there was relatively high correlation between the standard diagnostic variables iron, SAA and fibrinogen, whereas the correlation with d-dimer and *S. vulgaris* antibody titer was low. The correlations between SAA and the other standard diagnostic variables is presented in Table 8s, which suggests that imputing SAA values from the remaining diagnostic values was reasonable. Table 9s shows the observed standard diagnostic values from group 2 (*S. vulgaris* NSII negative) along with the imputed data points highlighted. None of the imputed values gave any reason for concern, as they did not deviate markedly from the remaining values observed in the group.

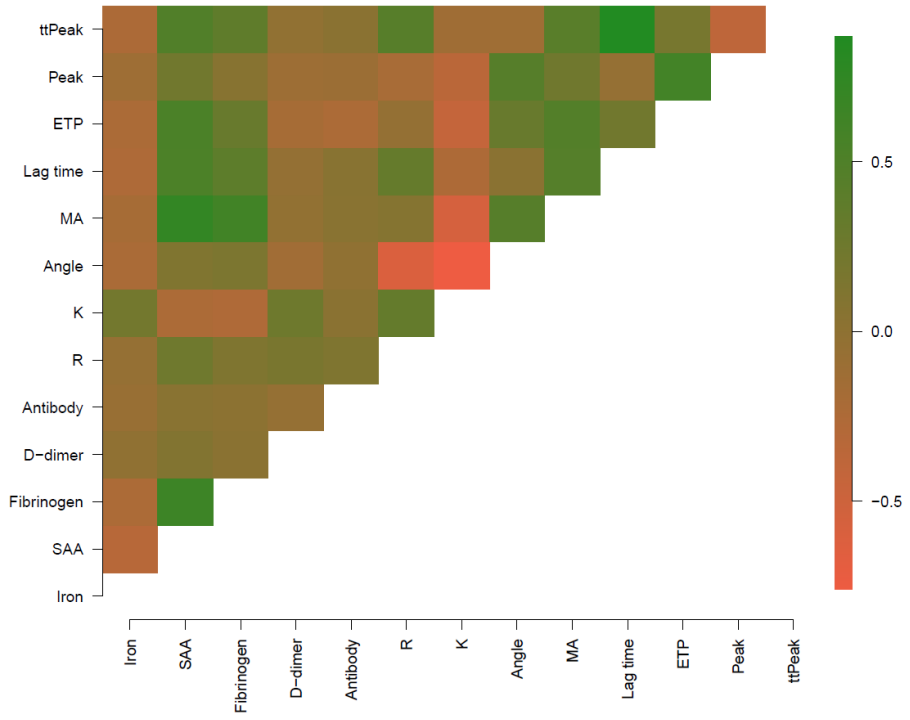


Figure 2s. Correlation structure of the variables. There was a substantial correlation between the standard diagnostic variables iron, serum amyloid A (SAA), and fibrinogen, whereas the correlation with d-dimer and *Strongylus vulgaris* antibody (antibody) titer was low. ttPeak: time to peak, ETP: endogenous thrombin potential, MA: maximum amplitude, K: clotting time, R: reaction time.

Table 8s. Correlations between serum amyloid A (SAA) and the other standard variables (iron, fibrinogen, d-dimer, and *Strongylus vulgaris* antibody titer).

Variable	**Correlation**
Iron (umol/L)	-0.324
Fibrinogen (g/L)	0.646
D-dimer (mg/L)	0.097
S. vulgaris antibody titer (%)	0.041

Table 9s. Standard diagnostic observations from group 2 (*Strongylus vulgaris* non-strangulating intestinal infarction (NSII) negative) with imputed values in red. None of the values was extreme or gave cause for concern. The imputed fibrinogen value was the median of the non-missing values. The imputed serum amyloid A (SAA) values were imputed using a random forest algorithm. KY SVP: *S. vulgaris* NSII negative group.

ID no.	Group	Iron (umol/L)	SAA (mg/L)	Fibrinogen (g/L)	D-dimer (mg/L)	*S. vulgaris* antibody titer (%)
4 KY SVP	2	11.10	0.00	4.24	0.22	30.11
5 KY SVP	2	18.80	0.00	4.43	0.27	13.81
6 KY SVP	2	9.20	0.00	3.95	0.24	42.05
7 KY SVP	2	11.50	0.00	3.41	0.19	29.05
13 KY SVP	2	11.20	0.00	4.28	0.20	34.27
14 KY SVP	2	19.20	0.00	4.61	0.19	57.70
1 KY SVP	2	14.00	1.90	4.06	0.19	77.75
3 KY SVP	2	20.20	2.40	4.79	0.23	45.19
17 KY SVP	2	25.50	2.50	3.36	0.52	103.57
8 KY SVP	2	12.60	9.70	4.15	0.39	114.47
11 KY SVP	2	16.00	27.60	3.31	0.25	59.86
9 KY SVP	2	20.40	33.70	3.84	0.27	85.37
10 KY SVP	2	13.20	133.60	3.82	0.21	59.17
16 KY SVP	2	12.20	0.00	4.06	0.22	1.29
12 KY SVP	2	25.00	6.51	3.97	0.46	46.05
2 KY SVP	2	11.50	9.59	4.41	0.28	81.46
18 KY SVP	2	11.40	12.42	4.24	0.20	75.98
15 KY SVP	2	13.30	38.84	3.77	0.46	118.87

Logistic regression

Using the data including the imputed values we performed a logistic regression on the groups, with the *S. vulgaris* NSII postive group (group 3) as y = 1 and the remaining groups as y = 0. We initially included all variables, except K, which was excluded. In order to reduce the model, we performed stepwise selection using the AIC to evaluate each model fit. Based on this, the final model included iron, SAA, angle and ETP. Intuitively this made good sense, since this retained variables from the standard diagnostic measures along with a single summarizing variable from both the TEG and CAT assays.

Figure 3s plots the predictors against the model predictions (in log-odds). There were no clear signs of non-linear patterns. For SAA there were a significant amount of 0 values. This variable could in practice have been log-transformed, but it would not have changed the pattern much and the 0 values would become -∞ if unchanged. Hence, we deemed the linearity assumptions to be fine.

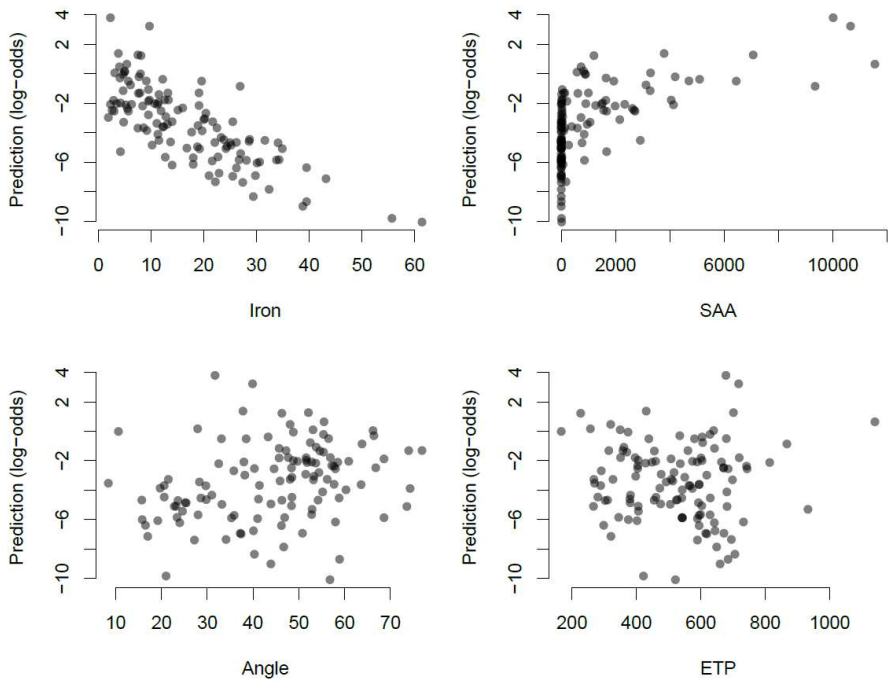


Figure 3s. Linearity of the variables included in the final model against the log-odds model predictions. There were no significant non-linear trends. Considering the (serum amyloid A (SAA)) variable a log-transformation could be used, however it would not remedy the large point mass at 0, which will be -∞ with a log-transformation. ETP: endogenous thrombin potential.

Furthermore, a Hosmer-Lemeshow test for goodness-of-fit of the binary logistic regression did not raise any concerns regarding the fit. Using various numbers of groups for the test, we found the lowest p-value at 0.118 with 10 groups.

References

[1] Daniel J. Stekhoven (2013) missForest: Nonparametric Missing Value Imputation using Random Forest, R package version 1.4.

[2] R Core Team (2020) R: A Language and Environment for Statistical Computing, Addison R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/

Appendices

Appendix I

The consensus statement given to horse owners with horses being admitted to the Large Animal Teaching Hospital at the University of Copenhagen.

KØBENHAVNS UNIVERSITET
DET SUNDHEDSVIDENSKABELIGE FAKULTET

Samtykkeerklæring

Forbedret diagnostik af hestens blodorm

Hestens blodorm (*Strongylus Vulgaris*) har igennem de seneste år været årsag til flere syge heste end tidligere på Universitetshospitalet for Store Husdyr. Derfor har vi i januar 2018 startet et flerårigt forskningsprojekt, der skal bidrage med ny viden om, samt forbedre diagnostikken af sygdomme forårsaget af hestens blodorm. Alle hesteejere, hvis hest indlægges med kolik og/eller bughindebetændelse (peritonitis) på Universitetshospitalet for Store Husdyr i Taastrup, vil blive tilbudt at kunne deltage i projektet.

I forbindelse med at din/jeres hest er blevet indlagt på hospitalet er der udtaget blodprøver til almindelige diagnostiske formål. Vi vil gerne anmode om jeres tilladelse til at anvende en mindre del af dette blod til analyser af nye diagnostiske biomarkører i vores forskningsprojekt. Analyserne i forbindelse med projektet foretages naturligvis uden beregning.

Vi vil desuden bede dig/jer besvare nogle enkelte spørgsmål vedr. hestens tidligere ormebehandlinger, opstaldning, symptomer, mm. Spørgeskemaet findes ved at følge linket i mailen.

De indsamlede data vil blive anonymiseret og vil udelukkende blive anvendt til forskningsbrug. I 2018 til 2021 indsamles prøver og øvrige data, der så bearbejdes de kommende år, du vil derfor ikke kunne få oplyst resultaterne for netop din hest. De samlede resultater af forskningsprojektet forventes offentliggjort både internationalt og i Danmark og det er forventningen, at resultaterne af forskningsprojektet vil forbedre diagnostikken af hestens blodorm samt muligheden for at forudsige patientens prognose og overvåge effekten af behandlingsforløbet. Projektet vil dermed forbedre hestenes chancer for overlevelse.

Ved at besvare den fremsendte e-mail med en positiv tilkendegivelse giver du/I tilladelse til, at vi må anvende de udtagne blodprøver, og at resultaterne må publiceres i forskningsøjemed.

Venlig hilsen
Dyrlæge, Ph.d., Lektor Tina Pihl
Dyrlæge, Ph.d.-studerende Marie Louise Honoré Jørgensen

Blodorm og kolik

Larver af hestens blodorm vandrer i ca. 5 mdr. i tarmens blodkar og i denne periode af sin livscyklus kan den ikke diagnosticeres ved hjælp af en gødningsundersøgelse.

Blodormens vandring i tarmens blodkar kan forårsage blodpropper, hvorved der kan opstå koldbrand i tarmvæggen, der blandt andet viser sig som koliksymptomer.

Kun hvis tilstanden hurtigt diagnosticeres og hesten tilbydes operation, har den en chance for at overleve.

Projektet er, igangsat netop med henblik på at undersøge en række biomarkører i blodet, således, at larvernes vandring i blodkarrene kan opdages inden de forårsager skade.

Vi håber derfor meget at du/I vil deltage i undersøgelsen.

The consensus statement given to horse owners with horses being admitted to the University Animal Hospital at the Swedish University of Agricultural Sciences and Evidensia Helsingborg Equine Referral Hospital.

NY DIAGNOSTIK FÖR STOR BLODMASK

Bakgrund och syfte

Hästens parasiter har utvecklat resistens mot avmaskningsmedel vilket är ett växande hot mot hästars hälsa. I likhet med antibiotika är det därför nödvändigt att vara restriktiv med användning av avmaskningsmedel. Sedan ungefär tio år rekommenderas att avmaskning utförs beroende på resultat av träckprovsundersökning (så kallad riktad avmaskning), och inte som tidigare då avmaskning skedde rutinmässigt flera gånger per år.

Tyvärr har vi visat att hästens mest fruktade parasit, stor blodmask (*Strongylus vulgaris)*, har ökat i Sveriges sedan 1999. Syftet med det här projektet är att utvärdera nya markörer för migrerande larver av stor blodmask som tidig indikation på kolik orsakad av denna parasit. Det här är ett samarbete mellan UDS/SLU och Köpenhamn. En sammanställning från projektet kommer att delges er via email under 2020.

Provtagningar

Blodprov kommer tas för utvecklande av nya markörer.

Plats för etikett

STIFTELSEN HÄSTFORSKNING

Den här studien är finansierad av Stiftelsen Hästforskning

Djurägarmedgivande

Jag har tagit del av ovanstående information samt fått muntlig information om studien. Härmed godkänner jag att min häst deltar i studien. Jag är införstådd med att deltagandet är frivilligt och att jag när som helst kan ta min häst ur studien. Uppgifterna avidentifieras och kommer inte att kopplas till enskild häst eller person.

_____

Hästens namn

_____

Hästägarens namn, adress och telefon

_____

Hästägarens email

_____

Ort, datum

_____

Namnunderskrift djurägare **Namnförtydligande djurägare**

Jag godkänner även att överblivna blod- och/eller vävnadsprov sparas för att läggas i en biobank.

JA **NEJ**

Kontaktpersoner

Projektledare: **Eva Tydén**, docent, forskare vid inst biomedicin och veterinär folkhälsovetenskap, SLU (eva.tyden@slu.se), **Ylva Hedberg Alm**, veterinär (ylva.hedberg.alm@uds.slu.se).

Appendix II

Table 14. The laboratory protocol used for the vascular cell adhesion molecule 1 (vCAM-1) assay.

Step	*Procedure*	*Description*
Step 1	Preparation of reagents, standards and samples	• All reagents were prepared according to manufacturer's instructions. • Serum samples were prediluted 1:50 with assay buffer. • Standard dilutions were prepared by adding assay buffer to the manufacturer provided human vCAM-1 standard in external tubes. • Dilutions ranging from 100.0 to 3.1 ng/ml (standard 1-6).
Step 2	Wash-step	• Micro-wells were washed twice with 400 µl wash buffer.

Step 3	Application of standards, blanks and samples	• 100 µl of:
		  • Each standard dilution
		  • Assay buffer (blank)
		  • Prediluted serum samples
		• Were added to micro-wells.

Step 4	Application of conjugate mixture	• 50 µl of conjugate mixture (biotin-conjugated anti-human sVCAM-1 antibody and Streptavidin-HRP) was added to each well.
Step 5	Incubation-step	• Micro-wells were incubated at room temperature for 2 hours on a plate shaker at 300 round per minute (rpm).
Step 6	Wash-step	• Micro-wells were washed three times with 400 µl wash buffer.
Step 7	Application of substrate solution	• 100 µl substrate solution (tetramethyl-benzidine) was added to each well.
Step 8	Incubation-step	• Micro-wells were incubated at room temperature for 10 min. (color development were continuously monitored) covered with foil.
Step 9	Application of stop solution	• 100 µl stop solution (phosphoric-acid) was quickly added to each well.
Step 10	Plate reading	• Absorbance was immediately read on a spectrophotometer at 450 nm (620 nm as reference wavelength).

Table 15. The laboratory protocol used for the P-Selectin assay.

Step	*Procedure*	*Description*
Step 1	Preparation of reagents, standards and samples	• All reagents were prepared according to manufacturer's instructions. • Standard dilutions were prepared by adding sample diluent to the manufacturer provided human P-Selectin standard in external tubes. • Dilutions ranging from 4000.0 to 125 pg/ml (the sample diluent serves as the 0 pg/ml standard).

Step 2	Application of standards, controls and samples	• 100 µl of:
		  • Each standard dilution
		  • Control
		  • Serum samples
		• Were added to micro-wells.

Step 3	Incubation-step	• Micro-wells were incubated at 37°C for 1.5 hours.
Step 4	Wash-step	• Micro-wells were washed four times with 350 µl wash buffer.
Step 5	Application of conjugate mixture	• 100 µl of biotin-conjugate mixture (biotin-conjugated anti-human P-selectin antibody) was added to each well.
Step 6	Incubation-step	• Micro-wells were incubated at 37°C for 1 hour.
Step 7	Wash-step	• Micro-wells were washed four times with 350 µl wash buffer.
Step 8	Application of Streptavidin-HRP	• 100 µl of working solution of Streptavidin-HRP was added to each well.
Step 9	Incubation-step	• Micro-wells were incubated at 37°C for 30 min. covered with foil.
Step 10	Wash-step	• Micro-wells were washed four times with 350 µl wash buffer.
Step 11	Application of substrate solution	• 100 µl of substrate solution was added to each well.
Step 12	Incubation-step	• Micro-wells were incubated at 37°C for 20 min. (color development were continuously monitored) covered with foil.
Step 13	Application of stop solution	• 100 µl stop solution was quickly added to each well.
Step 14	Plate reading	• Absorbance was immediately read on a spectrophotometer at 450 nm (620 nm as reference wavelength).

Appendix III

Table 16. Plasma-thromboelastography variables for the clinically healthy horses without *Strongylus vulgaris* and the *S. vulgaris* non-strangulating intestinal infarction (NSII) negative group. R: reaction time, K: clotting time, MA: maximum amplitude, G: shear elastic force. Values are median with 1st and 3rd quartile.

Horses		Plasma-thromboelastography				
		R (min.)	K (min.)	Angle (degree)	MA (mm)	G (dynes/cm^2)
Clinically healthy		13.5 (12.8-15.4)	11.85 (9.8-16.7)	18.1 (15.8-21.6)	18.45 (14.85-22.3)	1.1 (0.9-1.4)
S. vulgaris NSII negative horses	February	17.25 (15.5-18.2)	6.1 (3.6-8.5)	30.2 (23.3-42.8)	30.8 (27.25-36.1)	2.25 (1.85-2.8)
	March	12.25 (9.9-21.8)	3.55 (2.7-6.9)	45.7 (25.3-52.5)	37.9 (30.7-44.5)	3.05 (2.3-4.05)
	April	10.9 (8.6-18.8)	3.2 (2.1-4.8)	46.8 (35.3-57.7)	39.0 (34.2-40.6)	3.2 (2.6-3.4)
	May	11.9 (8.2-18.2)	3.8 (2.8-6.7)	48.75 (27.7-53.7)	26.1 (22.2-29.1)	1.75 (1.5-2.1)
	June	15.7 (13.2-19.03)	5.35 (4.6-5.7)	37.95 (27.3-40.6)	25.85 (18.95-28.6)	1.75 (1.15-2.05)
	July	16.8 (15.7-20.2)	9.7 (3.2-16.2)	24.1 (19.2-31.6)	18.3 (14.7-23.1)	1.1 (0.9-1.5)
	August	8.8 (7.4-10.5)	4.2 (3.2-8.2)	39.65 (37.6-50.7)	21.05 (18.03-27.9)	1.3 (1.1-1.9)
	September	12.4 (8.4-13.8)	4.35 (3.1- 8.4)	44.95 (27.1-53.2)	29.1 (22.1-33.55)	2.05 (1.4-2.5)
	October	22.9 (19.98-32.5)	15.1 (11.9-22.6)	18.2 (11.03-24.5)	21.3 (17.1-22.2)	1.35 (1.1-1.4)
	November	15.15 (12.3-17.4)	4.4 (3.2-7.73)	42.6 (39.3-47.8)	28.85 (21.6-34.8)	2.05 (1.4-2.7)
	December	11.05 (9.2-12.6)	3.75 (2.7-4.9)	52.6 (44.9-57.03)	29.0 (24.05-32.8)	2.05 (1.6-2.45)
	January	8.6 (7.03-14.8)	3.85 (2.2-5.03)	45.05 (30.5-54.5)	24.6 (17.5-30.8)	1.65 (1.1-2.25)

Table 17. Calibrated Automated Thrombogram variables for the clinically healthy horses without *Strongylus vulgaris* and the *S. vulgaris* non-strangulating intestinal infarction (NSII) negative group. ETP: endogenous thrombin potential, ttPeak: time to peak. Values are median with 1st and 3rd quartile.

Horses		**Calibrated Automated Thrombogram**			
		Lag time (min.)	ETP (nM/min.)	Peak (nM)	ttPeak (min.)
Clinically healthy		4.05 (3.6-4.6)	333.1 (296.2-386.2)	32.7 (27.3-36.01)	11.9 (11.3-12.7)
S. vulgaris NSII negative horses	February	6.7 (5.1-7.02)	409.3 (390.6-456.7)	39.7 (35.5-45.2)	13.9 (12.45-14.75)
	March	7.4 (6.02-9.4)	404.0 (322.8-450.0)	42.8 (33.3-52.1)	14.5 (12.9-16.6)
	April	5.95 (5.7-9.1)	454.3 (390.9-479.1)	47.6 (38.4-6.4)	12.2 (11.1-16.6)
	May	6.5 (5.5-10.7)	474.5 (364.6-602.8)	54.4 (44.3-81.3)	13.3 (10.8-17.2)
	June	9.1 (6.45-13.45)	479.0 (453.6-519.1)	52.45 (47.85-55.9)	16.6 (14.3-19.9)
	July	8.8 (6.3-13.5)	345.1 (319.3-424.5)	35.02 (31.6-47.1)	17.0 (14.0-20.5)
	August	6.95 (5.3-12.1)	576.6 (525.1-609.5)	80.1 (74.6-84.8)	12.3 (10.6-17.4)
	September	9.4 (6.0-13.4)	658.4 (602.7-717.8)	83.1 (57.85-90.5)	17.45 (11.6-19.5)
	October	8.5 (6.95-15.01)	582.8 (521.8-616.5)	51.9 (39.96-63.7)	17.4 (14.4-24.11)
	November	6.5 (6.2-11.5)	545.8 (507.7-656.8)	63.1 (56.95-75.1)	14.4 (12.9-18.97)
	December	5.3 (4.9-9.2)	554.4 (468.9-596.5)	74.6 (55.99-93.5)	11.95 (10.5-15.45)
	January	4.5 (3.97-7.5)	529.5 (508.0-574.0	68.5 (63.98-78.8)	10.4 (9.99-13.4)