Norwegian University of Life Sciences
Faculty of Veterinary Medicine
Department of Companion Animal Clinical Sciences

Philosophiae Doctor (PhD)
Thesis 2021:71

Clinical and clinicopathological characteristics in canine *Vipera berus* envenomation

Kliniske og klinisk-patologiske aspekter ved hoggormbitt hos hund

Hannah Jayne Harjén

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Ås 2021

Thesis number 2021:71
ISSN 1894-6402
ISBN 978-82-575-1847-9

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Acknowledgements

The work in this PhD project was carried out at the Department of Companion Animal Clinical Sciences, Faculty of Veterinary Science at the Norwegian University of Life Sciences (NMBU) and was generously funded by The Raagholt Research Foundation, Arkitekt Finn Rahn's Fund, Dyrlege Smiths Fund and The Agria and SKK Research Fund for Pets.

PhDs are about teamwork, and I have been fortunate to have a great team of colleagues, family and friends helping me along the way, all of whom I would like to thank:

Firstly, my supervisors: **Runa Rørtveit**, puppy talk, parenting talk, zoom chats about everything and nothing, with the occasional wee accident and people crawling around on the floor. Thank you for choosing me for this project and for your continuous encouragement and friendship. **Kristin Paaske Anfinsen**, thank you for always making time for me both academically and socially. I hope we will still manage the odd trip around Sognsvann, wherever my post PhD journey takes me. **Elena Regine Moldal**, thank you for always being so positive and encouraging. **Carola Henriksson,** I'm so glad you were involved in this project. Your enthusiasm for all things coagulation is infectious. You made it clear early on that there is no such thing as a stupid question, so I asked them all and learned all the more for it.

Thank you also to **Marit Hellum,** for your patience with my endless thrombin generation questions and graph-pad struggles. **Sandip Kanse**, for welcoming me into your lab. **Tone Grøn, Malin Oscarson and Josefin Hultman** for getting the project off to a flying start, and **Bente Sævik**, for valuable clinical pathology discussions.

Henriette Solberg Jæger, thank you for rescuing me in the lab when serial dilutions and pipetting technique were alien concepts to me. **Liva Ihle Vatne** and **Jo Harris** for repeatedly rescuing a floundering non-cardiologist. **Kjell Henrik Ophus** and **Thor Håkonsen** at the Norwegian Herpetological Society, for your enthusiasm and help with venom collection and amazing photos of *Vipera berus*. **Janicke**

Nordgreen, for your patience and help with statistics. **Karin Zimmer** and **Michael Tranulis**, for reviewing my thesis and for your valuable feedback.

To the **staff and students** at Anicura Dyresykehus Oslo, Anicura Jeløy Dyresykehus Evidensia Oslo Dyresyekhus and NMBU Dyresykehus Smådyr, thank you for welcoming this project in through your doors and for remembering to call me every time you had a snakebite patient. Most importantly, thank you to the **dogs and their owners**, without whom none of this would have been possible.

Keith and Kate, you said it yourselves: friends are the family you choose. Thank you for ensuring that living in different countries doesn't matter and always being there to listen to PhD and life frustrations, and making me laugh along the way. **Mum**, thank you for always being there for a chat and pep talk when needed. **Dad**, thank you for your continuous support throughout my veterinary degree and the PhD and especially for the childminding, DIY, car mending, endless supply of tea, and the dad jokes to snap me out of a bad mood. **Ellie**, an all-round super person, thank you for all the long distance moral support and for somehow always finding time for my problems when you had more than enough on your plate already.

"For better, for worse, in sickness and in health, and through PhDs and pandemics". **Magnus**, thank you for your endless encouragement, support and pep talks. I'm not sure where I'd be without you (other than probably not in Norway!). Everyone needs a Magnus in their lives.

And last but not least, **Freya and Marcus**, thank you for putting up with a busy, tired and sometimes stressed mum, particularly over the last year. You inspire me and make me smile every day. Follow your dreams, whatever they may be and whichever country they may take you to.

Hannah Harjen

Oslo, June 2021

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1 Abbreviations

AECG	Ambulatory electrocardiogram/electrocardiography
AIVR	Accelerated idioventricular rhythm
AKI	Acute kidney injury
ALP	Alkaline phosphatase
APC	Activated protein C
aPTT	Activated partial thromboplastin time
AV	Atrioventricular
CAT	Calibrated automated thrombogram
CRP	C-reactive protein
cTnI	Cardiac troponin I
DAMPs	Damage-associated molecular patterns
ECG	Electrocardiogram
ETP	Endogenous thrombin potential
EVs	Extracellular vesicles
FDP	Fibrin degradation products
GFR	Glomerular filtration rate
GGT	Gamma glutamyl transferase
HMW	High molecular weight
IL-8	Interleukin-8
KIM-1	Kidney injury molecule 1
LAAO	L-amino acid oxidase
LLOQ	Lower limit of quantification
LMW	Low molecular weight
LOQ	Limit of quantification
LT	Lag time
MCP-1	Monocyte chemoattractant protein 1
NGAL	Neutrophil-gelatinase associated lipocalin
OPN	Osteopontin
PLA_2	Phospholipase A_2
PS	Phosphatidylserine
PT	Prothrombin time
ROS	Reactive oxygen species
SA	Sinoatrial

sCr	Serum creatinine
SDMA	Symmetric dimethylarginine
Snaclecs	Snake venom C-type lectins
SSS	Snakebite severity score
SVMP	Snake venom metalloprotease
SVSP	Snake venom serine protease
TAT	Thrombin-antithrombin
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TM	Thrombomodulin
uClusterin	Urine clusterin
uCr	Urine creatinine
uCysB	Urine cystatin B
UPC	Urine protein to creatinine ratio
USG	Urine specific gravity
VEC	Ventricular ectopic complex
VPC	Ventricular premature complex
VT	Ventricular tachycardia

2 List of papers

I. **Ambulatory electrocardiography and serum cardiac troponin I measurement in 21 dogs envenomated by the European adder *(Vipera berus).***
Harjen HJ, Bjelland AA, Harris J, Grøn TK, Anfinsen KP, Moldal ER, Rørtveit R. J Vet Intern Med. 2020 Jul;34(4):1369-1378.
https://doi.org/10.1111/jvim.15817

II. **Serial serum creatinine, SDMA and urinary acute kidney injury biomarker measurements in dogs envenomated by the European adder *(Vipera berus)*.**
Harjen HJ, Nicolaysen TV, Negard T, Lund H, Sævik BK, Anfinsen KP, Moldal ER, Zimmer KE, Rørtveit R. BMC Vet Res. 2021 Apr 12;17(1):154.
https://doi.org/10.1186/s12917-021-02851-8

III. **Evaluation of urinary clusterin and cystatin B as biomarkers for renal injury in dogs envenomated by the European adder *(Vipera berus).***
Harjen, HJ, Anfinsen, KP, Hultman, J, Moldal, ER, Szlosek, D, Murphy, R, Friis, H, Petersen S, Rørtveit, R.
Manuscript in review.

IV. **Persistent hypercoagulability in dogs envenomated by the European adder *(Vipera berus berus).***
Harjen, HJ, Hellum, M, Rørtveit, R, Oscarson, M, Anfinsen, KP, Moldal, ER, Solbak, S, Kanse, S, Henriksson, CE.
Manuscript in review.

3 Summary

Envenomation of dogs by the European adder (*Vipera berus* (*V. berus*)) is a common emergency in veterinary practice in Europe. Despite its common nature, little research exists regarding the clinical effects of this type of snakebite in dogs.

In this thesis, clinical and clinicopathological effects were prospectively and longitudinally assessed in dogs envenomated by *V. berus* with an emphasis on cardiac, renal and haemostatic systems. Whether severity of initial clinical signs might give an indication as to the subsequent development of effects on these body systems, was also of interest.

Dogs naturally envenomated by *V. berus* and presenting to the small animal clinics at NMBU Oslo, Evidensia Oslo Dyresykehus, Anicura Dyresykehus Oslo and Anicura Jeløy Dyresykehus, during 2017 and 2018, were recruited to the studies. A total of 60 envenomated dogs were included in different parts of the project and were assessed at five timepoints after bite, from presentation to the clinic to two weeks after bite. Data collection involved severity scoring of clinical signs, continuous 48-hour ambulatory electrocardiography (ECG), serum cardiac troponin I (cTnI, a cardiac injury biomarker) measurement, serum and urine biomarkers for kidney function and injury, and plasma coagulation parameters.

The main findings were:

- During the first 48 hours after envenomation, a large proportion of dogs developed myocardial injury, detected as a ventricular arrhythmia (12/21, 56%) or increased cTnI concentrations (17/21, 81%). Severe arrhythmias were detected in 6 of 21 (29%) dogs. Myocardial injury appeared to have resolved 14 days after bite (Paper I).

- There was evidence of mild, transient, non-azotaemic acute kidney injury (AKI) in dogs after *V. berus* envenomation, as measured by novel urinary AKI biomarkers and compared to a group of healthy control dogs. However, further assessment of many of the urinary biomarkers used in these studies

is needed to elucidate their specificity for AKI , especially in the face of concurrent systemic inflammation (Papers II and III).

- Envenomated dogs in this cohort were hypercoagulable already at presentation and still at 15 days after *V. berus* envenomation, as measured using a thrombin generation assay, thrombin-antithrombin complexes and phosphatidylserine equivalents, and compared to a group of healthy controls. Dogs that that receive antivenom treatment might be less hypercoagulable than their non-antivenom treated counterparts (Paper IV).

- The severity of clinical signs at presentation was not generally a useful indicator for the subsequent development of clinical or clinicopathological effects in this cohort of envenomated dogs (Papers I-III).

This thesis provides new insights into the effects of *V. berus* envenomation in dogs and contributes to the generally sparse evidence base upon which treatment and monitoring decisions of these patients can be made. Based on the cardiac and renal effects observed in this cohort of envenomated dogs, prolonged ECG monitoring for ventricular tachycardia and hospitalisation with supportive intravenous fluid therapy, appear to be sensible recommendations after *V. berus* bites in dogs. The clinical significance of the hypercoagulable state detected is unclear, but coagulation parameters measured in paper IV may serve as laboratory endpoints in future randomised controlled trials of antivenom efficacy.

4 Norsk sammendrag

Sykdom forårsaket av bitt av hoggorm (*Vipera berus*) er vanlig blant hunder i store deler av Europa. På tross hyppig forekomst er de kliniske effektene av hoggormbitt hos denne arten mangelfullt beskrevet.

I dette doktorgradsprosjektet ble kliniske og klinisk-patologiske effekter av hoggormbitt hos hund studert prospektivt, med hovedvekt på bittets effekter på hjerte, nyrer og koagulasjon. I tillegg ble det undersøkt om alvorlighetsgraden av kliniske sykdomstegn ved ankomst til dyreklinikk kunne forutsi i hvilken grad de studerte organsystemene ville rammes.

Hunder med hoggormbitt som ble behandlet ved smådyrklinikken ved Norges miljø og biovitenskapelige universitet (NMBU), Evidensia Oslo Dyresykehus, Anicura Dyresykehus Oslo og Anicura Jeløy Dyresykehus i løpet av 2017 og 2018 ble inkludert i studien, og totalt deltok 60 hunder i de ulike delene av prosjektet. Data ble samlet ved fem ulike tidspunkter, fra presentasjon på klinikk og frem til to uker etter bitt, og inkluderte en klinisk vurdering ved hjelp av et scoringssystem, kontinuerlig 48-timers ambulatorisk elektrokardiografi (EKG), serumnivåer av troponin I (cTnI, markør for hjerteskade), biomarkører for nyrefunksjon og akutt nyreskade (AKI) i serum og urin, samt koagulasjonsparametere i plasma.

Hovedfunnene i forskningsprosjektet kan oppsummeres i de følgende punktene:

- En betydelig andel av hundene utviklet myokardskade i løpet av de første 48 timene etter hoggormbittet, definert som ventrikulære arytmier (12/21, 56%) eller forøkede cTnI konsentrasjoner (17/21, 81%). Alvorlige arytmier ble oppdaget hos 6 av 21 (29%) av hundene. Etter 14 dager var avvikene normaliserte (Artikkel I).

- Ved hjelp av nyere biomarkører i urin for AKI, ble en mild, kortvarig, ikke-azotemisk AKI identifisert blant hundene som var bitt av hoggorm, ved sammenligning mot data fra en gruppe friske kontrollhunder. Videre studier er nødvendig for å klargjøre spesifisiteten til flere av disse

urinmarkørene, særlig i pasienter med systemisk inflammasjon (Artikkel II og III).

- Ved målinger av trombingenerering, trombin-antitrombinkomplekser og fosfatidylserin ekvivalenter, ble det funnet at hunder med hoggormbitt var hyperkoagulable allerede ved ankomst til klinikk, og fremdeles etter 15 dager, sammenlignet med en gruppe friske kontrollhunder. Hunder som ble behandlet med antivenin var mindre hyperkoagulable sammenlignet med hunder som ikke mottok denne behandlingen (Artikkel IV).

- Alvorlighetsgrad av kliniske sykdomstegn ved ankomst til klinikk samsvarte ikke med grad av myokard- eller nyreskade i denne kohorten med hunder bitt av huggorm (Artikler I-III).

Denne avhandlingen gir ny innsikt i effektene av huggormbitt hos hund, og bidrar til økt kunnskap som behandling og overvåkning av disse pasientene kan baseres på. Ut ifra effektene på hjerte og nyrer observert i denne studien, vil innleggelse og observasjon, inkludert EKG-overvåkning, samt intravenøs væskebehandling være et hensiktsmessig behandlingsregime ved denne tilstanden. Den kliniske betydningen av den hyperkoagulable tilstanden til hunder bitt av hoggorm er foreløpig usikker, men koagulasjonsparametere brukt i artikkel IV kan være til nytte i randomiserte kontrollerte kliniske studier med hensikt å vurdere klinisk effekt av antivenin-behandling ved huggormbitt hos hund.

5 Introduction

"It is the bright day that brings forth the Adder, and that craves wary walking"

William Shakespeare, 1599.

5.1 Background

A recent systematic review described envenomation by *Vipera species* in Europe as a neglected disease and highlighted the need for standardised protocols for snakebite management in humans (Paolino et al., 2020). A similar situation exists in veterinary medicine.

Accidental envenomation of dogs by the European adder (*Vipera berus, V. berus)* is a common seasonal emergency. Recording of snake envenomation is not mandatory in most European countries, including Norway, and the true incidence of *V. berus* envenomation in humans and dogs is therefore likely underestimated. However, data obtained from the recently developed national veterinary diagnosis register indicate that the incidence of *V. berus* envenomation could be as high at 150 cases per 100 000 dogs per year in Norway (private communication, Pyramidion, DyreID AS).

Despite the relative frequency of this emergency, studies investigating the clinical effects of this type of snakebite are sparse both in human and veterinary medicine, and evidence-based guidelines for the treatment of dogs bitten by *V. berus* are consequently lacking. Evidence-based treatment protocols and recommendations would allow clinicians to optimise the management of these patients and to better inform dog owners of the expected disease course and prognosis.

The theme for this thesis is broad: **What are the effects of *V. berus* bites in dogs, and what implications do they have in terms of treating and monitoring envenomated dogs?**

5.2 *Vipera berus (berus)*

Vipera berus is a venomous snake of the Viperidae family (subfamily Viperinae, 'true' vipers). Of the European vipers, *Vipera berus* is the most geographically widespread (Chippaux, 2012) and is estimated to be responsible for over 60% of human viper envenomations in Europe (Paolino et al., 2020). The World Health Organization classifies *V. berus* as a category two, medically significant snake, defined as:

''*Highly venomous snakes capable of causing morbidity, disability or death, for which exact epidemiological or clinical data may be lacking; and/or are less frequently implicated (due to their activity cycles, behavior, habitat preferences or occurrence in areas remote to large human populations)*''

(World Health Organization, 2017)

Two subspecies of *V. berus* are recognised: *V. berus berus* (the common European adder) and *V. berus bosniensis* (the Balkan cross adder). *Vipera berus bosniensis* is found only in the Balkans, whereas *Vipera berus berus* is widely distributed throughout Europe and is the only native venomous snake in Scandinavia (Mallow et al., 2003, World Health Organization, 2017, Chippaux, 2012). This thesis concerns *V. berus berus* envenomation of dogs in Norway, hereafter referred to as *V. berus,* for simplicity.

Vipera berus is found in a variety of habitats including woodland, rocky hillsides, grassland and the banks of lakes and ponds (Mallow et al., 2003). *Vipera berus* is often recognized by its dorsal zigzag pattern and a distinctive V or X on the head, although melanistic individuals with an indiscernible dorsal pattern also exist and rarely the zigzag may be absent or replaced with a continuous stripe (Mallow et al., 2003) (Figure 1). *Vipera berus* has an inactive period of winter dormancy or "brumation", triggered by a fall in maximum daily temperature to 13°C and ending in the spring when maximum daily air temperatures reach 8 -12°C (Mallow et al., 2003). Incidents of envenomation by this species of snake therefore show a seasonal distribution with most cases in Scandinavia occurring between April and October and peaking in June and July (Hermansen et al., 2018, Karlson-Stiber et al., 2006, Kängström, 1989).

Figure 1. Pattern and colour variation in Norwegian *Vipera berus*. (Photos: Thor Håkonsen)

5.3 Venom

The word “venom” is generally associated with negative attributes and is thought to be derived from the Latin “venenum” (a magic substance or love potion) and Middle English words “venomous”, “venyme” or “venome” (meaning malice, poison or bitterness) (Weinstein, 2015). The poisonous properties of snakes have long been recognised, and used in warfare, such as the putrefied mixture of decomposing viperids used to coat arrowheads by the Scythians, circa 440 BC (Weinstein, 2015). A direct association between a yellow liquid originating from the fangs of Italian vipers, and the pathophysiological effects of snakebite were first described in 1664 by Francesco Redi, dispelling the commonly held belief that bad spirits formed by snakes were the cause of poisoning (Habermehl, 1994, Redi, 1664) . These findings were confirmed by Abbé Felice Fontana in 1764, who further described a specialised venom delivery apparatus in the European viper (*V. berus* or *Vipera aspis* (*V. aspis*)) and the pathophysiological effects of its venom (Hawgood, 1995, Fontana, 1781).

From a modern perspective, venoms are probably best described as complex mixtures of bioactive toxins designed to acquire and digest prey and to defend against threat through a variety of biological targets and functions (Casewell et al., 2020). Snake venom is produced by secretory cells in the venom gland and delivered to the victim through a wound created by specialised fangs (Fry, 2015).

5.3.1 Snake venom composition and biological effects

Snake venom composition and its biological effects is an extensive and complex topic. Many studies describe modes of action and biological effects of venom toxins in general terms, but few studies demonstrate specific effects of individual components of *V. berus* venom. Unless otherwise stated, references to mode of action and biological effects in this thesis are therefore generalised for the toxin group rather than specifically demonstrated effects of isolated components in *V. berus* venom.

Snake venoms are broadly described as exerting haemotoxic, cytotoxic or neurotoxic effects (World Health Organization, 2010). *Vipera berus* venom has mostly been associated with haemotoxicity (Bocian et al., 2016) and some

cytotoxicity, although individual case reports also describe neurotoxicity in humans (Malina et al., 2008, Malina et al., 2013). The most recent proteomic study of *V. berus* venom identified more than 80 distinct proteins from 15 toxin families in the venom of Russian *V. berus* (Al-Shekhadat et al., 2019). Percentages of the various components in *V. berus* venom vary considerably between proteomic (venomic) studies, likely due to intra-species variation and differences in methodology. However, phospholipases A_2 (PLA_2), snake venom metalloproteinases (SVMPs) and snake venom serine proteases (SVSPs), have consistently been identified as the dominant venom protein families across these studies. An overview of the components identified in *V. berus* venom, with possible associated modes of action and related biological effects, are presented for reference, in Table 1.

Whilst it follows that the major protein groups are the most medically relevant components of *V. berus* venom, the importance of some of the quantitatively minor components should not be overlooked. Hyaluronidases for example are generally found in low concentrations but have been associated with increased venom lethality in other types of snake (Bordon et al., 2012).

The potential effects of *V. berus* venom are diverse and paradoxical, hindering predictions as to their net clinical effects, especially when other factors such as the amount of venom injected, are considered. The majority of *V. berus* venom toxins have coagulopathic potential through direct pro- and anticoagulant effects, effects on the fibrinolytic pathway and promotion or inhibition of platelet activity. Local tissue damage, haemorrhage, myonecrosis and oedema are described in murine models of *V. berus* envenomation, albeit from a global venom perspective rather than specific pathophysiological contributions from individual venom components (Calderón et al., 1993). Figure 2 summarises the main possible effects of *V. berus* envenomation on different body systems.

Table 1: Protein families identified in three *V. berus* venomics studies* with relative abundance (%), mode of action for the general toxin group and possible biological effects. Studies specific to *V. berus* venom components are referenced in bold.

Component	%	Modes of action	Examples of associated biological effects
Phospholipase A_2 (PLA_2) (type II)	10-59	Hydrolysis of ester bonds in membrane phospholipids. Membrane damage and release of arachidonic acid. Formation of pro and anti-inflammatory mediators and oxidative injury. Inhibition of prothrombinase and tenase complexes. Inhibition of platelet aggregation. (Burke and Dennis, 2009, Teixeira et al., 2003, Markland, 1998) .	Inflammation and tissue injury. Pain, oedema, haemolysis, neurotoxicity, myotoxicity, cardiotoxicity, anticoagulant effects, hypotension (**Karabuva et al., 2017, Boffa and Boffa, 1976**, Teixeira et al., 2003, Kini, 2003, **Zanetti et al., 2018)**.
Snake Venom Serine Proteases (SVSPs)	15-31	Hydrolysis of peptide bonds in pro-enzymes in the coagulation cascade. Thrombin-like fibrinogenolytic activity. Prothrombin, factor V and factor X activation. Platelet activation. Inhibition of the intrinsic tenase complex. Plasminogen activation. Protein C activation. (Latinović et al., 2018, Serrano and Maroun, 2005, Slagboom et al., 2017).	Pro- and anticoagulant activity. Hypotension. (Péterfi et al., 2019, Serrano and Maroun, 2005).
Snake Venom Metalloproteases (SVMPs)	3-19	Proteolytic degradation of basement membranes and extracellular matrix. Vessel wall disruption. Factor X activation or degradation, prothrombin activation or degradation, platelet activation or inhibition, fibrinogenolysis **(Samel et al., 2003, Samel and Siigur, 1995, Latinovic et al., 2016,** Calvete et al., 2009).	Pro-and anticoagulant activity. Local and systemic haemorrhage. Haemolysis. Inflammation, oedema and tissue necrosis. (Gutiérrez and Rucavado, 2000, Slagboom et al., 2017)

Natriuretic peptides	7.8-11	Angiotensin-converting enzyme inhibition. Increase in cyclic GMP signaling (St Pierre et al., 2006).	Vasodilation, diuresis and natriuresis leading to hypotension (Péterfi et al., 2019).
L-Amino acid oxidases (LAAOs)	2-9	Oxidative stress through oxidative deamination of L- amino acid (Fox, 2013).	Tissue injury, apoptosis. Inhibition of platelet aggregation **(Samel et al., 2006).**
Cysteine-Rich Secretory Proteins (CRISPs)	6-8	Blockage of cyclic nucleotide -gated and voltage gated calcium channels (Yamazaki and Morita, 2004).	Inhibition of smooth muscle contraction (Yamazaki and Morita, 2004).
Snake venom C-type lectins (Snaclecs)	2-6	Non enzymatic inhibition and activation of platelet membrane receptors and coagulation factors (Lu et al., 2005, Arlinghaus and Eble, 2012).	Procoagulant and anticoagulant activity. Activation or inhibition of platelets. (Lu et al., 2005, Arlinghaus and Eble, 2012).
Kunitz -type protease inhibitors	0.07- 2.6	Inhibition of thrombin and plasmin (Slagboom et al., 2017).	Anticoagulant and antifibrinolytic activity (Mukherjee and Mackessy, 2014, Slagboom et al., 2017).
Disintegrins	1-1.6	Non-enzymatic. Bind to glycoprotein IIIb/IIa (integrin) on activated platelets and inhibit of their interaction with fibrinogen (Kamiguti et al., 1998).	Inhibition of platelet aggregation (Kamiguti et al., 1998).
Hyaluronidase	0-0.1	Degradation of hyaluronic acid leading to structural alteration to the extracellular matrix (Girish et al., 2002).	Increased permeability of tissue at bite site ''spreading factor''(Girish et al., 2002).

*(Al-Shekhadat et al., 2019, Bocian et al., 2016, Latinovic et al., 2016)

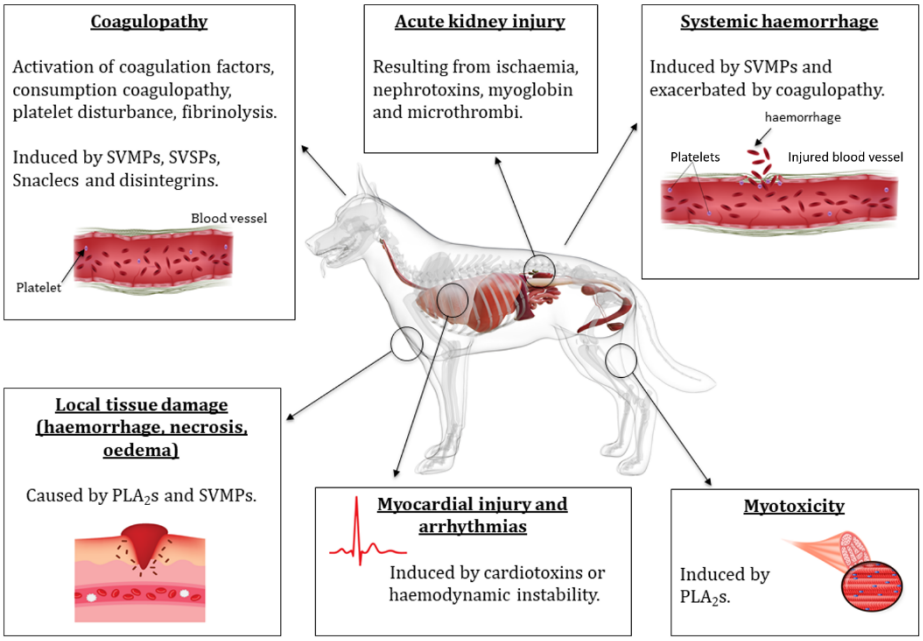


Figure 2: The possible actions of *V. berus* venom components on different body systems. PLA_2s; phospholipases A_2, Snaclecs; snake venom C-type lectins, SVMPs; snake venom metalloproteases, SVSPs; snake venom serine proteases. *Based on information and a similar figure in Snakebite envenoming Gutiérrez, J. M. et al. (2017) Nat. Rev. Dis. Primers.*

5.3.2 Snakebite-related inflammation and oxidative stress

Snakebite effects on the body are not only mediated directly by venom proteins, but also by the reaction of the individual to the venom (Bickler, 2020). Snake venom PLA_2s, L-amino acid oxidases (LAAOs), SVMPs and hyaluronidases can lead to a multitude of downstream inflammatory and oxidative stress-related effects (Sunitha et al., 2015). Whilst a detailed description of the possible inflammatory and oxidative stress-related mechanisms of snakebite injury is beyond the scope of this thesis, a brief overview is useful to help understand the possible pathophysiological effects of *V. berus* bites beyond direct cytotoxic and haemotoxic effects.

In basic terms, inflammation is a response made by the body in reaction to infection or tissue injury, the cardinal signs of which are pain, swelling, heat and redness. This response is intended to eliminate the inciting cause and initiate healing and a return

to normal function by increasing vascular permeability, vasodilation and recruiting leucocytes (primarily neutrophils) to the site of injury through chemotaxis. Chemical mediators originating from plasma (such as complement and proteins derived from the coagulation system) and cellular mediators (such as cytokines, chemokines and histamine), drive this process and have been shown to be increased in humans envenomated by other snake types (Teixeira et al., 2003, Stone et al., 2013). Upon reaching afflicted tissues, neutrophils become activated and release toxic granules, including reactive oxygen species (ROS) aimed at removing the inciting cause of inflammation but that can also damage host tissues (Medzhitov, 2008). Although initially intended as protective, inflammation can become maladaptive if left unchecked.

Oxidative stress may be defined as a "state harmful to the body, which arises when oxidative reactions exceed antioxidant reactions''. Oxidative metabolism releases inflammatory and cytotoxic ROS, the effects of which are normally counteracted by antioxidant defence mechanisms. If ROS are produced in excess, these defence mechanisms become overwhelmed, leading to oxidative stress (Yoshikawa and Naito, 2002).

Venom PLA_2s trigger a marked inflammatory response through cell membrane degradation and release of the potent inflammatory agent, lysophosphatidylcholine (Sunitha et al., 2015). Oxidative stress may ensue as a result of ROS production from arachidonic acid metabolism, but also from free iron released from the haemolytic and rhabdomyolytic effects of PLA_2 and SVMPs (Sunitha et al., 2015). Haemorrhage and tissue injury caused by snake venom toxins can lead to the release of damage-associated molecular pattern molecules (DAMPs) which in turn can contribute to oxidative injury and inflammation through activation of target cells and upregulation of inflammatory cytokine production (Sunitha et al., 2015). Non-enzymatic venom components such as Snaclecs might contribute to inflammation by inducing neutrophilic infiltration and subsequent release of proinflammatory cytokines (Elifio-Esposito et al., 2011). A basic overview of the mechanisms by which snake venom components can lead to inflammation and oxidative stress, is shown in Figure 3.

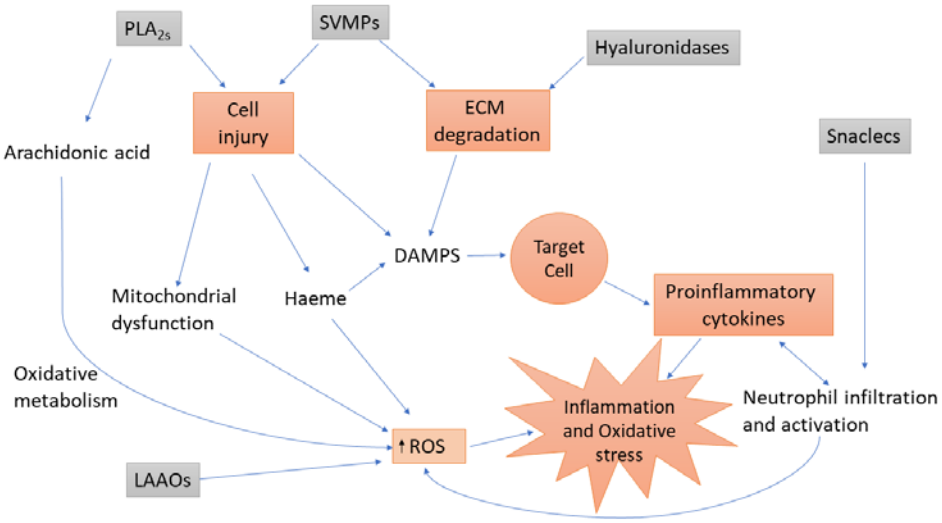


Figure 3: Simplified mechanisms by which select venom components can induce inflammation and oxidative injury. DAMPs; danger-associated molecular patterns, ECM; extracellular matrix, LAAOs; L-amino acid oxidases, PLA_2s, phospholipases A_2, Snaclecs; snake venom C-type lectins, SVMPs; snake venom metalloproteases, ROS; reactive oxygen species. *Based on information and figures in Inflammation and oxidative stress in viper bite: an insight within and beyond. Sunitha K. et al. Toxicon. 2015; 98:89-97.*

Increased concentrations of the positive acute phase inflammatory markers C-reactive protein (CRP) and serum amyloid A (SAA), have been measured in dogs bitten by *V. berus* (Christensen et al., 2014, Langhorn et al., 2014), confirming that *V. berus* envenomation induces a systemic inflammatory response. Oxidative stress is considered a highly relevant but somewhat overlooked mechanism of snake venom cytotoxicity which may be particularly important when considering systemic sequels to envenomation such as kidney injury (Mukhopadhyay et al., 2016). Furthermore, oxidative stress and inflammation are reciprocally related (Lugrin et al., 2014) as are inflammation and coagulation (O'Brien, 2012), thus the effects of envenomation may be wide ranging and self-perpetuating.

5.3.3 Venom variation

Snake venom composition varies at both the interspecies and intraspecies level (Tasoulis and Isbister, 2017, Chippaux et al., 1991). The finding of a neurotoxic component in the venom of a Hungarian population of *V. berus* (Malina et al., 2017) and differences in proportions of toxin groups found in venomics studies, indicate a population-level variation in venom composition for *V. berus*. Age, sex and individual-level variations in venom have also been described for this species (Malina et al., 2017) and are likely related to ontogenetic dietary shifts and adaptation to specific ecological niches (Casewell et al., 2020).

Given the inter- and intraspecies differences in venom composition, care should be taken not to unequivocally extrapolate findings from studies of other snake species, including other European vipers, and apply them to *V. berus* and the clinical effects of this type of snakebite. Where published data for *V. berus* is lacking, other snake species are referred to in this thesis, with the caveat that the comparison may give an indication only.

5.3.4 Venom pharmacokinetics

Venom is usually injected subcutaneously or intramuscularly and subsequently absorbed via the lymphatic and vascular systems. Higher molecular weight toxins, predominant in *Vipera* venoms, are primarily absorbed via the lymphatics, whereas smaller toxins may be absorbed via the vasculature. Absorption is dependent on the anatomical site of the bite, molecular weight of the individual venom molecules and other factors such as movement and limb position in the immediate post-bite period (Fry, 2015, Paniagua et al., 2017). The bioavailability of snake venoms varies considerably and may be as low as 4% in some cases, indicating that not all venom proteins are absorbed from the bite site and that some might be retained and contribute to local tissue damage (Sanhajariya et al., 2018).

Pharmacokinetic studies of snake venom in the absence of antivenom are generally sparse and, to the author's knowledge, have not been published specifically for *V. berus* venom other than a report of local oedema reaching a maximum 0.5-1 hours after envenomation in a murine model (Calderón et al., 1993). An estimated maximum plasma venom concentration 0.5-4 hours after bite and a biological half-

life of 6-16 hours has been described for *V. berus* venom in one review paper, but the primary source appears to be a single clinical case report and this data therefore needs further validation in larger, pharmacokinetic studies (Czajka et al., 2013).

Pharmacokinetic data has been published for *V. aspis.* Venom was detected in plasma as soon as 0.5 hours after bite, and for up to 36 hours, with an apparent half-life of 8 hours in a study of humans envenomated by *V. aspis* (Audebert et al., 1994a). In a rabbit model, initial absorption of *V. aspis* venom occurred within 10 minutes of intramuscular injection and persisted up to 72 hours, with a terminal half-life of 32 hours, indicating that high venom concentrations may be maintained for several days after bite. Studies of other types of snake have demonstrated a suspected depot effect of venom allowing toxins to persist and exert effects for weeks after envenomation (Seifert et al., 1997, Judge, 2013), although it is not known whether this also applies to *V. berus* venom.

Less than 5% of *V. aspis* venom was detected in the urine of envenomated rabbits, indicating that this is unlikely to be the main route of venom excretion (Audebert et al., 1994b). Smaller venom molecules may be eliminated more rapidly by the kidneys whereas larger molecules are likely to be cleared more slowly via the reticuloendothelial system (Fry, 2015).

5.4 *Vipera berus* envenomation in dogs

Given the diversity of toxins in *V .berus* venom and their direct and indirect biological actions, it follows that a range of clinical effects may be induced in the envenomated dog. It also follows that some of these effects may be more subtle and thus, overlooked. Furthermore, venom elimination rates, a possible depot effect and secondary self-amplifying mechanisms may lead to effects that persist beyond the immediate envenomation period.

Inter- and intra-snake variation in venom composition, differences in volume of venom injected both in terms absolute dose and relative to body weight, anatomical location of the bite and individual susceptibility to venom are all factors that may influence the clinical effects observed in the envenomated individual. The currently reported effects of *V. berus* bites in dogs are summarised in this section.

5.4.1 Clinical signs and clinical pathology findings

An overview of clinical signs observed in dogs after *V. berus* envenomation are presented in Table 2, and photographic examples from this thesis work, in Figure 4. Dogs are most often bitten in the head or neck (> 70% of cases) or in a limb (approximately 20% of cases) (Bolon et al., 2019, Lervik et al., 2010, Kängström, 1989, Lund et al., 2013). Presenting clinical signs most commonly include local swelling, pain, tachycardia, lethargy or depression, bruising, hypersalivation, hyperthermia, collapse and vomiting (Sutton et al., 2011, Turkovic et al., 2015, Lervik et al., 2010). A combination of local and systemic effects appears most common, although these effects may also occur in isolation (Sutton et al., 2011). Of particular note is the finding by Sutton et al (2011) that systemic effects occurred in the absence of local effects in 7% of cases, which may cloud the clinical picture.

Table 2. Clinical signs reported in 422 dogs bitten by *V. berus* (Sutton et al., 2011)

Clinical Signs	**Number of cases (n= 422)**
Oedema	92.4% (n=379)
Lethargy	14.4%
Depression	11.2%
Pain	11.2%
Hyperthermia	10.7%
Tachycardia	10%
Bruising	9.8%
Hypersalivation	8.8%
Collapse	8.3%
Vomiting	7.6%
Tachypnoea	5.1%
Haemorrhage	4.9%
Lameness	4.4%

Haematological findings of leucocytosis, haemoconcentration and thrombocytopenia are reported in *V. berus* envenomated dogs (Kängström, 1989, Turkovic et al., 2015), but are not consistent findings (Brandeker et al., 2015). Biochemistry findings after *V. berus* bite are generally mild and include hyperglycaemia, hypoalbuminaemia, increases in the liver enzyme activities alkaline phosphatase (ALP) and alanine transaminase (ALT), increased CRP and SAA, and creatine kinase (CK) (Lervik et al., 2010, Turkovic et al., 2015, Kängström, 1989, Christensen et al., 2014). Thus, indicating stress, inflammation, hepatic and muscular injury after this type of snakebite in dogs.

Cardiac arrhythmias and myocardial injury have been reported in several studies (Pelander et al., 2010, Kängström, 1989, Vestberg et al., 2017, Lervik et al., 2010, Brandeker et al., 2015, Langhorn et al., 2014). Renal and coagulopathic effects are also reported, albeit less frequently (Sutton et al., 2011, Gordin et al., 2020, Palviainen et al., 2013, Brandeker et al., 2015, Turkovic et al., 2015). These effects are described in more detail in the dedicated sections that follow.

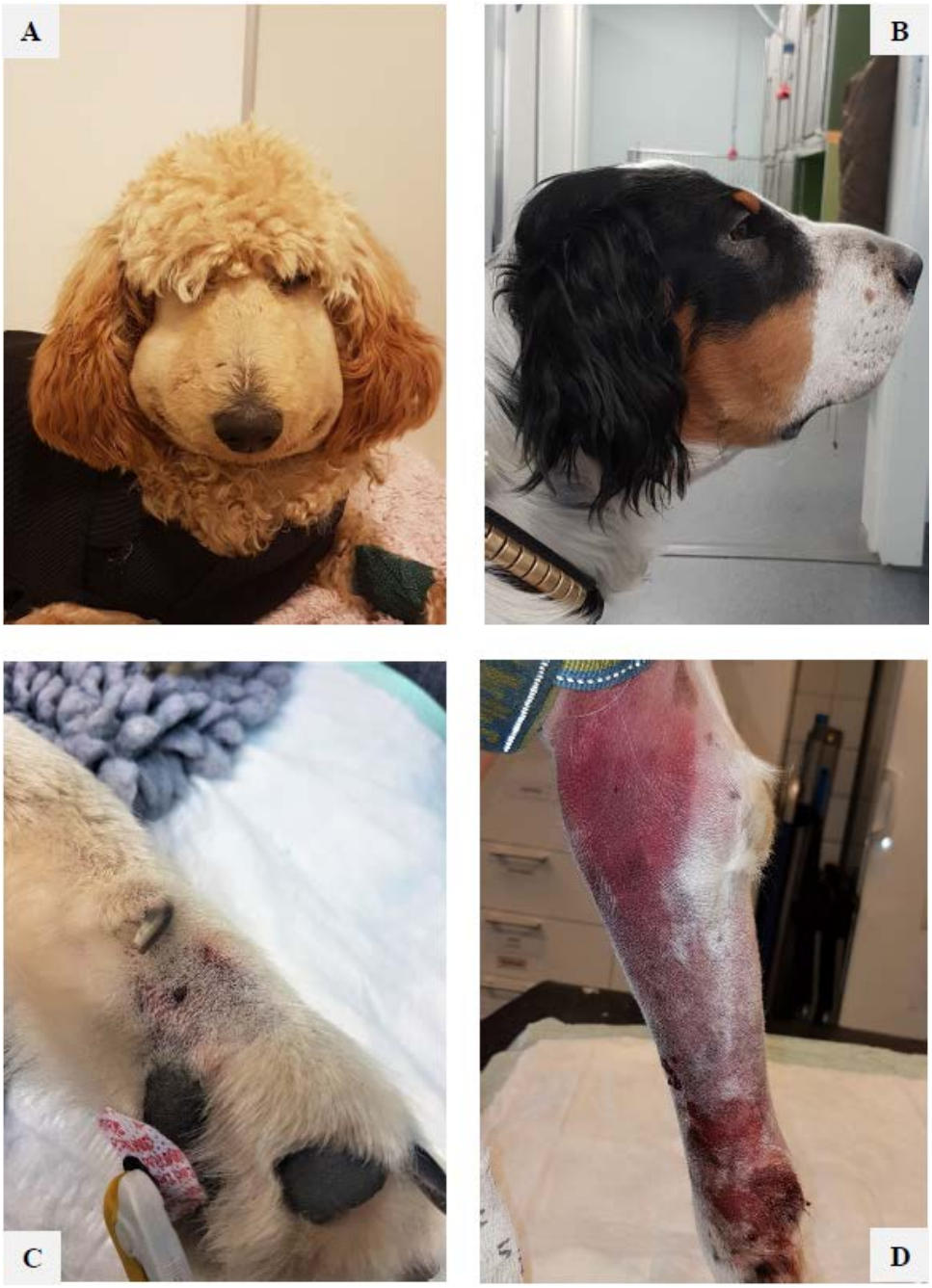


Figure 4. Clinical findings in dogs bitten by *Vipera berus*. Facial swelling after a bite to the head (A and B), fang marks (C) and extensive bruising/extravasation of blood from a bite to the limb with persistent bleeding from the bite wound, 24 hours after bite (D). (Photos taken by the author, with permission from dog owners).

5.4.2 Diagnosis

There are currently no commercially available tests to definitively diagnose *V. berus* envenomation. Immunoassays have been used to measure *V. berus* and *V. aspis* venom concentrations in research settings (Sjostrom et al., 1996, Audebert et al., 1992) and more recently a PLA_2 assay has shown promise as a tool for diagnosing snakebite (Maduwage et al., 2014), but these assays have not been developed for clinical use. A diagnosis of *V. berus* envenomation in dogs is therefore generally made based on a history of a geographical location and seasonal timing compatible with the presence of *V. berus*, and clinical signs consistent with envenomation such as acute onset swelling and pain. The snake is sometimes witnessed by the dog owner and fang marks may be observed on clinical examination. However, a study of dogs envenomated by *Vipera palestinae* found fang marks in only 50% of cases (Segev et al., 2004), indicating that this should not be relied upon as a diagnostic criterium for snakebite in dogs.

It is estimated that up to 30% of *V. berus* bites are void of venom, referred to as "dry" bites (Persson and Irestedt, 1981), and thereby do not result in significant signs of envenomation. It is unclear how long patients should be monitored before a snakebite can be declared "dry" and thereby not of clinical concern. Some human studies suggest patients have not been envenomated if there is a lack of local swelling within two hours of the bite (Reading, 1996, Karlson-Stiber et al., 2006). However, since dogs may develop systemic signs of envenomation in the absence of local signs (Sutton et al., 2011), monitoring for 12-24 hours after the bite is likely more appropriate (Pucca et al., 2020, Naik, 2017). In humans, stress and anxiety associated with a snakebite, albeit a dry bite, can lead to clinical signs that may be confused with envenomation such as tachycardia, tachypnoea, syncope and vomiting (Pucca et al., 2020), although this is less likely to be an issue in canine snakebite patients.

5.4.3 Treatment

Treatment of *V. berus* bites in dogs consists of opioid analgesia, intravenous fluid therapy, and in many cases, antivenom. The only currently available specific treatment for snakebite is antivenom therapy. In Norway, an equine derived $F(ab')_2$ antivenom is available for use in dogs. This type of antivenom is associated with

adverse reactions in approximately 10% of dogs and humans (Lund et al., 2013, Karlson-Stiber et al., 1997), although these effects generally appear mild and self-limiting. Bearing this in mind, antivenom administration is based on an individual cost-benefit assessment. Guidelines for antivenom use in canine *V. berus* envenomation are based on recommendations adopted from human medicine. Broadly speaking, indications for antivenom treatment include severe local or systemic signs or a lack of improvement or deterioration despite supportive treatment in dogs with mild local or systemic signs. In humans, UK national guidelines (TOXBASE, NPIS, obtained from (Lamb et al., 2021), state that antivenom is indicated if the patient has any of the following:

1. Anaphylactic reaction to the venom
2. Hypotension persisting for more than 10 minutes
3. Abdominal pain or diarrhoea
4. Leucocytosis
5. ECG abnormalities
6. Metabolic acidosis
7. Increased creatine kinase
8. Severe local envenoming such as swelling spreading beyond the next major joint; with or without systemic features
9. Evidence of systemic envenoming such as haemorrhage or pulmonary oedema

There is currently insufficient evidence to indicate a positive effect of antivenom treatment on time to recovery in *V. berus* envenomated dogs (Hodgson and Brambilla, 2017). Randomised controlled trials for the efficacy of antivenom treatment after *V. berus* envenomation are lacking from both human and veterinary medicine. Such studies would help to clarify the questions surrounding antivenom treatment, but first more objective endpoints for such studies need to be identified and this may be achieved through observational studies.

Corticosteroids have historically featured in the treatment of *V. berus* envenomation in both humans and dogs, with owners even being provided with tablets for emergency administration to their dog in the event of snakebite. However, studies have since shown the clinical benefit of corticosteroid administration to be doubtful, and thus, corticosteroid treatment is no longer common practice (Karlson-Stiber et al., 2006, Brandeker et al., 2015).

5.4.4 Mortality rate

Abbé Felice Fontana described the first experimental injection of European viper venom into a dog in 1781 and concluded that the viper ejected insufficient venom to kill a large dog in a single bite (Hawgood, 1995) . This has since been disproved with mortality rates after *V. berus* envenomation in dogs estimated to be in the region of 3-4% (Sutton et al., 2011). Representative mortality rate data is however difficult to obtain due to factors such as acute deaths or mild cases not presenting for treatment and a lack of diagnostic recording.

5.4.5 Post-mortem findings

Very few studies have reported post-mortem findings in dogs bitten by *V. berus*, presumably due to the relatively low associated mortality rate. Findings described include subcutaneous and intramuscular haemorrhage, thrombosis, disseminated intravascular coagulopathy (DIC); hepatocellular degeneration, necrosis and icterus; glomerulopathy, thrombosis of vessels at the renal cortico-medullary junction with tubular degeneration and necrosis; myocarditis, coronary artery thrombosis and myocardial petechiae; gastric bleeding, venous thrombosis and necrosis of the colon, and melaena (Kängström, 1989, Kolbjørnsen, 2014). Thus, illustrating the diverse effects of envenomation.

Based on the knowledge of the potential effects of *V. berus* venom on the various body systems and clinical studies and observations to date, three key areas were chosen for the studies that form this thesis:

1. Cardiac effects
2. Renal effects
3. Coagulopathic effects

In the following sections, these three areas are introduced in more detail, with specific reference to venom effects, pathophysiology, current knowledge, and identification of knowledge gaps.

5.5 Cardiac effects of envenomation

Based on knowledge of venom contents and its biological actions, there are several mechanisms by which the heart may be affected in *V. berus* envenomation. The cardiotoxin, ammodytin L, has been identified in *V. berus* venom suggesting a possible direct cardiotoxic effect (Karabuva et al., 2017, Latinovic et al., 2016). Hypotension, hypovolaemia, ischaemia and myocardial infarction are other possible mechanisms (Gutiérrez et al., 2017). Additionally, snakebite-induced systemic inflammation could play a role in cardiac injury (Langhorn et al., 2014, Hamacher et al., 2015). Post-mortem findings of myocarditis, myocardial infarcts and coronary vessel thrombosis in dogs envenomated by *V. berus*, are suggestive of inflammatory, ischaemic and thrombotic causes of cardiac injury (Kängström, 1989).

Cardiac effects of envenomation may manifest as electrophysiological changes detected clinically as arrhythmias or other changes on an electrocardiogram (ECG), increased levels of cardiac injury biomarkers, or both (Pelander et al., 2010, Langhorn et al., 2014). Cardiac arrhythmias are a complex subject. Given the broad nature of this thesis it is impossible to cover all aspects of this topic. Thus, the focus of the following section is a basic overview of snakebite-relevant arrhythmias and their clinical importance, as well as diagnosis of arrhythmias and cardiac injury.

5.5.1 Arrhythmias in a nutshell

The term “cardiac arrhythmia” refers to any changes in the normal electrical activity in the heart and may include abnormalities in impulse formation and propagation. Arrhythmias include ectopia, abnormalities in rhythm, rate and conduction (Martin, 2002), of which ectopia is the most commonly reported after *V. berus* envenomation and which therefore forms the main focus of this introductory section.

Electrical depolarisations originating from a location other than the dominant cardiac pacemaker cells in the sinoatrial (SA) node are termed ectopic beats, observed as abnormal “ectopic” complexes on an ECG. Ectopic complexes may be ventricular or supraventricular in origin. Various causes and mechanisms of ectopia exist, examples of which include cardiac pathology, myocardial hypoxia and electrolyte disturbances (Martin, 2002).

Ventricular ectopic complexes (VECs) do not follow the normal, fast cardiac electrical conduction pathways and therefore result in a depolarisation wave that is both slow and in an abnormal direction, observed on an ECG as an abnormally shaped ”wide and bizarre” QRS complex (Figure 5). Ventricular ectopic complexes may occur as a rescue mechanism by subsidiary pacemaker cells (in the bundle of His or Purkinje fibres) with a slow intrinsic rate, after a pause in conduction between the atria and ventricles (such as atrioventricular (AV) block or sinus arrest), called ventricular escape complexes. If the ectopic complex occurs before the next normal complex is expected, it is termed a ventricular premature complex (VPC). Ventricular premature complexes may occur singly, in couplets or in triplets, and sometimes in a set ratio of one normal sinus complex to one ectopic (bigeminy) or one ectopic to two normal sinus complexes (trigeminy). Four or more consecutive VPCs are known as ventricular tachycardia (VT). Accelerated idioventricular rhythm (AIVR), sometimes referred to as slow VT, is a term used to describe a ventricular arrhythmia with a rate between that of a ventricular escape rhythm (< 60/min) and VT (> 180/min). The rates used to define AIVR and VT vary in the veterinary literature, a subject returned to later.

Supraventricular ectopics are categorized as escape complexes (junctional escapes, from the AV node) or premature supraventricular premature complexes (SVPCs) of which a run of four or more is known as supraventricular tachycardia (SVT). Atrial fibrillation is probably the most commonly diagnosed supraventricular tachyarrhythmia in dogs. Arrhythmias may also be caused by faulty impulse generation from the SA node or a fault in conduction of the impulse from the SA node through the specialized conduction system (AV node, bundle of His and Purkinje fibres). Such arrhythmias include sinus bradycardia, sinus arrest (a total failure of the SA node to generate an impulse), and AV block (a failure of normal conduction through the AV node).

Ventricular arrhythmias and AV block have been described in dogs envenomated by *V. berus* (Pelander et al., 2010, Vestberg et al., 2017). Supraventricular arrhythmias have not been reported in association with *V. berus* envenomation but did feature in a study of dogs envenomated by *Vipera palaestinae* (Segev et al., 2008).

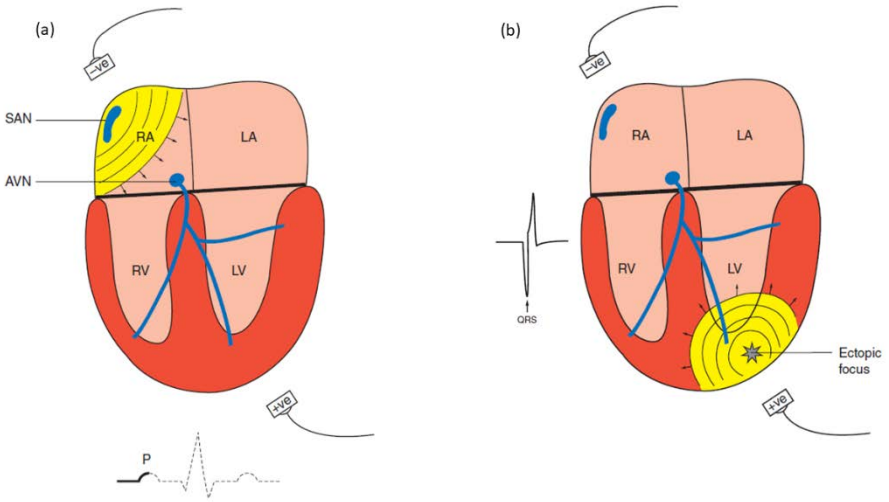


Figure 5. Cardiac depolarisation with associated ECG complexes. (a) Under normal circumstances, a wave of atrial depolarisation is triggered by dominant pacemaker cells in the sinoatrial node (SAN) (seen as a P wave on an ECG) and transmitted via the atrioventricular node (AVN) to the cardiac conduction tissue in the ventricles, forming the QRS complex observed on ECGs. (b) An ectopic focus in the ventricle triggers a wave of slow depolarisation in an abnormal direction, appearing as an abnormal QRS complex on ECG with an absent P wave. RA; right atrium, LA; left atrium, RV; right ventricle, LV; left ventricle. +ve and -ve represent ECG electrodes*. Reproduced from Small animal ECGs: an introductory guide. Martin, M. John Wiley & Sons, 2015. With permission via Copyright Clearance Center.*

5.5.2 Clinical relevance of arrhythmias

Many arrhythmias are well tolerated and do not require specific treatment other than identifying and addressing a possible underlying cause. Arrhythmias are likely to be clinically significant if they result in haemodynamic compromise (observed clinically as hypotension, reduced mentation, weak pulses, mucous membrane pallor or syncope), or if they have the potential to cause sudden death. As a general rule, the significance of ventricular arrhythmias increases with the rate and complexity of the ectopic beats (Lown and Wolf, 1971). Accelerated idioventricular rhythm is generally considered benign since it is not normally associated with significant hemodynamic compromise and is therefore well tolerated (Mavropoulou, 2018). Ventricular tachycardia on the other hand, can lead to significant

haemodynamic derangement and has been associated with sudden death (Klüser et al., 2016, Wiberg et al., 2019). Ventricular fibrillation is a terminal arrhythmia, often preceded by VT.

5.5.3 Cardiac effects associated with *Vipera berus* envenomation in dogs

A handful of studies describe cardiac effects of *V. berus* envenomation including arrhythmias or other ECG findings and increased cardiac troponin I (cTnI) concentrations, with incidences ranging from 11 to 58% (Lervik et al., 2010, Sutton et al., 2011, Langhorn et al., 2014, Pelander et al., 2010, Vestberg et al., 2017, Kängström, 1989). Only three studies have prospectively examined dogs specifically for the presence of arrhythmias and other ECG changes after *V. berus* envenomation (Vestberg et al., 2017, Pelander et al., 2010, Langhorn et al., 2014), all three report predominantly ventricular arrhythmias in the form of VPCs, AIVR and VT, although the incidence detected was still very variable (12.5- 47%). This begs the question of why such variation in arrhythmia rate exists between studies. Whilst there are many factors at play, the answer may lie in the sensitivity of the methods used to detect arrhythmias and myocardial injury in these dogs.

5.5.4 Diagnosis of arrhythmias and myocardial injury

Cardiac auscultation, and short (2-5 minute) resting ECGs are routinely used for assessment of cardiac rhythm. Arrhythmias may be auscultated as “beat tripping” with pulse deficits (pulse rate slower than the heart rate).

5.5.4.1 Continuous ambulatory ECGs

Continuous ambulatory electrocardiography (AECG) is a method of arrhythmia diagnosis and monitoring, becoming more widely used in the clinical setting. Continuous ambulatory ECG devices confer the advantage of being able to monitor cardiac rhythm for an extended period of time, and whilst normal activities are performed. A number of AECG devices exist that can be broadly categorised as loop recorders or continuous recorders. Loop devices such as event recorders, implantable loop recorders and smart phone devices intermittently record short

sections of ECG whereas continuous recorders such as the Holter monitor, patch electronic monitors (PEMs) and telemetry, are able to record for the whole duration they are connected to the patient. Of the continuous monitors, Holter and telemetry allow multichannel monitoring and are therefore better suited to generating full quantitative data such as ectopic beat counts, than single channel PEMs (Sanders et al., 2019). The continuous ECG is either recorded onto a device worn on the dog (e.g. Holter monitor) or transmitted wirelessly via a telemetric system to a nearby computer or mobile device. Continuous ambulatory ECGs are more sensitive for arrhythmia detection than auscultation and short resting ECGs (Wess et al., 2010, Meurs et al., 2001).

5.5.4.2 Cardiac troponins

Cardiac troponins, measured in serum or plasma, can also offer insights into myocardial injury after snakebite. Contraction and relaxation of myocardial muscle cells (cardiomyocytes) are mediated by a calcium and a troponin "switch", consisting of cardiac troponin T, (cTnT), cardiac troponin I (cTnI) and cardiac troponin C (cTnC). The majority of troponin is structurally bound to the contractile unit, but a small amount is present in a free cytosolic form that is released quickly upon cardiomyocyte injury followed by a slower release of the structurally bound form. Of these troponins, cTnI is cardiac specific (Bodor et al., 1995) and although cardiac isoforms of TnT may also be expressed in damaged skeletal muscle, cardiac specific TnT assays have been developed (Müller-Bardorff et al., 1997). Cardiac troponins T and I are therefore sensitive and specific markers of myocardial injury (Langhorn and Willesen, 2016).

Cardiac-specific troponin I has been measured in *V. berus* envenomated dogs, alone or in combination with ECG recordings, and with variable findings. Two studies have serially measured cTnI in combination with two-minute ECGs in 24 dogs bitten by *V. berus* , and report increased cTnI in 33% and 58% of cases, with or without ECG findings (Langhorn et al., 2014, Pelander et al., 2010). Another study of 17 dogs found that cTnI was not increased at presentation in any dog (Vestberg et al., 2017). Significant ECG findings have also been reported in the absence of increases in cTnI (Pelander et al., 2010).

Combining serial cTnI measurements and continuous AECG is likely to give the most comprehensive overview of the cardiac effects of *V. berus* envenomation and their duration. However, such a study does not yet exist.

5.6 Renal effects of envenomation

Relative to size, the kidneys receive the greatest percentage of cardiac output compared to any other organ, rendering them particularly susceptible to toxic injury. Renal tubular epithelial cells are vulnerable to ischaemic injury due to their high metabolic rate and may also be directly exposed to nephrotoxins in the glomerular filtrate (De Loor et al., 2013).

5.6.1 Pathophysiology of snakebite-related kidney injury

Specific studies of the nephrotoxic effects of *V. berus* venom have not been published, but several mechanisms of kidney injury are possible. Knowledge of venom toxins present in *V. berus* venom and cell culture studies using other types of snake venom, suggest a likely direct nephrotoxic effect of SVMPs and PLA_2 on the glomerular basement membrane and proximal tubular epithelial cells (Willinger et al., 1995, de Castro et al., 2004). In addition to direct toxin effects, renal damage may be induced by ischemia, inflammation and oxidative injury (Sitprija and Sitprija, 2012). Renal ischaemia may result from haemodynamic alterations caused by haemorrhage, vasoactive toxins and inflammatory cytokines. Thrombotic microangiopathy resulting from the actions of procoagulant toxins and inflammatory cytokines, is also possible (Noutsos et al., 2020). Haemoglobinuria and myoglobinuria resulting from haemolysis and rhabdomyolysis can also cause direct tubular toxicity and obstruction of tubules with casts, and secondarily decrease glomerular filtration rate (Zager, 1996, Denis et al., 1998, Shaver et al., 2019, Plewes et al., 2017).

5.6.2 Acute kidney injury

The term “acute kidney injury” (AKI) encompasses a spectrum of acute onset kidney disease, from mild clinically inapparent insult to overt azotaemic kidney dysfunction. Four phases of AKI are described: 1) Initiation: the ischaemic or toxic injury itself occurs, lasting from minutes to hours; 2) Extension: an inflammatory

response and ongoing hypoxia or toxaemia lead to continuation of the injury. This phase lasts from hours to days and is associated with the most cellular damage; 3) Maintenance: ongoing cellular injury, often with concurrent repair. This stage represents peak injury which may or may not be reversible; 4) Recovery: cellular differentiation and recovery of function. In severe AKI, this phase is characterised by fibrosis and loss of function (Sutton et al., 2002, Furuichi et al., 2009).

The initiation phase of AKI often goes undetected and yet identification of and treatment at this stage, is likely to yield better clinical outcomes. Importantly, AKI may also progress to chronic kidney disease (Ishani et al., 2009, Coca et al., 2012, Cowgill et al., 2016). Research in recent years has therefore focused on identifying biomarkers of kidney injury able to diagnose early and milder stages of AKI (Yerramilli et al., 2016).

5.6.3 Kidney effects associated with *Vipera berus* envenomation

Renal histopathology reports of dogs envenomated by *V. berus* and *V. aspis* describe thrombosis of vessels at the renal cortico-medullary junction with tubular degeneration and necrosis (Kolbjørnsen, 2014, Puig et al., 1995, Kängström, 1989), indicating that kidney effects occur after envenomation by these snake species.

Only a few studies report clinical kidney effects in dogs after *V. berus* bite, and with variable findings. In general, conventional tests of kidney function (serum urea and creatinine) have been measured. Occasional findings of azotaemia (increased creatinine and/or urea) exist (Palviainen et al., 2013, Kängström, 1989), whilst other studies report serum creatinine and urea values within the reference range after envenomation (Lervik et al., 2010, Gordin et al., 2020, Turkovic et al., 2015). In a large retrospective study, kidney effects ranging from haematuria to overt kidney failure, were reported in 3.4 % of dogs after *V. berus* bite (Sutton et al., 2011).

Similarly, only occasional reports of azotaemia and AKI after *V. berus* bite can be found in the human literature (Persson and Irestedt, 1981, Garkowski et al., 2012, Lamb et al., 2021, Reid, 1976). Snakebite-related AKI is however, well documented in humans after bites by other snake species and has been attributed to venom-induced consumptive coagulopathy (VICC), haemoglobinuria, myoglobinuria, direct

effects of venom on renal tubular epithelium, and systemic hypotension (Adukauskiene et al., 2011, Czajka et al., 2013, Aye et al., 2017, Sitprija, 2006).

Despite knowledge of *V. berus* venom indicating that the kidneys could be affected after envenomation by this species, and histopathological indications of kidney injury in *V. berus* and *V. aspis* envenomation, renal effects are relatively rarely reported in *V. berus* envenomation of dogs and humans. This begs the question of whether AKI is rare, or whether it is under-recognised in these patients either due to insufficient studies or insensitivity of the diagnostic methods used. This brings us to the limitations of the routinely measured kidney parameters in diagnosing AKI, and the distinction between kidney function and kidney injury.

5.6.4 Laboratory assessment of acute kidney injury

Kidney injury and function tests can be broadly divided into indirect markers of glomerular filtration rate (GFR) and thus, overall kidney function, and urinary protein markers of tubular and glomerular pathology. Conventional tests of kidney function and injury include serum creatinine (sCr), serum urea, urine protein to creatinine ratio (UPC), and urine specific gravity (USG). However, these tests have limitations which are best understood alongside a basic introduction to kidney anatomy and physiology.

5.6.4.1 Glomerular filtration rate

The nephron is the functional unit of the kidney, comprised of the glomerulus (formed by Bowman's capsule surrounding a nest of capillaries), a proximal convoluted tubule, loop of Henle, distal convoluted tubule, and collecting duct. Urine formation starts with filtration of plasma at the glomerulus. Fluid and small molecules able to pass through pores in the capillary walls are forced into Bowman's space and subsequently selectively reabsorbed in the tubular system (Wallace, 1998).

Glomerular filtration rate is the rate at which this plasma filtration occurs, and measurement of which is considered the gold standard for assessment of kidney functional mass. Due to practical and financial reasons, GFR studies are not

generally performed as part of first-line diagnostics, instead methods that indirectly approximate GFR are widely used to estimate kidney function.

5.6.4.2 Indirect serum markers of glomerular filtration rate

Serum creatinine

Creatinine is a small molecule derived from enzymatic cleavage of creatine, 95% of which originates from skeletal muscle. Since creatinine is almost completely freely filtered at the glomerulus and undergoes minimal tubular reabsorption and secretion, it correlates inversely with GFR and is widely used as a marker of kidney function. However, serum creatinine (sCr) does not increase above the reference interval until approximately 75% of nephron function is lost, and its relationship with GFR is non-linear such that large reductions in GFR result only in small increases in circulating creatinine in the early phase of dysfunction (Braun et al., 2003). These characteristics make sCr insensitive for the diagnosis of early or mild kidney injury. Non-renal factors including muscle mass, diet, age and breed can influence sCr concentration thereby further limiting its use (Hall et al., 2015, Braun et al., 2003).

The sensitivity of sCr for detecting early or mild kidney disease can be improved through serial measurements, also called "trending creatinine" (Nabity et al., 2015). Small but clinically relevant increases in sCr that remain within the reference interval can thereby be detected. Trending creatinine is included in the AKI grading system recommended by the International Renal Interest Society (IRIS), whereby an increase in sCr ≥ 26 μmol/L in a 48 hour period, is a criterium for AKI grade I and II (International Renal Interest Society (IRIS), 2016).

Symmetric dimethylarginine

Symmetric dimethylarginine (SDMA), is an endogenous amino acid that is freely filtered across the glomerulus and not subject to tubular reabsorption. Serum SDMA has been proposed as a more sensitive and specific surrogate marker of GFR, in part because unlike sCr it is not influenced by extrarenal factors such as muscle mass, sex or age (Hall et al., 2015, Yerramilli et al., 2016, Nabity et al., 2015). The use of SDMA in kidney function assessment is still relatively new to veterinary medicine but it is becoming more widely used, and most often in the context of chronic kidney disease. Serum SDMA can be used to detect overt AKI in dogs (Dahlem et al., 2017),

but further studies are needed to fully elucidate its use in diagnosing early or milder forms of AKI and whether it confers an advantage over sCr in this setting.

Whilst overwhelming kidney injury often results in decreased functional nephron mass and thus, decreased GFR, kidney damage may also occur in the absence of kidney dysfunction (Haase et al., 2011). Reliance on serum estimates of GFR alone may therefore lead to under-recognition of AKI which although subclinical may still be of importance (Ronco et al., 2012). The measurement of biomarkers of glomerular and tubular injury in urine, is potentially a more sensitive tool with which to diagnose AKI. These biomarkers are usually proteins which due to the selective permeability of the glomerular filtration barrier and tubular reabsorption, are not normally present or only present in small amounts in the urine of healthy individuals. An overview of renal protein handling is necessary to understand the role of such urinary biomarkers in diagnosing AKI.

5.6.4.3 Renal protein handling

The glomerular capillary wall acts as a selective filtration barrier, restricting the passage of plasma proteins into the glomerular filtrate, based on their molecular weight and charge. Low molecular weight (LMW) proteins and some larger positively charged proteins, are freely filtered and fully reabsorbed in the proximal tubule where they are either metabolised or returned to the plasma. Intermediate weight (IMW) proteins (mostly albumin), are subject to fractional filtration and reabsorption. Proteins as large or larger than albumin (≥ 69 kilodaltons (kDa)), are not filtered across the glomerulus (Wallace, 1998). Negatively charged heparan sulphate in the glomerular basement membrane also impedes the passage of negatively charged proteins (Tryggvason and Wartiovaara, 2001).

Changes in the structure of this barrier affect the selective permeability of the glomerulus, resulting in the passage of intermediate and HMW proteins into the glomerular filtrate, saturation of reabsorption mechanisms and thus, proteinuria (Figure 6A). Tubular dysfunction can result in LMW and IMW proteinuria due to decreased absorbance of filtrated proteins, or release of intracellular proteins from injured cells in the brush border (Figure 6B). Since albumin is both fractionally filtered and reabsorbed, its excess presence in urine can indicate glomerular or tubular dysfunction.

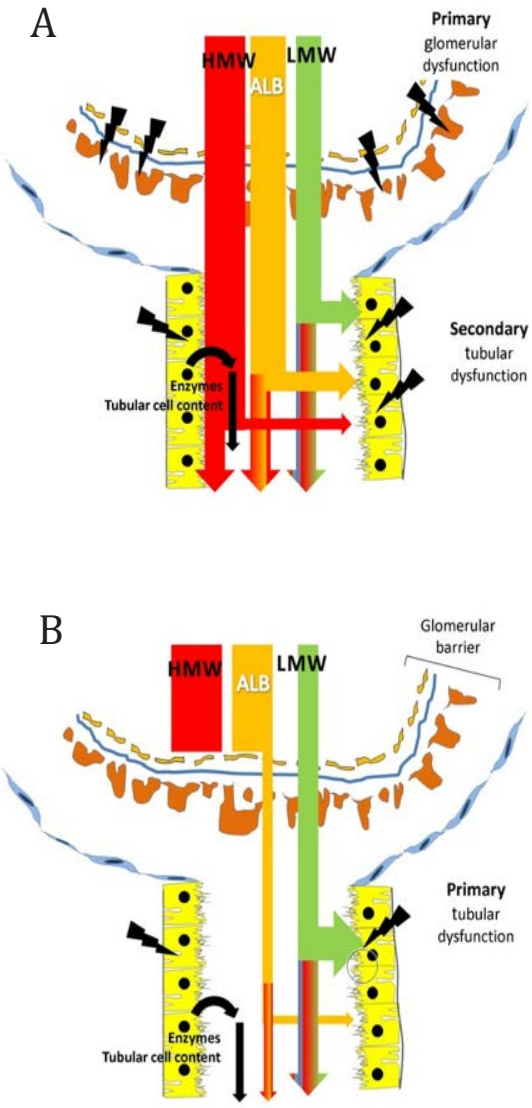


Figure 6. Glomerular damage resulting in filtration of intermediate and high molecular weight proteins and saturation of reabsorption mechanisms (A) and tubular dysfunction resulting decreased reabsorption of intermediate and LMW proteins (B).
Reproduced from "Urinary biomarkers for acute kidney injury in dogs'', De Loor et al, J Vet Intern Med. 2013 Sep-Oct;27(5):998-1010, based on an adaptation from "Pathophysiology of proteinuria", D'Amico G, Bazzi C, Kidney Int. 2003 Mar;63(3):809-25 With permission via copyright clearance center.

Proteinuria

The term 'proteinuria' simply refers to the presence of protein in urine, irrespective of cause or source which may be physiological or pathological, and renal or extrarenal.

Proteinuria is commonly evaluated as part of initial urinalysis, using a urine dipstick colourimetric test. The dipstick test primarily measures overt albuminuria (> 30 mg/dL) and results of ≥ 2+ are generally considered likely to reflect true proteinuria (Lyon et al., 2010). The dipstick test is a useful screening test but is poorly specific and thus, positive results without an obvious pre or post renal cause, require further quantification through measurement of urine protein to creatinine ratio (UPC) (Lyon et al., 2010).

Urine protein to creatinine ratio allows quantification of urine protein whilst accounting for urine flow rate and volume but does not generally allow differentiation between renal proteinuria and extrarenal causes of proteinuria, such as haemolysis, myoglobinaemia, and urinary tract inflammation (Vaden et al., 2004). Results therefore need to be considered alongside urine sediment examination and other clinical findings. In dogs, UPC values of < 0.2 are considered negative and values ≥ 0.5 are considered positive for proteinuria, and values ≥ 2 are usually associated with glomerular disease (Center et al., 1985, Lees et al., 2005).

Dipstick tests and UPC are more sensitive for the detection of albuminuria than other proteins, but they suffer from poor sensitivity at lower protein concentrations and are therefore insensitive for the detection of microalbuminuria (1-30mg/dL) (Lyon et al., 2010). More sensitive albumin assays exist, better suited for this purpose.

Urinary AKI biomarkers

Research into diagnosis of AKI is focused on identifying specific protein markers in urine that are both sensitive for early and mild kidney injury but also able to localise the injury within the nephron (glomerulus, proximal or distal tubules).

Increased urinary albumin can indicate glomerular injury (increased filtration) or tubular injury (decreased reabsorption) and has previously been shown to be

increased in dogs after *V. berus* bite, although the study only included four dogs (Palviainen et al., 2012).

A wide range of urinary protein biomarkers for the detection of acute kidney tubular injury are reported in various studies, and reviewed in a number of papers (Yerramilli et al., 2016, Hokamp and Nabity, 2016, De Loor et al., 2013). An introduction to all of these biomarkers is beyond the scope of this thesis. This introduction therefore restricts itself to the urinary biomarkers pertinent to this thesis work , as summarised in Table 3. This table is by no means intended to be a detailed description of all available biomarkers and the studies in which they are reported, but rather to be a brief overview, including select reference examples.

Various sources and mechanisms of altered urinary excretion of biomarkers are possible, including upregulation of HMW renoprotective proteins such as kidney injury molecule 1 (KIM-1) and clusterin (Rosenberg and Silkensen, 1995, Tanase et al., 2019), upregulation of inflammatory cytokines and chemokines such as interleukin 8 (IL-8), osteopontin (OPN) and monocyte chemoattractant protein 1 (MCP-1)(Schmouder et al., 1992, Haller et al., 2016, Xie et al., 2001), leakage of intracellular tubular proteins such as alkaline phosphatase (ALP), gamma glutamyl transferase (GGT) and cystatin B (Whiting and Brown, 1996, Yerramilli et al., 2016), decreased reabsorption of freely filtered LMW proteins such as cystatin C (Conti et al., 2006), and combined upregulation and decreased reabsorption of neutrophil-gelatinase associated lipocalin (NGAL) (Mori et al., 2005).

Table 3. Urinary kidney tubular injury biomarkers with source and proposed mechanisms of altered urinary excretion.

Biomarker	Size	Source	Mechanism of altered excretion	Example references for association with AKI in dogs
ALP **GGT**	HMW IMW	Proximal tubular cells.	Release from brush border upon tubular injury.	Spontaneous AKI (Heiene et al., 1991), pyometra (Heiene et al., 2001), *V. berus* bite (Palviainen et al., 2013)
Cystatin B	LMW	All nucleated cells.	Tubular cell rupture.	*V. berus* bite (Gordin et al., 2020)
Cystatin C	LMW	All nucleated cells.	Decreased reabsorption by proximal tubular cells.	Leishmania (García-Martínez et al., 2015)
NGAL	LMW	Renal tubular cells, small intestine, prostate, bronchi, stomach.	Increased production (distal tubular cells) and decreased reabsorption (proximal tubular cells).	Spontaneous AKI (Steinbach et al., 2014) Gentamicin model (Kai et al., 2013, Zhou et al., 2014)
Clusterin	HMW	Proximal and distal tubular cells.	Increased local production.	Gentamicin model (Zhou et al., 2014) *V. berus* bite (Gordin et al., 2020)
KIM-1	HMW	Proximal tubular cells.	Increased local production .	Spontaneous AKI (Lippi et al., 2018) Haemorrhagic shock (Boyd et al., 2019)
MCP-1	LMW	All nucleated cells.	Increased local or systemic production.	Haemorrhagic shock (Boyd et al., 2019)
IL-8	LMW	Inflammatory and epithelial cells.	Increased local or systemic production.	Cisplatin model (McDuffie et al., 2016)
OPN	LMW	Distal tubular cells and inflammatory cells.	Increased local or systemic production.	

ALP, alkaline phosphatase; GGT, gamma glutamyl transferase; NGAL, neutrophil-gelatinase associated lipocalin; KIM-1, kidney injury molecule 1; MCP-1, Monocyte chemoattractant protein 1; IL-8, interleukin-8; OPN, osteopontin.

Urinary AKI biomarkers in *Vipera berus* envenomation

Three studies have reported findings of increased urinary biomarkers of AKI in dogs bitten by *V. berus*, including UPC (Palviainen et al., 2013, Gordin et al., 2020); ALP, GGT and CRP (Palviainen et al., 2013); alpha-1-antitrypsin and beta-2-microglobulin (Palviainen et al., 2012); and clusterin and cystatin B (Gordin et al., 2020). Thus, indicating tubular injury.

Serum creatinine was measured in two of these studies with contrasting findings. In one study, azotaemia was reported although the exact number of dogs affected is unclear (Palviainen et al., 2013). In the other study neither sCr nor SDMA were increased in any dog (n=25) after bite, despite biomarker evidence of tubular injury (Gordin et al., 2020). Thus, suggesting that AKI does occur in the absence of a detectable decrease in GFR in these dogs. All of these studies restrict themselves to a single timepoint after bite. Further studies, including more biomarkers and longitudinal measurements, would be useful to both confirm AKI in these dogs and its duration.

Snakebite-associated AKI is not a new concept, but it is unclear to what extent it plays a role after *V. berus* envenomation. Furthermore, since dogs envenomated by *V. berus* generally present rapidly for veterinary treatment, they provide a rare opportunity to study biomarkers of AKI in a group of dogs, immediately after a possible nephrotoxic insult, and thus, in the key initiation phase of AKI.

5.7 Coagulopathic effects of envenomation

"In this disease an extreme dissolution of a part of this humour [blood], exuding everywhere through the vessels, takes place; and, at the same time, a coagulation of another part, which fixes and condenses in a few moments . . . Every advance I made, in this new career of experiments, presented me either with something paradoxical, or with a novel and unexpected circumstance".

Abbé Felice Fontana
Treatise on the venom of the viper, 1781

Coagulopathy is a widely recognised and clinically significant effect of snake envenomation (Slagboom et al., 2017, Warrell, 2010). Although the term "coagulopathy" is often associated with bleeding disorders, in its most literal sense, the word means a disease affecting blood coagulation and thus includes conditions of increased or decreased clotting. It is this broad meaning of the word that is employed in this thesis.

The majority of toxins identified in *V. berus* venom have coagulopathic potential through direct pro- and anticoagulant effects, but toxins may also affect haemostasis through activation or inhibition of platelet aggregation, fibrinolysis and also exert indirect inflammatory effects (summarised in Table 1). In order to understand these effects, we start with a basic overview of haemostasis.

5.7.1 Haemostasis in a nutshell

Haemostasis is a highly regulated process designed to protect against blood loss by sealing sites of vascular injury through clot formation, followed by restoration of blood flow through clot dissolution once the injury is healed. The haemostatic process involves interactions between the vascular endothelium, platelets and coagulation factors, and can be divided into the following key stages: (1) vasoconstriction, (2) primary haemostasis, (3) secondary haemostasis and (4) fibrinolysis. Under normal circumstances, healthy vascular endothelium maintains an anticoagulant surface, ensuring that blood remains in a fluid state. During blood vessel injury, exposure of substances such as collagen and von Willebrand factor in the subendothelium leads to adherence, and subsequently, activation and

aggregation of circulating platelets and the formation of a primary platelet plug, otherwise known as primary haemostasis. Secondary haemostasis results in the formation of fibrin on the platelet plug and is achieved through a series of coagulation factor activations, a process often referred to as the coagulation cascade. Fibrinolysis is initiated as soon as the fibrin clot starts to form and is an essential counteracting mechanism to prevent excessive blood coagulation and vessel occlusion. Plasmin, a fibrinolytic protease, cleaves fibrin, thereby dissolving the fibrin clot and releasing fibrin degradation products (FDPs) such as D-dimers. Disruptions in the finely tuned balance of haemostasis can manifest as haemorrhage or inappropriate blood clotting, known as thrombosis (Brooks and Catalfamo, 2013).

5.7.2 The cascade and cell-based models of coagulation

Secondary haemostasis involves a series of coagulation factors that sequentially and enzymatically cleave and activate one another, culminating in fibrin formation (Davie et al., 1991). Activated platelets expose negatively charged phospholipids (PLs) such as phosphatidylserine (PS) and provide a surface for coagulation factors to assemble and establish complexes such as the tenase and prothrombinase complexes that are able to activate factor X and prothrombin (factor II), respectively. A key step in secondary haemostasis is the generation of large amounts of thrombin (factor IIa (FIIa)) by activated factor X (FXa), on the surface of activated platelets. Thrombin subsequently converts fibrinogen to fibrin, forming a lysis-resistant fibrin-covered clot.

The series of enzymatic coagulation factor activations was first described as a “cascade” or ”waterfall” in the 1960s and is known today as the coagulation cascade (Davie and Ratnoff, 1964, Macfarlane, 1964). This cascade model went on to be further described as Y shaped, with intrinsic and extrinsic activation pathways that converged into a common pathway. Both the intrinsic and the extrinsic pathway are able to generate activated factor X (factor Xa) (Figure 7).

During vessel injury, tissue factor (TF) in the subendothelium, i.e. extrinsic (external) to the blood vessel, is exposed and can bind FVII/FVIIa, thus activating the extrinsic pathway. Activation of the intrinsic pathway occurs on the surface of platelets within the blood vessel, hence the name “intrinsic.” Calcium and

phospholipids (PLs), especially phosphatidylserine (PS), are essential components in the activation of many of the coagulation factors (Davie et al., 1991).

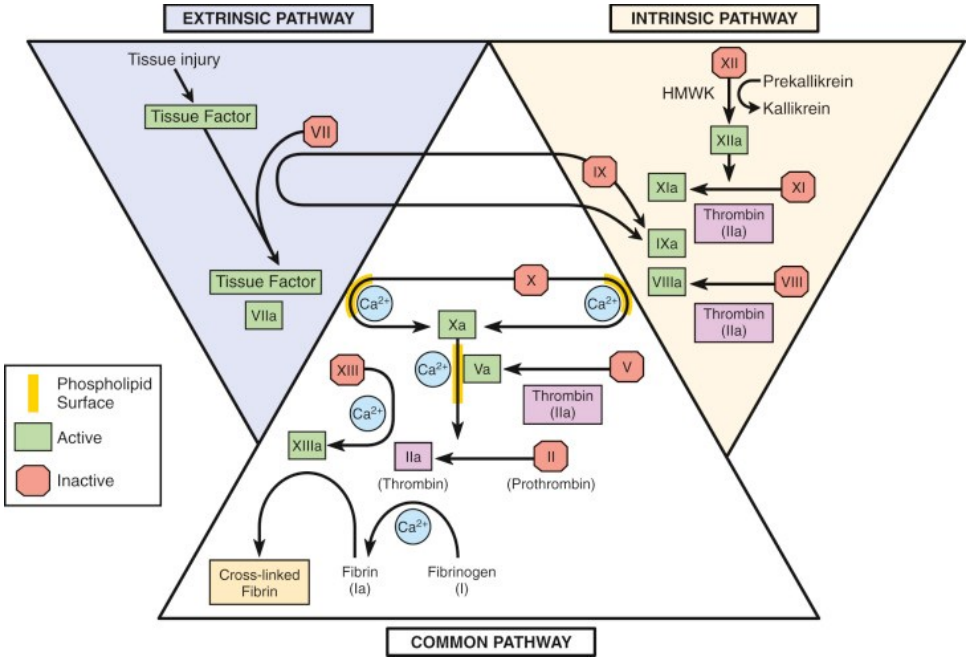


Figure 7. The classic coagulation cascade. The intrinsic and extrinsic pathways produce intrinsic (FIXa-FVIIIa) and extrinsic (TF-FVIIa) tenase complexes that activate factor X. HMWK, high molecular weight kininogen. TF, tissue factor. *Reproduced from Chapter 2-Vascular Disorders and Thrombosis. Mosier, D.A. In: Pathologic Basis of Veterinary Disease (Sixth Edition). Zachary JF, editor. Mosby; 2017. With permission via Copyright Clearance Center.*

The cascade model is useful in understanding the principles of measurement of laboratory coagulation analyses such as prothrombin time (PT) and activated partial thromboplastin time (aPTT), and the interpretation of abnormal test results. However, this model has several weaknesses. In 1977, the intrinsic and extrinsic pathways were found to interact at an earlier stage than FX, with the extrinsic tenase complex (TF-FVII/FVIIa) able to activate not only FX but also FIX, a coagulation factor located in the intrinsic pathway (Osterud and Rapaport, 1977).

The cascade model also failed to adequately explain why patients with severe haemophilia A, i.e. a severe deficiency of FVIII (a coagulation factor located in the intrinsic pathway) have an increased bleeding tendency. In these patients, the intrinsic tenase complex (FIXa-FVIIIa) cannot optimally generate FXa and the generation of thrombin is subsequently markedly reduced. Factor Xa generated by the extrinsic tenase complex (TF-FVII/FVIIa) does not compensate for the inability of the intrinsic tenase complex (FIXa-FVIIIa) to generate FXa in these individuals (Hoffman, 2003).

The failure of the cascade model to explain the *in vivo* bleeding tendency in humans with severe haemophilia A and the realisation that the intrinsic and extrinsic pathways interact at an earlier stage than FX, led to the development of the cell-based model of coagulation. This model recognises TF on subendothelial cells as the main physiological activator of haemostasis and the importance of cell surfaces in assembly of coagulation factor complexes. In the cell-based model, coagulation factors of the extrinsic pathway initiate coagulation on TF-bearing cells, and coagulation factors of the intrinsic pathway are activated on PS-exposing platelets, both of which are necessary for normal haemostasis (Hoffman and Monroe, 2001).

The cell-based model is described as three overlapping phases: initiation, amplification and propagation (Figure 8). The initiation phase occurs on a TF-bearing cell and leads to activation of small amounts of FIX and FX. Factor Xa activity is restricted to the TF-bearing cell through the actions of the coagulation inhibitors tissue factor pathway inhibitor (TFPI) and antithrombin (AT). Small amounts of thrombin, generated on the TF-bearing cell diffuse to and activate adjacent platelets. Thrombin also amplifies the coagulation process by activating cofactors FV and FVIII, and also FXI, and initiates a positive feedback mechanism that serves to generate more thrombin (Hoffman, 2003). The amplification and propagation phases occur on the surface of an activated platelet, culminating in the production of large amounts of thrombin that subsequently converts fibrinogen to fibrin, forming a resistant fibrin network on the platelet plug (Hoffman and Monroe, 2001). This burst of platelet-produced thrombin is also able to stabilise the fibrin network through activation of factor XIII (FXIII) that crosslinks fibrin threads, and activation of the antifibrinolytic protein, thrombin activable fibrinolysis inhibitor (TAFI) (Bajzar et al., 1995, Maurer et al., 2006).

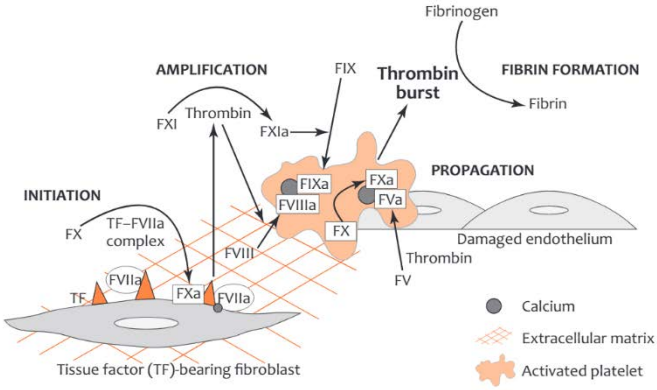


Figure 8. The cell-based model of coagulation with initiation, amplification and propagation phases. *Reproduced with permission from the BSAVA Manual of Canine and Feline Clinical Pathology, 3rd edition © BSAVA. Artwork by Samantha J Elmhurst www.livingart.org.uk.*

5.7.2.1 Regulation and control of coagulation

Localisation and control of coagulation factor activity is essential to prevent excessive fibrin generation and thrombosis. Some coagulation factors inevitably diffuse away from the site of injury but are rapidly inactivated. Coagulation inhibitors such as protein C, protein S, AT and TFPI are able to inactivate different coagulation factors, thereby restricting thrombin generation. Free thrombin is either inhibited by AT or binds to thrombomodulin (TM) in the endothelium. The thrombin-TM complex is able to generate activated protein C (APC) which binds with its cofactor protein S and subsequently inactivates FVa and FVIIIa (Dahlbäck, 2005).

5.7.3 Mechanisms of coagulopathy in *Vipera berus* envenomation

5.7.3.1 Venom effects

Numerous pathological mechanisms are at play after *V. berus* envenomation that may contribute to disruption of haemostasis. Procoagulant FX activators and fibrinogenases (enzymes that convert fibrinogen to fibrin) have been identified specifically in *V. berus* venom (Samel and Siigur, 1995, Samel et al., 2003, Latinovic et al., 2016). Conversely, anticoagulant PLA_2 activity (Boffa and Boffa, 1976), and

LAAO platelet aggregate inhibitors (Samel et al., 2006), are also present in the venom. Other venom toxin families present in *V. berus* venom, have been shown to have procoagulant FV and prothrombinase activity, plasminogen activating (fibrinolytic) activity, platelet activating properties and anticoagulant activity through protein C activation (outlined earlier in Table 1), and thus likely also contribute to coagulopathy after envenomation by *V. berus*. Additionally, SVMPs may cause spontaneous haemorrhage due to endothelial membrane damage (Gutiérrez et al., 2016) which could be exacerbated by venom-induced coagulopathies.

5.7.3.2 Crosstalk between inflammation and coagulation

Snakebite-induced systemic inflammation may also contribute to procoagulant activity. Examples of interactions between inflammation and coagulation systems include: inflammatory cytokine stimulation of platelet production and activity, downregulation of protein C and TM expression, CRP-induced TF expression and complement activation which leads to increased PL exposure on cell surfaces, and consumption or inactivation of AT (Esmon, 2005, Wolbink et al., 1998, Cermak et al., 1993, Rao and Pendurthi, 2012). Inflammatory cytokine induction of TF expression on monocytes and subsequent release of extracellular vesicles (EVs) is another described link between inflammation and coagulation.

Extracellular vesicles and circulating tissue factor

Extracellular vesicles are membrane-derived particles capable of transferring cellular components and thus act as a form of intracellular communication (van Niel et al., 2018). Under normal circumstances, PS is contained to the inner leaflet of cell membranes. When cells are activated, or during apoptosis, PS may be exposed on the cell surface through membrane flipping and incorporated onto EVs that are released from the cell. PS-positive EVs are procoagulant since they provide a circulating PL surface upon which coagulation factors may assemble (Owens and Mackman, 2011). In addition to extravascular expression, TF may also be exposed on the surface of circulating monocytes activated during inflammation and on EVs derived from these cells. The most procoagulant EVs, expose both PS and TF (Owens and Mackman, 2011). Apoptosis, endotoxins, DAMPs and inflammatory cytokines are all possible stimuli for PS and TF expression on monocytes, and subsequent EV formation and release (Rivers et al., 1975, Rao and Pendurthi, 2012, Ito et al., 2007).

Snake venom metalloproteases from other snake species have been associated with increased plasma TF activity (Yamashita et al., 2014). EVs expressing PS or TF or both may therefore play a procoagulant role in snake envenomation.

5.7.4 *Vipera berus* envenomation: a coagulopathic conundrum?

Vipera berus venom has been shown to be procoagulant *in vitro* (Latinovic et al., 2016, Chowdhury et al., 2021), but the net *in vivo* clinical effect is unclear. The clinical picture in humans and dogs is complicated by occasional reports of prolonged clotting times (Turkovic et al., 2015, Brandeker et al., 2015, Dyląg-Trojanowska et al., 2018) contrasted with findings of thrombosis in other studies (Gary et al., 2010, Jørgensen et al., 2014, Lamb et al., 2021, Kängström, 1989, Kolbjørnsen, 2014).

There are no published studies specifically assessing the coagulation status of dogs after *V. berus* envenomation. Prolonged PT and aPTT times indicating hypocoagulability, thrombocytopenia, and increased D-dimers indicating hyperfibrinolysis or increased fibrinolysis as a counteracting response to hypercoagulability, are occasionally reported in dogs (Brandeker et al., 2015, Turkovic et al., 2015, Kängström, 1989). Human studies also report infrequent findings of prolonged PT and aPTT, increased D-dimers, increased FDPs and decreased fibrinogen (Dyląg-Trojanowska et al., 2018, Karlson-Stiber and Persson, 1994).

5.7.4.1 Venom-induced consumptive coagulopathy

Studies of other snake species describe a dominant procoagulant effect of venom, leading to consumption of coagulation factors and subsequent hypocoagulability which may manifest clinically as bleeding and prolonged clotting times (Warrell, 2010, Berling and Isbister, 2015, White, 2005). Consumption coagulopathy has been reported after envenomation of dogs by other *Vipera* species (Aroch et al., 2010, Puig et al., 1995, Segev et al., 2004).

The term “venom induced consumptive coagulopathy” (VICC) is often used to describe this consumption phenomenon. Anticoagulant and fibrinolytic venom toxins and impaired platelet activity may further exacerbate VICC. In humans, VICC is a more common sequel to the hypercoagulable state induced by *Vipera* species

envenomation, than thromboembolic complications (White, 2005). Patients with VICC can develop unexpected fatal haemorrhage despite antivenom treatment (White, 2005), thus early identification of a hypercoagulable state would be of benefit.

Given the low number of studies and often small sample size, the incidence and nature of coagulopathy in dogs after *V. berus* envenomation is unclear: Do pro-or anticoagulant mechanisms dominate after *V. berus* envenomation and if procoagulant mechanisms dominate, do they lead to VICC?

A major limitation of studies reporting coagulation status in dogs after *V. berus* envenomation to date, is the use of the clot-based assays: PT and aPTT.

5.7.5 Laboratory evaluation of coagulation: limitations and new concepts

5.7.5.1 Prothrombin time and activated partial thromboplastin time

Prothrombin time and aPTT are commonly used coagulation assays in veterinary medicine. Prothrombin time measures the collective activity of coagulation factors in the extrinsic and common coagulation pathways as the time from the addition of reagent containing TF, PLs and Ca^{2+}, to fibrin formation. Activated partial thromboplastin time measures the collective activity of the coagulation factors in the intrinsic and common pathway as the time from initiation of FXII activation using a contact-activating reagent, until fibrin formation. Deficiency or dysfunction of one or several of the coagulation factors in the extrinsic and common pathways, or intrinsic and common pathways, may prolong PT and aPTT respectively (Winter et al., 2017).

Both PT and aPTT measure time to fibrin formation and as such do not reflect total haemostatic and thrombotic potential. A key concept in understanding the limitations of these commonly used clotting analyses, is that the initial fibrin clot is formed when less than 5% of thrombin has been generated, and thus, only represents a minute amount of the total thrombin produced during haemostasis (Mann et al., 2003).

The exogenous trigger reagents used in PT and aPTT are also added in such excess that the assays are insensitive to small amounts of procoagulant factors in circulation. These assays are therefore generally considered insensitive for the detection of hypercoagulable states such as may occur after snake envenomation (Brooks and Catalfamo, 2013). Milder hypocoagulable states may also go undetected using these conventional clot-based assays (Duarte et al., 2017). Instead they may be useful in identifying moderate or severe hypocoagulability that may be associated with anticoagulant venom effects or consumption of coagulation factors following an initial hypercoagulability (Berling and Isbister, 2015). Furthermore, these clotting analyses do not always correlate well with clinical phenotype. The anticoagulant drivers in plasma cannot fully exert their effects in PT and aPTT assays, especially the TM-protein C-protein S pathway which is reliant on the presence of an endothelial membrane.

Prothrombin time and aPTT can therefore provide information on the deficiency or dysfunction of one or several coagulation factors but not whether the defect is counteracted by decreased anticoagulant forces (Tripodi, 2016). These commonly used assays are therefore not optimal tests for assessing the overall coagulation status of dogs after *V. berus* envenomation, or its expected clinical effects.

5.7.5.2 Markers of fibrinolysis, hypercoagulability, and factor consumption

D-dimers and FDPs are markers of fibrinolysis and thus increased concentrations can indicate primary hyperfibrinolysis or increased fibrinolysis secondary to a hypercoagulable state. These parameters are sensitive but not specific markers of increased fibrinolysis and are therefore of limited use when assessed in isolation (Brooks and Catalfamo, 2013). Furthermore, since they are dependent on fibrinolytic processes, D-dimers and FDPs are relatively late markers for detecting an initial hypercoagulability.

Fibrinogen concentration and AT activity may be decreased in consumptive coagulopathy, but these markers also suffer from a lack of specificity. Fibrinogen is a positive acute phase inflammatory protein (Stockham and Scott, 2008b) and AT may

be decreased in in protein losing nephropathies and enteropathies (Stockham and Scott, 2008a).

5.7.5.3 Thrombin-antithrombin complexes

Thrombin-antithrombin (TAT) complexes can provide an insight into *in vivo* coagulation status. Thrombin-antithrombin complexes are formed when AT binds and inhibits thrombin. Increased TAT complex concentrations may thereby represent active thrombin formation *in vivo* and are associated with hypercoagulability. In veterinary medicine, increased TAT complexes have been measured in hypercoagulable states associated with hyperadrenocorticism and neoplasia (Maruyama et al., 2005, Jacoby et al., 2001) but not in the context of *V. berus* envenomation. Since TAT assays are expensive, they are generally restricted to research settings although a point of care TAT test has shown promise in a recent canine study (Kato et al., 2020), and TAT assays may therefore become more widely available.

5.7.5.4 Thrombin generation assays

Thrombin generation assays are better able to simulate the coagulation process by incorporating the generation of thrombin and the inhibitory effects of coagulation inhibitors such as AT and TFPI into the measurements. Such assays measure a broader aspect of secondary haemostasis and may therefore aid the detection of both hypo- and hypercoagulable states, in the clinical setting (Duarte et al., 2017).

Thrombin is both pro- and anticoagulant, making it a key player in haemostasis and a useful target for assessing overall coagulation status. In human medicine, the generation of thrombin appears to be a better marker than clot-based assays to estimate the risk of bleeding or thrombosis (Dave et al., 2021, Besser et al., 2008). Thrombin generation assays that measure the total amount of thrombin generated were first described in the 1950s (Macfarlane and Biggs, 1953, Pitney and Dacie, 1953) and after various refinements over the decades, are becoming more widely used in human medicine.

The calibrated automated thrombogram (CAT), is one such thrombin generation assay. The CAT assesses the overall thrombin generating potential in plasma under the relative contributions from pro-and anticoagulant drivers and is therefore better

suited to assessing the balance between pro- and anticoagulant forces in plasma than PT and aPTT assays (Tripodi, 2016). The use of CAT is relatively new to veterinary coagulation research and has been described in clinical studies including canine blastomycosis, canine gastroenteropathies and to predict and monitor therapy efficacy in a canine haemophilia model (McMichael et al., 2015, Krogh et al., 2020, Madsen et al., 2017). The use of CAT in the context of snake envenomation research is limited to one *in vitro* study of Brazilian snake venoms where it was shown to be more sensitive than PT and aPTT in detecting hypercoagulability in pooled human plasma exposed to venom (Duarte et al., 2019). The CAT is a promising tool with which to assess coagulation status after canine *V. berus* envenomation and is discussed in more detail in the methodology chapter of this thesis.

This chapter started with a quote from Fontana's experiments, highlighting the paradoxical nature of the clinical effects of European viper venom. Whilst understanding of this field has of course advanced since 1781, more work is clearly needed to elucidate the global coagulation status of dogs bitten by *V. berus*, especially when bearing in mind the outlined limitations of the commonly used clotting analyses in detecting hypercoagulable states.

5.8 Knowledge gaps

Although *V. berus* bites are common in dogs, surprisingly few studies exist describing the clinical and subclinical effects of such bites or the duration of these effects. More specifically, prospective longitudinal studies focused on individual aspects of envenomation, are absent from the literature. There are therefore several knowledge gaps in this field, the key ones of which are summarised below.

Studies report cardiac effects after *V. berus* envenomation in dogs, but the true incidence, nature and duration of these effects are not known due to the low number of studies, small sample sizes, and a lack of studies combining cTnI measurements and AECG. ***What proportion of dogs suffer myocardial injury after envenomation, how long does it last, and is it clinically important?***

When this project was initiated there was a general paucity in studies reporting kidney effects and more specifically only one study that had measured urinary AKI biomarkers (ALP, GGT and CRP) in more than four dogs bitten by *V. berus* (Palviainen et al., 2013). Given the knowledge of venom toxins and some reports of overt kidney effects detected using conventional tests of GFR, combined with the knowledge that these tests are insensitive for the detection of early or mild AKI, it is quite possible that AKI is under-recognised in dogs bitten by *V. berus*. Another research group has, during the course of this thesis work, published findings of increased urinary AKI biomarkers (clusterin and cystatin B) in dogs bitten by *V. berus*, at a single timepoint after bite, further supporting this theory (Gordin et al., 2020). However, it is unknown what the incidence and duration of AKI is in these dogs due to the low number of studies, few biomarkers examined, and lack of serial sampling. ***Do dogs develop AKI after V. berus envenomation, and if so, how long does the injury last?***

Despite coagulopathy being the most widely recognised effect of snake envenomation, prospective studies investigating the coagulopathic effects of *V. berus* envenomation are lacking from both the human and canine literature. Given that *V. berus* venom has the potential to induce a range of paradoxical coagulopathic effects both directly and indirectly, and that routine clot based tests are insensitive for the detection of the procoagulant effects in particular, the global coagulation status of dogs after this type of snakebite is unknown. ***What is the net effect of V. berus envenomation on the coagulation status of dogs?***

There are few studies evaluating the development of complications after *V. berus* bite, in relation to severity of clinical signs of envenomation. ***Is the severity of clinical signs at presentation a useful indicator for the development of more severe complications of snakebite envenomation during hospitalisation after V. berus bite?***

All of these are clinically relevant questions that if answered, would contribute significantly to the evidence base upon which *V. berus* envenomation treatment and management decisions are made.

Additionally, there are no randomised controlled trials (RCTs) of the efficacy of antivenom treatment in dogs or humans envenomated by *V. berus*. Due to a lack of knowledge of the extent of clinical and clinicopathological effects of this type of snakebite, there are currently few objective parameters that could serve as laboratory endpoints in such studies.

6 Aims of the thesis

The overall aim of this thesis was to contribute to filling the identified knowledge gaps regarding the clinical and clinicopathological effects of *V. berus* envenomation in dogs with an emphasis on three organ systems: cardiac, renal and haemostasis. This was implemented through the specific objectives of the four papers included in this thesis:

- To describe the incidence, nature and duration of arrhythmias in dogs during the first 48 hours after envenomation using continuous AECG monitoring and investigate associations with serial serum cTnI concentrations. **(Paper I).**

- To evaluate the presence and duration of AKI in dogs bitten by *V. berus* using markers of renal function and measurements of more specific urinary AKI biomarkers. **(Papers II and III).**

- To evaluate associations between severity of clinical signs at presentation and the development of cardiac and renal effects of envenomation **(Papers I-III).**

- To longitudinally describe the global coagulation status of dogs bitten by *V. berus* using CAT, and measurements of TAT complexes and PS equivalents. A sub aim of this paper was to compare the coagulation status of dogs treated with and without antivenom **(Paper IV).**

7 Materials and methods

7.1 General (papers I-IV)

Study design

All studies included in this thesis are prospective longitudinal cohort studies, based on convenience sampling of dogs presenting for veterinary treatment after snakebite.

Animals

A total of 60 dogs bitten by *V. berus* and presenting to the small animal clinics at the Department of Companion Animal Clinical Sciences at the Norwegian University of Life Sciences (NMBU), Evidensia Oslo Dyresykehus, Anicura Dyresykehus Oslo and Anicura Jeløy Dyresykehus between 2017 and 2019, were included.

Forty healthy control dogs were included in papers II-IV. The control group was recruited through stratified sampling by weight and age to match the cases in papers II and III. Relationships between study populations are illustrated in Figure 9.

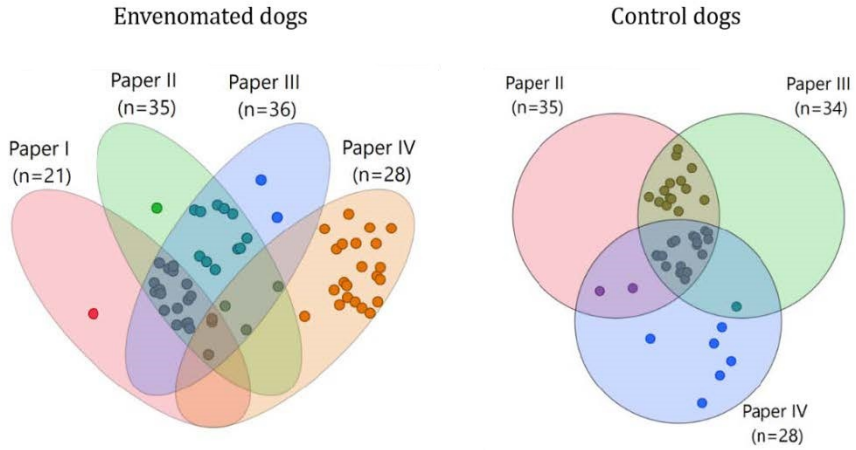


Figure 9. Venn diagrams illustrating the relationship between populations of envenomated dogs in papers I-IV, and control dog populations in papers II-IV. Paper I did not include a control group.

General inclusion and exclusion criteria

Diagnosis of snakebite and thus inclusion in each of the studies was based on a history and clinical signs compatible with snake envenomation (fang marks, local swelling or systemic signs of envenomation). Dogs were excluded if they presented more than 24 hours after a bite and if they lacked clinical signs of envenomation within 12 hours of the bite. Dogs were also excluded on the basis of any pre-existing conditions and medications, with a few exceptions. More detailed inclusion and exclusion criteria are described in the individual papers.

For the control dogs, healthy status was defined as an unremarkable history including no chronic disease or any disease in the preceding 14 days, an unremarkable physical examination, normal urinalysis and no clinically significant haematology or biochemistry abnormalities.

Sampling timeline

All papers followed the same basic sampling timeline as outlined in Figure 10. Envenomated dogs were examined, and blood (serum and citrated plasma) and free-catch urine samples taken, at five timepoints after bite: **T1**: presentation (0.5-7.5 hours, h), **T2**: 12 (±2) h, **T3**: 24 (±2) h, **T4**: 36 (±2) h and **T5**: 14 days, with ranges of 10-21 days (paper I) and 10-23 days (papers II and III). In paper IV, T2 and T4 had a greater time range than in the other papers (±4 hours) and T5 ranged from 10-32 days. Control dogs (papers II-IV) were sampled at a single timepoint.

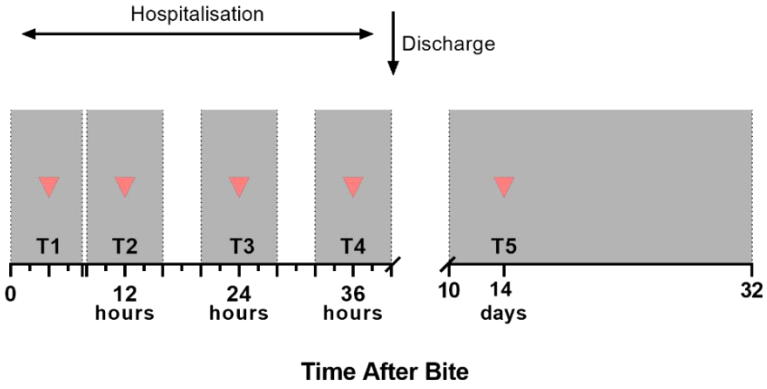


Figure 10. Sampling timeline for envenomated dogs in papers I-IV. T1 (presentation), T2 (12 ± 2 hours), T3 (24 ± 2 hours) and T4 (36 ± 2 hours) and T5 (10-32 days). In paper IV, T2 and T4 corresponded to 12 ± 4 hours, and 36 ± 4 hours.

The reader is referred to the individual papers in this thesis for a comprehensive overview of the methodologies employed. Specific methods worthy of extra attention are described below.

Actual sample size

For various reasons (outlined in the methodological considerations section), samples size varied at each timepoint, in each paper. An overview of actual sample numbers at each timepoint, for each study, is provided in Table 4.

Table 4. Overview of actual numbers of samples included in statistical analyses for each paper, for control dogs and envenomated dogs at each timepoint.

			Controls	T1	T2	T3	T4	T5
Paper I			-	21	21	21	20	19
Paper II	**serum**		35	23	30	30	26	30
	urine	maximum*	35	-	20	28	24	25
		minimum*	34	-	16	25	22	19
Paper III	**serum**		36	24	33	32	26	31
	urine		34	5	22	28	24	27
Paper IV	maximum*		28	25	27	26	23	21
	minimum*		13	17	25	22	21	20

* In papers II and IV sample numbers varied for the different analyses. Maximum and minimum sample numbers are therefore provided.

Snakebite severity scoring (SSS)

In papers I-III, scoring systems were used to record severity of clinical signs. In paper I a simple three-point scoring system was adapted from a grading system for *V. berus* and *V. aspis* envenomation in humans (Audebert et al., 1992) and previously adapted for a canine study (Palviainen et al., 2013). The original grading system was shown to correlate with serum venom concentrations in humans (Audebert et al., 1994a) and thus thought to be good indicator of venom dose and thereby, severity.

The scoring is based on the degree of swelling and systemic signs of envenomation with grade one being local swelling only and grade three being extensive swelling with marked systemic signs of envenomation (see paper I for full details).

Since there is no consensus for severity scoring after *V. berus* envenomation in dogs and the original scoring system by Audebert et al (1992) is based on somewhat subjective criteria, papers II and III made use of an adapted version of a more comprehensive grading system, previously validated for crotalid snakebites in humans (Dart et al., 1996). This grading system includes more specific assessment of the local wound as well as respiratory, cardiovascular, gastrointestinal, haemostatic and central nervous systems. Given the differences in expected venom effects between *V. berus* and crotalids and the need for a system not reliant on laboratory parameters of coagulation, central nervous system parameters were changed to general demeanour parameters, and haematological parameters were removed. Thus, adapting the scoring from a 20-point to a 16-point grading system (see papers II and III for full details).

Blood pressure measurement

Papers I-III included indirect blood pressure measurements using an oscillometric technique (Cardell®). The blood pressure measurement procedure was standardised using a cuff size of approximately 40% limb circumference, placed on the distal radius or metatarsus with the dog in lateral recumbency, 12 measurements were made and systolic (SAP), diastolic (DAP) and mean (MAP) arterial pressures recorded. The first two measurements and any obvious outliers were excluded, and the mean of the remaining values used in analyses.

Antivenom treatment

Due to the observational nature of this study, treatment decisions were made at the discretion of the attending clinician. Antivenom was administered to 16/21 dogs in paper I, 27/35 dogs in paper II, 28/36 dogs in paper III and16/28 dogs in paper IV.

Ethics

All studies included in this thesis complied with the national animal welfare rules and regulations for the use of animals in research. Written owner consent was obtained for all participating dogs, prior to enrolment.

7.2 Paper I

Ambulatory electrocardiography and serum cardiac troponin I measurement in 21 dogs envenomated by the European adder (Vipera berus).

This paper used Holter AECG and serial cTnI measurements to give a comprehensive overview of the cardiac effects of envenomation. Serum was obtained from the dogs according to the sampling timeline in Figure 10, and a Holter AECG placed as soon as possible after presentation and prior to the first blood sampling event.

The Holter AECG

The Holter AECG system (Lifecard CF Holter recording system) was used to monitor cardiac rhythm in dogs in this study. A modified bipolar orthogonal lead system (X,Y,Z) was used with electrodes placed as demonstrated in Figure 11, after clipping and skin cleaning with alcohol. Once the electrodes were in place, the Holter device was attached to the dogs back using an adjustable Holter vest. Data was recorded for up to 51 hours and analysed by an RCVS recognised specialist in cardiology, using commercially available software (Pathfinder Digital V9.019). This software was programmed using previously agreed measurement criteria, adapted for dogs (Harris et al., 2017) and categorises beats as normal or abnormal in morphology.

In this paper, arrhythmias were graded 0-3 (with subgrading of grades 1 and 2), based on their type, frequency and severity. Grade 0 was considered normal and grades 1-3 were increasingly abnormal. The grading system employed in this study was based on a previously described grading system (Meurs, 2004, Palermo et al., 2011). Since this previously described system is largely based on screening for ventricular arrhythmias associated with cardiomyopathy and not designed for acute cardiotoxicity, the literature regarding expected cardiac arrhythmias in envenomated dogs and a previously described system for grading after *Vipera palaestinae* envenomation (Segev et al., 2008), were reviewed, and the previous systems adapted accordingly. The main difference being the inclusion of SVPCs, AV block and VEC complexity in the grading system in this paper, compared to Meurs et al (2004) and Palermo et al (2011).

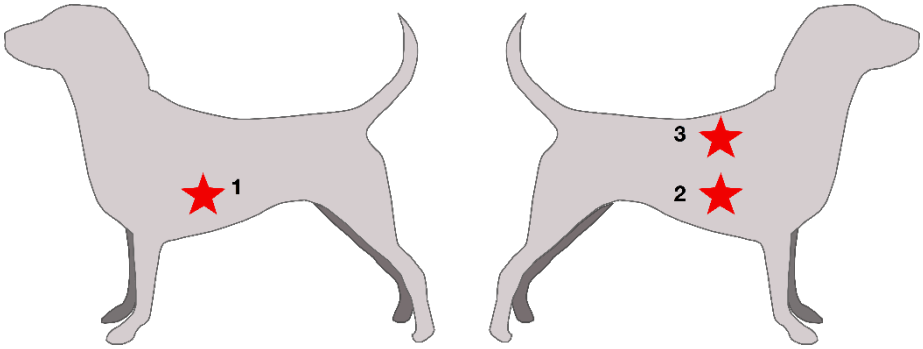


Figure 11. Electrode placement used for AECG recording in the dog. 1. Left lower: immediately caudal to the apex beat. 2. Right lower: immediately caudal to the apex beat 3. Right upper: at the caudal border of the right scapula. *(Image reproduced with permission from HeartVets, UK).*

Cardiac troponin I analysis

Serum cTnI concentrations were measured at a commercial laboratory (IDEXX bioanalytics), using an ultrasensitive chemiluminescence assay (Siemens ADVIA Centaur® CP, TnI -UltraTM), validated for use in dogs (Langhorn et al., 2013). This assay is a three-site sandwich immunoassay comprised of two monoclonal biotinylated anti-cTnI antibodies and an acridinium ester-labelled goat anti- cTnI antibody. Cardiac troponin I in the sample is bound by these antibodies and the biotin in the immune complex then bind to streptavidin- labelled magnetic particles. Light emission from the reaction of the acridinium ester label with hydrogen peroxide is then measured and cTnI quantified by interpolation from a standard curve. The laboratory reference threshold of <0.06ng/mL was used to define values as normal or increased.

7.3 Paper II

Serial serum creatinine, SDMA and urinary acute kidney injury biomarker measurements in dogs envenomated by the European adder (Vipera berus).

Multiplex immunoassays

Research into biomarker analysis has expanded over the years and is facilitated by assays able to simultaneously quantify multiple analytes in a single sample that increase speed of analysis and decrease the volume of sample needed (Christopher-Hennings et al., 2013). In paper II, a multiplex assay was used for simultaneous analysis of several urinary AKI biomarkers (albumin, KIM-1, MCP-1, IL-8, OPN, NGAL, cystatin C). Similar to the principle of a sandwich immunoassay, this assay uses Luminex Xmap bead-based technology that couples capture antibodies to fluorescently dyed magnetic beads (microspheres) with different emission spectra (Figure 12). Beads are fluorescently coded according to analyte. Detection antibodies are also fluorescently labelled. The amount of light emitted from the fluorochromes is proportional to the number of antibodies bound to the target analyte. Beads are excited and identified with a 635 nm wavelength, and a 525 nm wavelength is used to excite and quantify the amount of target analyte for each bead type and measured using a spectrofluorometer.

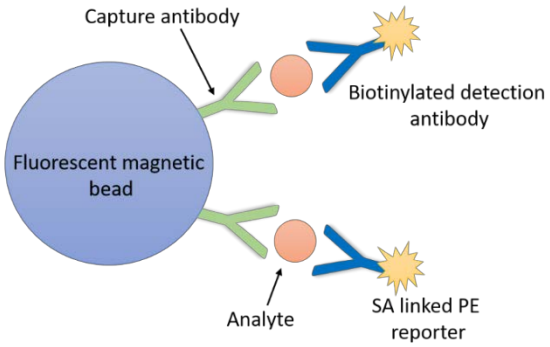


Figure 12. Principle of the bead-based multiplex immunoassay. Analytes are detected by capture antibodies coupled to fluorescently dyed magnetic beads and fluorescently labelled detection antibodies. The magnetic beads are fluorescently coded according to analyte, allowing simultaneous analysis of several biomarkers. SA; streptavidin. PE; phycoerythrin.

Other biomarkers

Serum SDMA was measured at IDEXX Laboratories Inc, using a high throughput competitive chemiluminescence assay, validated for use in dogs (Patch et al., 2015, Prusevich et al., 2015). Serum and urine creatinine were measured at IDEXX laboratories Inc or the central laboratory (CL) at NMBU, using Jaffe's reaction with picrate at alkaline pH whereby creatinine reacts with picric acid to form a yellow-orange complex and the rate of change in absorbance is measured by spectrophotometry and is proportional to the creatinine concentration in the sample (Stockham and Scott, 2008c).

Urinary ALP and GGT activity were measured at CL using colorimetric assays. The hydrolysis of colourless para-nitrophenyl phosphate to yellow para-nitrophenol is catalysed by ALP, the rate of absorbance of which is measured (Schumann et al., 2011). In the GGT assay, glycylglycine acts as an acceptor for the transfer of the gamma-glutamyl group from a synthetic substrate (L-gamma-glutamyl-3-carboxy-4-nitroanilide), catalysed by GGT. 5-amino-2-nitrobenzoate (ANB) is released, leading to proportional colour development and absorbance is measured (Schumann et al., 2002).

All urinary analytes in paper II were normalised to urine creatinine to adjust for urine volume and flow rate and expressed as ng/mg creatinine except for ALP and GGT that were expressed as U/g creatinine and albumin expressed as mg/g. Urinary albumin creatinine ratios of 30-300 mg/g were considered to be positive for microalbuminuria (Sabharwal et al., 2008).

7.4 Paper III

Evaluation of urinary clusterin and cystatin B as biomarkers for renal injury in dogs envenomated by the European adder (Vipera berus).

This was a collaborative study with IDEXX Laboratories, Inc., to evaluate the novel urinary AKI biomarkers urinary cystatin B and the kidney-specific isoform of urinary clusterin. The assays used in this study are pre-commercial research sandwich immunoassays using monoclonal capture antibodies (Yerramilli et al., 2016). Reference intervals for these biomarkers were based on urine from 78 apparently healthy dogs.

Urine protein was measured at IDEXX laboratories Inc, using a pyrogallol red molybdate (PRM) method whereby red PRM dye forms a blue purple-coloured complex upon binding with protein (Fujita et al., 1983). A shift in maximum absorbance from 470 nm to 600 nm is measured using spectrophotometry. Urine protein was then normalised to urine creatinine.

Serum creatinine, urine creatinine and serum SDMA was measured as for paper II. In this paper both absolute urinary biomarker values and values normalised to urinary creatinine, are reported. There is currently no consensus regarding the normalisation of urinary biomarkers to urinary creatinine in AKI, a subject that will be discussed in more detail later.

7.5 Paper IV

Persistent hypercoagulability in dogs envenomated by the European adder (Vipera berus berus).

In paper IV, three assays were used to assess coagulation status in envenomated dogs from presentation to 15 days after bite, compared to a group of healthy control dogs.

Sampling technique

Blood was collected from the cephalic, saphenous or jugular vein using a winged catheter with extension (all control dogs and 22/28 envenomated dogs), via an indwelling venous catheter (3/28 envenomated dogs), or a combination of the two (3/28 envenomated dogs), into Vacuette® collection tubes containing 3.2% sodium citrate. Samples were centrifuged within 15 minutes at 2700 x g, and top layer plasma was pooled for each dog and stored at -80°C until analysis.

Thrombin generation assay

There are no haemostatic pathways that bypass thrombin, making it a central enzyme in coagulation and the assessment thereof. The CAT is a thrombin generation assay that simply put, measures the net thrombin generating capacity in

plasma under the influences of pro- and anticoagulant drivers , thus representing the balance between these effects (Baglin, 2005).
In this assay, thrombin generation is continuously measured after coagulation is triggered by a reagent containing Ca^{2+} and variable concentrations of TF and PLs. The basis of this assay is the cleavage of a slow-acting fluorogenic thrombin substrate. As the substrate is split by thrombin, a fluorescent signal is generated. Dedicated software records the fluorescent signal and calculates thrombin generation compared to an internal thrombin standard, traces a thrombogram and calculates relevant parameters (Tripodi, 2016). A typical thrombogram is shown in Figure 13.

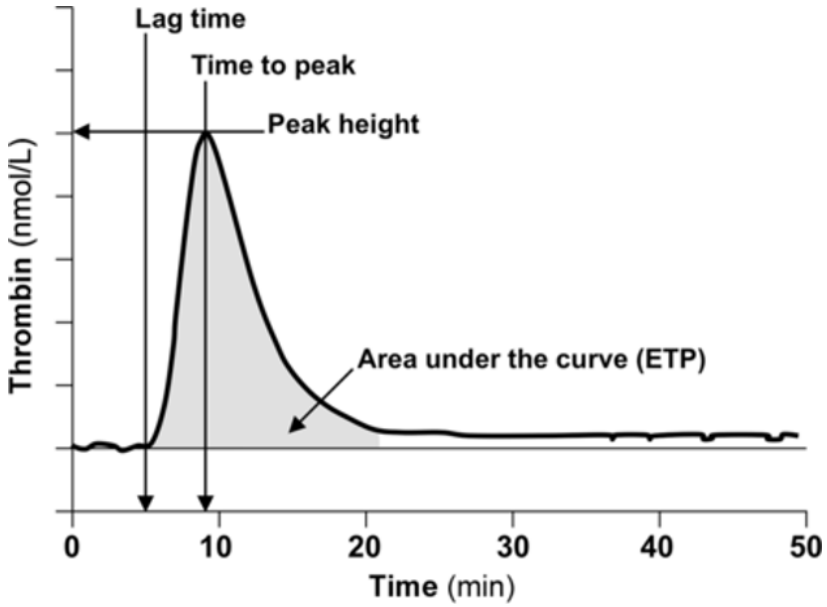


Figure 13. Typical thrombogram with relevant parameters.
Reproduced from: "Thrombin generation assay and its application in the clinical laboratory". Tripodi A. Clinical chemistry (2016), 62 (5) 699-707. Oxford University Press. With permission via Copyright Clearance Center.

Thrombogram parameters used in this study were:
Lag time: the time in minutes from addition of the trigger to the start of thrombin generation.
Peak height: the highest thrombin concentration that can be generated.

Endogenous thrombin potential (ETP): the area under the curve (nM thrombin x time), i.e. the total amount of thrombin that a plasma sample can generate over time. In this study, thrombin generation was measured in platelet poor plasma. Thrombin generation in plasma is dependent upon the trigger used (Berntorp and Salvagno, 2008). Three different triggering reagents were used in this study, as outlined in Table 5.

Table 5. Overview of triggering reagents used in paper IV

Reagent	TF (pM)	PL (μM)	Comments
PPP low/2* (+Ca^{2+})	0.5	4	Small amounts of TF, and PLs in excess render the assay sensitive to pro- and anticoagulant factors in the patient's plasma. The measurement may therefore be influenced by venom components (such as FX activating enzymes), changes in the activity of coagulation factors (e.g. reduced due to consumption) and changes in the activity of specific coagulation inhibitors such as AT and TFPI.
PRP/2* (+Ca^{2+})	0.5	-	The absence of PLs in the exogenous trigger reagent increases the assay sensitivity to PLs in the sample, i.e. includes the activity of PS-positive EVs.
Ca^{2+} alone	-	-	No exogenous trigger added, other than Ca^{2+}, gives the "native" thrombin generating capacity of the sample.

*in pilot studies, thrombin generation using trigger reagents provided by the manufacturer, i.e. PPP low (1pM TF, 4 μM PLs) and PRP (1pM TF, no PLs), was excessively rapid. Tissue factor concentrations were therefore halved by diluting the PPP low reagent 1+1 with a triggering reagent containing only PLs, i.e. 4 μM PLs, and the PRP reagent was diluted 1+1 with buffer.

Thrombin-antithrombin complexes

Thrombin-antithrombin complexes were measured as an indication of preceding *in-vivo* thrombin generation, using a sandwich enzyme immunoassay (ELISA) (Enzygnost® TAT micro). In brief, TAT complexes in the plasma samples bind to AT antibodies coating the ELISA plate, unbound constituents are washed away, and

peroxidase-conjugated AT antibodies are added that bind to the retained AT determinants. Excess enzyme conjugated antibodies are washed away, and a chromogen added that reacts with hydrogen peroxide. The resulting colour intensity is proportional to TAT complex concentration and is measured at 492nm wavelength on a spectrophotometer.

Phosphatidylserine-dependent thrombin generation

The PS-dependent procoagulant activity in plasma was measured as PS-equivalents, using a commercially available, capture based prothrombinase activity assay (ZymuphenTM MP activity assay). This assay uses biotinylated annexin V (AV) to capture PS-positive EVs in the sample, in the presence of Ca^{2+} (Figure 14). After incubation, unbound material is washed away and the ability of the AV -bound PS-positive EVs to generate thrombin is measured. Prothrombinase complexes are formed on the PS surface of bound EVs after addition of FVa, FXa, prothrombin (FII) and Ca^{2+}. Conversion of FII to thrombin by the prothrombinase complexes is dependent on PS, and thus the concentration of PS-positive EVs in the sample. The amount of thrombin formed is measured using a chromogenic substrate that releases free para-nitroaniline (pNA), upon cleavage by thrombin. The colour development caused by free pNA is measured at 405 nm wavelength on a spectrophotometer. Results are read from a standard curve and expressed ad PS equivalents (nM).

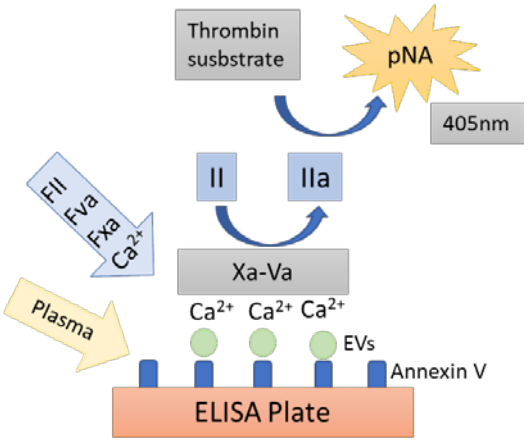


Figure 14. The prothrombinase assay. Annexin V antibodies capture extracellular vesicles (EVs), the thrombin generating capacity of which is measured after addition of FVa, FXa, prothrombin and Ca^{2+}. pNA = para-nitroaniline, FIIa = thrombin.

7.6 Statistical methods

Data was visually assessed for normality (papers II-IV) or by using the Shapiro-Wilk W test (paper I). The majority of data in this thesis was not normally distributed and thus generally reported as median and range with non-parametric statistical tests applied. Statistical software programs used were: JMP Pro 14.3.0, Stata SE 15.1 and 16.0, R 4.0 and GraphPad Prism 8.3.1.

Paper I used largely descriptive statistics and non-parametric tests of cTnI concentrations between arrhythmia grades (Wilcoxon exact test), cTnI concentrations between SSS scores (Steel Dwass for multiple comparisons) and SSS between arrhythmia grades (Fischers exact test). Serum cTnI concentration in the first 12 hours after bite was also evaluated as an indicator for arrhythmia development using a non-parametric receiver operating characteristics (ROC) curve analysis. Different cut off values for cTnI and their sensitivity and specificity for the different arrhythmia grades were assessed.

In papers II-IV, comparisons of outcome variables were made between a control group and envenomated dogs at each timepoint, using Wilcoxon rank sum test with Bonferroni correction, or Steel Dwass test to adjust for multiple comparisons. Correlations between SSS and AKI biomarker concentrations were evaluated using Spearman's rank correlation coefficient, in papers II and III.

An important feature of the studies in this thesis is their repeated measures design. Generalised linear mixed model analysis was used in papers II and IV, to assess the effect of time on the outcome variables whilst also factoring in the within dog variation over time. In these models, "dog" was a random effect and "timepoint" a fixed effect. In paper II additional fixed effects of age, sex and weight were included. In paper IV, antivenom treatment was included as a fixed effect in addition to timepoint.

All of the studies included in this thesis are non-interventional and thus the number of dogs with and without antivenom treatment varied. In paper I-II, the majority of dogs received antivenom treatment thus precluding statistical evaluation of outcome variables between treatment group. However, in paper IV the treated and untreated groups were more evenly matched and thus compared at each timepoint (Wilcoxon rank sum test).

8 Results (summary)

An overview of research questions, materials and methods, and a summary of the results for each of the four papers included in this thesis, are provided in Tables 6-9.

Paper	Research questions	Materials and Methods	Statistics	Main Limitations	Key findings
I	***1. What is the incidence, nature and duration of myocardial injury in dogs bitten by V. berus?*** ***2. Can SSS at presentation indicate the development of myocardial injury?*** ***3. Can cTnI concentrations measured early after bite be used as an indicator for the development of arrhythmias?***	21 dogs bitten by *V. berus* in 2018. 3-point SSS at presentation. Holter AECG from presentation to up to 48 hours after bite, and a 5 min ECG at T5. Arrhythmias were graded 0-3 based on frequency and severity, where 0 was considered normal. Serial cTnI measurements T1-T5. Indirect blood pressure measurements T1-T5.	Comparison of cTnI concentrations between arrhythmia grades 0 vs 1-3, at each timepoint: *Wilcoxon exact test.* Comparison of cTnI concentration between SS scores at presentation: *Steel Dwass for multiple comparisons*. Comparisons between SSS and arrhythmia grade: *contingency table analysis: Fishers exact test.* Assessment of cTnI concentrations in relation to arrhythmia grade: *Receiver operating characteristics (ROC) curve analysis.*	Small sample size. Confounding effect of antivenom treatment.	**1.** Arrhythmias were detected in 12/21 dogs (57%). All arrhythmias were ventricular in origin. VT was observed in 6 dogs (29%). No arrhythmias were detected 14 days after bite. Increased cTnI concentrations were found at one timepoint or more, in 17 dogs (81%). cTnI was normalised in all but one dog, 14 days after bite. **2.** SSS at presentation was not a useful indicator of cardiac effects. **3.** cTnI concentrations ≥ 1.89 ng/mL, 12 hours after bite may indicate the development of VT.

Table 6. Summary of paper I.

Paper	Research question	Materials and Methods	Statistics	Main Limitations	Key findings
II	***1. Do dogs bitten by V. berus sustain an acute kidney injury?*** ***2. How long does the injury last last?*** ***3. Does the severity of clinical signs give an indication of AKI?***	35 dogs bitten by *V. berus* in 2018 and 35 healthy control dogs. Urine samples at T2-T5. Serum samples at T1-T5. Control dogs were sampled at a single timepoint. Urinary biomarkers normalised to creatinine: - **albumin** - **OPN** - **KIM-1** - **MCP-1** - **IL-8** - **cystatin C** - **NGAL** - **ALP** - **GGT** Serum biomarkers: **SDMA** and **creatinine** Other parameters: 16-point **SSS** at T1-T5 and indirect **blood pressure** measurements at T1-T5.	1. Comparisons of biomarker/Cr ratios between cases and controls: *Wilcoxon rank sum test or t-test with Bonferroni correction.* 2. Repeated measurements of biomarker/Cr ratios in envenomated dogs: *Mixed model analysis* *Fixed effects*: time, age, weight, sex *Random effect*: dog. 3. Correlations between SSS and peak biomarker ratios: *Spearman's rank correlation*.	High number of samples with values below the limit of quantification (LOQ) for the assay, for ALP, IL-8, and albumin. This resulted in unusable ALP results. Uncertain specificity of OPN, NGAL, MCP-1 and Il-8, for AKI. Confounding effect of antivenom treatment.	**1**. There was evidence of non-azotemic kidney tubular injury during the first 36 hours after *V. berus* bites in dogs. **2.** Urinary biomarker/creatinine ratios were normalised 14 days after bite. **3.** SSS was not a useful indicator of increased urinary AKI biomarker/creatinine ratios.

Table 7. Summary of paper II.

Paper	Research question	Materials and Methods	Statistics	Main Limitations	Key findings
III	***1. Do dogs bitten by V. berus sustain an acute kidney injury*** ***2.How long does the injury last?*** ***3. Does the severity of clinical signs give an indication of AKI?***	36 dogs bitten by *V. berus* in 2018 and 34 healthy control dogs. Urine samples and serum samples at T1-T5. Control dogs sampled at a single timepoint. <u>Urine biomarkers</u>: - **clusterin** (with and without normalisation to urine creatinine). - **cystatin B** (with and without normalisation to urine creatinine). - **UPC** <u>Serum biomarkers</u>: **Creatinine** and **SDMA.** <u>Other parameters</u>: 16-point **SSS** at T1-T5 and indirect **blood pressure** measurements T1-T5.	1. Comparison of biomarkers concentrations between cases and controls: *Wilcoxon rank sum test.* 2. Proportions of samples with biomarker concentrations over the reference interval compared to controls, at each timepoint: *Fishers exact test with Bonferroni correction*. 3. Correlations between SSS and biomarker concentrations: *Spearman's rank correlation.*	High number of samples with clusterin and cystatin B below the LOQ for the assay. Uncertain specificity of cystatin B for AKI. Confounding effect of antivenom treatment.	**1**. Absolute urine cystatin B concentrations and values normalised to urinary creatinine were higher in envenomated dogs than in controls at T1-T4. When clusterin was normalised to urinary creatinine, levels were significantly higher at T3 and T4 in envenomated dogs compared to controls. **2**. Urinary cystatin B values were not significantly different to controls at T5. **3**. SSS correlated with absolute cystatin B concentrations at T1.

Table 8. Summary of paper III.

.Paper	Research question	Materials and Methods	Statistics	Main Limitations	Key findings
IV	***1. What is the global coagulation status of dogs envenomated by V. berus?*** ***2. Is there a difference in coagulation status between dogs treated with and without antivenom?***	28 dogs bitten by *V. berus* in 2017 and 28 healthy control dogs. Citrated plasma samples taken at T1-T5 in envenomated dogs and a single timepoint in controls. Thrombin generation measured by **CAT** (lag time, peak and ETP). **TAT** complexes. **PS equivalents**.	1. Comparisons of CAT parameters, TAT and PS equivalents between cases and controls: *Steels test for multiple comparisons.* 2. Comparisons between dogs treated with and without antivenom at each timepoint: *Wilcoxon rank sum test.* 3. Analysis of proportions of dogs with peak, ETP and LT values above or below the control group at presentation: *Fishers exact test.* 4. Repeated measurements of CAT parameters, TAT and PS equivalents between envenomated dogs: *Mixed model analysis* *Fixed effects:* timepoint and antivenom treatment. *Random effect*: dog.	Large number of samples excluded due to haemolysis.	**1**. Envenomated dogs were hypercoagulable compared to controls, already at presentation and still at 15 days after bite. **2.** Dogs treated with antivenom may be less hypercoagulable than non-antivenom treated dogs. Lag time might serve as a diagnostic test for envenomation.

Table 9. Summary of paper IV.

9 Methodological considerations

The studies presented in this thesis work have limitations which should be taken into consideration when interpreting results and the conclusions that may be drawn.

9.1 Validity in observational studies

Clinical research strives to make valid inferences about associations between exposure and outcome in a study group and generalise them to a wider 'target' population of clinical interest (Vetter and Mascha, 2017). Internal and external validity are essential components in the design, analysis, and inference of studies. **Internal validity** refers to the strengths of inference that can be made in a given study (Carlson and Morrison, 2009). In this case, *"did the exposure (snakebite) cause the outcome (cardiac, renal or coagulation effect)"?* **External validity** is the ability to generalise study findings to a wider population (Carlson and Morrison, 2009). In this case, *"are the conclusions from these studies likely to hold true for all dogs bitten by V. berus"?*

Systematic error or "bias" can lead to an incorrect estimation of an association between an exposure and outcome, thereby compromising study validity. Three types of systematic bias exist: (1) selection bias, (2) information bias and (3) confounding bias (Dohoo et al., 2009b).

9.1.1 Selection bias

Selection bias results from a difference in composition between the study group and target population. The convenience sampling used in the studies in this thesis comes with an inherent selection bias which may compromise **external validity.** These studies relied on dogs presenting to the clinics after snakebite. This may have resulted in a bias towards including more severely affected dogs since these would be more likely to present for treatment within the inclusion timeframe for this project. Combined with the non-interventional nature of the studies, this means that the findings can be tentatively generalised to **dogs presenting for veterinary care within 24 hours of a *V. berus* bite and managed under similar conditions to the**

dogs in this thesis. This selection bias is not considered a significant limitation since this is also the group of most clinical interest.

Longitudinal studies such as these are often subject to loss to follow-up or **"attrition".** Loss to follow-up at T5 was low in these studies, most likely due to the relatively small number of dogs and short interval between sampling times. A total of 8/60 (13%) dogs did not return (n=5) or returned beyond the allocated timeframe (n=3) for sampling at T5. However, attrition due to missing data was a feature at other timepoints for reasons including dogs presenting nine hours or more after bite, resulting in lack of a T1 (presentation) sample; occasional missed or delayed sampling; a lack of sufficient urine in papers II and III, and haemolysis and analysis-level factors resulting in exclusion of samples in paper IV.

Selection bias can occur if data is missing for a non-random reason (Vetter and Mascha, 2017). Every attempt was made to sample all snake-bitten dogs presenting to the four clinics during the timeframe for this project, but a reliance on the investigator being informed by attending clinicians, manpower and equipment considerations meant that occasionally dogs were not included due to Holter equipment being in use or a presentation sample being missed. These are however considered to be random events, not linked to severity of disease, and thus are not considered a source of significant bias.

Although oliguria was not confirmed since urine production was not quantified, it is a possible explanation for inadequate urination and subsequent missing urine samples in papers II and III. Missing data in papers II and III might therefore be a surrogate indicator of AKI severity (Vaden et al., 1997). However, since this missing data is likely to bias the findings in a conservative direction, it is not thought to significantly affect the conclusions in these studies.

Similarly, in paper IV, a large number of samples were excluded due to haemolysis. This was suspected to be due to sampling technique in the majority of cases (Grant, 2003), but haemolysis is also a feature of *V. berus* envenomation (Garkowski et al., 2012) and could be associated with increased severity of disease (i.e., a non-random effect). Since haemolysis itself is a procoagulant condition (Stief, 2007), the inclusion of haemolytic samples would have introduced a confounding effect on the outcome parameters.

Another challenge encountered in papers II-IV was that of outcome variable measurements below the limit of quantification (LOQ) of the assay. There are various ways to tackle this (discussed under "statistical considerations"), of which a conservative form of imputation was generally used and thereby these data were not lost. However, rapid initiation of, or prolonged thrombin generation (TG) not reaching baseline led to a lack of start or end point for the software to calculate peak and ETP in a number of samples. Consequently, this data is missing and may have contributed to **selection bias.** This was partly compensated for by the use of three different trigger reagents. Individual samples with rapid, unregistrable thrombin generation with PPP low/2 reagent, could be measured when the process was slowed using the other reagents, meaning that not all data was lost. In fact, there was only one individual for which ETP could not be measured using any of the reagents, but information was still gained for this dog through TAT complex and PS equivalents concentrations. Thus, missing data affected the comparisons of different reagents but is unlikely to have widely affected the conclusion of a hypercoagulable state in these dogs. The issue of data loss due to rapid thrombin generation and future alternative approaches are discussed under the specific considerations for paper IV.

9.1.2 Information bias

Information bias refers to the incorrect classification of exposure, extraneous factors or outcome status (Dohoo et al., 2009b). Since there is no available diagnostic test for *V. berus* envenomation in dogs, the diagnosis of snakebite is presumptive in this thesis work and might therefore be considered a source of information or **misclassification bias** which could affect both the **internal and external validity** of the study. The snake and/or fang marks were observed in 66.3% (40/60) of included dogs. Inclusion of dogs where the snake or fang marks were not observed, was restricted to those with a history, clinical signs and geographical location compatible with snakebite, all of which were similar to those dogs where the snake or fang marks were witnessed. The most likely differential diagnoses for per acute swelling and pain in a dog being walked in the summer months without other obvious trauma, are snakebite or bee or wasp (Hymenoptera) stings. Clinical signs of Hymenoptera envenomation in dogs are generally restricted to local erythema, pain and swelling, and rarely, anaphylaxis. Rhabdomyolysis, myocardial infarction and acute kidney injury have also been sparsely reported but only after multiple stings (Fitzgerald and Flood, 2006, Shimada et al., 2005). Dog

walkers would most likely have noticed a swarm of wasps or bees and thus it seems unlikely that any observed cardiac, renal and coagulopathic effects are a result of a misclassification bias, in this study.

9.1.3 Confounding bias

Confounding bias is a major concern in observational studies such as these. A confounding factor is a variable that may compete with the exposure of interest in explaining an outcome variable, such as age, sex, breed, environment, and treatments (Skelly et al., 2012).

A literature review identified weight and age as possible confounders for some of the variables measured in papers II and III (Trachtenberg and Barregård, 2007), and their effect was mitigated by matching the control group to the envenomated group through stratified sampling by weight and age, with reasonable success. Multivariate statistical models are also able to "control" for such factors when they are included as explanatory variables (Skelly et al., 2012), a technique which was employed in papers II and IV when comparing the envenomated dogs across timepoints.

Additionally, in paper II, previous studies suggested a possible confounding effect of pyuria or urinary tract infection on urinary GGT, NGAL, KIM-1 and IL-8 (Lippi et al., 2018, Nivy et al., 2017, Daure et al., 2013, Rao et al., 2001). This effect was mitigated by excluding individual urine samples with > 5 leucocytes per high power microscopy field from statistical analysis of these four biomarkers. As already mentioned, the confounding effect of haemolysis on coagulation parameters in paper IV (see section on preanalytical considerations in coagulation assays), was mitigated by excluding samples with gross haemolysis, this did however come at a cost of sample size.

The majority of assessments and sampling were carried out by a single investigator in these studies in an attempt to minimise a potential confounding effect of investigator, particularly on subjective measure such as SSS. Four dogs in papers II and III were recruited and evaluated by a different (single) investigator. Because of the small number, this is not considered a major limitation. Samples in paper IV were the result of a sampling period in 2017 and although several investigators were involved, all cases were from a single clinic and sampled as per a

predetermined protocol, thus minimising the potential confounding effect of clinic, treatment and veterinarian. Venom dose, inter-individual venom variation and individual dog susceptibility to venom effects are other variables likely to influence results that could not be controlled for.

One advantage of prospective cohort studies is that since exposure occurs before the outcome being measured, associations (but not necessarily causation) between exposure and outcome can be established (Euser et al., 2009). However, since these studies were observational (without intervention by the researcher), antivenom treatment is another possible confounding factor which can compromise both the **external and internal validity** of the study, and one that unfortunately could not be controlled for and may have impacted the results obtained.

9.2 Control groups

Internal validity may be compromised by the lack of a control group or the lack of a suitable control group. For papers II-IV, a single sample control group was used to mitigate the effects of identified or suspected confounders, as previously discussed. Ideally, a longitudinally sampled control group of hospitalised healthy dogs, exposed to the same environment to allow assessment of the degree of individual variation over time on the outcome parameters measured, would also have been included. Hospitalising privately owned healthy dogs for the sole purpose of this project was unfeasible. One option may have been to use a cohort of research dogs, although any effect of environment or stress on the outcome parameters may not be tested in a group of dogs that are normally kennelled. One example of this is a possible association between hospitalisation and increased UPC compared to outpatients (McCaw et al., 1985). Dogs in boarding kennels would have been a reasonable option, had manpower and financial constraints not been an issue.

In contrast to many of the outcome variables in papers II-IV, the cTnI assay used in paper I has an established reference threshold of < 0.06 ng/mL in dogs and concentrations of cTnI in envenomated dogs were therefore defined as normal below this limit. However, since age, breed and possibly exercise level are associated with increased cTnI concentration (Ljungvall et al., 2010, LaVecchio et al., 2009, McKenzie et al., 2007), it would have been useful to have a matched control group for this study also. Equally, for normal AECG variation and the effects of

hospitalisation on AECG findings would ideally have been controlled for by comparing findings in envenomated dogs to a group of healthy hospitalised dogs.

9.3 Statistical considerations

The data sets in this thesis involve repeated measures: outcome variables were measured several times in each envenomated subject with the intention to evaluate the trend over time. Repeated measures observations are non-independent: data from each subject may be correlated due to within-subject random effects that should not be ignored (Dohoo et al., 2009a).

There are several ways in which to tackle analysis of repeated measures data including (1) univariate methods such as analysing the timepoints separately or using a summary statistic such as peak concentration; (2) repeated measures analysis of variance (ANOVA) or Friedman's ANOVA (non-parametric data); (3) regression analysis using a random effects model such as a generalised linear mixed model (GLMM)(Dohoo et al., 2009a).

Repeated measures ANOVAs requires a balanced data set with the same number of observations per subject (Dohoo et al., 2009a). Missing values precluded this type of analysis and is one of the reasons that this method has been superseded by GLMM which provides more flexibility in the type of data set to which it can be applied. Thus, univariate and GLMM methods were used in these studies.

Univariate techniques avoid modelling the repeated measures either through reducing the number of observations to one, such as the peak cTnI comparisons between arrhythmic and non-arrhythmic dogs in paper I and the use of peak SSS and peak AKI biomarker values in paper II, or analysis by separate timepoint such as comparing individual timepoints from envenomated dogs to the control group in papers II-IV. In papers II and IV GLMM was used to evaluate the effect of timepoint on outcome parameters in the envenomated dogs, in addition to univariate analysis of each timepoint against the control group. The success of these methods varied and although attempted in paper III, did not result in a reliable model, as discussed below.

The majority of data in this thesis work was non-normally distributed. Generalised mixed models allow analysis of such data; thus, this method was suitable for assessing the effect of time after bite on the outcome parameters in papers II-IV. Fixed and random predictor variables can be included in GLMM modelling. Random effects are categorical variables that can be viewed as a sample from a larger population and not of direct interest themselves, such as individual dogs in these studies. One of the most common uses of a random effect is to control for non-independence such as in repeated measures designs. Fixed effects are of interest in their own right, such as timepoint after bite in this case (Grafen and Hails, 2002).

Specific statistical assumptions need to be considered in GLMM modelling. In brief, model assumptions of (1) homogeneity of variance (similar variance at all levels of a predictor variable), (2) linearity and (3) normally distributed residuals, should be checked and where these assumptions are not met, various techniques can be used to transform the data in an attempt to meet them (Harrison et al., 2018). Ignoring these assumptions is likely to increase the type I error rate (erroneously concluding that results are significant) (Harrison et al., 2018). In paper III (cystatin B and clusterin), and some variables in paper II (cystatin C, IL-8 and albumin), a satisfactory model fit was not obtained, likely due to the high number of AKI biomarkers values below the LOQ for the assays skewing the data and which were therefore imputed (see next section for an explanation of imputation). Since a satisfactory transformation of the data to normalise the residuals was not achieved, the models were not trustworthy and were therefore not reported for these variables. Therefore, in paper III only individual timepoints against the control group are reported, as for cystatin C, IL-8 and albumin in paper II.

One of the other general statistical considerations relevant to papers II-IV, was how to handle values below the limit of quantification (LOQ) for the assay. In papers II and III there were a high number of samples with AKI biomarker values below the lower LOQ (LLOQ). The LLOQ is the lowest concentration of an analyte that can be reliably quantified with acceptable precision and accuracy (Armbruster and Pry, 2008). The question is how to tackle values that fall below this limit and might thereby be unreliable. The best option would be to re-analyse using lower dilution factors. However, the amount of material available as well as concerns over the effect of freeze-thaw cycles on biomarker concentrations (Herrington et al., 2016), precluded this approach. Elimination of the unreliable values is an option, but this would have resulted in a large amount of data loss in these studies. Another option

is to assign an arbitrary value to the data below the LOQ, also known as **imputation**, for which several techniques exist.

Three single imputation techniques were considered:

1. The LLOQ value
2. 0
3. A mean value between LLOQ and 0 (LLOQ/2)

A disadvantage of simple imputation is that since all values < LLOQ are replaced with the same value, the variance between these values is lower than it should be, thereby creating bias. Using the mean value (option 3) assumes a linear relationship between the LLOQ and 0, and this method was therefore not used. In papers II and III, the LLOQ value was imputed for all measurements < LLOQ. The choice of LOQ rather than 0 came down to choosing the most conservative option for the research question: ***"is there a difference in AKI biomarker concentration between envenomated dogs and controls?".*** The choice of the maximised value (the LLOQ) was the most conservative option, thereby increasing confidence in any significant findings and decreasing the chance of type 1 error (erroneously concluding there is a difference). In paper IV, missing lag time data was also imputed. Since these values were missing due to rapid thrombin generation, imputation using the lowest registered lag time was also a conservative approach.

For TAT complexes and PS equivalents, values above the upper standard for the assay were reported as extrapolated concentrations to graphically demonstrate the diversity in the data. These values are therefore less accurate but since both these values and a conservatively imputed value (set to the highest standard concentration) produced the same statistical results, the use of extrapolated values does not change the conclusions of the study.

Given the equipment, time, manpower and financial constraints, the numbers of subjects included in these studies is not unreasonable, but as with many veterinary clinical studies, sample size is a limiting factor in their interpretability and is reflected in the relatively wide 95% confidence intervals in paper I in particular.

9.4 Paper I

Biological variation is an important consideration for any assay and determines whether a population-based or subject-based reference interval is most appropriate for evaluation of individual values. Biological variation data can be used to help determine whether an observed change is greater than expected physiological change (Campora et al., 2018). As with many analytes, the degree of intra-individual variation in cTnI is lower than the inter-individual variation, resulting in a high inverted index of individuality and meaning that subject based reference intervals would be better suited to detection of disease (Ruaux et al., 2015). Subject-based reference intervals using a reference change value may therefore have been a better method of evaluating marginally increased cTnI concentrations in this study, than using the reference threshold. However, the use of such a method is complicated by not knowing which value is ”normal” for each individual and although the best guess in this study would be T5, such a retrospective analysis would be of very little practical use in the clinical setting.

In this study, the Holter AECG was chosen for ease of post-hoc single blinded analysis by a cardiologist. Whilst this was a good choice for research purposes, a real-time telemetric method would be more useful in the clinical setting. This would allow immediate identification of any potentially life-threatening arrhythmias such VT. Since AECGs in this study were assessed post hoc and blood pressure measurement was not continuous, haemodynamic compromise was not fully evaluated.

Definitions of true VT versus AIVR are variable in veterinary medicine, with many cardiologists defining a ventricular rate >160 bpm as VT. In the human literature there are also various definitions, including AIVR defined as a rhythm arising below the AV node at a rate within 10% of the underlying sinus rhythm, and above this being VT (Katritsis et al., 2012). It has been suggested that the same system could be adopted in dogs (Santilli et al., 2019). Snake envenomation can result in pain, stress, and varying degrees of hypovolaemia, and it is therefore reasonable to assume a higher than normal sinus rate in *V. berus* bitten dogs. For this reason, and to define VT as a rhythm which was likely to be dangerous and with an indication to treat, the definition chosen in this study was a minimum of four consecutive VECs at an instantaneous rate of ≥ 200 bpm. The grading system used in this study was therefore conservative in terms of VT definition compared to other canine snake

envenomation studies where VT has been defined at a minimum of 3 VECs at ≥100 bpm or ≥160 bpm (Vestberg et al., 2017, Segev et al., 2008).

Common to many grading systems is the occasional marginal values upon which an individual's grading is based. The arrhythmia grading system used in this paper was based on both number and complexity of VECs. For the vast majority of dogs in this study results were very clear cut with either high numbers and complexity of VECs or few, single VECs. However, two dogs had very low numbers of VECs (3 in 24 hours and 4 in 19 hours) and were defined as arrhythmic because of a single couplet in that time. This raised the question of whether a single couplet might be a normal finding on a 24-hour AECG. Since the literature regarding AECG findings in healthy dogs is sparse and in a number of cases, hampered by the inclusion of breeds predisposed to ventricular arrhythmias such as Boxers and Dobermanns, this stimulated a post-hoc decision to obtain 24-hour AECGs on five of the arrhythmic dogs, one year after bite. Unfortunately, the two individuals with a single VEC couplet after envenomation were not available for follow-up at this timepoint. Nevertheless, no VECs were found in the dogs available for one-year follow-up, thereby increasing the confidence in the association between snakebite and arrhythmias observed during the first 48 hours after envenomation. The marginal classification of these two individuals is not considered a major limitation in this study since all other results were unequivocal. However, this does highlight the importance of considering clinical relevance when interpreting results from paper I, a subject discussed in more detail in chapter 10.

9.5 Papers II and III

9.5.1 To normalise or not to normalise, that is the question

Other than handling values below the LOQ, the main consideration in these two papers was whether to normalise urinary biomarker concentrations to urinary creatinine (uCr). Many papers report urinary biomarkers normalised to uCr, such that this approach is generally expected and one that is probably appropriate in chronic kidney disease (Goldstein, 2010). However, whether this technique is appropriate for AKI, is a subject of some debate and one for which a consensus has yet to be reached. This is reflected in the different primary approaches used in papers II and III.

Urinary biomarker concentrations may be affected by time of urine collection and urine concentration as a result of urine flow rate. Normalisation of urinary biomarkers to uCr is a technique used to control for urine flow rate. However, this technique assumes a steady state of creatinine production and excretion both within and between individuals, or a linear relationship between biomarker and uCr excretion rate, which is not necessarily the case since creatinine concentration are known to vary with age, diet and muscle mass (Waikar et al., 2010).
Urine creatinine concentration is determined by:

$$[sCr] \times GFR + TScr - Bcr = [uCr] \times UFR$$

Where *[sCr]* = serum creatinine concentration, *GFR*= glomerular filtration rate, *TScr* = rate of tubular secretion of creatinine, *Bcr* = rate of creatinine backleak, *UFR*= urine flow rate (Waikar et al., 2010).

Bearing in mind the determinants of uCr concentration, urinary AKI biomarkers can be under- or overestimated using this normalisation method, depending on the setting. In the human literature, clinical situations in which normalisation of urinary biomarkers to uCr is considered misleading, includes individuals with: (1) different rates of creatinine generation and extrarenal degradation, (2) where GFR is changing and has not yet reached a stable state (such as in AKI), (3) where secretion rates of creatinine differ, and (4) where tubular backleak differs (Waikar et al., 2010).

Backleak results from damaged renal tubular epithelium allowing the passage of creatinine in the glomerular filtrate back into systemic circulation and may be relevant to the envenomated dogs in this study (Basile et al., 2012). Tubular secretion of creatinine is however, negligible in dogs compared to humans and thus was not considered to significantly influence uCr in these studies (Braun et al., 2003).

Absolute concentrations of urinary biomarkers are being increasingly reported in studies of AKI, instead of normalising to uCr. However, this method is not without its own limitations. Absolute urinary biomarker values are increased by oliguria and decreased by polyuria. Whilst a number of studies have reported little difference in normalised and absolute values (Han et al., 2008, Du et al., 2011), the individual setting needs to be considered. Envenomated dogs in papers II and III were

compared to healthy controls and the effect of intravenous fluid therapy (IVFT) on GFR and subsequent urine flow rate, needed to be considered. The control group did not receive IVFT and thus the envenomated dogs were comparatively polyuric.

Despite the weaknesses of normalisation, this technique was deemed appropriate to control for a suspected large discrepancy in urine flow rate between cases and controls due to IVFT and not AKI itself. Furthermore, normalising to uCr has been shown to decrease intraindividual variability for some AKI biomarkers which was also a consideration due to the longitudinal design of these studies and the desire to minimise the confounding effect of variability due to normal (i.e. non-snakebite related) intraindividual variation (Delanaye et al., 2011). Normalisation to uCr was therefore the chosen technique for paper II.

A different approach was employed in paper III. A similar study of *V. berus* envenomated dogs (Gordin et al., 2020) suggested that presenting normalised values of urinary clusterin and cystatin B, conferred no advantage over absolute values and thus the results are primarily presented as absolute values although normalised data is also included and showed in this instance that there was a difference between the conclusions drawn from the different techniques, highlighting the need for further studies before a consensus regarding the use of normalisation or absolute values, can be reached.

Other options for controlling for urine flow rate and thus concentration, include standardising the specific gravity in urine samples through dilution, or adjusting for urine osmolality as was employed in a recent canine study (Boyd et al., 2019). Urine osmolality is considered the best method of measuring urine concentration since it takes into account all solutes, irrespective of molecular mass, and thus could have been a good alternative in this study (Dossin et al., 2003).

9.6 Paper IV

Pre-analytical variables are an important consideration in the analysis of coagulation parameters. Pre-analytical factors include all steps from sample collection until analysis and is the phase in which most analytical errors occur (Lippi et al., 2011).

9.6.1 Blood collection

The choice of blood collection system including the tube type, is an important pre-analytical consideration due to potential contact activation of the intrinsic pathway and haemolysis-associated thrombin generation. An increased rate of haemolysis when drawing blood through indwelling catheters or winged needle catheters compared to a convention straight needle approach, has been demonstrated in some studies whilst others have failed to find a significant difference between these techniques (Lippi et al., 2005, Kennedy et al., 1996). Direct venepuncture has shown better repeatability compared to winged-needle venepuncture for CAT in dogs (Cuq et al., 2018), but the effect of different blood collection systems on the degree of thrombin generation, has produced contradictory results in human studies and the situation is therefore unclear (Spronk et al., 2009, Loeffen et al., 2012). For practical reasons, the majority of dogs in this study were blood sampled using a winged-needle with extension into the collection tube (82% of samples from cases and 100% of controls), although due to ethical and licencing considerations, samples were taken via an indwelling venous catheter from dogs in the 2018 cohort. Since every attempt was made to standardise the blood sampling method and only a small number of samples were taken via an indwelling catheter, results are considered comparable between dogs in this study.

9.6.2 Corn trypsin inhibitor

Factor XII may undergo autoactivation to FXIIa after contact with surfaces such as the blood collection tube (Silverberg et al., 1980). A consideration often mentioned in the context of thrombin generation assays, is the use of corn trypsin inhibitor (CTI) in the collection tube to inhibit blood collection system-dependent activation of the intrinsic pathway. Corn trypsin inhibitor is a FXII inhibitor able to prevent the activation of plasma coagulation initiated by contact activation (Luddington and Baglin, 2004). The use of CTI is discussed in the proposed guidelines for

standardisation of pre-analytical conditions in thrombin generation by the International Society on Thrombosis and Haemostasis (Dargaud et al., 2017), but is a subject of continued debate. Thrombin generation in the absence of, or at low triggering TF concentrations, has been shown to be increased compared to that in samples with CTI addition, indicating contact activation of coagulation and thus, falsely increasing thrombin generation in humans (Loeffen et al., 2012, Rodgers et al., 2014). However, the use of CTI hinders evaluation of the contribution of molecules such as PS positive EVs that are able to activate the intrinsic pathway, and can result in inadequate thrombin generation curves (Hellum et al., 2017, Van Der Meijden et al., 2012). Furthermore, other studies have concluded that the use of CTI may not be strictly necessary, and cost and availability of CTI loaded tubes have restricted its general use (Spronk et al., 2009, Mohammed et al., 2014). For these reasons, and also considering a lack of evaluation specifically in canine thrombin generation, CTI was not used in this study.

9.6.3 Centrifugation, storage and freeze-thaw effects

Blood samples were centrifuged only once, which might have resulted in residual platelets that fragment during a freeze-thaw cycle and may subsequently affect phospholipid-dependent thrombin generation and PS equivalents (Helmond et al., 2013). Double centrifugation (2x 2500 g for 15 minutes) is recommended to minimise this effect (Lacroix et al., 2012). However, residual PS-positive fragments are less likely to influence the measurement when thrombin generation is initiated with excess PLs in the exogenous reagent such as when using the PPP/2 low reagent (Gerotziafas et al., 2005). When triggered with PPP/2 low reagent, all thrombin generation parameters were indicative of hypercoagulability in envenomated dogs. Thus, increasing the confidence in the overall conclusion of snakebite-associated hypercoagulability, despite the possible effects of this pre-analytical variable on some of the thrombin generation parameters.

The number of freeze-thaw cycles in this study were kept to a minimum since platelet derived EVs may increase with repeated freeze-thaw cycles, although the literature regarding this effect is contradictory (Lacroix et al., 2012). However, as is often inevitable with limited sample material, TAT complex and PS-equivalents were analysed after a second freeze-thaw cycle.

There was a disparity in plasma storage time for analysis of thrombin generation between cases (four months) and controls (15 months). Previous studies suggest that lag time and peak height are unlikely to be significantly affected by storage and that ETP and number of EVs are more likely to have decreased with longer storage times (Cuq et al., 2018, Ayers et al., 2011). Since ETP and PS equivalents were higher in the envenomated dogs than the controls in this study, storage time is considered unlikely to have had a significant impact on the overall findings.

9.6.4 Tissue factor concentration

The concentration of TF used to trigger the CAT assay significantly influences its sensitivity for individual coagulation factors and is an important source of interlaboratory variation (Kristensen et al., 2018). When higher TF concentrations are used, only changes in the activity of the coagulation factors of the extrinsic pathway exert an influence (Duarte et al., 2017). The low amounts of TF used in this study rendered the thrombin generation measurement sensitive to changes in the activity of all coagulation factors, thus, allowing a broader evaluation of secondary haemostasis. Furthermore, between-subject variation in thrombin generation measurements, and thus, discrimination between individuals, is higher at low TF concentrations compared with high TF concentrations (Kristensen et al., 2018).

9.6.5 Rapid thrombin generation and missing data

A number of ETP and peak height values could not be calculated due to rapid thrombin generation or the thrombin generation curve not reaching the baseline. Whilst this is unfortunate, it does suggest that the findings in this study might actually underestimate the thrombin generation potential in dogs bitten by *V. berus* and does not alter the conclusion of a hypercoagulable state in this study. Thrombin generation increases with decreasing temperature (Hemker and Kremers, 2013) and it is therefore possible that thrombin generation could be slowed by increasing the incubation temperature during analysis. Bearing in mind the results from paper IV and considering that dogs have a higher baseline body temperature than humans, future studies using CAT in envenomated dogs could consider increasing the temperature at which thrombin generation is measured in an attempt to slow thrombin generation and minimise data loss due to rapid lag times. One other canine thrombin generation study used higher CAT incubation temperatures than

employed in paper IV (39°C versus 37°C) (Krogh et al., 2020). The same study also performed CAT readings every 10 seconds as opposed to the 20 seconds employed in paper IV. This may also decrease data loss due to rapid thrombin generation but does significantly increase the cost and time of analysis.

9.6.6 Coagulation inhibitors affecting thrombin generation

Thrombin generation measured in this study provides information on the global haemostatic status of dogs after snakebite. Thrombin generation measurements are influenced by the activity of the coagulation inhibitors TFPI and AT (Dielis et al., 2008). In this study, measurements of TFPI and AT were not performed due to scarcity of patient material. Reduced activity of TFPI and AT may increase thrombin generation and measurements of the activity of these coagulation inhibitors could have further clarified the mechanism behind the increased thrombin generation detected in envenomated dogs. However, thrombin generation measurements do not assess the anticoagulant contribution from APC, due to the absence of TM. The addition of TM in thrombin generation measurements, is not standardised, nor has the addition of TM been evaluated in dogs and was therefore not performed in this study. An anticoagulant contribution from TM-independent protein C activators has been described in venom from other snake species (Murakami and Arni, 2005), although such activators have not yet been demonstrated in *V. berus* venom.

10 General discussion*: Vipera berus* envenomation in dogs

The topic of this thesis stemmed from a lack of evidence-based guidelines for the treatment and management of dogs bitten by *V. berus* due to a general paucity in research in this field despite dogs commonly being envenomated by this species of snake.

The broad questions posed in the introduction to this thesis, are addressed in this section:

1. ***What are the effects of V. berus bites in dogs?***
2. ***What implications do these effects have in terms of treatment and management of envenomated dogs?***

10.1 What are the effects of *Vipera berus* bites in dogs?

Having identified key areas that were feasible to research within the timespan available, this thesis work focused on cardiac, renal and coagulopathic effects after *V. berus* envenomation. Thus, this work gives insights into the effect of envenomation on these three body systems specifically.

10.1.1 Cardiac effects

Paper I evaluated serial cTnI measurements in combination with 48-hour AECGs for the first time in dogs bitten by *V. berus* and was chosen to give a more comprehensive overview of the cardiac effects of envenomation and their duration, than previous studies. In accordance with other studies, cardiac effects were identified in a high proportion of dogs as demonstrated by ventricular arrhythmias of varying severity and increased cTnI concentrations. Additionally, paper I demonstrated that arrhythmias may still be present 48 hours after envenomation.

Arrhythmia grades 1-3 were considered increasingly pathological, although all appeared to be well tolerated in dogs in this study. Risk associated with ventricular arrhythmias has not been evaluated in *V. berus* envenomated dogs, but arrhythmias are known to be a risk factor for sudden death in other canine cardiac disease and may be occult (Meurs et al., 2016). In a study of Doberman pinschers with dilated cardiomyopathy, the presence of fast VT (> 260 bpm) and increased cTnI concentrations were both predictors of sudden death (Klüser et al., 2016). Dogs envenomated by *V. berus* with these findings might therefore be at similar risk. Grade 1 and 2 arrhythmias are considered likely to be well tolerated and not thought to be a significant clinical concern. An important limitation to this study is the lack of full evaluation of haemodynamic compromise since only spot blood pressure measurements were made. Since clinical significance of arrhythmias is based in large part on the presence of haemodynamic compromise, the significance of some arrhythmias in paper I might have been underestimated.

Although generally well-tolerated, frequent VECS (>25% of all QRS complexes in a 24-hour period) are occasionally associated with ectopy-mediated cardiomyopathy in humans, even in the absence of sustained tachycardia (Lee et al., 2019). The VEC burden associated with such complications is not described in dogs but it is reasonable to use human figures as a guideline. Whilst five dogs had a high VEC burden after *V. berus* bite, given that these arrhythmias were of relatively short duration, ectopy-mediated cardiomyopathy is not considered a concern in these dogs.

Equipment and manpower for prolonged ECG monitoring is unlikely to be available for all dogs bitten by *V. berus*, since it is common to have several envenomated dogs hospitalised concurrently. Thus, an ability to ascertain relative risk of developing a clinically significant arrhythmia, would be of benefit in order to prioritise allocation of equipment and patient monitoring. With this in mind, severity of clinical signs at presentation was evaluated as an indicator for the subsequent development of VT, and in accordance with one other study, severity of clinical signs of envenomation was not found to be a useful parameter in this context (Vestberg et al., 2017).

On the other hand, results of paper I suggest that cTnI concentrations measured 12 hours after bite might be useful in predicting the development of VT. Cardiac troponin I concentrations have previously been described as having a positive predictive value for the development of clinically significant arrhythmias after blunt

trauma in dogs (Biddick et al., 2020), and is an aspect worthy of more investigation, especially given the availability of a point of care cTnI test which would allow immediate decision making (Porter et al., 2016). Due to sample size in paper I, results from the ROC curve analysis regarding a potential cut-off cTnI concentration to guide monitoring for VT should not be generalised but do give an indication as to where future research resources might be focused.

10.1.2 Renal effects and utility of acute kidney injury biomarkers

In accordance with previous studies, paper II and III in this thesis are indicative of mild, non-azotaemic AKI in *V. berus* envenomated dogs. Previous studies have been restricted to a single, non-standardised timepoint and one of them included only four envenomated dogs (Gordin et al., 2020, Palviainen et al., 2013, Palviainen et al., 2012). Gordin et al (2020) specifically identified the need for longitudinal evaluation of AKI in *V. berus* envenomated dogs.

The novel aspect of papers II and III is the longitudinal evaluation of AKI and thus the additional conclusions that AKI in the cohort of dogs in this thesis was present 36 hours after bite and can be considered transient since it resolved by 14 days. These papers also evaluated a larger panel of urinary AKI biomarkers than previous studies. This is important considering the multifactorial aspect of AKI (aetiologies, severity, location of injury) and that it is unlikely that a single biomarker will be able to universally diagnose AKI.

Urinary clusterin and uCysB have been shown to be sensitive markers for proximal tubular injury in a canine gentamicin model (Yerramilli et al., 2016). In a recent study, both absolute and normalised uClusterin and uCysB values were higher in *V. berus* envenomated dogs compared to controls (Gordin et al., 2020). In accordance with Gordin et al (2020), absolute and creatinine-normalised urinary cystatin B values were increased in envenomated dogs compared to controls in paper III.

Interpretation of the uClusterin results is more challenging. In the cohort of dogs in paper III, absolute uClusterin values were not increased at any timepoint compared to controls, this, in contrast to findings by Gordin et al (2020). However, when normalised to creatinine, uClusterin was significantly higher 24 and 36 hours after bite compared to controls. Given the IVFT treatment and likely relative polyuria of envenomated dogs compared to controls, use of the normalised values might be

more appropriate in dogs in paper III. Whilst studies of AKI in humans suggests that normalisation of urinary biomarkers to creatinine is not necessary and might even be contraindicated (Waikar et al., 2010, Han et al., 2008, Du et al., 2011), the clusterin results in paper III indicate that more work is clearly needed to evaluate the use of absolute versus creatinine-normalised values in situation such as these where a big disparity in urine concentration is expected.

Five of the LMW urinary biomarkers measured in papers II and III need further evaluation to ascertain their specificity for diagnosing AKI, particularly in the presence of systemic inflammation. **Cystatin B** is a LMW (11 kDa) ubiquitous intracellular protein (Ochieng and Chaudhuri, 2010). It is currently unknown whether serum cystatin B concentrations are increased after snakebite and thus a systemic contribution to uCysB through glomerular filtration rather than due to AKI, cannot be ruled out. The same applies to the LMW cytokines and chemokines **MCP-1**, **IL-8** and **OPN** as well as **NGAL** measured in paper III. Serum concentrations of MCP-1, IL-8, OPN and NGAL are known to increase in various inflammatory states (Monari et al., 2020, Phillips et al., 2016, Galán et al., 2018, Barraviera et al., 1995, Tidball, 2005).Increased urinary values of these biomarkers may therefore not be specific for AKI but could reflect systemic inflammation which is known to occur after *V. berus* bite (Langhorn et al., 2014, Christensen et al., 2014). There are two forms of NGAL, of which the monomeric form is kidney specific (Hsu et al., 2014, Cai et al., 2010). An assay for monomeric kidney specific NGAL is not currently available, but if developed in the future, such an assay would increase the diagnostic utility of uNGAL. The conclusion that mild AKI was present after snakebite, is therefore largely based on the remaining urinary biomarkers that do not suffer from this limitation.

Of the urinary AKI biomarkers reported in this thesis work, only uGGT/uCr and uCysB concentration were correlated with SSS at presentation. The clinical utility of these correlations is unknown and due to the uncertainty regarding the specificity of uCysB, it is possible this correlation reflects systemic inflammation rather than AKI. One other study found a correlation between urinary AKI biomarkers and severity of clinical signs (Palviainen et al., 2013), but results so far are by no means compelling. More work is needed to fully evaluate this relationship, especially since it is not known whether uCysB and uGGT/uCr correlate with severity of renal injury in these dogs. The lack of correlation between SSS and the other biomarkers in these

studies, suggests that severity of clinical signs is not generally a useful indicator for the presence of or development of AKI.

With the exception of cystatin C, all urinary biomarkers (albumin, MCP-1, KIM-1, GGT, NGAL, OPN and Il-8) in paper II were higher in envenomated dogs at one timepoint or more, compared to controls. Cystatin C has been associated with severity of AKI in humans and dogs (Herget-Rosenthal et al., 2004, García-Martínez et al., 2015) and it is therefore possible that mild AKI explains the lack of uCysC findings in this paper. Although speculative, the same might apply to uClusterin since it is upregulated upon injury (Rosenberg et al., 2002) and it is possible the degree of injury was not sufficient to cause upregulation in these dogs.

Of the remaining urinary biomarkers measured, uAlb might reflect tubular or glomerular injury but increases have also been reported in association with extreme exercise, macroscopic haematuria and urinary tract infection, thereby limiting its specificity for AKI (Gary et al., 2004, Vaden et al., 2004). False positive enzymuria (uGGT) can potentially occur with severe glomerular damage resulting in increased filtration of serum enzymes (Grauer, 2005) and although glomerular injury markers were not fully evaluated in this thesis, severe glomerular injury seems unlikely given the lack of UPC values > 2 and comparatively low uAlb/uCr values (Center et al., 1985, Hrovat et al., 2013). Thus uGGT/uCr and uKIM-1/uCr are perhaps the most promising indicators of AKI in the envenomated dogs in this thesis. Urinary KIM-1 in particular is a highly sensitive and early marker for proximal tubular injury in rats and humans although it has been suggested that it may not be as early or sensitive a marker for canine AKI (Wagoner et al., 2017, Sasaki et al., 2014) and this might explain it not being increased until 36 hours after bite in paper II.

In accordance with other studies, neither serum creatinine nor SDMA were increased in envenomated dogs (Gordin et al., 2020, Lervik et al., 2010, Aroch et al., 2010), in fact they were significantly decreased during hospitalisation, compared to controls. This probably reflects a lack of overt renal dysfunction but haemodilution from IVFT could also have contributed to the lack of findings. It is possible these dogs might have been azotaemic in the absence of IVFT. A decrease in GFR may also have gone undetected due to insensitivity of sCr and SDMA to detect such states. Whilst the use of IVFT does limit the conclusions regarding sCr and SDMA, dogs in papers II and III are representative of a clinical situation in which assessment of AKI

needs to be made and it can be concluded that sCr and SDMA are of limited use in this particular context.

Vipera berus envenomated dogs were thought to provide a rare opportunity to evaluate biomarkers in the early "initiation" phase of AKI, in a natural model. Unfortunately, low samples size prohibited the evaluation of AKI biomarkers at presentation in paper II , but urinary albumin, uMCP-1 and uNGAL were increased 12 hours after bite. Additionally, uCysB was increased in envenomated dogs at presentation, but this particular result should be interpreted with care since only five samples were included. Future studies might therefore focus on a timepoint between presentation and 12 hours after bite, to evaluate whether AKI might be detected earlier using these biomarkers. This would be useful from a broader AKI perspective and is not limited to *V. berus* envenomated dogs.

The limitations of a number of AKI biomarkers highlights the benefits of using of a panel of biomarkers, to look for parallel increases and thus aid confidence in diagnosing AKI. Considered together, the results of papers II and III suggest the presence of AKI in envenomated dogs. The subclinical AKI (tubular injury biomarker positivity without dysfunction) identified in dogs in these studies, may be of clinical importance given the potential for AKI to progress to chronic kidney disease (Coca et al., 2012, Cowgill et al., 2016, Ronco et al., 2012).

10.1.3 Coagulopathic effects

This thesis longitudinally evaluated the coagulation status of dogs envenomated by *V. berus*, for the first time. The combined methodologies of thrombin generation that assesses changes in plasma coagulation factors and inhibitors and also procoagulant activity associated with EVs, TAT complexes and PS equivalents, allowed the detection of a hypercoagulable state, not previously described. The findings in paper IV contrast previous sparse reports of apparent hypocoagulability measured as prolonged PT and aPTT in both humans and dogs (Dyląg-Trojanowska et al., 2018, Turkovic et al., 2015, Brandeker et al., 2015). It therefore seems likely that due to lack of studies and a reliance on insensitive tests, that hypercoagulability has previously been overlooked in these patients.

The addition of PRP/2 reagent or only Ca^{2+} to patient samples, makes the measurement of thrombin generation dependent on PL. Findings in paper IV

indicate that PL-dependent thrombin generation was increased in envenomated dogs compared to controls. These results combined with findings of increased PS-equivalents, indicate that EVs likely contribute to thrombin generation after *V. berus* bite, and that thrombin generation is not dependent on changes in plasma coagulation factors and coagulation inhibitors alone. The most pertinent finding in this study was that dogs were hypercoagulable already at presentation and that this state persisted 15 days after bite.

There are two main considerations when interpreting these results: (1) ***how well does an in-vitro thrombin generation test reflect in-vivo thrombin generation?*** and (2) ***does a hypercoagulable state translate into risk of thromboembolic disease?*** i.e., what is the clinical significance of the hypercoagulable state detected in paper IV?

The short answer to this is that the clinical significance is unclear. Virchow's triad describes the three factors that predispose to pathological thrombus formation: a hypercoagulable state, blood stasis and endothelial damage (Kushner et al., 2021). This study demonstrated hypercoagulability, and endothelial damage and blood stasis are also possible after snake bite and subsequent hospitalisation. No dogs in in this study were suspected to have thromboembolic disease, but antemortem diagnosis of thromboembolic disease is challenging in dogs due to non-specificity of clinical signs (Johnson et al., 1999). This combined with a lack of histopathological examination of organs likely to be affected by thromboemboli such as the kidney and lungs, means that such complications were not evaluated in this cohort of envenomated dogs. However, previous post-mortem reports in dogs and human case reports indicate that thrombosis is a feature of *V. berus* envenomation (Jørgensen et al., 2014, Kolbjørnsen, 2014, Kängström, 1989).

A number of studies suggest a use for thrombin generation assays in predicting thrombotic risk in humans (Besser et al., 2008, Hron et al., 2006, Tripodi et al., 2008) and more specifically, the finding of concurrent abnormalities in lag time, peak height and ETP can help identify patients at risk of venous thromboembolism (Tripodi et al., 2009). Species differences in patterns of thrombotic disease mean that care should be taken in extrapolating findings between humans and dogs (deLaforcade et al., 2019). Thrombotic risk associated with CAT parameters, has not been fully evaluated in dogs, most likely due to its novel nature and general paucity of studies using this methodology. One study does however report a lack of

difference in thrombin generation measurements between healthy dogs (n=23) and dogs with thrombosis (n=10) (Dengate et al., 2016). It is therefore not known to what extent hypercoagulability in the envenomated dogs in paper IV, reflects ***actual risk*** of thromboembolic disease, although it is a ***risk factor*** as per Virchow's triad.

As mentioned, another consideration is how accurately *in vitro* assays reflect *in vivo* coagulation. The weaknesses of the routinely used clot-based assays (PT and aPTT) have already been discussed and it is unlikely that any *in vitro* test will be able to fully evaluate the complex interplay of all contributory factors involved in the haemostatic process. Thus, a more realistic aim is to use assays, or a combination of methods that will allow as close an approximation as possible to *in vivo* haemostasis. Hence the research interest in global coagulation assays such as CAT and viscoelastic methods.

Thrombin-antithrombin complexes are rapidly formed after thrombin generation and thus this assay can give insights into *in vivo* TG. The TAT assay has been described as the gold standard for detection of in vivo thrombin generation in veterinary medicine (Herring and McMichael, 2012) and thus the increased TAT complex concentrations in envenomated dogs compared to controls in paper IV are supportive of an *in vivo* hypercoagulable state. The finding of increased thrombin generation using CAT, but normal concentrations of TAT complexes 15 days after bite in paper IV might reflect a difference in potential (*in vitro*) and actual (*in vivo*) thrombin generation. However, parameters used to identify *in vivo* thrombin generation such as TAT complexes and D-dimers may also be influenced by factors other than thrombin generation, such as product clearance times and fibrinolytic activity (Baglin, 2005).

Phospholipid-dependent thrombin generation and especially the parameter lag time, clearly differed between control dogs and envenomated dogs. It would therefore be worth pursuing this as a potential diagnostic test for snakebite. Since dogs were already hypercoagulable at presentation, measuring lag time after addition of Ca^{2+} to patient plasma has the potential to be a rapid test for a venomous *V. berus* bite. Such a test would allow resources to be focused and might prevent unnecessary use of antivenom which is both costly and not without side effects.

10.2 Implications for the treatment and management of envenomated dogs

This thesis work has identified cardiac, renal and coagulopathic effects that may be occult and do not necessarily correlate with severity of clinical signs. These are important features for clinicians to be aware of, both in terms of patient monitoring and communicating the importance of monitoring dogs bitten by *V. berus* despite relatively mild clinical signs, to dog owners.

The cardiac arrhythmias detected in paper I appeared to be well-tolerated and thus their clinical relevance is unclear. However, given the previously discussed concerns regarding VT, findings in this thesis work suggest that prolonged (48 hour) ECG monitoring for the development of VT in particular, is a sensible recommendation in dogs bitten by *V. berus.* This which would allow rapid initiation of antiarrhythmic treatment where necessary. Rapid VT occurs after *V. berus* bite and its presence does not appear to correlate with severity of clinical signs of envenomation. Arrhythmias may also last into day two after bite. A general recommendation after *V. berus* bite has been for the dog to avoid exertion in the time between hospital discharge (typically 36-48 hours after bite) and a re-examination 14 days later. Bearing in mind the incidence of myocardial injury and that a number of dogs in paper I were still arrhythmic at discharge, this recommendation also appears to be justified in want of studies specifically examining arrhythmias and myocardial injury in the period between discharge and re-examination.

The absence of azotaemia does not rule out the presence of AKI in envenomated dogs. These studies suggest a likely mild transient AKI. Whilst all dogs in papers II and III received IVFT and knowledge regarding the severity of AKI in the absence of IVFT, is therefore not known, supportive, targeted IVFT would appear to be a sensible recommendation (Prowle et al., 2014, Brienza et al., 2009).

Dogs are persistently hypercoagulable after *V. berus* envenomation. Although the significance of the procoagulant state is unclear and diagnosis of thromboembolic disease is difficult in dogs, it would seem wise to be aware of the potential for thromboembolic disease and monitor for associated acute neurological and respiratory signs.

Based on this work, recommendations for the treatment and management of *V. berus* envenomated dogs include: 36-48-hour hospitalisation including IVFT and monitoring with a focus on arrhythmias and clinical signs of thromboembolic disease.

In many ways these recommendations were already implemented in the clinics involved in these studies, prior to initiation of the work included in this thesis. This work contributes to the general evidence base for the treatment and management of *V. berus* envenomation in dogs. More specifically, an added focus on arrhythmia monitoring and an awareness of AKI and hypercoagulability, have been achieved.

10.3 Antivenom or no antivenom, that is another question

Whilst a detailed discussion of antivenom treatment is beyond the scope of this thesis, it is an important topic worthy of some attention. There is currently insufficient evidence to indicate a positive effect of antivenom treatment on time to recovery in *V. berus* envenomated dogs (Hodgson and Brambilla, 2017). Mental status may be improved, and local oedema may be decreased if antivenom is administered within the first 24 hours but the evidence for this is relatively weak and contradictory (Hodgson and Brambilla, 2017, Lund et al., 2013, Turkovic et al., 2015). However, lack of evidence does not mean lack of effect, and may simply reflect a paucity of canine studies.

In an experimental model, antivenom treatment prevented mortality in monkeys exposed to lethal doses of *V. berus* venom (Theakston and Reid, 1976) and in humans antivenom treatment has been associated with shorter hospital stays and improvement in clinical signs (Lamb et al., 2021, de Haro et al., 1998, Boels et al., 2012, Karlson-Stiber and Persson, 1994, Karlson-Stiber et al., 2006, Karlson-Stiber et al., 1997). Given that venom effects are diverse, and some are indirect, it seems unlikely that any single antivenom will be able to neutralise all effects associated with envenomation.

Randomised controlled trials for the efficacy of antivenom treatment after *V. berus* envenomation are lacking from both human and veterinary medicine. Such studies would help to clarify the questions surrounding antivenom treatment, but it would be useful to first identify more objective endpoints for such studies through

observational studies. Paper IV in particular, is one such study. Antivenom-treated dogs appeared to be less hypercoagulable than their non-antivenom treated counterparts. Whilst study design does not allow causation to be implied, it does suggest that thrombin generation could be a laboratory parameter of interest in future RCTs and especially the measurement of PL-dependent lag time. However, the since the clinical significance of the hypercoagulability detected in dogs in paper IV is unclear, the relative importance of the neutralisation of this particular effect of *V. berus* envenomation, is also unclear.

10.4 Dogs as a model for human envenomation?

One of the considerations throughout this thesis work has been whether dogs might be used as a model for human *V. berus* envenomation. Since dogs are commonly bitten, they could be a natural and accessible model for *V. berus* envenomation. However, there are a few considerations which might restrict the applications of findings in dogs to humans.

Bite site may play a role in the severity of clinical effects of envenomation in dogs, with some studies reporting increased severity associated with a bite to the limb (Segev et al., 2004, Vestberg et al., 2017). Whilst the literature is somewhat contradictory on this point and canine studies have not been of sufficient size to fully evaluate the effect of bite site, there is a clear difference in distribution of bite site in dogs compared to humans. Dogs are most frequently bitten in the head or neck (Gordin et al., 2020, Vestberg et al., 2017, Lervik et al., 2010) whereas humans are unsurprisingly most commonly bitten in an extremity (Paolino et al., 2020, Gronlund et al., 2003, Hermansen et al., 2018). Therefore, the effect of bite site could be important when considering dogs as a model for humans.

In a recent comparative study, dog plasma was hypercoagulable compared to human plasma, but perhaps contrary to expectations, when baseline coagulation was corrected for, dog plasma was less affected by procoagulant snake venom toxins than cat and human plasma (Zdenek et al., 2020). It is not known if the same effect is observed after *V. berus* envenomation, but this interspecies difference is worthy of note when considering dogs as a model for the coagulopathic effects of *V. berus* venom in humans.

Nevertheless, research into *V. berus* envenomation in humans and dogs are likely to complement each other, as long as potential interspecies differences are considered before extrapolating findings from individual studies.

11 Concluding remarks

The main findings from this thesis were:

- A large proportion of dogs envenomated by *V. berus* develop myocardial injury with ventricular arrhythmias including ventricular tachycardia which is considered clinically significant. These arrhythmias may last at least 48 hours. Cardiac troponin I concentrations might be useful in predicting the development of ventricular tachycardia in envenomated dogs. These findings demonstrate the need for prolonged ECG monitoring of these patients.

- Mild non-azotaemic AKI appears to be a feature after *V. berus* envenomation. New knowledge regarding AKI after *V. berus* bite is its presence 36 hours after bite and apparent transient nature with resolution observed by 14 days after bite, in dogs treated with IVFT, as per these studies. These findings indicate that supportive treatment with IVFT is a sensible recommendation in these dogs.

- Severity of clinical signs of envenomation does not appear to be a useful indicator for the development of arrhythmias or AKI. This is useful information to impart to dog owners when explaining the recommendation to hospitalise and monitor these patients.

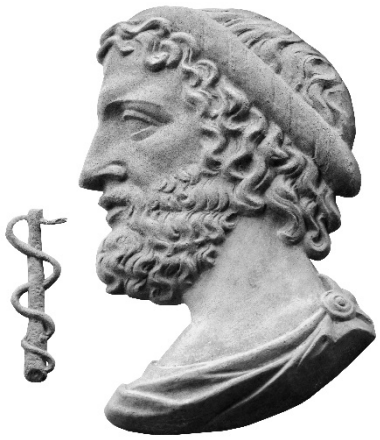
- Dogs were hypercoagulable already at presentation and still at 15 days after bite. The coagulation parameter lag time might be a useful tool in diagnosing *V. berus* envenomation. Antivenom-treated dogs were less hypercoagulable than those not treated with antivenom, but an RCT is needed to evaluate a causal link between antivenom treatment and outcome.

This thesis advances the understanding of the effects of *V. berus* bites in dogs and thereby contributes to the evidence base upon which treatment and monitoring decisions can be made in these patients. From a broader perspective this thesis also contributes to the evaluation of novel biomarkers that may have a future role in AKI

diagnosis. Parameters were also identified that may be explored for diagnosis of *V. berus* envenomation and implemented in the design of future RCT for antivenom efficacy.

11.1 A final note on *V. berus*: friend or foe?

This thesis started with *V. berus* and it therefore seems fitting that it should also end with *V. berus*. Throughout history and mythology snakes have been depicted as cruel and cunning, intelligent and immortal, symbols of healing and life or chaos and death. Whilst this thesis focuses on the clinically concerning aspects of *V. berus* envenomation in canine patients, it is worth bearing in mind that although important, many of these effects appear relatively mild compared to envenomation by other snake species. As much as snakebites are justifiably feared, snake venom has also been the source of several therapeutics including antihypertensives, antithrombotics, anticoagulants, platelet aggregate inhibitors, HIV treatments, anti-cancer agents and analgesics (Waheed et al., 2017, Koh and Kini, 2012). The prothrombin activating properties of snake venoms and the FV and FX activating properties of Russell's viper (*Daboia russelli*) venom in particular are also used in coagulation assays (Marsh, 2001). It is perhaps easy to become caught up in a fear of snakebites and to overlook the important benefits the medical field has gained from snake venom. It is, after all, not a coincidence that the rod of Asclepius is frequently used as a symbol of medicine.



Asclepius (the Greek god of medicine)

12 Future perspectives

As with many research projects, this thesis has answered some questions but has probably generated even more.

Snakebite diagnosis

There is no definitive diagnostic test for *V. berus* envenomation in dogs. An ability to identify envenomated dogs or rule out envenomation would allow resources to be prioritised and could have economical and treatment benefits in terms of the need for antivenom administration and hospitalisation. A study has been initiated to evaluate the use of PCR to detect snake DNA in blood samples from dogs presenting to clinics with suspected snakebite. This technique has shown promise for other types of snakebite in humans (Pucca et al., 2020). Given the significant difference in PL-dependent lag time between envenomated dog and controls in paper IV, this coagulation parameter might also be worth pursuing as a diagnostic tool for snakebite.

Venom content

Proteomic analysis of Norwegian *V. berus* venom has not been performed. Given the knowledge of intraspecies venom variation, this is of interest as differences in venom content could account for different findings between studies and might allow better prediction of expected effects. In conjunction with the aforementioned snakebite diagnosis study, the author has collected venom from 10 adult, wild *V. berus* in Norway which is awaiting proteomic analysis.

Antivenom and other venom-neutralising treatments

To date, RCTs of antivenom treatment for *V. berus* envenomation, do not exist. This thesis has identified objective parameters that might be measured in such a study. *In vitro* neutralising effects of antivenom and a metalloproteinase inhibitor on coagulotoxic effects of venom collected from Norwegian *V. berus* during this project, has been demonstrated (Chowdhury et al., 2021). As previously discussed, it seems unlikely that any antivenom will be able to neutralise all venom effects. A multimodal treatment approach might be a better option, whereby the main toxins (PLA_2, SVMPs and SVSPs) are specifically targeted. Further research focusing on specific toxin inhibitors such as the metalloproteinase inhibitor, prinomastat hydrocloride, would therefore be of interest.

AECG findings in healthy dogs

This project raised the question of what a "normal" AECG is (and particularly whether a single couplet in 24 hours should be considered abnormal). More research is needed into findings in healthy dogs in order to guide interpretation of AECG findings and ideally, more breed-specific findings should be published. A project is therefore already underway to evaluate 24-hour AECGs and cTnI concentrations in healthy Labrador retrievers.

AKI biomarker specificity

As identified, a number of proposed urinary AKI biomarkers need further study to ascertain their specificity for AKI. Low molecular weight biomarkers known to be ubiquitous, might be increased in urine secondary to systemic increases for example, in response to inflammation. Whilst this might be difficult to fully evaluate, paired serum and urine samples could give some insights. Further evaluation of glomerular injury would also be of interest.

Evaluation of platelet function, the TM-APC pathway and fibrinolysis

Changes in platelet number and function, TM-APC pathway activity and fibrinolysis were not evaluated in this study and are of interest given the knowledge that *V. berus* venom may also exert effects at these levels of the haemostatic process. Viscoelastic methods such as thromboelastography would allow assessment of fibrinolysis but require a strictly standardised procedure, including measurement of fresh whole blood samples and properly trained analysers. Such a study is currently in the early planning phase and is likely to provide an even more comprehensive overview of the effects of *V. berus* venom on haemostasis.

The interim period

An evaluation of the period between hospital discharge and recheck at 14 days, is lacking. With the exception of hypercoagulability, the effects of envenomation in these studies appeared to have resolved by 14 days after bite but it is not known at what point in these interim 12 days, the resolution occurred. It could therefore be of interest to evaluate this time period more specifically.

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Papers I-IV

Paper I

Received: 4 December 2019 | Accepted: 11 May 2020

DOI: 10.1111/jvim.15817

STANDARD ARTICLE

Journal of Veterinary Internal Medicine Open Access

ACVIM American College of Veterinary Internal Medicine

Ambulatory electrocardiography and serum cardiac troponin I measurement in 21 dogs envenomated by the European adder (*Vipera berus*)

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Abstract

Background: Envenomation by the European adder (*Vipera berus*) is common in dogs in Europe. Cardiac arrhythmias occur but clinical studies of envenomated dogs are limited.

Objectives: To describe arrhythmias in dogs within 48 hours of envenomation, and investigate associations between arrhythmia grade, serum troponin I (cTnI), and snakebite severity score (SS score).

Animals: Twenty-one client-owned dogs bitten by *V berus*.

Methods: Prospective cohort study of envenomated dogs. Ambulatory electrocardiograms were recorded from presentation to 48 hours after snakebite, and arrhythmias graded 0 to 3 based on frequency and severity. Serum cTnI was measured at presentation, 12 hours, 24 hours, 36 hours, and 14 days after bite. An SS score of 1 to 3 was recorded at admission and based on clinical examination.

Results: All dogs survived. Twelve dogs (57%) developed arrhythmias, all of which were ventricular in origin. Severe complex ventricular arrhythmias (VAs) were observed in 6 dogs (29%). Eighty-one percent of dogs (n = 17) had increased cTnI concentrations at 1 or more time points. Dogs that developed arrhythmias had significantly higher concentrations of cTnI at 12 hours (1.67 [0.04-32.68] versus 0.03 [0.01-0.052]; $P = .002$), 24 hours (1.88 [0.2-14.23] versus 0.06 [0.01-2.06]; $P = .009$), and 36 hours (3.7 [0.02-16.62] versus 0.06 [0.01-1.33]; $P = .006$) after bite compared to those that did not. Contingency table analysis showed that SS score was not significantly associated with arrhythmia grade ($P = .9$).

Abbreviations: AECG, ambulatory electrocardiography; AVB, atrioventricular block; cTnI, cardiac-specific troponin i; DAP, diastolic arterial pressure; HR, heart rate; MAP, mean arterial pressure; NMBU, Norwegian University of Life Sciences; SAP, systolic arterial pressure; SE, snake envenomation; VA, ventricular arrhythmia; VEC, ventricular ectopic complex; VT, ventricular tachycardia.

Conclusions and Clinical Importance: Myocardial cell injury, reflected by increased cTnI concentrations and VAs, is common after *V berus* envenomation in dogs. Prolonged electrocardiography monitoring is advised, particularly where cTnI is increased.

KEYWORDS

adder, ambulatory ECG, arrhythmia, canine, common adder, cTnI, ECG, envenomation, Holter monitoring, myocardial injury, snake bite, troponin, ventricular arrhythmia

1 | INTRODUCTION

Envenomation by the European adder (*Vipera berus*) is a common seasonal presentation in small animal practice in Europe.[1] During 2014 to 2018, a yearly average of 38 dogs were diagnosed with snake envenomation (SE) at the Faculty of Veterinary Medicine at the Norwegian University of Life Sciences (NMBU), and over 200 SE-related claims were reported yearly by the 2 largest pet insurance companies in Norway (Agria Dyreforsikring, Mo i Rana, Norway; Gjensidige Forsikring ASA, Oslo, Norway). *Vipera berus* is the only venomous snake in Norway. Signs of SE can therefore be attributed to this species alone.

Clinical signs of envenomation include edema, lethargy, pain, collapse, tachycardia, and cardiac arrhythmias.[1] Electrocardiographic findings, including sinus bradycardia, sinus arrest, supraventricular and ventricular premature complexes, accelerated idioventricular rhythm, and ventricular tachycardia (VT), occur in dogs bitten by *V berus* and *Vipera palaestinae*.[2-6] However, studies describing the time course and severity of these arrhythmias are limited.

Clinical grading systems are used to describe severity of snake bites in both humans and dogs.[7-9] In dogs envenomated by *V berus*, severity score correlates with renal injury.[8] However, a scoring system has not yet been assessed in relation to cardiac disease after SE.

Vipera berus venom contains several hemotoxic and cytotoxic proteases including phospholipase A2, serine proteases, and metalloproteinases.[10,11] Myocardial cell injury might occur secondary to systemic inflammation induced by these toxins.[3,4,12] Ammodytin L, a direct cardiotoxin, is present in *V berus* venom.[11,13] Myocardial cell injury could manifest as an arrhythmia.

Commonly used cardiac monitoring methods, including auscultation and short resting ECGs, are insensitive for the detection of arrhythmias when compared to continuous ambulatory ECGs (AECGs).[14-16] As such, the incidence of arrhythmias after SE might be underestimated.

Cardiac-specific troponin I (cTnI) is a sensitive and specific marker of myocardial cell injury and necrosis in dogs.[17] Troponin I increases both in primary cardiac disease and in myocardial injury secondary to systemic inflammation.[17,18]

Arrhythmias occur in 11% to 47% of dogs bitten by *V berus*.[2,4,5] Increased cTnI concentrations are reported in 33% to 58% of dogs and do not always correlate with the presence of arrhythmias.[2-4] However, the number and timing of sample collections vary between these studies and only 1 used AECG. Thus, the association between arrhythmias and cTnI concentrations is not fully established in these dogs. There are to date no studies combining AECG monitoring for longer than 24 hours and serial cTnI measurements in dogs bitten by *V berus*.

The primary aim of our study was to describe the incidence, nature, and duration of arrhythmias in dogs during the first 48 hours after envenomation and investigate associations with serum cTnI concentrations. A secondary aim was to investigate any association between a snakebite severity score (SS score) assigned at presentation and cTnI or arrhythmia grade. Such information could help to optimize treatment protocols and management of this group of dogs.

2 | MATERIALS AND METHODS

This prospective cohort study was approved by the ethical committee at NMBU. Written owner consent was obtained for all dogs before inclusion in the study.

2.1 | Animals

Twenty-six dogs presenting with a *V berus* bite to the small animal hospital at the Faculty of Veterinary Medicine at NMBU and Anicura Dyresykehus Oslo between April and October 2018 were evaluated for enrollment to the study. Diagnosis of snakebite and thus inclusion in the study was based on history and presence of consistent clinical signs at presentation (fang marks, local swelling, or systemic signs of envenomation). Five dogs were excluded from analyses for the following reasons: a previous history of cardiac disease (n = 1), a murmur detected at presentation (n = 1), treatment with antiarrhythmic medication before recruitment (n = 1), lack of clinical signs within 12 hours of the bite (n = 1), and presentation more than 24 hours after a snakebite (n = 1). Additional exclusion criteria included any preexisting disease and medications (other than levothyroxine [n = 1] and nonsteroidal anti-inflammatory drugs [n = 1]). Cases presented to the first opinion emergency service either directly (n = 16) or were transferred from clinics without an out-of-hours service (n = 5).

2.2 | Physical examination and blood sampling

All dogs underwent physical examination including demeanor assessment (normal, lethargic, or markedly lethargic) and blood sampling for cTnI analysis at the following time points after bite: T1: presentation (2-7.5 hours), T2: 10 to 14 hours, T3: 22 to 24 hours, T4: 34 to 38 hours,

and T5: 10 to 21 days. All examinations and blood sampling for project purposes were conducted by a single veterinarian (H.J. Harjen) except for 2 dogs at T5. Treatment decisions were made by the attending clinician.

Whole blood was collected through a venous catheter in the cephalic (n = 16) or saphenous vein (n = 5), into serum tubes and centrifuged at 2700*g* for 10 minutes, 30 to 60 minutes after sampling. Serum was pipetted into cryotubes and frozen within 15 minutes. Samples were stored in −80°C for a maximum of 200 days before transportation on dry ice to a reference laboratory (Idexx BioAnalytics, Vet Med Labor GmbH, Ludwigsburg, Germany).

Serum cardiac troponin I (cTnI) was measured using an ultrasensitive chemiluminescence assay (Idexx BioAnalytics, Vet Med Labor GmbH, Ludwigsburg, Germany), validated for use in dogs.[19] A serum cTnI concentration of up to 0.06 ng/mL was considered normal.

2.3 | Snakebite severity score

Each dog was assigned an SS score at presentation, using an adaptation of a previously described grading system.[7] All scores were assigned by the same veterinarian (H.J. Harjen). Grading criteria are described in Table 1.

2.4 | Ambulatory electrocardiography

An ambulatory electrocardiogram (Lifecard CF Holter recording system, Spacelabs Healthcare, Snoqualmie, Washington) was placed on each dog at presentation, before blood sampling, and removed after a minimum of 40 hours of hospitalization. A modified bipolar orthogonal lead system (X, Y, Z) was used (see Supporting Information). Electrodes were placed after shaving and skin cleaning with alcohol. A bespoke Holter vest (HeartVets, Exeter, UK) was used to minimize movement artifact.

Quantitative AECG analysis was performed by a blinded, single operator with experience in canine AECG analysis (J. Harris) using commercially available computer software (Pathfinder Digital V9.019, Spacelabs Healthcare Ltd, Hertford, UK). The analysis system was programmed using agreed measurement criteria adapted for dogs in the absence of published criteria, as previously described.[20] Beats were categorized as normal or aberrant morphologies. Ventricular tachycardia was defined as a minimum of 4 consecutive ventricular ectopic complexes (VECs) at ≥200 beats/min (bpm).

Arrhythmias were graded based on type, frequency, and severity, using previously described grading systems, modified to reflect clinical significance of previously reported arrhythmias in canine SE (Table 2).[2,3,6,21,22] Grades 1 to 3 were considered increasingly abnormal. Definitions of AECG arrhythmia criteria are presented in the Supporting Information.

Ambulatory ECG recordings were tabulated, analyzed, and graded according to time after SE as this was considered most clinically useful and allowed comparisons to be made between individuals. Thus, day 1 and 2 correspond to the first and second 24-hour periods after SE.

2.5 | Electrocardiography follow-up

A 5-minute, 6 lead ECG was performed on each dog upon reexamination (T5). A total of 5 dogs were available for 24-hour home Holter analysis 1 year after SE. Owners were instructed to carry out normal activities with the dog, except swimming.

2.6 | Blood pressure measurement

Indirect blood pressure (Cardell; Midmark, Versailles, Ohio) measurements were recorded at T1 to T5. A cuff size of approximately 40%

TABLE 1 Snakebite Severity Score definitions

Snakebite Severity Score		Clinical features
1	Mild	Local swelling around the bite, no systemic signs.
2	Moderate	Extensive swelling extending beyond the immediate bite site or mild systemic signs (lethargy, isolated episode of vomiting).
3	Severe	Pain and extensive swelling progressing beyond the limb or head, with marked systemic signs (collapse, cardiac arrhythmia, repeated vomiting, diarrhea, bleeding).

TABLE 2 Arrhythmia grading criteria

Arrhythmia grade		ECG criteria per 24-h period
0	Normal	• <50 VECs • <50 SVPCs • No complex arrhythmia (couplets, triplets, AIVR/VT, bigeminy, or trigeminy) • No high grade AVB
1a	Mild	• 50-1000 VECs • No couplets, triplets or AIVR/VT, bigeminy, or trigeminy • No high grade AVB
1b		• 50–1000 VECs • Any couplets, triplets, bigeminy/trigeminy, or AIVR • No VT • No high grade AVB
2a	Moderate	• Grade 1a + >1000 VECs
2b		• Grade 1b + >1000 VECs
3	Severe	• Presence of any VT (>200 bpm)-irrespective of VEC number, and/or high grade 2DAVB/3DAVB

Abbreviations: 2DAVB, 2nd degree atrioventricular block; 3DAVB, 3rd degree atrioventricular block; AIVR, accelerated idioventricular rhythm; AVB, atrioventricular block; ECG, electrocardiography; SVPCs, supraventricular premature complexes; VECs, ventricular ectopic complexes; VT, ventricular tachycardia.

limb circumference was placed on either the distal radius or metatarsus with the dog in lateral recumbency. Twelve serial measurements of systolic arterial pressure (SAP), diastolic arterial pressure (DAP), and mean arterial pressure (MAP) were recorded per time point. The first 2 measurements and any obvious outlying values were discarded. The mean of the remaining measurements was used.

2.7 | Statistical analysis

Statistical analysis was conducted using commercially available statistical software packages (JMP Pro 14.3.0, SAS Institute, Inc, Cary, North Carolina and Stata/SE 15.1, StataCorp, College Station, Texas). Data were tested for normality using the Shapiro-Wilk *W* test. Fisher's exact test was used to compare categorical variables. For nonparametric data, Wilcoxon exact test and Steel-Dwass test for multiple comparisons were used to analyze cTnI concentrations between arrhythmia grade and SS scores. Nonparametric receiver operating characteristic curves were used to analyze potential cTnI cut-off values for diagnosis of arrhythmia grades at T1 and T2. For all analyses, a *P* value of <.05 was considered significant. Where relevant, 95% confidence intervals (CIs) are presented. For parametric data, mean values ± SD are reported and a 2-tailed *t* test was used for between group comparisons.

3 | RESULTS

3.1 | Animals

Twenty-one dogs were included in the final study group. The snake, snakebite, or fang marks were observed in 17 dogs. In the remaining 4, the diagnosis was made based on the presence of clinical signs and history

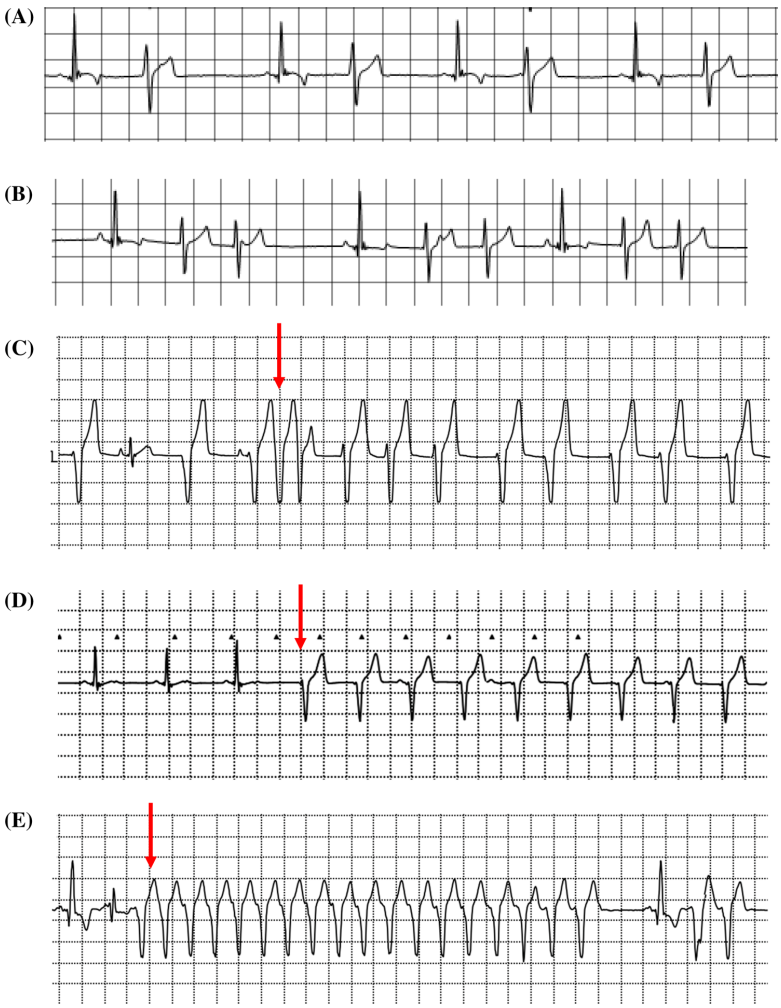

FIGURE 1 Extracts from ambulatory ECG recordings showing: A, Bigeminy (dog number 10); B, Trigeminy (nr 18); C, Triplet demonstrating R-on-T (the R wave of one ventricular premature complex occuring at the same time as the R wave of the preceding T wave with no return to baseline) (red arrow) (nr14); D, Accelerated idioventricular rhythm at a rate of 130 bpm (from red arrow) (nr 21); E, Nonsustained ventricular at a rate of 280 bpm (from red arrow) (nr 18)

consistent with *V berus* envenomation. Fifteen dogs were female and 6 were male. Median age was 3 years (range 7 months to 18.5 years). Median weight was 19 kg (range 5.5-43 kg). Breeds included 6 crossbreeds and 1 each of Border Collie, English Setter, Miniature Schnauzer, Samoyed, Nova Scotia Duck Tolling Retriever, Boston terrier, Cavalier King Charles Spaniel, Australian Kelpie, Standard Poodle, Flat Coated Retriever, Shetland Sheepdog, Staffordshire Bull Terrier, Akita, Kleiner Münsterländer, and Toy Poodle.

Median time from estimated snakebite to presentation was 1.5 hours (range 0.5-0.9 hours). Missing data included 1 T1 sample and 1 T4 sample, in separate dogs. Two dogs were lost to follow up at T5. All dogs were examined, and blood sampled, at a minimum of 4 time points.

3.2 | Treatment

All dogs received treatment of crystalloid fluid IV (Ringer-acetate, (n = 21), NaCl (n = 1, day 1) for the entire hospitalization period. Median fluid rate during the sampling period was 4 mL/(kg hr) (range 2.7-6.3 mL/(kg hr).

Analgesics used included buprenorphine (Vetergesic vet, Ceva Santé Animale, France) at a dose of 0.01 to 0.02 mg/kg IV or IM q8h (n = 3) and methadone (Metadon, Norges Apotek, Norway) at a dose of 0.1 to 0.2 mg/kg IV q4h (n = 13). Five dogs received methadone on day 1 and subsequently buprenorphine on day 2.

Lidocaine (Xylocain, Aspen Pharma trading Ltd, Ireland) was administered at dose of 2 mg/(kg hr) (continuous rate infusion) to 1 dog 39 hours after envenomation because of a ventricular arrhythmia (VA) observed on resting ECG by the attending clinician.

Sixteen dogs received intravenous equine $F(ab')_2$ antivenom (Viper Venom Antitoxin, SIS Biomed, Warsaw, Poland) IV, 7 of which received it before recruitment to the study. Median time from snakebite to antivenom treatment was 3.8 hours (range 0.75-0.24 hours).

Other treatments before presentation included penicillin and streptomycin (n = 1), prednisolone (n = 1), and dexamethasone (n = 1).

3.3 | Ambulatory electrocardiography

Median time from estimated SE to the start of AECG recording was 3.5 hours (range 2 hours 45 minutes to 9 hours) and median total AECG recording duration was 41 hours 43 minutes (range 24 hours 15 minutes to 50 hours 58 minutes). Recordings were standardized for analysis according to time after SE. Median duration of analyzed recordings for days 1 and 2 were thus 20 hours (range 14-21) and 23.5 hours (19–24), respectively. Day 2 AECG data were excluded for 3 dogs in which only 5 hours of recordings were available.

Nine dogs (42.9%, CI 24.5%-63.5%) were classified as not having an arrhythmia (grade 0). Arrhythmias were detected in 12 dogs (57%, CI 34%-76%), of which 5 were classified as grade 1b, 1 as grade 2b, and 6 as grade 3. All arrhythmias were ventricular in origin. Extracts from AECG recordings are presented in Figure 1. Overall grade was consistent across day 1 and day 2 in 13 dogs. Two dogs progressed

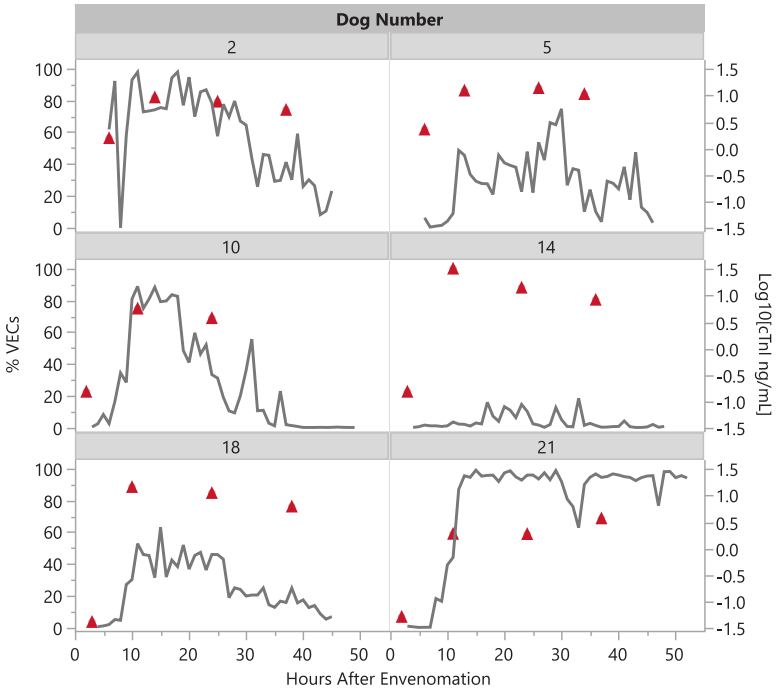

FIGURE 2 Percentage ventricular ectopic complexes (VECs) and log10 cardiac troponin I (cTnI) concentrations by hours after envenomation in six dogs with grade 3 arrhythmias. Dog number 10 was not available for cTnI analysis at time point 4 (36 hours). Percentage VECs showed a gradual decrease from approximately 24 hours after envenomation in dogs 2, 5, 10, and 18. In dog number 21, high VEC rates were still detected 50 hours after envenomation. Dog 14 had an overall low VEC rate but due to ventricular tachycardia, was classed as having a grade 3 arrhythmia

from grade 0 on day 1 to grade 1b on day 2. Three dogs showed an improvement on day 2 compared to day 1, from grade 1b to 0 (n = 1) and 3 to 2b (n = 2). Continuous percentage VECs and cTnI concentrations are presented for 6 dogs with grade 3 arrhythmias, in Figure 2. Arrhythmia grade was not significantly different between dogs with and without antivenom treatment ($P = .6$).

No arrhythmias were detected on resting ECG at T5. Five dogs with arrhythmias (≥grade 1) were available for 1-year follow-up AECG. Mean AECG recording duration at that time was 19.3 hours in these dogs, and no VECs were detected.

3.4 | Cardiac troponin I

Sixty-seven percent (CI 45%-83%) of dogs (n = 14) had increased serum cTnI concentrations at 3 or more time points, and 81% of dogs (n = 17) had increased cTnI at 1 time point or more. Five dogs (24%, CI 1.6%-45.1%) had cTnI concentrations above the reference cut-off value at all time points from T1 to T4. Three dogs had T5 cTnI concentrations of 0.06 ng/mL, 0.07 ng/mL, and 0.11 ng/mL, respectively. In 2 of these dogs, cTnI had decreased compared to all other time points.

The highest cTnI concentration of 32.68 ng/mL was observed in the oldest dog (18.5 years) at T2. This individual also had the highest T5 cTnI concentration (0.11 ng/mL) and was available for 1-year follow-up at which point cTnI was 0.12 ng/mL. Individual peak cTnI concentrations were observed across T2 to T4 and were significantly higher in dogs with arrhythmias (≥grade 1) compared to those without (Table 3). Cardiac troponin I concentrations were also significantly higher in dogs with arrhythmias (≥grade 1) than dogs without arrhythmias, at T2, T3, and T4 (Figure 3, Table 3). Peak cTnI concentration was not significantly different between dogs with and without antivenom treatment ($P = .7$).

Five dogs had increased cTnI concentrations at a minimum of 2 time points, in the absence of an arrhythmia. Troponin I concentrations of 0.52 to 2.06 ng/mL were detected in 1 of these dogs, whereas concentrations did not exceed 0.09 ng/mL in the other 4. One dog with a grade 2b arrhythmia had normal cTnI concentrations at all time points except T5 where it was marginally increased at 0.07 ng/mL

3.5 | Receiver operating characteristics curve analysis

Receiver operating characteristics curve analysis was used to investigate cTnI concentrations at T1 and T2 as an indicator of the presence of an arrhythmia ≥grade 1 at any time during hospitalization. Troponin I cut-off concentrations of 0.04 ng/mL (sensitivity: 75% [CI 46.8%-

TABLE 3 Summary of characteristics by arrhythmia group with relevant *P* values for comparisons between dogs with and without an arrhythmia ≥ grade 1

		Arrhythmia grade		
		Grade 0	≥Grade 1	*P* value
Number		9	12	
Age (y)		2 (0.58-11)	5 (0.58-18.5)	.25[a]
Weight (mean) (kg)		17.7 (±9.6)	25.2 (±9.8)	.36[b]
Sex (male/female)		2/7	4/8	.66[c]
Hours from bite to presentation		1.25 (0.75-6.5)	1.625 (0.5-5)	.89[a]
Bite location (head/limb)		6/3	10/2	.61[c]
Antivenom treatment (yes/no)		6/3	10/2	.61[c]
Treatment with antivenom before recruitment (yes/no)		4/5	3/9	.39[c]
Arrhythmia detected on initial examination (yes/no)		0/9	2/12	.49[c]
Buprenorphine (yes/no)		5/6	4/8	.67[c]
Methadone (yes/no)		9/0	9/3	.22[c]
SS score (n) (1/2/3)		2/6/1	2/8/2	.82[c]
Cardiac troponin I (ng/mL)	T1	0.02 [(n=8)] (0.01-0.08)	0.055 (0.01-2.27)	.086[a]
	T2	0.03 (0.01-0.52)	1.665 (0.04-32.68)	.002[a]
	T3	0.06 (0.01-2.06)	1.875 (0.02-14.23)	.009[a]
	T4	0.06 (0.01-1.33)	3.7 [(n=11)] (0.02-16.62)	.005[a]
	T5	0.02 [(n=8)] (0.01-0.06)	0.03 [(n=11)] (0.01-0.11)	.28[a]
Peak troponin I (ng/mL)		0.07 (0.03-2.06)	4.7 (0.07-32.68)	.001[a]

Note: Median and range values are presented for group data.
Abbreviation: SS score, snakebite severity score.
[a]Wilcoxon test (= Mann-Whitney test).
[b]*t* test.
[c]Fisher's exact test.

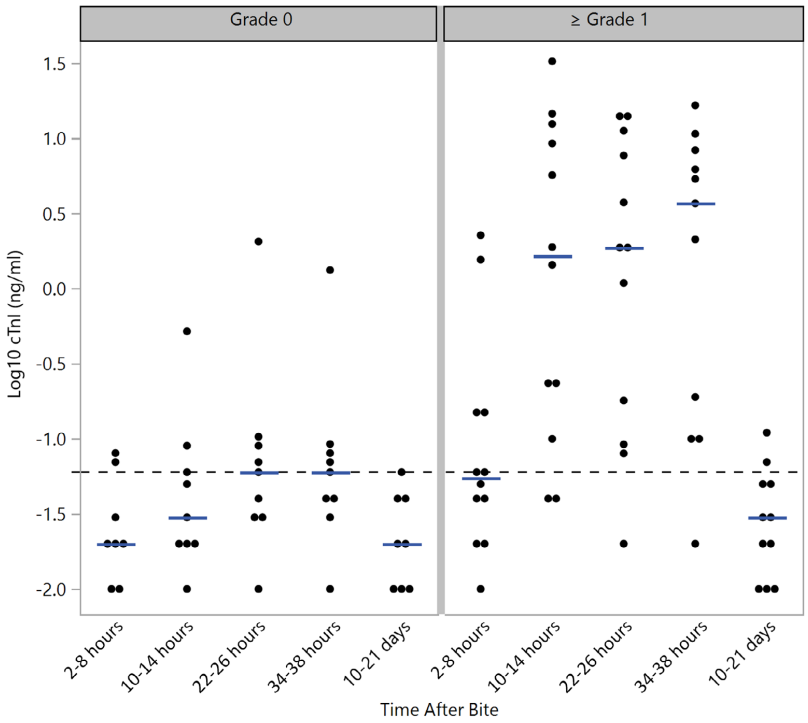

FIGURE 3 Logarithmic scale cardiac troponin I concentrations (cTnI) by time after bite for dogs with and without an arrhythmia ≥ grade 1. Bars indicate median. ---- denotes the reference cutoff value of 0.06 ng/mL

91.1%] and specificity: 75% [CI 4.9%-92.9%]) and 0.1 ng/mL (sensitivity: 83% [CI 55.2%-95.3%], specificity: 89% [CI 56.5%-98%]), for diagnosis of arrhythmias ≥ grade 1, were found for T1 (Area under the curve (AUC) 0.73, CI 0.49-0.96) and T2 (AUC 0.9, CI 0.77-1.0), respectively. For indication of a grade 3 arrhythmia, cTnI cut-off concentrations of 0.15 ng/mL (sensitivity: 66.6% [CI 30%-9.3%], specificity: 100% [CI 78.5%-100%]) and 1.89 ng/mL (sensitivity: 100% [CI 61%-100%], specificity: 100% [CI 79.6%-100%]), were found for T1 (AUC 0.9, CI 0.9-1.0) and T2 (AUC 1.0, CI 1.0-1.0), respectively.

3.6 | Snakebite severity score

Nineteen percent (CI 7%-40%) of dogs (n = 4) had an SS score of 1, 67% (CI 45.4%-82.9%) (n = 14) had a score of 2, and 14% (CI 4.98%-34.6%) (n = 3) had a score of 3. No significant associations were found between SS score and cTnI concentrations at presentation (P = .5) or SS score and arrhythmia grade (P = .9).

3.7 | Blood pressure

Blood pressure was measured in 20 dogs (metatarsus n = 18, forelimb n = 2) at T1 to T5. A mean of 10 measurements was used in all but 8 cases (single time point) in which 5 to 8 measurements were used. Overall mean SAP, DAP, and MAP during hospitalization (T1-T4) were 131.7 ± 14.6 mm Hg (range 104-169), 73.4 ± 13.5 mm Hg (range 52-119), and 95.6 ± 13.2 mm Hg (range 70-134), respectively. At T5, these values were 131.7 ± 12.8 mm Hg (117-154), 77.3 ± 13.9 mm Hg (55-103), and 98.4 ± 11.5 mm Hg (81-117), respectively. No significant difference in SAP, DAP, or MAP was found between dogs with or without an arrhythmia ≥ grade 1 at each given time point other than at T2 where SAP and MAP were significantly higher in dogs with an arrhythmia ≥ grade 1 (SAP 137 ± 14.8 mm Hg versus 123.9 ± 11.8 mm Hg, P = .04; MAP 99.6 ± 13.3 mm Hg versus 87.6 ± 1.6 mm Hg, P = .04).

3.8 | Other clinical findings

Overall median heart rate (HR) during hospitalization (T1-T4) was 90 bpm (range 40-200). At T5, median HR was 100 bpm (range 72-135). Heart rate was significantly higher in dogs with an arrhythmia ≥ grade 1 than those without at T2 (mean 106 ± 2.9 bpm versus 78.2 ± 22.8 bpm, P = .009) and T3 (mean 100 ± 29.2 bpm versus 77.2 ± 11 bpm, P = .03). Overall, no statistically significant difference in demeanor, mucus membrane color, capillary refill time (CRT), or femoral pulse quality was found between dogs with and without an arrhythmia ≥ grade 1. All dogs survived. All the dogs examined at T5 (n = 19) were assessed as being healthy. Both dogs that were unavailable for examination at T5 were reported as healthy by the owner.

4 | DISCUSSION

Our study shows that cardiotoxicity, evident as an arrhythmia or increased cTnI concentrations, is a common sequel to *V berus* envenomation in dogs. Previous studies using 2 to 5 minute ECGs reported arrhythmia incidences of 25% to 41.6%,[3,4,6] compared to 57% (CI 34%-77%) in our study. Cardiac effects were reported in 11% and 14% of dogs with *V berus* SE in 2 other studies.[1,5] However, ECGs were not performed in all dogs in these 2 studies, thereby likely underestimating the true arrhythmia incidence compared to the present study. The only other AECG study of *V berus* envenomated dogs reported a similar arrhythmia incidence of 47%.[2]

Ventricular tachycardia, present in 6 dogs in our study, occurs in SE dogs.[2,4,6,23] However, quantification of VT incidence is lacking from previous work, and differences in VT definition make comparisons between studies challenging. The grading system, and specifically the assignment of a grade 3 arrhythmia, in the current study is conservative in terms of VT definition (4 consecutive VECs at an instantaneous rate of ≥200 bpm) when compared to other SE studies where VT has been defined as a minimum of 3 VECs at ≥100 or ≥160 bpm.[2,6]

Perhaps surprisingly, no dogs developed atrioventricular block (AVB) in the current study, compared to 21% in a study of dogs with *V palaestinae* SE.[6] The lack of AVB and other previously described ECG findings, such as ST segment depression and sinus arrest, might reflect variations in venom dose and composition, individual response to venom, individual or species variation in response to myocardial injury, or different mechanisms of cardiotoxicity.[24]

Previous longitudinal studies of *V berus* SE describe cTnI concentrations suggestive of myocardial cell injury in 33% and 58% of dogs, respectively.[3,4] The equivalent figure appears higher in the current study (81%, CI 60%-92%) and could reflect higher assay sensitivity and a lower upper reference cut-off concentration than previous studies. The differences observed could also be an effect of small sample size.

Cardiac troponin I findings from the T1 time point in our study are comparable to another study that reported no significant difference in cTnI concentrations between dogs with and without arrhythmias, up to 8 hours after SE.[2] Serial cTnIs in our study demonstrate, as previously suggested, that insufficient time between SE and sampling is a likely explanation for the lack of cTnI increase at this time point and that measurement at the time of presentation is of limited value in separating dogs that develop arrhythmias from those that do not.[2]

Troponin I concentrations 12 to 36 hours after bite were higher in dogs with an arrhythmia grade ≥ 1 compared to those without an arrhythmia. However, normal cTnI concentrations did not rule out the presence of an arrhythmia and vice versa. Troponin I concentrations of ≥1.89 ng/mL, 12 hours after envenomation, were useful in predicting a grade 3 arrhythmia in our study. Given the small sample size in the current study, further studies of cTnI concentrations 12 hours after envenomation would be of interest to determine whether a true diagnostic cTnI cut-off can be established and to assess risk of sudden cardiac death in this population.

The clinical relevance of marginal increases in cTnI in dogs is not known. cTnI concentrations of up to 0.136 ng/mL have been described in dogs screened and found to be free of cardiac disease.[17] Interbreed variation, extreme exercise, and age are documented causes of mild cTnI increase in otherwise apparently healthy dogs.[17,25,26] Troponin I kinetics are reflected in the finding of peak cTnI concentrations 12 to 36 hours after SE in our study. Studies in humans describe biphasic cTnI release, with a rapid release of a small unbound cytoplasmic pool within 4 to 6 hours, peaking at 12 to 24 hours after insult, followed by a more gradual release of bound, structural cTnI creating a second peak 2 to 4 days after injury.[27] The duration of cardiomyocyte injury after SE is not fully documented. A previous study found that 28.6% of dogs have increased cTnI 5 to 10 days after SE.[4] In the current study, 3 dogs had abnormal cTnI 10 to 14 days after SE. Given the short half-life of cTnI in the dog (1.85 hours),[28] this might indicate on-going cardiac injury, but could also be explained by individual variation in baseline cTnI due to age, physical activity, or breed.[25,26,29] Five dogs had abnormal cTnI in the absence of an arrhythmia. Individual variation in baseline cTnI is a possible explanation for this finding. Myocarditis can be present in the absence of ECG changes[30] and could therefore also explain the finding of raised cTnI concentrations in the absence of arrhythmia in envenomated dogs.

The exact pathophysiology of arrhythmias after SE is unknown. Cardiomyocyte injury might occur secondary to a systemic inflammatory state induced by venom proteases.[10-12] Previously described findings of a correlation between concentrations of C-reactive protein and cTnI in *V berus* envenomated dogs[4] and hypersensitivity myocarditis after *V berus* SE in a human patient[31] support an inflammatory mechanism of myocardial injury. A direct cardiotoxin (Ammodytin L) has also been isolated in *V berus* venom.[11,13] Tachyarrhythmias themselves can also contribute to cTnI increase.[32,33]

Arrhythmias resolved without anti-arrhythmic treatment in 11 of 12 dogs in our study, similar to another study of 126 dogs envenomated by *V berus*.[23]

Snakebite severity score, based on clinical examination at presentation, was investigated as an indicator of arrhythmia development. Scoring was carried out by the same clinician, in order to maximize comparability between individuals. However, the results of our study suggest that it does not provide additional information regarding severity of cardiac effects of SE.

There are limitations to our study. Echocardiography would have been a useful addition before inclusion in the present study to rule out preexisting cardiac disease. However, as cTnI has a high specificity and sensitivity for cardiac injury, reduction in cTnI concentration from the initial 38 hours after SE to 14 days after SE was considered consistent with the absence of underlying heart disease. Antivenom treatment was an unavoidable confounding factor in our study. Arrhythmia occurrence and cTnI concentrations were, however, not significantly different between antivenom and non-antivenom treated dogs. Three dogs received anti-inflammatory medication before recruitment to our study. Given that mechanism of arrhythmia after SE is unknown, we cannot rule out that this might have impacted the findings in these individuals. Another previously mentioned limitation of our study was the sample size; more definite conclusions could likely be drawn from a larger study population.

While we have demonstrated that continuous ECG is a useful tool in detecting and quantifying arrhythmias in SE dogs, Holter AECG is not ideal for real-time arrhythmia monitoring due to the need for retrospective ECG analysis. Telemetric ECG, which combines continuous monitoring with real-time viewing, would be a more useful monitoring tool in SE dogs in the clinical setting.

Clinical effects of SE vary in severity from benign signs to sudden death[1,34] to which severe systemic inflammatory response, arrhythmia, and anaphylaxis are potential contributory factors.[35-37] Arrhythmias appeared to be well tolerated by dogs in our study, and therefore the clinical importance of the arrhythmias is unclear. The post hoc diagnosis and lack of continuous blood pressure measurement in our study might have resulted in an underestimation of hemodynamic effects. The finding of higher SAP and MAP at T2 in dogs with an arrhythmia ≥ grade 1 is contrary to what might be physiologically expected. However, given the small sample size and the marginal *P* value (.04), we suggest that this finding is likely incidental and not arrhythmia-related.

Risk associated with VA has not been assessed in SE in dogs; however, in Doberman pinschers with dilated cardiomyopathy, presence of fast VT (instantaneous rate > 260 bpm) and high cTnI were both predictors of sudden cardiac death.[38] Therefore, dogs with similar findings secondary to SE warrant monitoring and might be at similar risk. Given that increased cTnI and presence of VT are risk factors for sudden cardiac death in myocardial disease in dogs,[37-39] we suggest that continuous ECG monitoring of SE dogs is advisable.

ACKNOWLEDGMENTS

A poster of this study was presented at the 29th ECVIM-CA annual congress, Milan in 19th to 21st September 2019. The authors thank The Raagholt Research Foundation, Norway for funding this study and staff and students at the small animal hospital at the Faculty of Veterinary Medicine at NMBU and Anicura Dyresykehus, Oslo for the care and provision of dogs in this study.

CONFLICT OF INTEREST DECLARATION

Joanne Harris is a director of HeartVet Consultants Ltd who provided commercial Holter monitor rental and analysis. All Holter recordings in our study were analyzed on a research platform with J. Harris blinded to all patients details during analysis. The remaining authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

The study was approved by the Committee for Ethical Approval of Studies with Animal Patients at the Norwegian University of Life Sciences (NMBU).

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Harjen HJ, Bjelland AA, Harris J, et al. Ambulatory electrocardiography and serum cardiac troponin I measurement in 21 dogs envenomated by the European adder (*Vipera berus*). *J Vet Intern Med*. 2020;34:1369–1378. https://doi.org/10.1111/jvim.15817

Supporting information

Table S1. Definitions of AECG arrhythmia criteria

Criteria	Definition
Pause	≥ 4 s
Dropped beat	≥ 300% of R-R interval
Bradycardia	Minimum of 10 beats at ≤ 45bpm
Premature Normal	≤ 45 % of R-R interval
Premature aberrant	≤ 90% of R-R interval
Bigeminy	1:1 Ratio of VECs to normal complexes
Trigeminy	2:1 ratio of VEC to normal complexes (or vice versa)
Couplet	Two consecutive VECs
Triplet	Three consecutive VECs
Accelerated Idioventricular Rhythm (AIVR)	Minimum of 4 VECs occurring in a regular monomorphic rhythm <200bpm
Ventricular Tachycardia (VT)	Minimum of 4 VECs at ≥ 200 bpm
Supraventricular tachycardia (SVT)	Minimum of 3 beats at >200 bpm

VEC, Ventricular Ectopic Complex; bpm, beats per minute

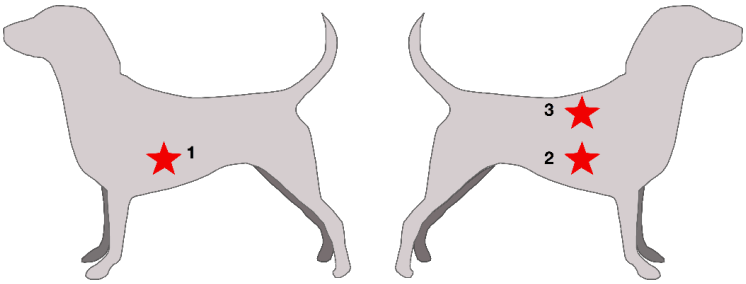

Figure S1. Electrode placement used for AECG recording in the dog. 1. Left lower: immediately caudal to the apex beat. 2. Right lower: immediately caudal to the apex beat 3. Right upper: at the caudal border of the right scapula.

Paper II

Harjen et al. BMC Veterinary Research (2021) 17:154
https://doi.org/10.1186/s12917-021-02851-8

BMC Veterinary Research

RESEARCH ARTICLE Open Access

Serial serum creatinine, SDMA and urinary acute kidney injury biomarker measurements in dogs envenomated by the European adder (*Vipera berus*)

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Abstract

Background: Acute kidney injury (AKI) is associated with high morbidity and mortality in dogs, but diagnosis may be impaired due the insensitivity of routine renal function biomarkers to detect earlier or milder forms of injury. Snake envenomation is one of several causes of AKI in dogs and humans. Dogs are commonly envenomated by the European adder (*Vipera berus*) between April and October each year, but few studies exist examining serial serum creatinine (sCr) and symmetric dimethylarginine (SDMA) measurements and AKI biomarkers in these dogs. Novel urinary biomarkers could improve clinical outcome by allowing earlier diagnosis of and intervention in AKI. The aim of this study was to assess the presence of AKI in dogs envenomated by *V. berus* at 12, 24 and 36 h after bite, as well as 14 days later, using sCr, SDMA and a panel of urinary AKI biomarkers normalised to urine creatinine (uCr), compared to a group of healthy control dogs.

Results: Thirty-five envenomated dogs and 35 control dogs were included. Serum creatinine did not exceed the upper reference limit at any time point in any dog after envenomation. Serum SDMA did not exceed 0.89 μmol/L in any dog. Compared to controls, urinary albumin/uCr, neutrophil gelatinase-associated lipocalin/uCr and monocyte chemotactic protein-1/uCr were significantly elevated 12 h ($P < 0.0001$, $P < 0.0001$, $P = 0.01$), 24 h ($P < 0.001$, $P < 0.001$, $P = 0.002$) and 36 h ($P < 0.001$, $P < 0.001$, $P = 0.0008$) after bite. Osteopontin/uCr was higher 24 and 36 h after bite ($P < 0.0001$), kidney injury molecule-1/uCr, interleukin-8/uCr and γ- glutamyl transferase/uCr were significantly higher 36 h after bite ($P = 0.003$, $P = 0.0005$, $P = 0.001$). Urinary cystatin C/uCr was not significantly different to controls at any timepoint. Biomarker/uCr ratios were not significantly different 14 days after envenomation compared to controls.

Conclusion: Urinary biomarker/Cr ratios are indicative of mild transient, non-azotaemic AKI in dogs envenomated by *V. berus*.

Keywords: Acute kidney injury, Adder, Viper, Snakebite, Biomarker, Renal

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Background

Acute kidney injury (AKI), historically defined as an abrupt reduction in renal function [1], carries a substantial risk of mortality in dogs [2, 3]. Acute kidney injury represents a wide spectrum of disease, from subclinical injury to overt kidney dysfunction, with associated clinical signs including anorexia, vomiting, diarrhea, lethargy, polyuria, oliguria, anuria and polydipsia [4]. Causes of AKI are varied and include ischemia, infection, drugs such as non-steroidal anti-inflammatory drugs and aminoglycosides, and toxins including snake venom [5, 6].

Snakebite-related AKI is described in humans and dogs [5–7]. Envenomation by the European adder *(Vipera berus)* is a common occurrence in dogs in Scandinavia during April to October. Studies to date have failed to show elevations in renal function markers such as serum creatinine (sCr) after *V.berus* envenomation, but measurements of urinary markers of AKI and histopathological findings are indicative of tubular injury in these dogs [5, 8, 9]. These studies are however limited, and AKI in dogs envenomated by *V. berus* therefore warrants further investigation. Snakebitten dogs often present for treatment within a short time of insult, prior to clinical signs referable to acute kidney dysfunction. Thus, *V.berus* envenomated dogs could also represent a model for studying kidney injury biomarkers in the early phase of AKI.

Serum creatinine, the surrogate marker of glomerular filtration rate (GFR) routinely used to assess kidney function in dogs, is insensitive for diagnosing early or mild dysfunction [10, 11]. Serum symmetric dimethylarginine (SDMA) is reportedly a more sensitive and specific marker for GFR than sCr in dogs [11]. The use of SDMA in diagnosing early or mild forms of AKI is not fully established. Furthermore, kidney damage can be present without subsequent loss of function [12], thus limiting the use of such functional biomarkers in AKI diagnosis. Emergency treatment of envenomated dogs often involves fluid therapy, which can further hinder the diagnostic use of serum biomarkers for AKI due to haemodilution. Despite its limitations, sCr forms the basis of current AKI grading systems. Diagnosis of AKI is therefore largely based on identifying dysfunction and not necessarily damage, meaning that early or milder stages of kidney injury may go undetected.

Various AKI grading systems based on sCr concentrations and urine output are used in human medicine [1, 13, 14] and have been adapted for veterinary patients [3, 15, 16]. AKI can progress to chronic kidney disease [17–19],and higher grades are associated with increased mortality in both humans and dogs [3, 15, 20, 21]. Earlier diagnosis of AKI could allow clinicians to intervene and prevent further progression of kidney disease. It would therefore be of benefit to develop more sensitive methods of diagnosing AKI, using biomarkers able to detect injury in the absence of altered GFR as measured by sCr. This would aid diagnosis of mild or early stage AKI which, although subclinical, might be of importance [22].

A variety of low and high molecular weight (LMW and HMW) urinary proteins have been proposed as more sensitive and location specific (glomerular versus tubular) biomarkers of AKI than sCr. Urinary albumin can be an indicator of early kidney disease in dogs and AKI in humans [23], but its use can be limited by lack of specificity [24]. Urinary alkaline phosphatase (uALP) and γ- glutamyl transferase (uGGT) are proximal tubule brush border enzymes previously shown to be elevated in dogs with histological evidence of AKI [25, 26]. Kidney injury molecule-1 (KIM-1), neutrophil gelatinase-associated lipocalin (NGAL), interleukin 8 (IL-8), osteopontin (OPN) and monocyte chemotactic protein-1 (MCP 1) are upregulated upon renal tubular injury with increased urinary concentrations found in AKI of various etiologies in both humans and dogs [27–32]. Urinary Cystatin C (uCysC), a freely filtered LMW protein metabolised by renal tubular cells, is reportedly elevated in dogs with proximal tubular injury [33].

The aim of this study was to investigate AKI in *V. berus* envenomated dogs using sCr and a panel of urinary biomarkers for glomerular and tubular injury in the initial 48 h after envenomation and 14 days later, compared to a group of healthy controls. A secondary aim was to investigate associations between severity of clinical signs of envenomation and AKI biomarker concentrations. We hypothesized that dogs sustain kidney tubular injury following *V. berus* envenomation and that biomarker concentrations correlate with clinical severity of envenomation.

Results

Control dogs

The control group comprised various breeds (additional file 1). Eighteen (51%) were female, of which 4 were neutered, and 17 (49%) were male, of which 1 was neutered. Median age was 5 years (range 8 months – 13 years). Median weight was 24 kg (range 5.2–50 kg).

Envenomated dogs

Thirty-five dogs of various breeds (additional file 1) envenomated by *V. berus* were included in the final study group. Twenty-two cases presented to the small animal hospital at the Norwegian University of Life Sciences (NMBU), three cases to Anicura Dyresykehus Oslo (ADO), six cases to Evidensia Oslo Dyresykehus (EOD) and four cases to Anicura Jeløy Dyresykehus (AJD). The snake or fang marks were witnessed in 25 dogs. Diagnosis was based on a history and clinical signs consistent

with envenomation in the remaining dogs. Twenty (57%) dogs were female, of which 3 were neutered, and 15 (42.8%) were male, of which 3 were neutered. Median age was 4.5 years (range 7 months-18 years). Median weight was 19 kg (range 5.5–46.5 kg).

Twenty-four dogs were bitten in the head, 10 in a limb, and one on the sternum. Median time from bite to initial presentation was 1.6 h (range 0.5–9 h). Due to transfer of veterinary care, five dogs presented more than 8 h after bite and thus lack a T1 blood sample. Two dogs had been diagnosed with a *V. berus* bite six and five years previously.

Treatment

All envenomated dogs were treated with crystalloid fluid therapy IV during hospitalisation (ringer-acetate (n = 34) or NaCl on day one followed by ringer-acetate on day two (n = 1)). Median fluid rate was 4 ml/kg/hour (range 2.7–6.3). Twenty-seven dogs received equine F(ab')2 antivenom IV (Viper Venom Antitoxin, SIS Biomed®, Warsaw, Poland). Median time from bite to antivenom treatment was 4 h (range 0.5–24 h). Analgesics included buprenorphine (Vetergesic vet®, Ceva Santé Animale, France) at a dose of 0.01–0.02 mg/kg IV, IM or SC q8 hours (n = 6) or methadone (Metadon, Norges Apotek, Norway) at a dose of 0.1–0.2 mg/kg IV or SC q 4 h (n = 22). Six dogs received a combination of methadone and buprenorphine. One dog received a combination of methadone and transdermal fentanyl at a dose of 4 μg/kg/h (Durogesic®, Janssen-Cilag AS). Lidocaine (Xylocain®, Aspen Pharma trading Ltd., Ireland) was administered at dose of 1 mg/kg to one dog within one hour of presentation due to a ventricular arrhythmia. Three dogs were treated with maropitant at a dose of 1 mg/kg IV due to hypersalivation or vomiting. One dog was administered furosemide (2 mg/kg IV) prior to T3 due to perceived oliguria. Other treatments included ampicillin (n = 1), streptomycin and penicillin (n = 1), clindamycin between T4 and T5 (n = 1), amoxicillin between T4 and T5 (n = 1) dexamethasone (n = 1), prednisolone (n = 1), pimobendan (n = 1) and levothyroxine (n = 1).

Blood pressure

Blood pressure was measured in 23/35 envenomated dogs. Overall median systolic arterial pressure (SAP) was 135 mmHg (range 104–196 mmHg), median diastolic arterial pressure (DAP) was 76 mmHg (range 48–154 mmHg) and median mean arterial pressure (MAP) was 97 mmHg (range 70–171 mmHg). No dogs were considered hypotensive (SAP < 90 mmHg or MAP < 60 mmHg and clinical signs of hypotension). Nine dogs had SAP values > 150 mmHg at one timepoint or more, of which four dogs had SAP values over 160 mmHg. Two of those four dogs had SAP values persistently > 160 mmHg, whereas two had SAP > 160 only at presentation, (including the individual with SAP of 196 mmHg).

Snakebite severity score (SSS)

Median SSS at presentation was 4/16 (range 0–12) and 4/16 (range 0–10) during T2-T4. At T5 all dogs had a score of 0, except for two dogs with scores of 1 and 2 respectively.

Urinalysis

Median USG in the envenomated dogs was 1.022 (range 1.006–1.058) at T2-T4, and 1.036 (range 1.005–1.053) at T5. Median urine pH in the samples analysed from envenomated dogs was 7 (range 5–9). Of the envenomated dogs, five had a positive blood dipstick in the absence of erythrocytes on urine microscopy, possibly suggesting the presence of haemoglobinuria, and 13 had varying degrees of haematuria (median 50 red blood cells (RBC) /μL (range 10–250), median 22.5 RBC/ HPF (range 3–100). Macroscopic haematuria was not observed. One dog had myoglobinuria at T2, diagnosed by brown urine in the absence of haemolysed serum or erythrocytes on microscopy. The same dog had pigmenturia at T4 with concurrent haemolysis, thus haemoglobinuria, myoglobinuria or both were present at that single timepoint. Cellular (1/HPF) and granular casts (1–3/HPF) were observed in one and two dogs respectively.

Median USG and urine pH were 1.045 (range 1.018–1.060) and 6.5 (range 5–8) respectively in the control dogs.

Biomarker analysis

Urinary ALP was deemed low and statistical analysis not performed due to 99% of samples having values under the limit of quantification (LOQ) for the assay. Urinary albumin, uIL-8, uMCP-1, uNGAL and uKIM-1 had 77.5, 68, 31, 20 and 3% of values under the LOQ for the assay, respectively. Values under the LOQ for these analytes were imputed using the lower LOQ (LLOQ) for the assay. For the remaining biomarkers, all values were over the LLOQ.

Serum creatinine, SDMA and urinary biomarker/uCr values are presented by timepoint for envenomated dogs and controls in Table 1. Serum creatinine did not exceed the upper reference limit at any time point in envenomated dogs. Two envenomated dogs did however have an increase in sCr of 26.5 μmol/L between T2 and T4 and T2 and T3 respectively. Symmetric dimethylarginine did not exceed the upper reference limit (> 0.74 μmol/L) in envenomated dogs at T1-T4. At T5 two dogs had SDMA concentrations of 0.79 and 0.84 μmol/L with corresponding USGs of 1.036 and 1.041 respectively. These were different dogs to those with increases in sCr.

Table 1 Median (range) biomarker values by timepoint after bite in envenomated dogs and healthy controls

	Control	T1 (presentation)	T2 (12 h)	T3 (24 h)	T4 (36 h)	T5 (14 days)
sCr (μmol/L)	88.4 (44.2–150.3)	70.7 (53–106)	61.88 (53–97.2)	66.3 (44.2–88.4)	70.7 (44.2–97.2)	79.6 (53–123.8)
SDMA (μmol/L)	0.54 (0.35–0.74)	0.5 (0.35–0.64)	0.47 (0.25–0.64)	0.45 (0.25–0.64)	0.4 (0.25–0.69)	0.54 (0.3–0.84)
uKIM-1/uCr (ng/mg)	0.13 (0.013–0.36)		0.13 (0.001–0.2)	0.15 (0.002–0.32)	0.20 (0.008–0.35)	0.14 (0.03–0.25)
uOPN/uCr (ng/mg)	1.86 (0.13–4.2)		2.54 (0.08–7.96)	5.22 (0.22–24.87)	6.34 (0.5–31.86)	1.88 (0.26–15.46)
uCysC/uCr (ng/mg)	0.95 (0.16–3.74)		0.66 (0.22–1.43)	0.57 (0.17–2.94)	0.56 (0.24–1.78)	0.58 (0.17–2.39)
uNGAL/uCr (ng/mg)	0.21 (0.02–2.94)		2.8 (0.1–15.78)	2.1 (0.07–3.02)	2.62 (0.09–21.66)	0.28 (0.03–23.3)
uMCP-1/uCr (ng/mg)	0.46 (0.05–1.97)		0.8 (0.15–5.44)	0.78 (0.23–7.24)	0.88 (0.27–5.58)	0.6 (0.09–6.14)
uGGT/uCr (U/g)	15.4 (0.95–75.26)		30.14 (3.96–83.44)	21.6 (0–85.65)	28.52 (9–120.5)	18.27 (0–48.83)
uALB/uCr mg/g	0.005 (0.002–6) 32/34 (94.1%) < LOQ*		0.019 (0.03–289) 9/16 (56%) < LOQ	0.036 (0.004–116) 16/25 (64%) <LOQ	0.025 (0.01–175) 17/22 (77%) < LOQ	0.007 (0.004–124) 16/19 (84%) < LOQ
uIL-8/uCr (ng/mg)	0.06 (0.02–0.9) 18/34 (52.9%) < LOQ**		0.08 (0.02–0.29) 10/16 (62.5%) < LOQ	0.14 (0.03–1.62) 21/24 (87.5%) < LOQ	0.14 (0.05–0.79) 18/22 (81.8%) < LOQ	0.07 (0.03–3.37) 14/20 (70%) < LOQ
uALP/uCr (U/g)	100% < LOQ***		100% < LOQ	100% < LOQ	96% < LOQ	100% < LOQ

** Limit of quantification (LOQ) = 9.6 ng/mL, **LOQ = 0.07 ng/mL, *** LOQ = 10 U/L*

Comparison of Envenomated dogs to control dogs

Serum creatinine (Fig. 1 A) was significantly lower in envenomated dogs at T2, T3 and T4 compared to controls ($P < 0.0001$). Symmetric dimethylarginine (Fig. 1 B) was significantly lower at T3 ($P = 0.0006$) and T4 ($P < 0.0001$), compared to controls. No significant difference in sCr And SDMA was found at T1 or T5 compared to controls. Urinary Alb/uCr and NGAL/uCr were significantly higher compared to the control group at T2, T3 and T4 ($P < 0.0001$, Fig. 2 A and B). Urinary MCP-1 /uCr was significantly higher at T2 ($P = 0.01$), T3 ($P = 0.002$) and T4 ($P = 0.0008$, Fig. 2 C) compared to

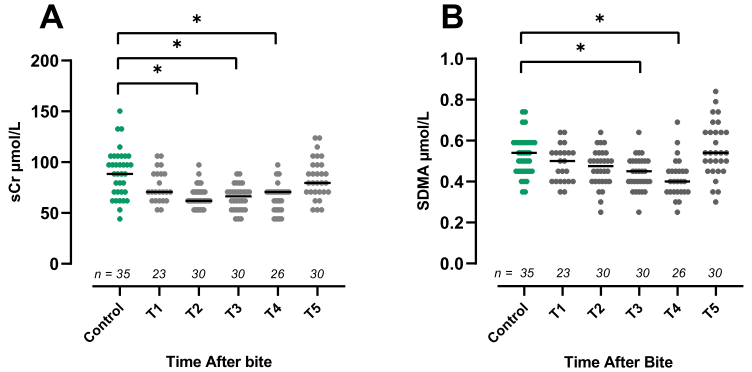

Fig. 1 Serum creatinine (sCr) (**a**) and SDMA (**b**) concentrations for control dogs and each timepoint after bite for envenomated dogs. T1 = 0–7.5 h, T2 = 10–14 h, T3 = 22–26 h, T4 = 34–38 h, T5 = 10–23 days. Bar denotes median. * indicates $P < 0.0001$

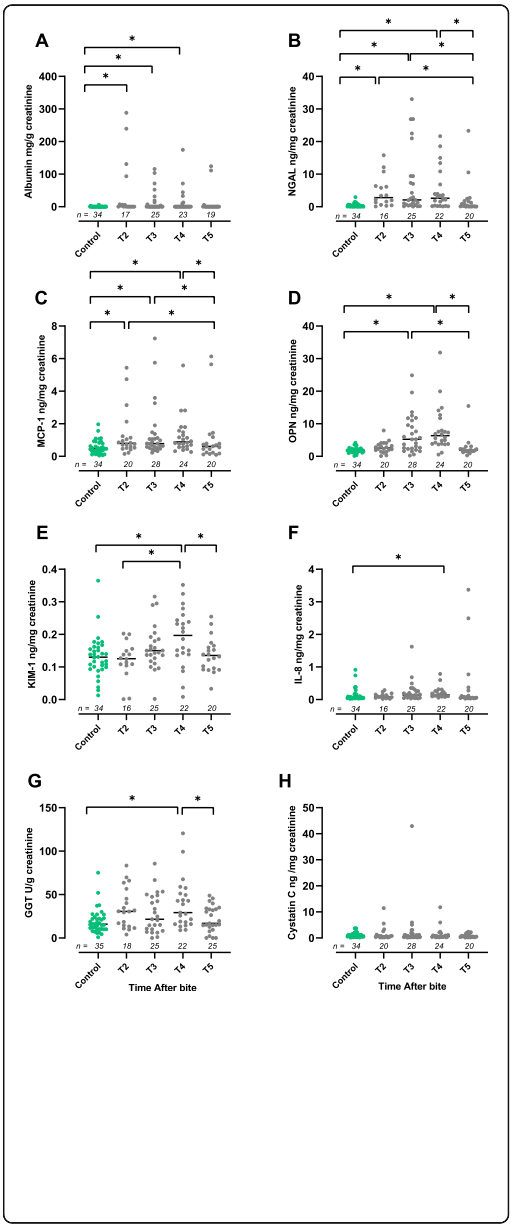

Fig. 2 Urinary biomarkers NGAL, KIM-1, OPN, CysC, MCP-1, IL-8, albumin and GGT normalised to urine creatinine in control dogs and each timepoint after bite for envenomated dogs. T = 0–7.5 h, T2 = 10–14 h, T3 = 22–26 h, T4 = 34–38 h, T5 = 10–23 days. Bar denotes median, * indicates $P < 0.0125$ for comparisons with the control group and $P < 0.008$ for timepoint comparisons for envenomated dogs. For Cystatin C, IL-8 and albumin, comparisons of time points within the envenomated group were not made due to poor fitting of the regression model

controls. Urinary OPN/uCr was significantly higher at T3 and T4 compared to controls ($P < 0.0001$, Fig. 2 D). Urinary KIM-1/uCr, uIL-8/uCr and uGGT/uCr were significantly higher at T4 compared to controls (P = 0.002 (Fig. 2 E*)*, P = 0.002, (Fig. 2 F*)* and $P = 0.003$, (Fig. 2 G*)* respectively). Urinary CysC/uCr was not significantly higher at any timepoint compared to control dogs (Fig. 2 H*)*.

Eight of the envenomated dogs had microalbuminuria at one time point or more. Two dogs had microalbuminuria at T5.

Comparison of time points for envenomated dogs

Urinary KIM-1/uCr was significantly higher at T4 compared to T5 ($P = 0.012$, Fig. 2 E), uOPN/uCr was higher at T3 and T4 compared to T5 ($P < 0.0001$) and at T3 and T4 compared to T2 ($P = 0.0003$ and $P < 0.0001$ respectively, Fig. 2 D), MCP-1/Cr was higher at T2, T3 and T4 compared to T5 ($P = 0.0012$, $p < 0.0001$ and $P = 0.0002$ respectively, Fig. 2), uNGAL/uCr was higher at T2, T3 and T4 compared to T5 (P = 0.0002, P < 0.0001 and P = 0.0002 respectively, Fig. 2 B) and uGGT/uCr was significantly higher at T4 compared to T5 (P < 0.0001, Fig. 2 G). Effects of explanatory variables within the model are reported in Table 2.

NS = not- significant.

Urinary biomarker correlations with SSS

In the envenomated dogs, SSS at presentation showed a strong positive correlation with peak uGGT/uCr (Spearman's ρ 0.62, $P = 0.0025$) and peak SSS was moderately correlated with peak uMCP/uCr (Spearman's ρ 0.43, $P = 0.02$). No other significant correlations were found between peak SSS or SSS at presentation and peak urinary biomarker/creatinine ratios.

Discussion

Results from this study are suggestive of mild transient non-azotaemic AKI in dogs envenomated by *V. berus*. These findings are supported by previous studies of snake-bitten dogs [5, 6].

Increased urinary AKI biomarkers have previously been reported in dogs envenomated by snakes compared to healthy controls [6]. Although significant differences

Table 2 *P* values for mixed model analysis for the effect of explanatory variables on urinary biomarker/creatinine ratios

Explanatory Variable	Response Variable				
	uKIM-1/uCr ng/mg	uOPN/uCr ng/mg	uMCP-1/uCr ng/mg	uNGAL/uCr ng/mg	uGGT /uCr U/g
Dog (random effect)	0.02	0.02	0.002	0.01	0.003
Age	NS	0.0001	< 0.0001	0.02	0.02
Weight	NS	NS	0.005*	NS	NS
Sex	NS	NS	NS	NS	0.04**
Time point after bite	0.005	< 0.0001	0.0001	< 0.001	0.003

**inverse i.e uMCP-1/uCr decreases with increasing weight*
*** lower in females*

in uAlb/uCr between cases and controls existed in this study, the ratios are lower than reported in previous studies [6], possibly due to a milder kidney insult following *V.berus* envenomation compared to other snake species, although an effect of the extra freeze-thaw cycle these samples were subjected to, cannot be ruled out [34]. Urinary albumin can reflect either glomerular or tubular injury [35] but lacks specificity, with increases also seen with extreme exercise [36], macroscopic hematuria and urinary tract infection [37]. Hematuria was detected in several samples in this study but did not exceed 100 RBC/HPF and was not observed to be macroscopic in any sample. We therefore consider hematuria unlikely to have influenced uAlb/uCr ratios in these individuals.

One other study has specifically examined urinary markers for AKI in *V.berus* envenomated dogs, and reported higher uGGT/uCr and uALP/uCr compared to controls, suggestive of renal injury [5]. Urinary GGT/uCr ratios in both cases and controls in our study were in accordance with the aforementioned study, but uALP/uCr was unexpectedly low. Given that ALP and GGT are both renal brush border enzymes, simultaneous leakage into urine upon tubular cell injury is expected. There are several possible explanations for a lack of increase in uALP in our study compared to others. Poor assay sensitivity might be considered most likely, but differences in timing and storage of samples are also possible explanations [38]. An optimal uALP detection window of less than 12 h after renal insult is reported in humans [39]; thus, the detection window may have been missed in our study. Urinary ALP can also originate from the epididymis and prostatic fluid, thus, differences in proportions of intact male dogs between studies might also influence results [40].

Urinary CysC was not significantly different between envenomated dogs and controls in this study. Urinary CysC increases as a result of decreased reabsorption after proximal tubular injury [41], and an association between uCysC concentration and severity of AKI has been described in humans and dogs [42, 43]. Mild AKI is therefore a likely explanation for the lack of increase in uCysC/uCr in envenomated dogs in the present study. A recent study indicates that the assay used in our study might measure lower concentrations of uCysC compared to other assays [44]. Care should therefore be taken in comparing results from different assays.

Urinary KIM-1 is an early and highly sensitive and specific diagnostic biomarker for proximal tubular injury, approved by the US Food and Drug Administration (FDA) as a marker for drug induced AKI in rodent models [45, 46]. Human studies describe upregulated uKIM-1 expression as early as 2 h, and lasting up until 48 h, after toxic or ischemic insult to the kidney [46]. In our study, significant differences in uKIM-1/uCr between envenomated dogs and controls were not observed until T4. Species differences, sample size, and different mechanisms of renal toxicity are possible explanations for the seemingly later induction of uKIM-1 in our study. However, it has also been suggested that uKIM-1 may not be as sensitive and early a marker of AKI in dogs compared to humans [47, 48]. Urinary uKIM-1/uCr and absolute uKIM-1 concentrations in healthy dogs in our study were lower than reported previously, also in one study using the same assay [33, 49]. The same studies reported comparatively higher uKIM-1/uCr and absolute uKIM-1 in dogs with AKI than found in the envenomated dogs in our study. This indicates that although uKIM-1/uCr was significantly higher at T4 compared to both controls and T5, these ratios are overall low and likely represent mild AKI given previous findings of correlations between KIM-1 and extent of injury [50]. All dogs in the present study had uKIM-1/uCr values below the cutoff of 0.75 ng/mg for diagnosing AKI proposed by one canine study [49], further supporting the theory that dogs bitten by *V. berus* might sustain a milder form of renal injury compared to AKI of other etiologies. A recent study suggests that uKIM-1 concentrations measured in the multiplex assay used in this study could be lower than those detected using other assays [44]. An assay specific reference interval is needed and direct comparisons between concentrations

detected in different studies should therefore be made with caution.

Neutrophil gelatinase-associated lipocalin is a ubiquitous LMW epithelial protein, subject to glomerular filtration and tubular reabsorption. Local NGAL production is induced in the distal tubule during renal injury [51]. Urinary NGAL may therefore result from either proximal or distal tubular injury. Higher uNGAL/uCr ratios have previously been reported in snake-bitten dogs that developed AKI compared to controls [52], but this is the first study describing increases in uNGAL in dogs envenomated specifically by *V.berus*. In accordance with another canine AKI study [30], uNGAL/uCr ratios were higher already from 12 h after bite in envenomated dogs, compared to controls, indicating its potential use as an early marker of AKI, as has previously been suggested [53]. A recent study indicated that systemic inflammation has a significant impact on uNGAL/uCr ratios [30]. Two forms of NGAL exist, of which the monomeric form is kidney specific, whereas increases in dimeric NGAL are seen in UTI and other inflammatory disease [54, 55]. Assays for detection of monomeric NGAL in canine urine are not currently available, thus the possible lack of specificity should be taken into consideration when interpreting results.

Upregulation of the inflammatory cytokines MCP-1 and IL-8 is described during renal injury in dogs [27, 33] and in human snake envenomation [56]. Few studies have measured uMCP-1 in dogs; hence, less is known regarding its kinetics. The finding of increased uMCP/uCr 12–36 h after envenomation compared to controls in our study, corresponds with previous nephrotoxicity studies [28, 33], indicating that MCP-1 might be useful as an early marker of AKI. An interesting finding in the present study was the inverse relationship between bodyweight and uMCP-1/uCr in envenomated dogs. Although a lower relative venom concentration in larger dogs is a possible explanation, this was considered more likely to be an incidental finding, since a similar effect was not observed for the other biomarkers. A high number of samples in this study had uIL-8 concentrations under the LLOQ. This was also described in another canine AKI model, despite elevations in other AKI markers [33], raising questions as to the sensitivity of uIL-8 as a marker for canine AKI.

The chemokine OPN has rarely been quantified in canine urine, thereby limiting comparisons with our study. Urinary OPN is a sensitive marker for renal tubular injury in rodent drug induced AKI models [57]. Absolute uOPN concentrations in the present study were comparable to those in a canine hemorrhagic shock model [33]. In the aforementioned study, absolute uOPN values did not increase significantly from baseline after induction and treatment of shock, whereas in our study, uOPN/uCr was significantly higher in envenomated dogs 24 and 36 h after bite, compared to controls. The difference in findings might be explained by difference in sample size, mechanism of AKI, or a lack or normalization to creatinine in the other study. A lack of specificity in the presence of inflammation is also possible and thus a systemic contribution to the uOPN measured, cannot be ruled out.

Serum concentrations of MCP-1, IL-8 and OPN can increase in various inflammatory states, including muscle injury and snake bite [57–60]. Given their LMW, we cannot rule out a systemic contribution via glomerular filtration during systemic inflammation induced by snake envenomation [61, 62], and further studies are therefore needed to ascertain the specificity of these markers.

Except for uCysC, all urinary biomarker/Cr ratios were higher 36 h after bite compared to controls. Whilst clearance kinetics of the various biomarkers may differ, our findings suggest that AKI might occur until at least 36 h after *V. berus* bite. A resolution of AKI by 14 days is suggested by a lack of significant difference between cases and controls at T5, but further studies are needed to clarify whether AKI is present beyond 36 h after bite. Although our study design does not allow us to establish a direct benefit of IVFT in the treatment of AKI in these dogs, hospitalization with monitoring and targeted IVFT is a sensible recommendation for dogs envenomated by *V.berus*, and results from our study indicate that this treatment should be implemented for a minimum of 36 h.

In accordance with previous studies [9, 63], sCr concentrations were within the reference interval for all dogs in this study, likely due to a lack of kidney dysfunction, although an effect of hemodilution due to IVFT cannot be ruled out. The increases in SDMA observed in two individuals at T5 were marginal and given that the two dogs also had high USG values at T5, we consider kidney dysfunction unlikely. According to veterinary AKI grading guidelines [16], two of the envenomated dogs in this study would be classified as having AKI grade I due to a non-azotaemic increase in sCr of 26.4 μmol/L within 48 h, and eight dogs due to microalbuminuria at one time point or more. A grading system incorporating novel renal injury biomarkers such as those measured in this study, to identify non-azotaemic AKI biomarker-positive individuals, would be of benefit by allowing early identification and treatment. More work is needed to establish the specificity of many of these biomarkers as well as to generate reference intervals for their clinical use.

In accordance with one other study [5], a positive correlation was found between severity of clinical signs at

presentation and uGGT/uCr in our study. Although SSS at presentation might thus be an indicator of peak uGGT/uCr after snake bite, further studies are needed to fully assess its use in this setting, especially since the relationship between uGGT/uCr and extent of injury is unknown. The lack of correlation of SSS with the other biomarkers in this study, suggests that severity of clinical signs after envenomation is of overall limited use in predicting which dogs are most likely to develop AKI.

There are limitations to this study. As previously mentioned, the specificity of uOPN, uNGAL, uIL-8, and uMCP-1 in the face of systemic inflammation needs clarification. However, the parallel increases in the urinary biomarkers evaluated in this study are suggestive of renal tubular injury in dogs envenomated by *V. berus*. Sample size limits the statistical inferences that can be made. Differences in assay sensitivity and imputation methods used for values under LLOQ hinder direct comparisons between our study and others for some of the biomarkers. A high number of samples had values under the LLOQ for uALP, uIL-8 and uAlb, thereby limiting their interpretation. Evidence suggests that uALb/uCr and uNGAL/uCr values are unlikely to be significantly affected by microscopic haematuria and haemoglobinuria, respectively [52]. Likewise, haematuria and haemoglobinuria are unlikely to significantly affect uGGT/uCr [37, 38], but it is unknown to what extent results for the other biomarkers in this study might be affected. Although the extent to which myoglobinuria might interfere with biomarker measurement is unknown, it is thought unlikely to influence the overall conclusions of this study since this was observed only in one dog. Urine samples were not subjected to bacterial culture, and thus subclinical bacteriuria may have been missed, although the extent to which this might influence results is unknown. Treatment with antivenom is a confounding factor, although unavoidable due to the non-interventional nature of this study. Differences in venom dose and individual susceptibly to venom are also factors that might impact our results. Hypotension was not apparent after envenomation in the 23 dogs for which data was available. Two dogs were at moderate risk of TOD due to persistent SAP values > 160 mmHg and we therefore cannot rule out that hypertension, albeit likely situational, may have contributed to proteinuria in these dogs [64]. Renal histopathology would have been a useful addition to this study but was not included due to ethical considerations. Finally, potential glomerular injury was not fully assessed in this study and further studies examining specific markers of glomerular injury in envenomated dogs would therefore be useful.

Conclusions

Increases in the urinary AKI biomarkers KIM-1, GGT, NGAL, IL-8, OPN, MCP-1 and albumin were indicative of renal tubular injury in dogs 12–36 h after envenomation by *V.berus* in this study, although further work is needed to ascertain the specificity of IL-8, NGAL, OPN and MCP-1. Serum creatinine and SDMA were of limited diagnostic use in this study. Overall, severity of clinical signs did not appear to be a useful indicator of urinary AKI biomarker/uCr ratios, suggesting that AKI can occur regardless of initial clinical assessment. The finding of increased AKI biomarkers 36 h after bite suggests that hospitalisation and supportive treatment of *V.berus* envenomated dogs is sensible.

Methods

Animals

Forty-one dogs presenting with a *V. berus* bite to the first opinion emergency service at the small animal hospitals at NMBU, EOD, ADO and AJD between April and October 2018, were evaluated for enrollment to the study. Diagnosis of snake bite and thus inclusion in the study was based on history and presence of consistent clinical signs at presentation (fang marks, local or systemic signs of envenomation). Six dogs were excluded for the following reasons: treatment with non-steroidal ant-inflammatory drugs (n = 1), presentation more than 24 h after snakebite (n = 2), dry bite (lack of clinical signs within 12 h of the bite) (n = 1), sampling occurring outside of the stipulated time frame (n = 1) and a lack of urine samples (n = 1). Permitted pre-existing diagnoses and treatments included hypothyroidism treated with levothyroxine (n = 1) and mitral valve insufficiency treated with pimobendan (n = 1).

Physical examination, urine and blood examination

All envenomated dogs underwent physical examination and blood sampling at the following time points post bite: T1 (presentation: 1–7.5 h), T2: 12 (± 2) hours, T3: 24 (± 2) hours, T4: 36 (± 2) hours and T5: 10–23 days. Urine samples were obtained at all timepoints except T1. Treatment decisions were made by the attending clinician.

Midstream voided urine samples were collected at each timepoint following cleaning of the vulva or prepuce with 0.3% chlorhexidine wipes (ICF, Palazzo Pignano, Italy). Macroscopic examination, dipstick analysis (IDEXX UA* strips, IDEXX Laboratories, Inc., Westbrook, Maine 04092) and specific gravity, measured by refractometry, were performed before centrifugation at 450 g for seven minutes. Supernatant was aliquoted into cryotubes and frozen at − 80 °C within 15 min. For all samples, sediment was resuspended in approximately 1 ml of supernatant and immediately examined in

unstained and stained (BD Clay Adams Sedi-Stain, Sparks, MD 21152 USA) wet slides. Where findings were made on wet slides, dry preparations were prepared (Hemacolor, Merck KgaA, 64,271 Darmstadt, Germany) and reviewed by two veterinarians. Where differences in cell and cylinder counts existed between veterinarians, the mean of the two observations was used.

Urine supernatant was aliquoted and stored at − 80 °C for a maximum of 9 months prior to ALP and GGT analysis and 17 months prior to multiplex immunoassay analysis of IL-8, OPN, KIM-1, NGAL, CysC, MCP-1 and Albumin. Samples were transported on dry ice to either the reference laboratory at NMBU: Sentrallaboratoriet (SL) (ALP, GGT and creatinine) or to a research laboratory at NMBU for immunoassay analysis.

Blood was collected at T1-T5 through a venous catheter in the cephalic or saphenous vein or using a 21-gauge needle with butterfly extension in the jugular vein into serum tubes and centrifuged at 1100 x g for 10 min, 30–60 min post sampling. Serum was pipetted into cryotubes and stored at − 80 °C for a maximum of 12 months prior to transportation on dry ice to a research laboratory (IDEXX Laboratories, Inc., Westbrook, Maine 04092) and a maximum of 20 months before sCr and SDMA analysis.

Blood pressure measurement

Indirect blood pressure (Cardell®, Midmark, Versailles, USA) measurements were recorded at T1-T5. A cuff size of approximately 40% limb circumference was placed on either the distal radius or metatarsus with the patient in lateral recumbency. Twelve serial measurements of SAP, DAP and MAP were recorded per time point. Mean SAP, DAP and MAP were calculated after discarding the first two measurements and any obvious outlying values.

Snakebite severity score (SSS)

Envenomated dogs were assigned a 16-point snakebite severity score (SSS) at each timepoint, using an adaptation of a previously described grading system [65]. Grading criteria are described in Table 3.

Control dogs

A cohort of 35 privately-owned healthy control dogs not previously bitten by *V. berus* was recruited using stratified sampling by age and weight. A single blood sample was collected into EDTA and serum tubes from the jugular vein using a 21-gauge needle with butterfly extension. Haematology and biochemistry were performed on EDTA blood and one serum aliquot on the day of collection. The remaining serum aliquot was stored and transported as above, for sCr and SDMA analysis. A single urine sample was collected and prepared as for the envenomated dogs. Healthy status was defined as an unremarkable history and physical examination, no history of chronic disease or any illness within the two weeks prior to recruitment, no clinically significant haematology or serum biochemistry abnormalities and an unremarkable urinalysis on voided urine including less than five leucocytes per high power field (HPF). A mild elevation in sCr (150 μmol/L) was permitted in one very muscled dog where urine specific gravity (USG) was 1.043 and SDMA was within the reference interval.

Laboratory analyses

Urinary ALP, GGT and creatinine were analyzed at SL on an automated biochemistry analyzer (Advia®1800, Siemens, Germany). Urinary ALP and uGGT were measured using a Modified International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) Method [66, 67]. Urinary and serum creatinine were determined by Jaffe's reaction using picrate at alkaline pH at SL and a research laboratory (IDEXX Laboratories, Inc., Westbrook, Maine 04092), respectively. Serum SDMA concentration was measured using a high-throughput immunoassay (IDEXX Laboratories, Inc., Westbrook, Maine 04092).

Concentrations of uCysC, uKIM-1, uIL-8, uNGAL, uMCP-1 and uOPN were measured using a 6-plex Luminex xMAP® assay (Milliplex® MAP Kit, Canine Kidney Toxicity Expanded Magnetic Bead Panel 1, EMD Millipore Corporation, MA, USA). Urinary albumin was measured as a single analyte in a Luminex xMAP® assay (Milliplex® MAP Kit, Canine Kidney Toxicity Expanded Magnetic Bead Panel 2, EMD Millipore Corporation, MA, USA), after a second freeze-thaw cycle. Both assays were previously validated by the manufacturer for use on canine urine. Samples were analyzed in duplicate and according to the manufacturer's instructions. Briefly, samples were thawed at room temperature, vortexed and centrifuged at 14000 RCF at 4 °C for 5 min and then diluted 1:2 in assay buffer for CysC, KIM-1, IL-8, NGAL, MCP-1 and OPN, and 1:500 for albumin. The plates were read on a Bio-Rad 200 instrument (Bio-Rad, USA) and data was processed using BioPlex Manager 6.1 software (Bio-Rad, USA). A 5-parameter logistic regression model was used to generate standard curves for each analyte. Standard curve outliers were excluded by the software if situated outside the standard's acceptable recovery range (70–130%). Sample duplicates were manually excluded where the coefficient of variation (CV) exceeded 40%. The standard curves were optimized by the software before calculating the concentration of each analyte. Values below the lower limit of quantification (LLOQ) were imputed using the LLOQ, including values below the limit of detection (LOD = mean blank + 3 standard deviations) since these were close in value to the LLOQ.

Table 3 16 -point snake bite severity score grading criteria

	Score
Respiratory system	
• Signs within normal limits	0
• Minimal: Mild tachypnea, consistent with pain response	1
• Moderate: Marked tachypnea and/or slightly increased respiratory effort	2
• Severe: Extreme tachypnea and or respiratory insufficiency/failure	3
Cardiovascular system	
• Signs within normal limits	0
• Minimal: Tachycardia consistent with pain, general weakness, hypertension	1
• Moderate: Tachycardia, hypotension with tarsal pulse still palpable	2
• Severe: Extreme tachycardia, hypotension with non-palpable tarsal pulse or systolic blood pressure < 80mmgHg, arrhythmia or cardiac arrest	3
Local wound	
• Signs within normal limits	0
• Mild: Pain, swelling, ecchymosis, erythema limited to bite site	1
• Moderate: Pain, swelling, or ecchymosis involving less than half the extremity and may be spreading slowly	2
• Severe: Pain, swelling, or ecchymosis involving most or all of one extremity and is spreading rapidly	3
• Very severe: Pain, swelling, or ecchymosis extending beyond affected extremity (in case of head: extending to the neck)	4
Gastrointestinal system	
• Signs within normal limits	0
• Minimal: Abdominal pain, tenesmus,	1
• Moderate: Vomiting or diarrhoea	2
• Severe: Repetitive vomiting, diarrhea, haematemesis, or haematochezia	3
Demeanor	
• Bright, alert, responsive (BAR)	0
• Minimal: Quiet, alert, responsive (QAR)	1
• Moderate: Lethargic	2
• Severe: Extreme lethargy, collapse	3
Total Score	/16

All urinary biomarkers were normalized to urinary creatinine and expressed as ng/mg creatinine except for ALP and GGT that were expressed as U/g creatinine and Albumin expressed as mg/g. Urinary Alb/Cr ratios of 30-300 mg/g were considered to be positive for microalbuminuria [68].

Statistical analysis

Statistical analysis was performed using commercially available statistical software packages (JMP Pro 14.3.0, SAS Institute Inc., Cary, NC and GraphPad Prism 8.3.1, GraphPad Software LLC, San Diego, CA). Data were visually assessed for normality. Most data were not normally distributed; thus, median and range are reported throughout. Comparisons of urinary biomarkers between the control group and each of the timepoints for the envenomated dogs were performed using a Wilcoxon rank sum test for non-parametric variables and a t-test for parametric variables. Bonferroni correction was applied to adjust for multiple comparisons.

Repeated measurements of urinary AKI biomarkers in envenomated dogs were analysed in a linear mixed model with age, weight and sex as fixed effects and dog as a random effect. Generalised linear model assumptions were checked by assessing the residuals in each model and data was log, square root or cube root- transformed where appropriate. Post hoc Bonferroni correction was applied. For uAlb, uIL-8 and uCysC a satisfactory transformation was not achieved; thus, ratios of these variables were not compared across timepoints for envenomated dogs.

Statistical analysis of albumin, GGT, NGAL, KIM-1 and IL-8 was performed after exclusion of 11 individual urine samples from five envenomated dogs with leucocyte counts > 5/ HPF due to evidence of an effect of pyuria or urinary tract infection on results in previous studies [49, 69–72].

Correlations between peak AKI biomarker/creatinine ratios and between peak AKI biomarkers and SSS at presentation and peak SSS were assessed using Spearman's rank correlation.

Abbreviations

AKI: acute kidney injury; ADO: Anicura Dyresykehus Oslo; AJD: Anicura Jeløy Dyresykehus; EOD: Evidensia Oslo Dyresykehus; HMW: high molecular weight; HPF: high power field; IFCC: International Federation of Clinical Chemistry and Laboratory Medicine; IRIS: International Renal Interest Society; LLOQ: lower limit of quantification; LMW: low molecular weight; LOD: limit of detection; LOQ: limit of quantification; NMBU: Norwegian University of Life Sciences; SDMA: Symmetric dimethylarginine; sCr: serum creatinine; SSS: snakebite severity score; uAlb: urinary albumin; uALP: urinary alkaline phosphatase; uCr: urine creatinine; uCysC: urinary cystatin C; uGGT: urinary γ-glutamyl transferase; uIL-8: urinary interleukin-8; uKIM-1: urinary kidney injury molecule − 1; uMCP-1: urinary monocyte chemoattractant protein-1; uNGAL: urinary neutrophil gelatinase-associated lipocalin; uOPN: urinary osteopontin; USG: urine specific gravity

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12917-021-02851-8.

Additional file 1. Breed overview for envenomated dogs and healthy control dogs.

Acknowledgements

The authors would like to thank IDEXX Laboratories, Inc., Westbrook, Maine 04092 for serum creatinine and SDMA analysis, Dr. Janicke Nordgreen for advice on statistical analysis and staff, students and dog owners at the small animal clinics at NMBU, ADO, AJD and EOD for the care and provision of dogs in this study.

Authors' contributions

HJH planned the study, collected and coordinated analysis of data, and was a major contributor in writing the manuscript. TVN analysed biomarkers in the urine samples, contributed to analysis and interpretation of data and the drafting of the manuscript. TN was a substantial contributor to data collection and project coordination at AJD. HL contributed to data analysis and interpretation and drafting of the manuscript. BKS was a contributor to data interpretation and manuscript revision. KA contributed to the planning of the study, data interpretation and manuscript revision. ERM contributed to the planning of the study, data interpretation and manuscript revision. KZ contributed to study planning, data interpretation and manuscript revision. RR was a major contributor to study planning, data interpretation and manuscript revision. All authors have read and approved the final manuscript. HH is the corresponding author.

Funding

This study was funded by the Agria and SKK Research Fund for Pets. The funding body played no part in the design, analysis or reporting of the study.

Availability of data and materials

The datasets used during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

This study was approved by the local ethical committee at the Norwegian University of Life Sciences (NMBU) and permitted by the Norwegian Research Authority. Written owner consent was obtained for all dogs prior to inclusion in the study. All methods were carried out according with relevant guidelines and regulations and in compliance with the ARRIVE guidelines.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

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Received: 17 December 2020 Accepted: 25 March 2021
Published online: 12 April 2021

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Publisher's Note

Additional File 1: Breed overview for envenomated dogs and healthy control dogs.

Breed	Envenomated Dogs	Control Dogs
Akita	1	
Australian kelpie	1	
Bichon frise	1	
Bodeguero		1
Border collie	1	
Border terrier		1
Boston terrier	1	
Boxer		1
Briard		1
Cavalier king Charles spaniel	1	2
Cocker spaniel	1	2
Crossbreed	8	3
Danish-Swedish farm dog		2
English setter	4	3
Finnish lapphund	1	
Flat coated retriever	2	
Golden retriever		4
Gordon setter	1	
Greyhound		1
Irish setter		1
Irish wolfhound		1
Japanese spitz		1
Kleiner Münsterländer	1	1
Labrador retriever	2	3
Malinois	1	
Miniature pinscher		1
Miniature poodle	1	
Miniature Schnauzer	1	
Nova scotia duck tolling retriever	1	
Pointer		2
Saluki		1
Samoyed	1	
Shetland sheepdog	1	1
Staffordshire bull terrier	1	1
Standard poodle	1	1
Toy poodle	1	

Paper III

Evaluation of urinary clusterin and cystatin B as biomarkers for renal injury in dogs envenomated by the European adder (*Vipera berus*).

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Abbreviations

AKI = acute kidney injury, ADO = Anicura Dyresykehus Oslo, AJD = Anicura Jeløy Dyresykehus, BCS = body condition score, EOD = Evidensia Oslo Dyresykehus, GFR = glomerular filtration rate, IQR = interquartile range, , LOQ = limit of quantification, MCS = muscle condition score, NMBU = Norwegian University of Life Sciences, sCr = serum creatinine, SDMA = serum symmetric dimethylarginine, SSS = snakebite severity score, uclusterin = urinary clusterin, uCysB = urinary cystatin B, UPC = urine protein to creatinine ratio, USG = urine specific gravity.

Abstract

Dogs are commonly bitten by the European adder (*Vipera berus*) but studies investigating the effects of envenomation are limited. Snakebite-related kidney injury is reported in dogs but diagnosis of acute kidney injury (AKI) might be limited by the insensitivity of routinely used renal function biomarkers. The aim of this study was to evaluate novel biomarkers of renal injury (urinary cystatin B and urinary clusterin) and biomarkers of renal function (serum creatinine and serum symmetric dimethylarginine), and urine protein to creatinine ratio in dogs envenomated by *V. berus*. Biomarkers were measured at presentation (T1), 12 hours (T2), 24 hours (T3), 36 hours (T4) and 14 days (T5) after snakebite and compared to a group of healthy control dogs. A secondary aim was to investigate the association between biomarker concentrations and severity of clinical signs of envenomation using a snakebite severity score (SSS).

Urinary cystatin B concentrations were significantly higher at all timepoints in envenomated dogs compared to controls ($P < 0.010$), except for T5 ($P = 0.222$). Absolute urinary clusterin concentrations were not significantly different to controls at any timepoint. Compared to controls, serum creatinine and serum symmetric dimethylarginine concentrations were significantly lower in envenomated dogs at T1-T4 ($P < 0.036$) and T2-T4 ($P< 0.036$), respectively. Urine protein to creatinine ratio was higher in envenomated dogs compared to controls at T2 and T3. Urinary cystatin B concentrations at T1 were correlated with SSS (Spearman's $\rho = 0.690$, $P < 0.001$). The increased urinary cystatin B concentrations observed in dogs envenomated by *V.berus* in comparison to controls may indicate renal tubular injury in these patients.

Introduction

Envenomation by the European Adder (*Vipera berus*) is a common reason for dogs to require emergency veterinary treatment during the spring and summer months in Europe [1]. Clinical signs of envenomation are variable and include oedema, pain, arrhythmias, and collapse [1, 2]. Signs of snake envenomation in Norway can be attributed to *V. berus* alone since it is the only venomous snake in Scandinavia.

Snakebite-related acute kidney injury (AKI) is reported in humans and dogs [3-7] and is associated with risk of mortality [1, 7-9]. Renal tubular injury has been described in dogs bitten by *V. berus* and *Vipera aspis* [5-7], but studies specifically focusing on renal injury after *V. berus* envenomation in dogs are limited. Irrespective of cause, AKI might be a mediator for progressive kidney disease [10-12]. Early identification and treatment of AKI could improve clinical outcome through attenuation of damage [10]. Thus, there is a need for early identification and treatment of snakebite-related AKI in dogs.

Serum creatinine (sCr), an indirect marker of glomerular filtration rate (GFR), is routinely used to assess renal function in dogs. However, sCr is an insensitive marker for diagnosing early stage AKI since increased concentrations are not observed until 75% of functional nephron mass is lost and extra-renal factors affect individual baseline levels [13, 14]. Furthermore, renal injury can be present without renal dysfunction [15]. AKI might therefore be underdiagnosed in dogs envenomated by *V. berus* when sCr is used as the sole biomarker for renal injury. Urinary parameters such as urine specific gravity (USG), sediment examination, and urine protein to creatinine ratio (UPC) can be useful adjuncts in AKI diagnosis, but their value is limited by a lack of sensitivity, specificity, or both [16-18].

Serum symmetric dimethylarginine (SDMA) is a more sensitive and specific endogenous marker of glomerular filtration rate (GFR) than sCr in dogs [14, 19]. Further studies are needed to fully establish its use in diagnosing early stage or mild kidney injury. In recent years, there has been a focus on identifying novel biomarkers for renal injury with the potential to both diagnose subclinical AKI in the absence of functional change, and to localise injury within the kidney.

Urinary clusterin (uClusterin) is a cytoprotective glycoprotein, approved by the US Food and Drug Administration and European Medicines Agency as a biomarker for monitoring drug-induced proximal tubular injury in rats [20]. Urinary clusterin is upregulated during kidney injury and is more sensitive than sCr for detecting renal injury in dogs [21, 22].

Urinary cystatin B (uCysB), an intracellular protein thought to be released into the urine upon tubular cell rupture, has also shown promise as an early marker for AKI in a canine gentamicin model as well as in human diabetic nephropathy [19, 23]. A recent study identified increased concentrations of uClusterin and uCysB in dogs sampled at a single timepoint after *V.berus* bite [24]. There is, however, a need for longitudinal evaluation of these biomarkers in order to elucidate their use in diagnosing and monitoring AKI in dogs as well as to further evaluate renal tubular injury after V.berus envenomation in dogs.

The primary aim of this study was to investigate urinary levels of uCysB and uClusterin in dogs envenomated by *V. berus* during the first 36 hours and 14 days after bite, as well as the surrogate markers for GFR (sCr and SDMA) and UPC. A secondary aim was to assess whether severity of clinical signs of envenomation correlate with the concentration of AKI biomarkers. Such information could be useful in a clinical setting and could help optimise treatment of these dogs.

Methods

Animals

Forty-one dogs presenting with a *V. berus* bite to the first opinion emergency service at the small animal hospitals at the Norwegian University of Life Sciences (NMBU), Evidensia Oslo Dyresykehus (EOD), Anicura Dyresykehus Oslo (ADO) and Anicura Jeløy Dyresykehus (AJD) between April and October 2018, were assessed for inclusion in the study. Diagnosis of snakebite was made based on the history and consistent clinical signs (fang marks, local swelling or systemic signs of envenomation). Sample size were determined by convenience sampling of snakebite cases presenting for treatment. Five dogs were excluded for the following reasons: long term treatment with non-steroidal anti-inflammatory drugs (n=1), lack of clinical signs within 12 hours of the bite (n=1), presentation more than 24 hours after the bite (n=2) and sampling occurring outside of the stipulated timeframe (n=1). Permitted pre-existing diagnoses and treatments included hypothyroidism treated with levothyroxine (n=1) and a systolic heart murmur treated with pimobendan (n=1).

A control group of 40 healthy privately-owned dogs that were not previously bitten by V. berus, was recruited via stratified sampling by age and weight, and matched in number to the envenomated dog group. Healthy status was defined as an unremarkable history and physical examination, no history of chronic disease or any disease in the last 14 days,

haematology and serum biochemistry within normal reference intervals and normal urinalysis (voided sample) including less than ten leucocytes per high power field. Of the initial group of 40 control dogs, one was excluded due to pyuria and five were excluded due to serum SDMA concentrations (n=4) or both serum SDMA and sCr concentrations above the reference interval (n=1).

Physical examination and snakebite severity scoring

Envenomated dogs underwent physical examination, blood and urine sampling at five timepoints post envenomation: T1: presentation (1.5-7.5 hours), T2: 10-14 hours, T3: 22-26 hours, T4: 34-38 hours, T5: 10-23 days. A 16-point snakebite severity score (SSS) was recorded at each timepoint, using an adaptation of a previously described grading system [25] *(Table 1*). Body condition score (on a scale of 1-9) [26] and muscle condition score (normal, mild, moderate or severe loss) [27] were recorded at T1 and T5. Control dogs underwent physical examination and sampling at a single timepoint.

Indirect blood pressure was measured at each timepoint in envenomated dogs (Cardell®, Midmark, Versailles, USA). A cuff size of approximately 40% limb circumference was placed on the metatarsus or distal radius. Mean systolic (SAP), diastolic (DAP) and mean (MAP) arterial pressure were calculated from twelve consecutive measurements after discarding the first two and any obvious outlying values.

Sample collection

Whole blood was collected through a venous catheter in the cephalic or saphenous vein or using a 21-gauge needle with butterfly extension in the jugular vein, into serum tubes and centrifuged at 1100 x g for 10 minutes, 30-60 minutes after sampling. Serum for AKI biomarker analysis was pipetted into cryotubes and frozen within 15 minutes.

Midstream naturally voided urine samples were collected at each timepoint following cleaning of the vulva or prepuce with 0.3% chlorhexidine wipes (ICF, Palazzo Pignano, Italy). Macroscopic examination, dipstick (IDEXX UA* strips, IDEXX Laboratories, Inc., Westbrook, Maine 04092) and specific gravity measurement were performed before centrifugation at 450g for seven minutes. Supernatant was aliquoted into cryotubes and frozen within 15 minutes. Sediment was resuspended in approximately 1mL of

supernatant and immediately examined in unstained and stained (BD Clay Adams Sedi-Stain, Sparks, MD 21152 USA) wet slides. Where abnormal findings were made on wet slides, dry preparations were made (Hemacolor, Merck KGaA, 64271 Darmstadt, Germany) and reviewed by two veterinarians. Where differences in cell counts existed between veterinarians, the mean was used.

All samples for biomarker analyses were stored in -80°C for a maximum of 12 months before transportation on dry ice to a research laboratory (IDEXX Laboratories, Inc., Westbrook, Maine 04092) and a maximum of 20 months before analysis. Serum and EDTA blood from control dogs for biochemistry and haematology screening were analysed on the day of sampling at the reference laboratory at NMBU.

Table 1. Criteria for 16-point snakebite severity score

Criteria	Score
Respiratory system	
• Signs within normal limits • Minimal: mild tachypnoea, consistent with pain response • Moderate marked tachypnoea and/or slightly increased respiratory effort • Severe: extreme tachypnea and or respiratory insufficiency/failure	0 1 2 3
Cardiovascular system	
• Signs within normal limits • Minimal: Tachycardia consistent with pain, general weakness, hypertension • Moderate: Tachycardia, hypotension with tarsal pulse still palpable • Severe: Extreme tachycardia, hypotension with non-palpable tarsal pulse or systolic BP < 80mmgHg, arrhythmia or cardiac arrest	0 1 2 3
Local wound	
• Signs within normal limits • Mild: Pain, swelling, ecchymosis, erythema limited to bite site • Moderate: Pain, swelling, or ecchymosis involving less than half the extremity and may be spreading slowly • Severe: Pain, swelling, or ecchymosis involving most or all of one extremity and is spreading rapidly	0 1 2 3 4
Gastrointestinal system	
• Signs within normal limits • Minimal: Abdominal Pain, tenesmus, • Moderate: Vomiting or diarrhea • Severe: Repetitive vomiting, diarrhea, hematemesis, or hematochezia	0 1 2 3
Demeanor	
• BAR • Minimal: QAR • Moderate: Lethargic • Severe: Extreme lethargy, collapse	0 1 2 3
Total Score	**/16**

Laboratory analysis

Serum and urinary creatinine concentrations were determined by a colorimetric method, Jaffe's reaction using picrate at alkaline pH (Beckman Coulter, Inc, Brea CA). Urinary protein concentration was measured using a pyrogallol red molybdate method (Beckman Coulter, Inc, Brea CA)[28]. Urine protein to creatinine ratio was calculated by dividing protein (mg/dL) by creatinine (mg/dL). Serum SDMA concentration was measured using a high-throughput immunoassay (IDEXX SDMA Test). Urinary clusterin and uCysB concentrations were measured using ELISA plate assays (currently in early development at IDEXX).

Other data recorded

For each dog, fluid type and rate were recorded as well as an estimate of total fluid volume administered during hospitalisation, type of analgesia, administration of antivenom and any other treatments.

Statistical Analysis

Statistical analysis was performed using a commercially available statistical software package [29]. Data were visually assessed for normality. For demographic variables, median and interquartile ranges are reported. Categorical and ordinal data are presented as a percent of the total. Comparisons of uClusterin, uCysB, UPC, serum SDMA and sCr between the control group and each of the timepoints for the snake bitten group were made using a Wilcoxon rank sum test. Fishers exact test with Bonferroni correction for multiple comparisons was used to compare the proportion over the reference interval to control at each timepoint. The 16-point SSS was collapsed into 5 groups (0, 1-3,4-7, 8-12, 13-16) to increase numbers within each group for statistical analysis and compared to the AKI biomarkers via the first timepoint for each subject. Correlations between AKI biomarkers and SSS were assessed using Spearman's rank correlation.

In this study population, 18/34 (53%) of the heathy controls and 66/106 (62%) of the snake bitten dog samples had uClusterin values below the limit of quantification (LOQ) for the assay. For uCysB, 28/34 (82%) of the controls and 52/106 (50%) of the snake

bitten dog samples had values below the LOQ for the assay. The values below the LOQ were imputed using the LOQ value (70ng/mL for uClusterin and 15ng/mL for uCysB).

Tentative upper reference intervals of > 350 ng/ml and > 50ng/mL were used for uClusterin and uCysB, respectively. These preliminary reference intervals were previously established based on urine samples from 78 apparently healthy dogs.

Results

Controls

The final control group consisted of 34 dogs. Eighteen (52.9%) were female and 16 (47.1%) were male. Median age was 5.2 years (IQR 2.0-8.0 years, Range: 6 months to 13 years). Median weight was 23.7 kg (IQR 10.1-28.5 kg).

Envenomated dogs

Thirty-six dogs envenomated by *V. berus* were included in the final study group. Twenty-two cases presented to NMBU, two cases to ADO and six cases each to EOD and AJD. The snake or fang marks were witnessed in 83.3% (30/36) of dogs. Diagnosis was based on history and clinical signs consistent with envenomation in the remaining six dogs. Twenty-one (58.3%) dogs were female and 15 (41.7%) were male. Median age was five years (IQR 2.0-7.0 years, Range: 6 months to 18 years). Median weight at presentation was 19.0 kg (IQR 9.9- 25.8 kg). Twenty-five dogs were bitten on the head, 10 on a limb, and one on the sternum. Median time from bite to initial presentation was 1.8 hours (IQR 1-6 hours). Two dogs were diagnosed with a *V. berus* bite six years and five years previously.

Treatment

All envenomated dogs were treated with crystalloid fluid therapy IV during hospitalisation (ringer-acetate (n=35) or NaCl on day one followed by ringer-acetate on day two (n=1)). Median fluid rate, recorded in 26 dogs, was 4ml/kg/hour (IQR 3.9-4.8). Twenty-eight dogs received equine F(ab')2 antivenom IV (Viper Venom Antitoxin, SIS Biomed®, Warsaw, Poland). Median time from bite to antivenom treatment was 4 hours (IQR 2.5-8.5 hours). Analgesics included buprenorphine (Vetergesic vet®, Ceva Santé Animale) (n=6) or methadone (Metadon, , Norges Apotek, Norway) (n=24). Six dogs received a combination of methadone and buprenorphine. Lidocaine (Xylocain®, Aspen Pharma trading Ltd) was administered to one dog within one hour of presentation due to

a ventricular arrhythmia. Three dogs were treated with maropitant due to hypersalivation or vomiting. One dog was administered furosemide prior to T3 due to perceived oliguria. Other treatments included ampicillin (n=1), clindamycin (n=1), dexamethasone (n=1), prednisolone, streptomycin and penicillin (n=1), pimobendan (n=1) and levothyroxine (n=1).

Urinalysis

Median urine specific gravity (USG) in the envenomated dogs was 1.022 (IQR 1.015-1.037) during hospitalisation (T1-T4) and 1.036 (IQR 1.025-1.045) at T5. Median USG was 1.044 (IQR 1.038-1.048) in the control dogs. Urinalysis findings of note were myoglobinuria (n=1), haemoglobinuria (n=6), varying degrees of haematuria (n= 16) and urinary casts (n=3).

Snakebite Severity Score

At the time of hospitalization (T1), the most common SSS (46.2%) was between 4 and 7 (Table 2). At T4 the most common SSS (50.0%) was between 1 and 3 (Table 2). By T5, all except three dogs had an SSS of 0.

Blood pressure

Blood pressure was measured in 22 envenomated dogs, as previously reported [2, 30]. Overall median (range) for SAP was 135mmHg (104-196mmHg), DAP 76mmHg (52-154mmHg) and MAP 135mmHg (104-196mmHg). No dogs were hypotensive (SAP < 90mmHg or MAP <60mmHg). Three individual had SAP values >160mmHg, two of which had persistent values >160mmHg. The individual with a SAP measurement of 196mmHg was hypertensive only at presentation.

Table 2. Snakebite Severity Score expressed as proportion of envenomated dogs per timepoint.

	Timepoint 1 (T1)	Timepoint 2 (T2)	Timepoint 3 (T3)	Timepoint 4 (T4)	Timepoint 5 (T5)
Category	Proportion (n/N)	Proportion (n/N)	Proportion (n/N)	Proportion (n/N)	Proportion (n/N)
Snakebite Severity Score (0)	0.04 (1/26)	—	0.06 (2/31)	—	0.90 (28/31)
Snakebite Severity Score (1-3)	0.35 (9/26)	0.48 (16/33)	0.48 (15/31)	0.50 (13/26)	0.10 (3/31)
Snakebite Severity Score (4-7)	0.46 (12/26)	0.39 (13/33)	0.42 (13/31)	0.42 (11/26)	-
Snakebite Severity Score (8-12)	-	0.15 (4/26)	0.12 (4/33)	0.03 (1/31)	0.08 (2/26)

Biomarker analysis

Comparison of Envenomated dogs to control

Urinary clusterin concentrations did not differ significantly from controls at any of the timepoints after envenomation (Figure 1A). Urinary cystatin B concentrations were significantly higher at all timepoints after envenomation compared to controls ($P < 0.010$) except for the 14-day post-hospitalization (T5) time point ($P = 0.222$, Figure 1B). The proportion of envenomated dogs with a uCysB concentration above the tentative reference interval (> 50 ng/mL) was significantly higher at T2 (45.4%, 10/22, $P < 0.001$) and T3 (33.3%, 9/27, $P = 0.006$) after bite compared to controls (0.0%, 0/34). Proportions of envenomated dogs with a uClusterin concentration above the tentative reference interval (> 350ng/mL) was not found to differ to the control dogs at any timepoint.

As an exploratory analysis uClusterin and uCysB were normalized by urinary creatinine. When normalized by urinary creatinine, uClusterin concentrations were significantly higher at both T3 and T4 after envenomation compared to controls ($P < 0.001$, Supplementary Figure 1). Normalized uCysB values were statistically significant at all time points after envenomation compared to controls ($P < 0.003$, Supplementary Figure 1).

Serum SDMA values were lower in envenomated dogs between T2 and T4 ($P < 0.036$, Figure 1C). Serum SDMA concentrations at T1 and T5 had similar values compared to the healthy control dogs ($P > 0.143$, Figure 1C). Serum creatinine values were lower in envenomated dogs between T1 and T4 timepoints ($P < 0.036$, Figure 1D). Serum creatinine concentrations at T5 had similar values compared to the healthy control dogs ($P = 0.681$, Figure 1D). Neither sCr nor SDMA were observed to have any values above the reference interval (sCr >1.5 mg/dL =133 µmol/L and SDMA > 14 µg/dL = 692 nmol/L) in controls and envenomated dogs between T1 and T4. At T5, 13% (4/31) of envenomated dogs had SDMA concentrations above the reference interval and 3% (1/31) had sCr concentrations above the reference interval, although no differences in proportions compared to controls were observed ($P > 0.976$). Urine protein to creatinine values were higher in envenomated dogs between T2 and T3 ($P < 0.015$, Figure 1E). Urine protein to creatinine values at T1, T4, and T5 were similar to the healthy control dogs ($P > 0.143$, Figure 1E).

AKI Biomarkers and Snake Bite Severity Score

The 16-point SSS was collapsed into four groups (0, 1-3, 4-7,8-12, 13-16). A weak correlation was found between uClusterin concentration at the first timepoint for each subject and SSS (Spearman's $\rho = 0.365$, $P < 0.034$, Figure 2A). A correlation was found between uCysB concentration at the first timepoint and SSS (Spearman's $\rho = 0.690$, $P < 0.001$, Figure 2B).

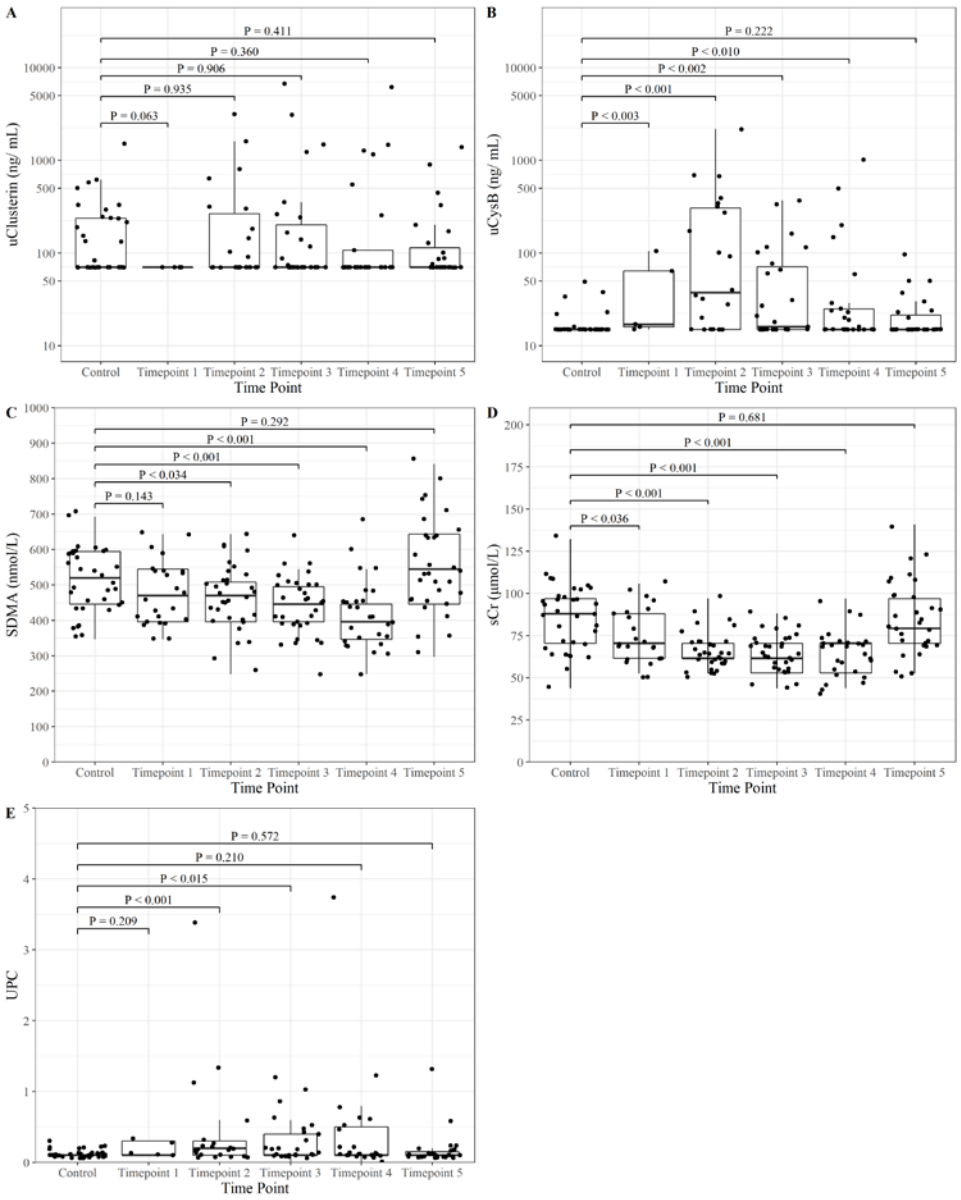

Figure 1. Boxplot of A) uClusterin, B) uCysB, C) SDMA, D) sCr, E) UPC values across time compared to control group

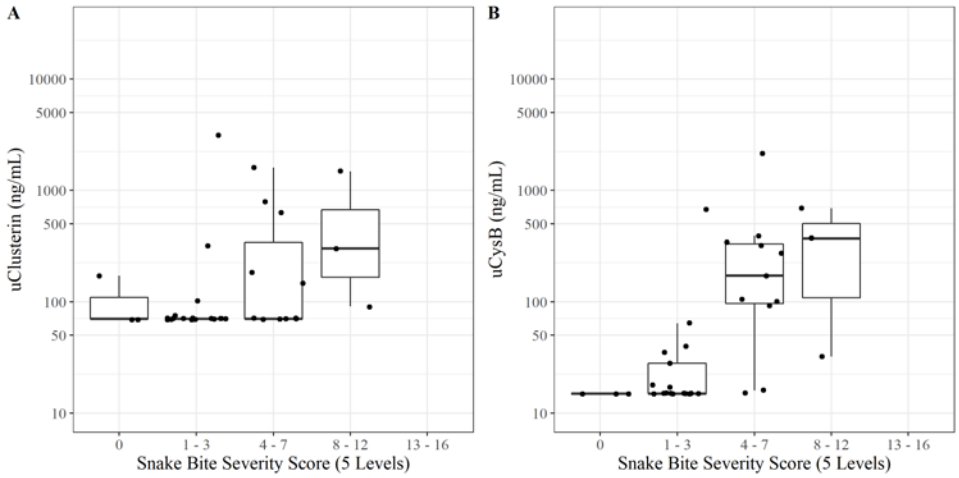

Figure 2. Boxplot of A) uClusterin and B) uCysB values at first available time point compared to 5-point SSS

Discussion

To our knowledge, this is the first study to evaluate serial uCysB and uClusterin concentrations in dogs envenomated by the European adder. The finding of increased uCysB levels in envenomated dogs during the first 36 hours after bite compared to healthy controls and normalised values after 14 days, might indicate transient renal tubular injury in these dogs in the absence of overt renal dysfunction. The finding of a possible renal tubular injury in this study is corroborated by elevations in biomarkers of renal injury in two previous studies of *V.berus* envenomated dogs [7, 24].

Absolute uClusterin concentrations were not significantly different in envenomated dogs compared to controls in our study. Urinary clusterin has better diagnostic performance for minimal tubular injury than sCr in rats [20], but studies evaluating the diagnostic performance of uClusterin compared to uCysB are lacking in that species. Clusterin is upregulated and released into the urine in response to renal damage [31], whereas CysB is an intracellular protein that leaks into urine upon tubular cell injury [19]. In rats, uClusterin concentrations are associated with the severity of tubular cell injury [32]. It is possible that a mild injury occurs after snakebite that causes leakage of cysB into urine but that is of insufficient magnitude to stimulate upregulation of clusterin expression, or that the time course of clusterin upregulation and appearance in urine was missed by the

sampling window in this study. Of note, many of the absolute uCusterin concentrations were below the LOQ, and these values were imputed as the LOQ value. When uClusterin values were normalised to urine creatinine, a significant difference was found between envenomated dogs and controls at T3 and T4, more in fitting with the uCysB findings and thus supporting a diagnosis of renal tubular injury. These normalised results should, however, be interpreted with caution given that they are driven in large part by the urine creatinine concentration alone due to the high number of uClusterin values below the LOQ for the assay. Thus, for uClusterin and uCystatin B values below the LOQ, all movement in the normalized markers was dictated by their urine creatinine values. Sample size is also a limitation of this study that may explain a lack of significant difference in uClusterin concentrations between cases and controls.

Interestingly, a recent study did find increased levels of non-normalized uClusterin in *V.berus* envenomated dogs compared to healthy controls [24]. There are several possible reasons for our contrasting uClusterin findings. The other study evaluating uClusterin in dogs bitten by *V.berus* included a single measurement only for each dog, and the timing after bite varied from 2.5 hours to 4 days [24], thus, more peak uClusterin measurements may have been included compared to our study where each standardised timepoint was compared to the control group. Other possible explanations for the contrasting findings may include differences in venom composition, dose and individual susceptibility may also account for the contrasting findings.

Cystatin B is a low molecular weight (11 kDa) ubiquitous intracellular protein [33]. Thus, we cannot absolutely rule out a systemic contribution via free glomerular filtration, to the increased uCysB concentrations observed in our study. Serum CysB measurement would help to establish whether there is a systemic contribution to uCysB, but such an assay has not yet been developed.

Urinary CysB concentrations were increased in the absence of a detectable decrease in GFR estimated using sCr and SDMA, with concentrations of these biomarkers lower in envenomated dogs compared to controls in our study. Haemodilution due to IVFT is the most likely explanation for the finding of lower SDMA and sCr concentrations in envenomated dogs 0-36 hours after bite compared to controls. This is supported by the fact that compared to controls, no significant difference was found 14 days after bite, when envenomated dogs were no longer receiving IVFT.

Similar to our study, studies of *V. berus* and *Vipera palaestinae* envenomated dogs have failed to show increases in sCr and SDMA after snakebite [24, 34, 35]. In our study, as well as others [24, 35], sCr and SDMA concentrations were likely influenced by treatment with IVFT and we cannot rule out that azotaemia may have been demonstrated should the dogs not have been receiving IVFT. One study of 12 dogs envenomated by *V.palaestinae,* measuring sCr concentrations prior to treatment, also found all dogs to have sCr levels within the reference range [34]. However, the small number and lack of serial measurements preclude generalisation of these results. In another study, one of 28 dogs envenomated by *V.palaestinae* had a sCr concentration above the reference interval within the first 24 hours after bite [36]. However, neither the nature (renal or prerenal) nor the magnitude of azotaemia were described. Results from our study, as well as others, indicate that sCr and SDMA levels have limited use in investigating AKI in envenomated dogs, possibly due to a lack of renal dysfunction or treatment with IVFT.

Urinary protein to creatinine ratio has low specificity for determining the source of proteinuria and injury severity, although UPC values of two or more are generally associated with glomerular rather than tubular disease in the dog [37]. Our finding of an increased UPC 12-24 hours after snakebite is similar to that of previous studies [7, 24], and likely encompasses the increase in uCysB, but not exclusively. Renal tubular injury can lead to increased UPC through decreased reabsorption of filtered proteins, leakage from damaged tubular cells or increased local production in response to injury [38]. Thus, the increased UPCs in our study may be supportive of tubular injury in envenomated dogs. However, prerenal causes of proteinuria cannot be ruled out, especially given the observation of haemoglobinuria and myoglobinuria in some dogs in this study. Whilst two dogs had UPC values above three, one was observed to have myoglobinuria, and the other haemoglobinuria. Thus, prerenal proteinuria rather than glomerular damage might have been the cause of increased UPC in these two dogs. Two dogs had persistent SAP values > 160mmHg and were therefore at moderate risk of target organ damage. We cannot rule out that hypertension may have contributed to proteinuria in these individuals.

The finding of a correlation between SSS and CysB in our study has a potential clinical application. One other study of *V. berus* envenomated dogs also found a correlation between severity of clinical signs and other AKI markers [7]. It would be useful to further investigate the potential of the SSS used in our study to predict AKI in *V. berus* envenomated dogs, and thus help guide treatment and monitoring.

There are limitations to this study. As already discussed, sample size and the high numbers of samples with uClusterin and uCysB concentrations below LOQ limit the statistical inferences that can be made, and particularly influencing the normalised values of uClusterin. Because values below the LOQ were censored, any potential differences in concentrations on the low end of the assay were unobservable. The validation work for the uClusterin and uCysB assays used in this study, is unpublished and although the refence intervals used are based on urine from 78 heathy dogs, they are preliminary intervals only and thereby not formulated according to the American Society for Veterinary Clinical Pathology (ASVCP) reference interval guidelines. Antivenom treatment is a confounder in our study. Given the small number of dogs that did not receive antivenom, statistical testing of renal biomarkers between treated and untreated groups was not performed. Renal histopathology would have been a useful addition to this study but was not performed due to ethical considerations. The systemic contribution to uCysB is not known. Further work is needed to establish whether cellular injury and systemic inflammation induced by a snake bite increase serum concentrations of cystatin B and thus increase uCysB, since it is freely filtered at the glomerulus. Additional limitations include the use of only one timepoint in the control group, and the small number of samples collected at time of presentation. These limitations restrict the interpretability of this study.

In the present study, we primarily report absolute values of uClusterin and uCysB. Urinary biomarkers are traditionally normalised to urinary creatinine to account for urine flow. Whilst a consensus on whether to normalise to creatinine does not currently exist, it has been suggested that this approach may be less than ideal in AKI where creatinine clearance might change acutely. Biomarkers normalised to creatinine and absolute values have shown good correlation in recent studies [24, 39, 40]. Given that the envenomated dogs in our study were receiving IVFT and were hyposthenuric compared to the control dogs, the magnitude of the difference in biomarker concentration between envenomated dogs and controls may be underestimated in our study. The difference in findings between absolute and creatinine-normalized uClusterin results may reflect this dilution effect, although it may also be due to the high number of values below the LOQ for the assay and highlights the need for further studies of early stage or mild AKI will help to establish a consensus regarding normalisation of urinary AKI biomarkers to urinary creatinine.

Conclusions

Urinary CysB concentrations were increased in dogs envenomated by *V.berus* in comparison to controls and may indicate renal tubular injury in these patients. However, the specificity of uCysB for diagnosing AKI needs clarification through further studies. Given the lack of absolute uClusterin findings in this study, the evidence for AKI is less compelling than in another study of *V.berus* dogs using the same biomarkers [24]. Additional studies are needed to elucidate the utility of uClusterin and uCysB in different settings and to definitively conclude whether dogs sustain renal tubular injury after *V.berus* envenomation.

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Declarations

Ethics approval and consent to participate

This study was approved by the local ethical committee at the Norwegian University of Life Sciences (NMBU) and permitted by the Norwegian Research Authority. Written owner consent was obtained for all dogs prior to inclusion in the study.

Availability of data and materials

The datasets used during the current study are available from the corresponding author on reasonable request.

Competing interests

Donald Szlosek, Rachel Murphy Hanne Friis and Sarah Peterson are employees at IDEXX Laboratories, Inc., Westbrook, Maine where the assays for urinary clusterin and urinary cystatin B are currently in development.

Funding

This study was funded in part by Arkitekt Finn Rahns Legat, Oslo, Norway and supported by IDEXX Laboratories, Inc., Westbrook, Maine.

Authors' contributions

HH planned the study, collected and coordinated analysis of data, and was a major contributor in writing the manuscript.

KPA contributed to the planning of the study, data interpretation and manuscript revision.

JH contributed to planning of the study, data collection and manuscript revision.

ERM contributed to the planning of the study, data interpretation and manuscript revision.

DS analysed and interpreted data and contributed to manuscript revision.

RM contributed to study planning, methodology and manuscript revision.

HF contributed to manuscript revision.

SP contributed to study planning and manuscript revision.

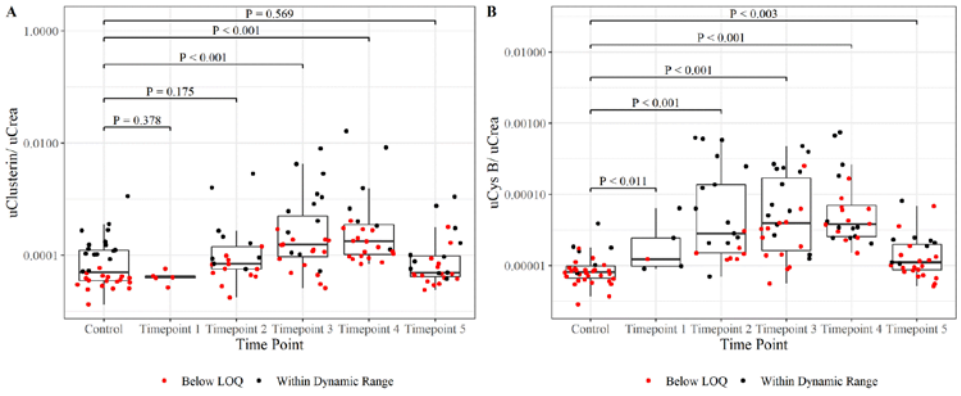
RR was a major contributor to study planning, data interpretation and manuscript revision.

All authors have read and approved the final manuscript. HH is the corresponding author.

Acknowledgements

The authors would like to thank staff, students and dog owners at the small animal hospital at the Faculty of Veterinary Medicine at NMBU, Evidensia Oslo Dyresykehus, Anicura Dyresykehus Oslo and Anicura Jeløy Dyresykehus for the care and provision of dogs in this study.

Appendix



Supplementary Figure 1. Boxplot of A) uClusterin and B) uCysB normalized by urinary creatinine values across time and study group.

Paper IV

Persistent hypercoagulability in dogs envenomated by the European adder (*Vipera berus berus*).

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Abstract

Background

Envenomation by the European adder, *Vipera berus berus (Vbb),* is a medical emergency. The overall *in vivo* haemostatic effects of pro- and anticoagulant toxins in *Vbb* venom, and the downstream effects of cellular injury and systemic inflammation, are unclear.

Objectives

To longitudinally describe the global coagulation status of dogs after *Vbb* envenomation and compare to healthy controls. A secondary aim was to investigate differences between dogs treated with and without antivenom.

Methods

Citrated plasma was collected at presentation, 12 hours (h), 24 h, 36 h and 15 days after bite from 28 dogs envenomated by *Vbb*, and from 28 healthy controls at a single timepoint. Thrombin generation (initiated with and without exogenous phospholipids and tissue factor), thrombin-antithrombin (TAT)-complexes and the procoagulant activity of phosphatidylserine (PS)-expressing extracellular vesicles (EVs), expressed as PS-equivalents, were measured.

Results

At presentation the envenomated dogs were hypercoagulable compared to controls, measured as increased thrombin generation, TAT-complexes and PS-equivalents. The hypercoagulability decreased gradually but compared to controls thrombin generation and PS-equivalents were still increased at day 15. The discrepancy in peak thrombin between envenomated dogs and controls was greater when the measurement was phospholipid-dependent, indicating that PS-positive EVs contribute to hypercoagulability. Lag time was shorter in non-antivenom treated dogs, compared to antivenom treated dogs <24 h after envenomation.

Conclusions

Hypercoagulability was measured in dogs up to 15 days after *Vbb* envenomation. Dogs treated with antivenom may be less hypercoagulable than their non-antivenom treated counterparts. Thrombin generation is a promising diagnostic and monitoring tool for *Vbb* envenomation.

Introduction

The European adder, *Vipera berus berus (Vbb),* is a category two medically important snake, responsible for approximately 60% of all *Vipera* species envenomations in humans in Europe [1, 2]. Dogs are more commonly bitten by *Vbb* than humans, and as such provide a natural, accessible model for the effects of envenomation. Since *Vbb* is the only venomous snake in Scandinavia [2, 3], clinical signs of snake envenomation in these countries can be attributed to this species alone. Pain, oedema, nausea, arrhythmia, acute kidney injury and coagulopathy have been reported in humans and dogs [1, 4-9], but studies of *Vbb* envenomation are limited, and the clinical consequences of envenomation by this species are not fully known. Case reports have described limb and pulmonary thrombosis in humans [6, 10] and microthrombi in the renal vasculature of dogs [11] bitten by *Vbb*. However, persistent bleeding from the snakebite wound, haematomas and ecchymoses have also been reported in dogs and humans bitten by this species of snake [4, 12, 13].

Vbb venom contains both pro- and anti-coagulant toxins such as phospholipase A_2 (PLA_2), thrombin-like enzymes, factor X (FX)-activating enzymes and platelet aggregate inhibitors [14-17], the net effect of which is not known. In addition to direct effects of toxins, downstream effects of systemic inflammation and cellular injury, including shedding of extracellular vesicles (EVs) from activated and apoptotic cells, may also lead to clinical and laboratory coagulation abnormalities. EVs carry procoagulant phospholipids (PLs) such as phosphatidylserine (PS), and some also carry tissue factor (TF). The most procoagulant ones carry both PS and TF [18]. As circulating entities, EVs are able to initiate and propagate thrombin generation systemically.

Coagulation analyses may help identify individuals at risk of haemorrhage or thromboembolic disease and thereby contribute to appropriate treatment, but studies reporting such analyses after *Vbb* envenomation are sparse and contradictory. Studies in humans and dogs have shown thrombocytopenia, prolonged prothrombin time (PT)/international normalized ratio (INR), and prolonged activated partial thromboplastin time (APTT), but hypercoagulability indicated by decreased INR and increased D-dimer concentrations has also been reported during the first 36 hours after *Vbb* bite [4, 12, 19]. Longitudinal studies assessing the time course of coagulation disturbances after envenomation by *Vbb* as well as the effect of antivenom treatment, are lacking.

Thrombin generation assays have proven useful to assess the *in vitro* thrombin-generating capacity of venoms, and the efficacy of antivenoms [20, 21]. However, to our knowledge, measurements of thrombin generation have not been performed in humans or dogs after envenomation by any snake species.

The primary aim of this study was to serially assess the global coagulation status of dogs envenomated by *Vbb,* compared to a group of healthy control dogs. We measured thrombin generation (initiated with and without exogenous phospholipids and TF), thrombin-antithrombin (TAT) complexes and the phospholipid-dependent activity of PS-exposing EVs. A secondary aim was to investigate differences in the global coagulation status of dogs treated with and without antivenom.

Materials and Methods

Study design and approval

A total of 49 dogs bitten by *Vbb* and presenting to the first opinion emergency services at the small animal hospitals at the Norwegian University of Life Sciences (NMBU), Evidensia Oslo Dyresykehus, and Anicura Dyresykehus Oslo between April and October in 2017 and 2018 were assessed for enrolment in the study. Inclusion in the study required a diagnosis of snake bite based on history, presence of consistent clinical signs at presentation (fang marks, local or systemic signs of envenomation), a minimum body weight of 10 kg, lack of significant chronic disease, and no medications.

Twenty-eight privately-owned dogs, not previously bitten by *Vbb,* were recruited as healthy control dogs. Inclusion in the study required a minimum bodyweight of 10 kg, lack of significant acute or chronic disease, and no medication in the preceding two weeks. Healthy status was determined based on information obtained from the owner, lack of clinically significant abnormalities on physical examination, haematology and biochemistry profiles, and a C-reactive protein concentration within the reference interval.

This study was approved by the local ethical committee at NMBU and permitted by the Norwegian Research Authority. Written owner consent was obtained for all dogs prior to inclusion in the study.

Sample collection and processing

Blood was drawn from all envenomated dogs at five timepoints (T) after bite. T1 presentation: median 3.5 hours (h) (range 0.5-7.5 h), T2: 12 h (9-16.5 h), T3: 24 h (22-25.5 h), T4: 36 h (32-39 h) and T5: 15 days (10-32 days). Blood was collected from the cephalic, saphenous, or jugular vein through an indwelling venous catheter or 21-G winged needle with extension, into citrate tubes and centrifuged within 15 minutes at 2700 x g for 15 minutes. Top layer plasma was pooled, aliquoted into cryotubes and stored at -80°C for a maximum of 24 months prior to analysis. In healthy control dogs citrated plasma was collected, prepared and stored as for the envenomated dogs, and analysed within 4 months.

Thrombin generation

Thrombin generation was measured using the calibrated automated thrombogram (CAT) [22]. Eighty µL of citrated plasma was mixed with 20 µL reagent in a 96-well plate (Thermo Immulon 2HB plate, Thermo Scientific). To measure the overall thrombin-generating capacity of different counteracting constituents of plasma, for example coagulation factors and inhibitors such as antithrombin (AT) and tissue factor pathway inhibitor (TFPI), pro- and anticoagulant content of the venom, and presence of TF- and PS-exposing EVs, we used different reagents: PPP low reagent (1 pM TF, 4 µM PLs), PRP reagent (1 pM TF, minimal amounts of PLs), and no exogenous reagent (Tris-buffered saline (TBS)), Sigma Aldrich (St. Louis, MO, USA). To avoid excessively rapid initiation of thrombin generation, we diluted the PPP low reagent 1+1 with MP reagent (no TF, 4 µM PLs) and the PRP reagent 1+1 with TBS, to obtain half the concentration of TF (but with sustained concentration of the PLs). Thrombin generation was initiated by automated addition of 20 µL FluCa buffer containing calcium (Ca^{2+}) and a fluorogenic thrombin substrate. Fluorescence was read for 60 min in a Fluoroscan Ascent microplate reader (Thermo Scientific, MA, USA), and the thrombin generation parameters lag time (LT), peak thrombin, and endogenous thrombin potential (ETP) were calculated by the Thrombinoscope software (Thrombinoscope BV, Maastricht, the Netherlands). All reagents for the thrombin generation experiments were from Thrombinoscope BV (Maastricht, Netherlands).

Procoagulant activity of phosphatidylserine (PS)-exposing extracellular vesicles (EVs)

Citrated plasma samples were thawed (10 min, 37°C) and lightly mixed before the activity of PS-exposing EVs was analysed with the Zymuphen MP activity kit (Hypen BioMed, Neuville-sur-Oise, France) according to the manufacturer's protocol. Plasma samples had been thawed once previously. In samples with values above the highest standard (60 nM), the software extrapolated the amount of PS equivalents (nM), and the absolute values above 60 nM should therefore be interpreted with caution.

Thrombin-antithrombin (TAT) complexes

Citrated plasma samples were thawed for 15 minutes at 37°C and gently mixed before the concentration of TAT complexes was determined with a sandwich enzyme immunoassay (Enzygnost®TAT micro, Siemens Healthcare Diagnostics) according to the manufacturer's protocol. Plasma samples had been thawed once previously. The standards included in the Enzygnost®TAT micro kit ranged from 2.0 to 60 µg/L. In samples with values above the highest standard, the software extrapolated the concentration of TAT, and the absolute values above 60 µg/L should therefore be interpreted with caution.

In vitro effect of venom and antivenom

To investigate whether the *in vitro* effect of *Vbb* venom was pro- or anticoagulant, and whether the presence of antivenom might interfere in thrombin generation measurements, venom or antivenom were added to pooled plasma from three to five healthy controls, and thrombin generation was immediately initiated. Pooled *Vbb* venom (Siberian Serpentarium Ltd, Novosibirsk, Russia) was added at concentrations of 0, 12.5, 25 and 50 µg/mL, and antivenom (Viper Venom Antitoxin, SIS Biomed®, Warsaw, Poland) at concentrations of 0, 0.25, 0.5 and 1 U/mL.

Statistical Analysis

Creation of figures and statistical analysis were performed using commercially available statistical software packages (JMP Pro 14.3.0, SAS Institute Inc, Cary, NC and GraphPad Prism 8.3.1, GraphPad Software LLC, San Diego, CA). Data were visually assessed for

normality. Most data were not normally distributed, thus median and range are reported throughout. Comparisons of LT, ETP, peak, PS equivalents and TAT complexes between the control group and each of the timepoints for the whole group of envenomated dogs and for dogs treated with and without antivenom, were performed using Steels test for multiple comparisons. Comparisons between dogs with and without antivenom treatment at each timepoint were made using Wilcoxon rank sum test. Fisher's exact test was used to compare proportions of values in envenomated dogs that were above (peak, ETP) or below (LT) the control group values at presentation, between reagents.

Repeated measurements of outcome variables (thrombin generation, PS and TAT complexes) in envenomated dogs were analysed using a generalized linear mixed model with dog as a random effect, timepoint and antivenom treatment as fixed effects, using Stata SE 16.0 (Statcorp LLC, Texas, USA). Data was analysed using gaussian, gamma and negative binomial distributions along with the appropriate link function. The distribution showing the best fit, determined by an examination of the residuals as well as information criterion (AIC/BIC), was used to analyse the data. Pairwise comparisons were made between adjacent timepoints for the envenomated dogs. Post hoc Bonferroni correction was applied.

Results

Demographic data

The study population consisted of 28 of the 49 dogs originally enrolled. Four dogs were excluded for the following reasons: treatment with non-steroidal anti-inflammatory drugs (n=1), presentation more than 24 hours after bite (n=2) and no clinical signs of envenomation within 12 hours after bite (n=1). Seventeen dogs, of which most had samples drawn from an indwelling venous catheter, were excluded due to macroscopic haemolysis at all sampling timepoints. Of the remaining 28 dogs, individual samples were excluded due to macroscopic haemolysis at the following timepoints: T3 (n=1), T4 (n=3) and T5 (n=5). Samples were collected through an indwelling venous catheter in 6/28 dogs and via a winged needle with extension in 22/28 dogs. Further demographic data for the snake-bitten and healthy control dogs are presented in table 1.

Table 1: *Demographic data (median and range) for envenomated dogs and the control group.*

	Envenomated dogs	Control group
Number	28	28
Age (years)	4.8 (0.3-13)	5 (0.8-12)
Weight (kg)	24.6 (13.5-43.0)	24.7 (11.0-50.0)
Sex (male:female)(n)	19: 9	15: 13
Bite to presentation (hours)	1.75 (0.25-8.50)	
Bite location (head:limb) (n)	22: 6	
Bite witnessed (n)[a]	17	
Antivenom treatment[b] (yes:no) (n)	16: 12	
Bite to antivenom (hours)	4.75 (1.0-20.50)	

[a]snake, snakebite or fang marks witnessed. For the remaining dogs, a diagnosis was made based on history and compatible clinical signs

[b]Equine F(ab')$_2$ antivenom intravenously (Viper Venom Antitoxin, SIS Biomed®, Warsaw, Poland)

Physical examination and treatment

Physical examination findings were recorded at T1, T3 and T5 for envenomated dogs. Varying degrees of local swelling were present in all dogs at T1. Swelling had resolved in one dog at T3 and in all but one dog at T5 in which a moderate swelling persisted. Haematomas and bleeding from the bite site were recorded in two dogs at T1. At T3, extensive subcutaneous extravasation of blood and ecchymoses were recorded in two dogs. Overt clinical signs of thromboembolic disease were not observed in any dog at any timepoint.

Envenomated dogs were treated using a routine protocol including crystalloid fluid therapy intravenously (IV) at a standard rate of 4 mL/kg/hour and adjusted according to individual requirements, and buprenorphine (Vetergesic vet®, Ceva Santé Animale, France) at a dose of 0.01-0.02 mg/kg IV, intramuscularly (IM) or subcutaneously (SC) or

methadone (Metadon, Norges Apotek, Norway) at a dose of 0.1-0.2 mg/kg IV or SC. Antivenom (Viper Venom Antitoxin, SIS Biomed®, Warsaw, Poland) was administered IV (n=16) over one to two hours, at a dose of 500 units diluted in 250 mL 0.9% NaCl, within the first 24 hours after bite (table 1). Indications for antivenom treatment included severe local or systemic signs or a lack of improvement or deterioration despite supportive treatment in dogs with mild local or systemic signs. Treatment decisions were made by the attending clinician.

Missing data

When thrombin generation was initiated without an exogenous reagent, the thrombin generation curve did not reach the baseline in nine samples from envenomated dogs and for 15 control dogs. These ETP values are therefore missing in statistical analysis and peak values may be slightly overestimated in these individuals. Likewise, with PRP reagent, ETP is missing for three samples from envenomated dogs and for eight control dogs. For PPP reagent, LT was too fast to be registered by the software in five T1 samples from envenomated dogs. These LT values were arbitrarily set to 1.33 minutes, i.e. the lowest LT registrable by the software, and included in statistical analysis. In these five cases peak thrombin and ETP could not be calculated due to the lack of a starting point and thus are missing. Statistical comparisons between antivenom and non-antivenom treated dogs at T1 and between antivenom treated dogs at T1 and controls were not made due to only two dogs in the antivenom treated group.

Thrombin generation in envenomated dogs and healthy controls

With PPP low reagent, LT was significantly shorter at T1 and T5 in envenomated dogs compared to controls. In the envenomated dogs, LT increased significantly from T3 to T4 and then decreased from T4 to T5. With PRP reagent, LT was significantly shorter at all timepoints (T1-T5) in envenomated dogs compared to controls and decreased significantly between T4 and T5. For no exogenous reagent, LT was shorter at T1-T3 and T5 in envenomated dogs compared to controls, with significant increases occurring from T1 to T2 and from T3 to T4 (figure 1A-C, supplementary table 1). With all reagents, including no exogenous reagent, peak and ETP were significantly higher at all timepoints for envenomated dogs than for controls, and decreased significantly between T3 and T4 (figure 1 D-I, supplementary table 1).

We also explored which reagent differentiated best between the control group and envenomated dogs at presentation. When thrombin generation was initiated with PRP or no exogenous reagent (i.e. both without PLs), proportions of envenomated dogs with peak thrombin values above the highest control value were significantly higher, compared to when thrombin generation was initiated with the phospholipid-containing PPP low reagent (both P = 0.003, table 2). For ETP, a similar pattern was observed, although proportions of dogs with values greater than the maximum control value did not differ significantly between the reagents after Bonferroni correction (both P = 0.07, table 2). For LT, the highest proportion of envenomated dogs with LT below the lowest control value was found with no exogenous reagent added, although proportions did not differ significantly between reagents.

***Table 2**: Proportion of envenomated dogs with values for lag time (LT) below the minimum value for the control group, and peak thrombin and endogenous thrombin potential (ETP) values above the maximum value for the control group, at presentation, for each reagent (PPP low, PRP and no exogenous reagent).*

	PPP low	PRP	No exogenous reagent
LT	68% (15/22)	68% (15/22)	77% (17/22)
Peak thrombin	18% (3/17)	73% (16/22)*	73% (16/22)*
ETP	24% (4/17)	62% (13/21)	62% (13/21)

* indicates a significant difference (P < 0.05) compared to PPP low, after Bonferroni correction.

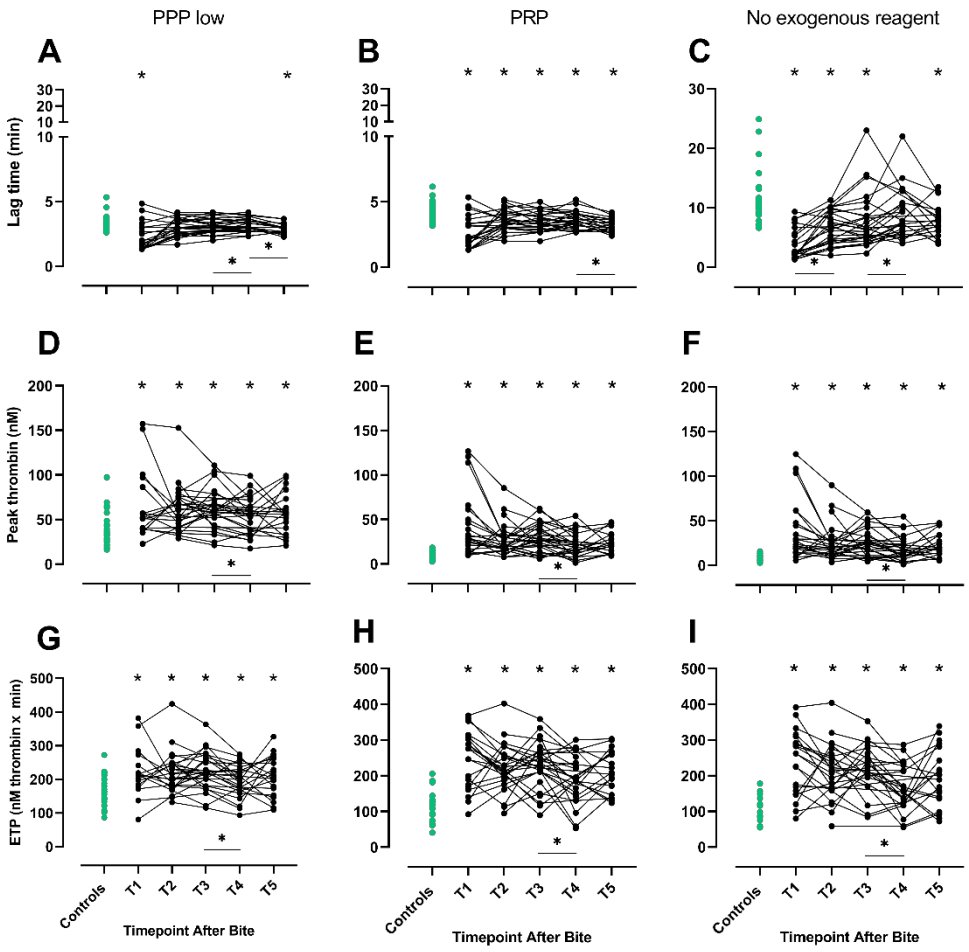

Figure 1: Thrombin generation parameters in envenomated dogs and controls.

*Lag time, peak thrombin and endogenous thrombin potential (ETP) measured by calibrated automated thrombography with three reagents, PPP low, PRP and no exogenous reagent, in envenomated dogs at five timepoints after bite (black dots) and healthy controls (green dots). T1 = presentation, T2= 12 hours, T3 = 24 hours, T4 = 36 hours and T5 = 15 days after bite. * indicates P < 0.05 for comparisons between envenomated dogs at each time point and the control group. Bar with star indicates a significant change between timepoints for envenomated dogs (P < 0.05).*

Thrombin generation in envenomated dogs with and without antivenom treatment

The difference between dogs treated with and without antivenom was best observed with LT. With all reagents, LT was significantly shorter in dogs not treated with antivenom compared to antivenom treated dogs at T2 and T3 (all P values ≤ 0.03, figure 2 A-C). In the antivenom treated dogs, LT did not differ significantly from the control group at these timepoints with any of the reagents (all P values ≥ 0.07). Statistical analysis was not performed at T1 due to only two dogs treated with antivenom at this timepoint. For peak thrombin and ETP, there were no differences between dogs with and without antivenom-treatment, except for at T4 with PPP low reagent, where peak was significantly higher for the antivenom treated dogs compared to dogs not treated with antivenom (P = 0.03, supplementary figure 1A), and at T2 with no exogenous reagent, where ETP was significantly higher in dogs that did not receive antivenom compared to those that did (P = 0.03, supplementary figure 1F).

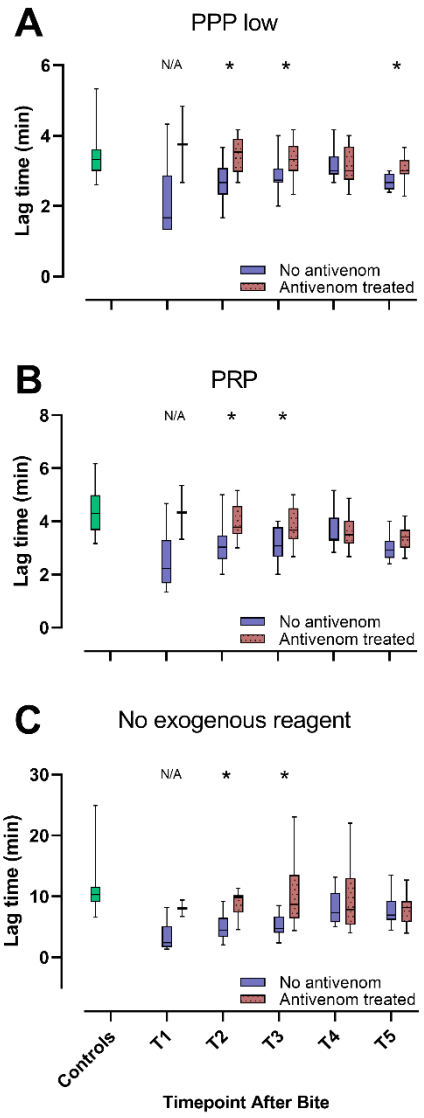

Figure 2 : Lag time in dogs treated with and without antivenom. *Box and Whisker plots for lag time with three different reagents (PPP low (A), PRP (B) and no exogenous reagent (C)), in dogs treated with (red) and without (blue) antivenom, and healthy controls (green). T1 = presentation, T2 = 12 hours, T3 = 24 hours, T4 = 36 hours and T5 = 15 days after bite.* indicates a significant difference (P < 0 .05) between treatment groups at a given timepoint. Statistical analysis was not performed at T1 due to a low number of antivenom treated dogs (n=2). N/A = not analysed.*

Phosphatidylserine (PS) equivalents

The procoagulant activity of PS-expressing EVs was measured as PS equivalents. Compared to the control group, PS equivalents were significantly higher in envenomated dogs at T1-T3 and T5 (figure 3, supplementary table 2). Levels of PS equivalents increased significantly from T4 to T5 (P = 0.024, figure 3). PS equivalents were significantly higher at T3 in dogs not treated with antivenom compared to those that received antivenom (P = 0.02, supplementary figure 2).

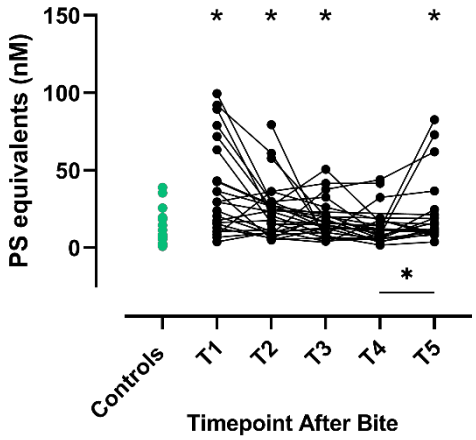

Figure 3: Phosphatidylserine equivalents in envenomated dogs and controls.

Phosphatidylserine (PS) equivalents in envenomated dogs at five timepoints after bite (black dots) and controls (green dots). T1 = presentation, T2= 12 hours, T3 = 24 hours, T4 = 36 hours and T5 = 15 days after bite. indicates P < 0.05 for comparisons between envenomated dogs at each time point and the control group. Bar with star indicates a significant change between timepoints for envenomated dogs (P < 0.05).*

Thrombin-antithrombin (TAT) complexes

Compared to the control group, concentrations of TAT complexes were significantly increased at T1-T4 (figure 4, supplementary table 2). In envenomated dogs, a significant decrease in TAT was observed between T4 and T5 ($P < 0.0001$, Figure 4), and at T5 the TAT concentration was similar to the controls. TAT values were not significantly different between treatment groups except at T3 where TAT was higher in the non-antivenom treated dogs ($P = 0.002$, supplementary figure 3).

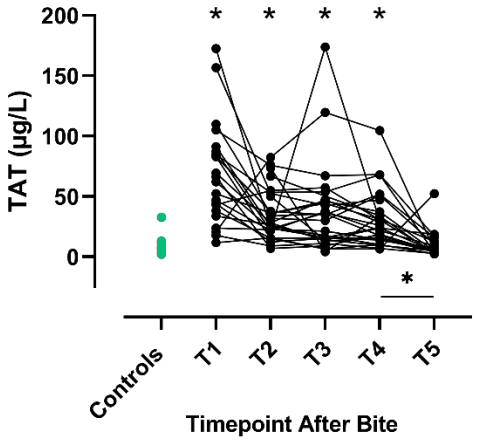

Figure 4 : Thrombin-antithrombin complexes in envenomated dogs and controls.
Concentrations of thrombin-antithrombin (TAT) complexes in envenomated dogs at five timepoints after bite (black dots) and controls (green dots). T1 = presentation, T2= 12 hours, T3 = 24 hours, T4 = 36 hours and T5 = 15 days after bite. indicates $P < 0.05$ for comparisons between envenomated dogs at each time point and the control group. Bar with star indicates a significant change between timepoints for envenomated dogs ($P < 0.05$).*

In vitro effect of venom and antivenom

Overall, antivenom did not appear to affect the thrombin generation curves (supplementary figure 4). When plasma was incubated with venom, thrombograms were unable to be generated due to rapid initiation of thrombin generation, even at the lowest venom concentration, indicating that the venom has procoagulant characteristics (data not shown).

Discussion

In this longitudinal study we measured the global coagulation status of dogs envenomated by *Vbb*, from presentation to median 15 days after bite. At presentation, the envenomated dogs were hypercoagulable compared to the healthy control dogs, as demonstrated by increased thrombin generation, TAT complexes and PS equivalents. Hypercoagulability gradually decreased but persisted at day 15, except for TAT complexes. LT was normalised in dogs treated with antivenom, suggesting that treatment may counteract hypercoagulability.

The most pertinent finding was that the envenomated dogs were hypercoagulable at presentation compared to the healthy control dogs. In contrast to our study, most previous studies using PT, INR and APTT are indicative of hypocoagulability after *Vbb* envenomation [4, 12, 19]. Prolongation of PT and APTT is used in human and veterinary medicine as a reliable indicator of envenomation in cases of suspected snake bite, although normal values do not exclude the possibility of envenomation [23]. One study reported short INR values at presentation in 2/119 humans bitten by *Vbb,* indicating hypercoagulability [4, 24]. Short PT and APTT values were also reported in one *in vitro* study of *Vbb* venom [25]. Due to the inherent insensitivity of PT and APTT to detect a procoagulant state, hypercoagulability after *Vbb* bite has likely been underestimated in previous studies using these routine clotting analyses [26]. Thrombin generation has not previously been measured in dogs after envenomation by *Vbb* or any other snake species*,* but one study has used thrombin generation to evaluate *in vitro* haemostatic changes caused by venom of several Brazilian snakes after addition to pooled normal plasma [20]. The authors concluded that PT and APTT were only able to detect mild hypocoagulable states, whilst thrombin generation measured by CAT could detect both hypo- and hypercoagulability. Measurement of thrombin generation may therefore give a

more complete picture of the global coagulation status after envenomation than routine clotting analyses.

Tissue injury and ongoing inflammation after snakebite [27, 28] may contribute to the release of procoagulant EVs [18]. The discrepancy in peak thrombin levels between healthy controls and envenomated dogs at presentation was greater when thrombin generation was phospholipid-dependent (i.e. using PRP reagent or no exogenous reagent), compared to phospholipid-independent (using PPP low reagent), indicating that PL-rich EVs may contribute to increased thrombin generation in envenomated dogs. This is also supported by the increased procoagulant activity of PS-exposing EVs (PS equivalents) in envenomated dogs at most timepoints, compared to the control group.

The repeated measurements in our study enabled us to detect a persistent hypercoagulability not previously described after *Vbb* envenomation. We demonstrated that *Vbb* venom was procoagulant *in vitro* but data regarding the distribution and half-life kinetics of *Vbb* venom have not been published. However, the venom composition of *Vipera aspis (V. aspis)* is similar to that of *Vbb* [29] and it is reasonable to assume they show similar elimination kinetics. The half-life of *V. aspis* venom in humans is eight hours [30], but case reports pertaining to other snake species have described a suspected depot effect of venom, allowing toxins to persist for weeks after envenomation [31, 32]. Thus, the venom could possibly contribute to the prolonged hypercoagulability observed in our study. Despite the persistent hypercoagulability in the plasma of dogs 15 days after bite in our study, we observed both clinical improvement and normalised TAT complex concentrations at this timepoint, indicating that there might be a difference between potential and actual thrombin generation in these dogs.

Clinical studies in rabbits and mice describe neutralisation of *Vbb* venom by antivenom [33, 34], including a reduction in local haemorrhagic effects, but there are no studies focusing on laboratory analyses of coagulation in the context of antivenom efficacy after *Vbb* bite. However, *in vitro* thromboelastography measurements in canine whole blood showed early hypercoagulable changes when exposed to venom of another viper, *Vipera palastinae* , that were annulled by addition of specific antivenom [35]. Antivenom treated dogs in our study had LTs similar to those of the control group, and significantly longer LTs than the non-antivenom treated dogs during the first 24 hours after bite. Whilst our study design does not allow us to imply causation, this is nevertheless a very interesting finding which warrants further investigation especially in light of the current lack of evidence supporting the use of antivenom in treating dogs with *Vbb* bites [36]. Significant

differences between the antivenom treated group and the non-treated group were almost exclusively observed with LT, and not peak or ETP, the reason for which is unknown. We cannot exclude that a confounding effect such as coagulation factor depletion may have been present in the envenomated dogs selected for treatment with antivenom. Selective depletion of coagulation factors important in the initiation phase of coagulation could potentially result in longer LTs without affecting peak and ETP. A neutralising effect of antivenom on venom components that specifically influence the initiation phase of thrombin generation is another possible explanation. A randomized control trial (RCT) of antivenom efficacy and further characterisation of the coagulation factor activities in envenomated dogs could clarify this question.

Phospholipid-dependent thrombin generation measurement, and especially the LT, may also be interesting to evaluate as markers to help confirm whether a dog has received a venomous bite or not. This could be particularly useful considering that fang marks may be absent in up to 50% of envenomated dogs [37] and it is estimated that up to 30 % of *Vbb* bites do not contain venom ('dry bites') [38]. Early confirmation or exclusion of *Vbb* envenomation could be of substantial clinical benefit considering the cost of treatment and potential for attenuation of venom-induced coagulation disturbances [35, 39].

There are some limitations to this study. Only two dogs had received antivenom treatment at T1 and statistical comparisons were therefore not made between dogs treated with and without antivenom at this timepoint. Further studies focusing on both the effects of antivenom treatment at this early timepoint and timing of treatment relative to time of bite, would be interesting. A lower bodyweight to venom ratio is possible in smaller dogs and could result in more severe envenomation effects [37]. Due to the volumes of blood collected in this study, only dogs over 10 kg bodyweight were included. We therefore cannot rule out the possibility of a selection bias resulting in some smaller, more severely affected dogs being excluded from this study. A large number of samples were excluded due to haemolysis since this is known to influence thrombin generation results [40]. However, the majority of excluded samples were from dogs sampled through an indwelling venous catheter and thus haemolysis was suspected to be due to sampling technique rather than an *in vivo* effect. Citrated plasma samples were centrifuged only once, which may lead to residual platelets that fragment during a freeze-thaw cycle, and may subsequently affect phospholipid-dependent thrombin generation and PS equivalents [41]. Double centrifugation (2x 2500 g for 15 minutes) is recommended to minimise this effect [42]. Finally, dogs and humans might respond

differently to snake venom coagulotoxins [43]. Thus, it is unclear to what extent findings in this study might be applicable to human *Vbb* envenomation.

In conclusion, repeated measurements of thrombin generation in plasma indicate a procoagulant state in dogs bitten by *Vbb* already at presentation that, although decreasing, is still present 15 days after bite. Whether the hypercoagulable state after *Vbb* bite has clinical implications remains to be determined. The thrombin generation parameter LT in dogs treated with antivenom reached similar levels to that of the healthy controls, indicating that antivenom treatment might counteract hypercoagulability. Thrombin generation measurement might serve both as a diagnostic and as a monitoring tool after *Vbb* envenomation and may be a suitable marker in future RCTs of antivenom efficacy.

Acknowledgements

The authors would like to thank students, staff and dog owners at the small animal clinics at NMBU, ADO, and EOD for the care and provision of dogs in this study.

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Supporting information

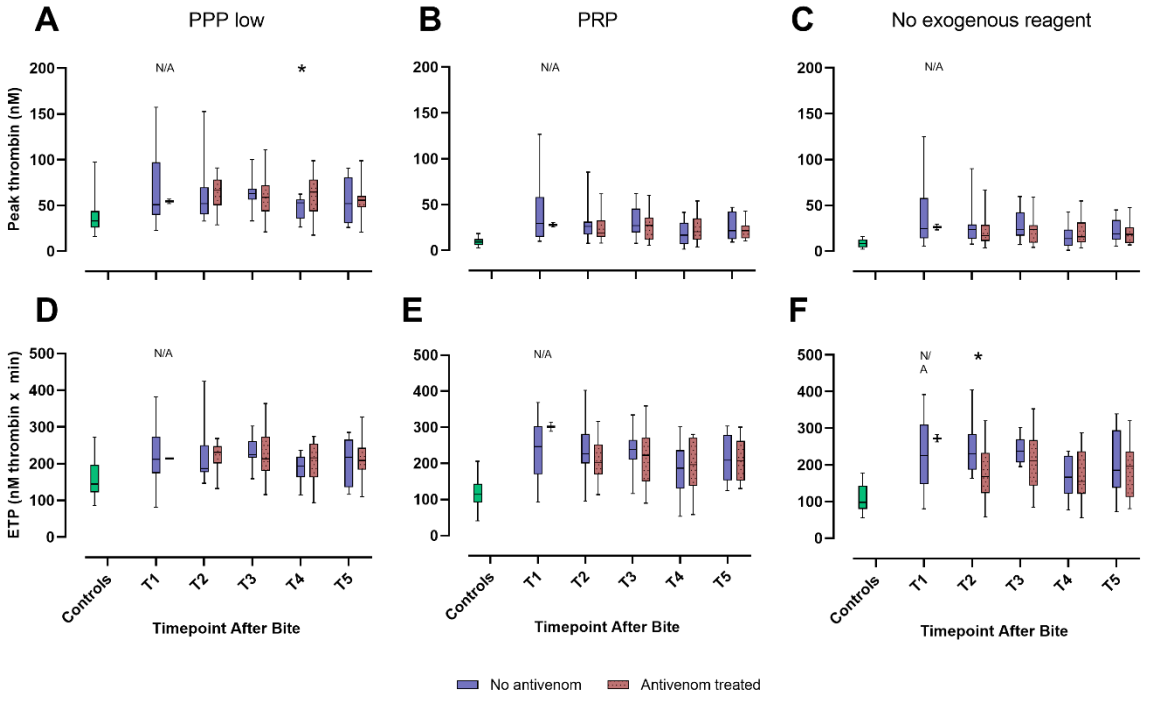
Supplementary table 1: *Thrombin generation parameters for three different reagents (PPP low, PRP and no exogenous reagent) in envenomated dogs at five timepoints (T1= presentation, T2= 12 h, T3= 24 h, T4 = 36 h, T5 = 15 days) after bite, and controls. Values are given as median (range) for lag time, peak thrombin and endogenous thrombin potential (ETP). P-value1 represents comparisons to controls. P-value2 represents comparisons to the subsequent timepoint. Significant P-values (P < 0.05) are in bold.*

		Lag time (min)		Peak (nM thrombin)		ETP (nM thrombin x min)	
		Median (range)	P-value[1] P-value[2]	Median (range)	P-value[1] P-value[2]	Median (range)	P-value[1] P-value[2]
PPP low	Controls	3.3 (2.6-5.3)		33 (16-97)		144 (86-272)	
	T1	1.7 (1.3-4.9)	**0.0006** > 0.99	52 (23-157)	**0.008** > 0.99	213 (81-382)	**0.008** > 0.99
	T2	3 (1.7-4.2)	0.45 > 0.99	61 (29-153)	**0.0002** > 0.99	217 (132-424)	**0.0002** > 0.99
	T3	3.0 (2.0-4.2)	0.7 **0.02**	59 (21-111)	**0.0007** **0.03**	221 (115-364)	**0.0001** **0.002**
	T4	3.0 (2.3-4.2)	0.83 **< 0.0001**	57 (18-99)	**0.01** > 0.99	202 (94-274)	**0.018** > 0.99
	T5	3.0 (2.3-3.7)	**0.02**	56 (21-99)	**0.027**	208 (110-327)	**0.013**
PRP	Controls	4.3 (3.2-6.2)		9 (3-19)		114 (41 – 206)	
	T1	2.3 (1.3- 9.4)	**< 0.0001** > 0.99	29 (10- 126)	**< 0.0001** 0.31	276 (92-369)	**< 0.0001** 0.73
	T2	3.5 (2.0-5.2)	**0.007** 0.56	22. (8-85)	**< 0.0001** > 0.99	213 (95-403)	**< 0.0001** > 0.99
	T3	3.3 (2.0-5.0)	**0.003** 0.23	27 (6-62)	**< 0.0001** **0.008**	233 (90-359)	**< 0.0001** **0.04**
	T4	3.4 (2.7-5.2)	**0.006** **< 0.0001**	20 (1-54)	**0.003** 0.56	189 (53-301)	**0.003** 0.4
	T5	3.2 (2.4-4.2)	**< 0.0001**	22 (9-47)	**< 0.0001**	206 (12- 303)	**< 0.0001**

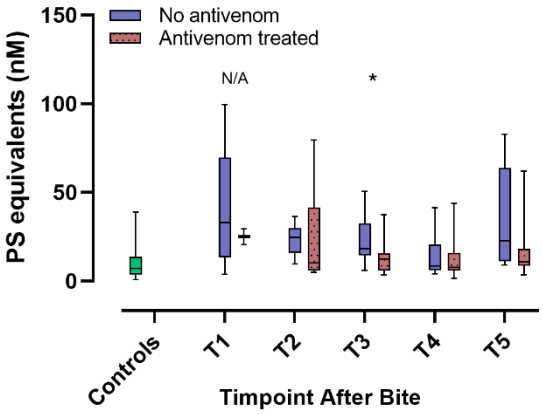
No exogenous reagent	Controls	10.3 (6.6-24.9)		8 (2-16)		97.9 (55-178)	
	T1	2.4 (1.3-4.8)	**< 0.0001 0.014**	25 (5-125)	**< 0.0001** 0.12	30 (4-100)	**0.0008** 0.05
	T2	6.8 (2.0-11.3)	**< 0.0001** > 0.99	21 (4-90)	**< 0.0001** > 0.99	215 (59-405)	**0.0003** > 0.99
	T3	6.6 (2.3-23.0)	**0.001 0.005**	24 (4-60)	**< 0.0001 0.023**	229 (84-353)	**0.0002 < 0.0001**
	T4	7.3 (4.0-22)	0.06 > 0.99	15 (1-55)	**0.03** 0.50	161 (56-287)	**0.04** 0.18
	T5	7.8 (4.0-13.5)	**0.003**	19 (5-48)	**0.0004**	195.5 (72-339)	**0.009**

Supplementary table 2: *Phosphatidylserine (PS) equivalents and thrombin-antithrombin (TAT) complex concentrations in envenomated dogs at five timepoints after bite (T1= presentation, T2= 12h, T3= 24h, T4 = 36 h, T5 = 15 days), and controls. Values are given as median (range). P-value1 represents comparisons to controls. P-value2 represents comparisons to the subsequent timepoint. Significant P-values are in bold.*

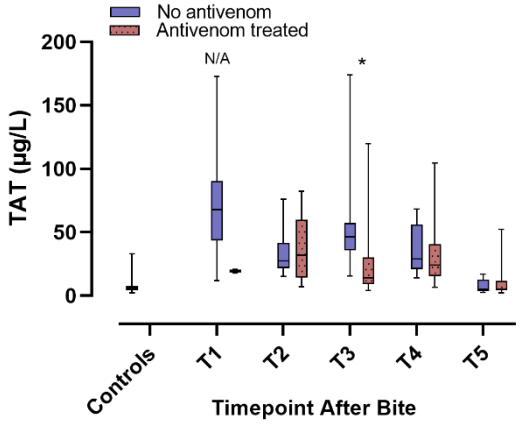
	PS equivalents (nM)		TAT complexes (µg/L)	
	Median (range)	P-value[1] P-value[2]	Median (range)	P-value[1] P-value[2]
Controls	7 (1-39)		6 (2-33)	
T1	30 (4-100)	**0.0001** 0.3	64 (12-173)	**< 0.0001** > 0.99
T2	21 (5-80)	**0.003** 0.79	28 (7-82)	**< 0.0001** > 0.99
T3	14 (4-51)	**0.03** 0.28	33 (4-174)	**< 0.0001** > 0.99
T4	8 (2-44)	0.66 **0.02**	29 (7-105)	**< 0.0001 < 0.0001**
T5	14 (4-83)	**0.007**	5 (2-52)	0.99



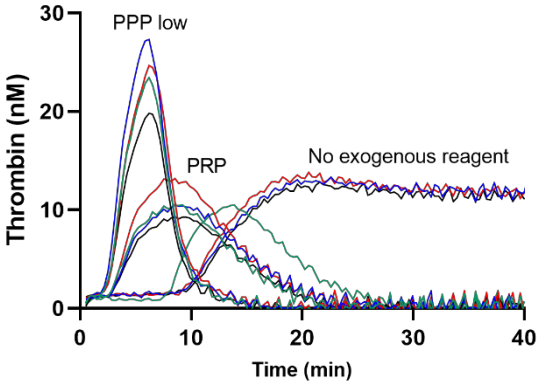
Supplementary figure 1: Thrombin generation parameters in dogs treated with and without antivenom. *Box and whisker plots for peak thrombin (A-C) and endogenous thrombin potential (ETP) (D-F) with three different reagents (PPP low, PRP and no exogenous reagent), in dogs treated with (red) and without (blue) antivenom, and controls (green). T1 = presentation, T2 = 12 hours, T3 = 24 hours, T4 = 36 hours and T5 = 15 days after bite. * indicates a significant difference (P < 0 .05) between treatment groups at a given time point. Statistical analysis was not performed at T1 due to a low number of antivenom treated dogs (n=2). N/A = not analysed*



Supplementary figure 2: Phosphatidylserine (PS) equivalents in dogs treated with and without antivenom. *Box and Whisker plots for PS equivalents in dogs treated with (red) and without (blue) antivenom, and controls (green). T1 = presentation, T2 = 12 hours, T3 = 24 hours, T4 = 36 hours and T5 = 15 days after bite. * indicates a significant difference (P < 0 .05) between treatment groups at a given time point. Statistical analysis was not performed at T1 due to a low number of antivenom-treated dogs (n=2). N/A = not analysed.*



Supplementary figure 3: Thrombin-antithrombin (TAT) complexes in dogs treated with and without antivenom. *Box and Whisker plots for TAT complexes in dogs treated with (red) and without (blue) antivenom, and controls (green). T1 = presentation, T2 = 12 hours, T3 = 24 hours, T4 = 36 hours and T5 = 15 days after bite. * indicates a significant difference (P < 0 .05) between treatment groups at a given time point. Statistical analysis was not performed at T1 due to a low number of antivenom-treated dogs (n=2). N/A = not analysed.*



Supplementary figure 4: Thrombograms with different concentrations of antivenom.

Thrombograms with three different reagents (PPP low, PRP and no exogenous reagent) with antivenom at concentrations of 0 (black), 0.25 (red), 0.5 (blue) and 1 U/mL (green).

Errata

Page	Line	Change made
7	16	'troponin I (markør for hjerteskade)' replaced with 'troponin I (cTnI, markør for hjerteskade)'
8	10	'hyperkoagulabilitet' removed
14	Table 1, column 4, line 11	'hemorrhage' replaced with 'haemorrhage'
43	20	'actor X' replaced with 'factor X'
57	Figure 9	formatting line removed from around figure 9
58	17	'T3: 36' replaced with 'T3: 24'
67	Table 5, column 4, line 6	missing bracket inserted after 'coagulation' 'coagulation'
72	Table 6, column 6, line 15	'≥ 1.89' replaced with '≥ 1.89 ng/mL'
74	Table 8, column 6, line 1	'absolut' replaced with 'absolute'
82	16	'in' removed
88	24	'take' replaced with 'takes'
96	33	'GGT/cr' replaced with 'uGGT/uCr'
108	19	'TEG' replaced with 'thromboelastography'
Paper I	Table 3, column 2	'buprenorphine 5/6' should read 'buprenorphine 4/5'
Paper III, page 16	15	font reduced from line 15 to match the rest of the paper

ISBN: 978-82-575-1847-9
ISSN: 1894-6402

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