Understanding the role of cell-free heme causing acute kidney injury following snakebite in India.

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Abstract

Snakebite is a neglected tropical disease and is estimated to cause 58 000 deaths/ year in India alone. Envenoming from *Viperidae* in India is characterized by venom induced consumption coagulopathy (VICC) and complications include rhabdomyolysis, microangiopathic haemolytic anaemia (MAHA) and acute kidney injury (AKI). The primary objective of this study was to determine whether protein porphyrin X-linked heme, a product of ferric-ferryl heme cycling in the kidney resulting in lipid peroxidation, was present in urine from patients with systemic haemotoxic snakebite envenoming.

A prospective observational study of 24 participants with systemic haemotoxic envenoming and three healthy control participants was performed. Urine was analyzed using reverse phase High Performance Liquid Chromatography (HPLC) to separate organic compounds. Clinical outcomes and serum biomarkers of participants were recorded for one week. Ten participants developed AKI, of which six required renal replacement therapy. Severe rhabdomyolysis was observed in five participants and MAHA was observed in four. Sixteen participants (16/22, 72.7%) had urine chromatograms consistent with X-linked heme compared to none of the control participants. Quantitative measurement of X-linked heme using logarithmic transformation of area under curve (AUC) correlated with creatinine phosphokinase (CPK) (a biomarker of myotoxicity) and schistocyte count (a marker of MAHA) with a Spearman's coefficients of 0.39, p= 0.07 and 0.37, p=0.07 respectively. The logarithmic AUC of X-linked heme was associated with a maximum serum creatinine with a Spearman's coefficient of 0.66, $p<0.01$.

The findings of X-linked heme in urine of patients with snakebite envenoming and clinical markers of haemolysis and or rhabdomyolysis support a hypothesis of oxidative heme-protein modification within the kidney. The correlation of X-linked heme with maximum serum creatinine may represent

lipid peroxidation and oxidative nephropathy. Demonstration of this pathophysiological process in *Viperidae* envenoming in India raises opportunities to consider therapeutic interventions, such as haptoglobin, desferrioxamine or paracetamol, that inhibit ferric-ferryl heme redox cycling reaction *in-vitro*.

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List of abbreviations

20WBCT	20-minute whole blood clotting test
AIN	Acute interstitial nephritis
AKI	Acute kidney injury
ATN	Acute tubular necrosis
AUC	Area under curve
CI	Confidence interval
CFH	Cell-free heme
CKD	Chronic kidney disease
CLS	Capillary leak syndrome
CMC	Christian Medical College
CPK	Creatinine phosphokinase
CRF	Case report form
CTL	C-Type lectins
ED50	Median effective dose
ELISA	Enzyme-linked immunosorbent assay
F_2-IsoPs	F_2-Isoprostanes
FHD	Ferryl-heme derivatives
Hb	Haemoglobin
HELLP	Haemolysis, elevated liver enzymes and low platelets
HUS	Haemolytic uraemic syndrome
INR	International normalized ratio
IRB	Institutional Review Board
JIPMER	Jawaharlal Institute of Postgraduate Medical Education and Research
KDIGO	Kidney Disease Improving Global Outcomes
LDH	Lactate dehydrogenase
LMIC	Low or middle-income country
MAHA	Microangiopathic haemolytic anaemia

Mb	Myoglobin
OR	Odds ratio
PfHRP2	*Plasmodium falciparum* histidine rich protein 2
PI	Principal investigator
PIS	Patient information sheet
PLA2	Phospholipase A2
RBC	Red blood cell
RCT	Randomized controlled trial
RP HPLC	Reverse phase High performance liquid chromatography
RRT	Renal replacement therapy
SAKI	Snakebite associated acute kidney injury
SDS PAGE	Sodium dodecyl-sulphate polyacrylamide gel electrophoresis
SNACLEC	Snake C-Type lectin-like proteins
SROC	Summary receiver operating characteristic
SSS	Snakebite severity score
SVDK	Snake venom detection kit
SVMP	Snake venom metalloproteinase
SVSP	Snake venom serine proteinases
TMA	Thrombotic microangiopathy
VICC	Venom-induced consumption coagulopathy
VEGF	Vascular endothelial growth factor
VEMAC	Vellore manual activated clotting time
WHO	World Health Organization

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Publications

Watson JA, Lamb T, Holmes J, Warrell DA, Thwin KT, Aung ZL, Oo MZ, Nwe MT, Smithuis F, Ashley EA. A Bayesian phase 2 model based adaptive design to optimise antivenom dosing: Application to a dose-finding trial for a novel Russell's viper antivenom in Myanmar. PLoS Negl Trop Dis. 2020 Nov 16;14(11):e0008109. doi: 10.1371/journal.pntd.0008109. PMID: 33196672; PMCID: PMC7704047.

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Preface

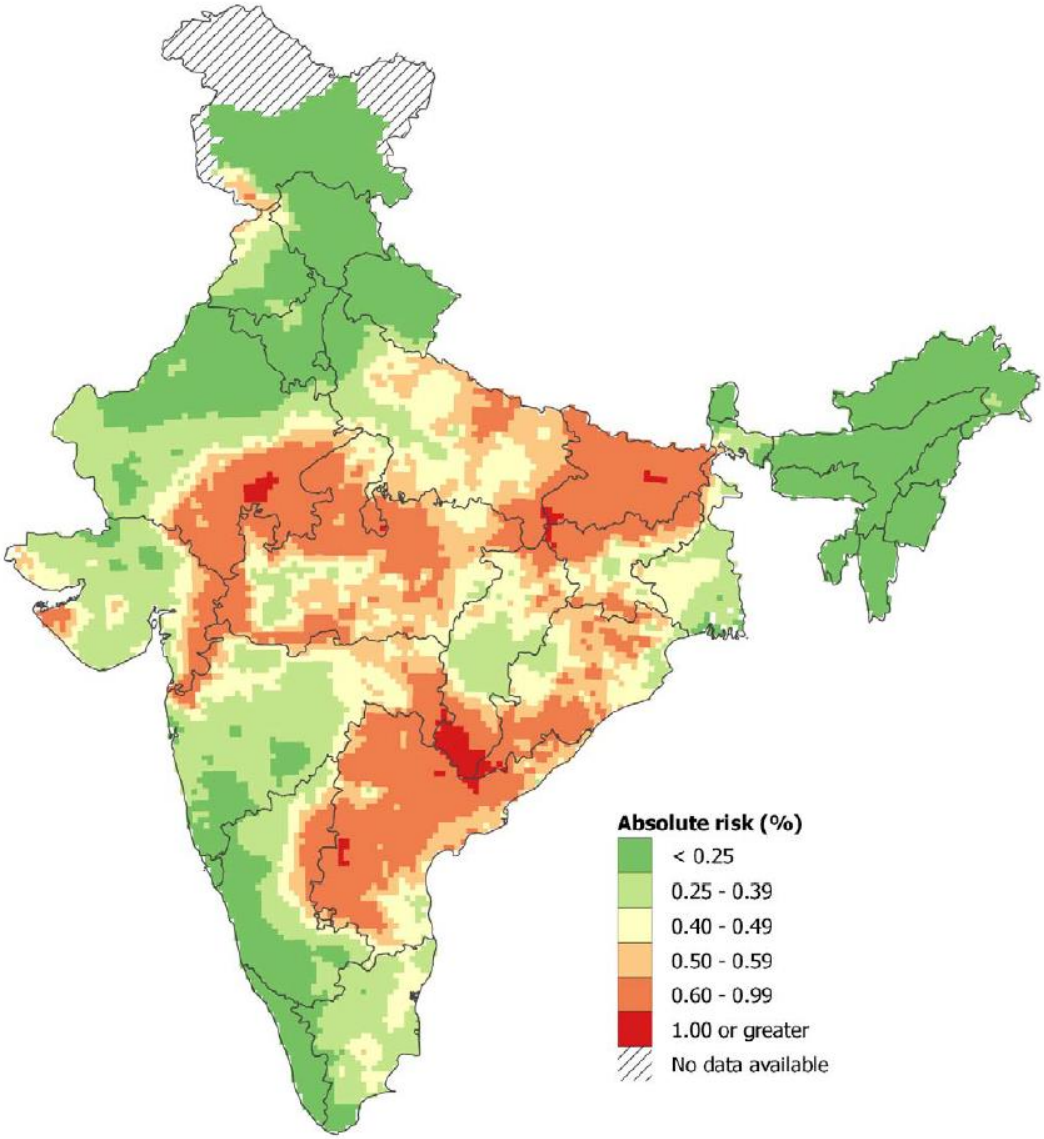
I arrived at the current subject of this thesis in a circuitous manner. Following a passion to improve outcomes for patients with snakebite, I was appointed as Research Coordinator with Myanmar Oxford Clinical Research Unit and first recipient of the Hamish Ogston Foundation scholarship. As Research Coordinator, my role was to develop and conduct a Phase II clinical trial to identify the optimum dose of antivenom for patients with *Daboia siamensis* envenoming in Myanmar. The project was awarded Doctor of Philosophy status at the University of Oxford. Six months after moving to Myanmar, the Covid-19 pandemic stopped research activity in Myanmar and closed international borders. I returned to the UK and paused the snakebite research so that I could contribute to the pandemic response in the NHS. I returned to Myanmar in August 2020 and over the following six months, obtained Institutional Review Board permission to commence the dose finding study. During this time, I also helped develop a protocol for antivenom dose finding trials in Vietnam and India, forging collaboration. On 1st February 2021, shortly before the trial was due to commence recruitment, Myanmar suffered a Coup d'état, resulting in significant political instability. It became apparent that the research was not viable and that my family and I needed to evacuate. Due to the ongoing disruption secondary to Covid, I placed the graduate studies on pause and resumed my medical training in the UK. Funding was later withdrawn following a scandal in the Hamish Ogston Foundation. After a period of 2 years suspension, I recommenced the graduate studies in 2022 as a part time student, switching program from DPhil to Masters' by research. This was made possible with financial support from the Lao-Oxford-Mahosot Wellcome Trust Research Unit PI fund. The current project is in collaboration with CMC Vellore, a relationship forged in 2020, and the University of Essex.

Chapter 1 Introduction

Snakebite epidemiology in India

Snakebite is a neglected tropical disease, predominantly affecting the rural population of tropical and subtropical regions of the world. Worldwide, there are an estimated 5.4 million episodes of snakebite/year resulting in 81, 000 – 138, 000 deaths (1). The wide variation in estimate reflects the considerable challenge of collecting reliable and up to date data on snakebite incidence and death. India is an anomaly in this respect due to the nationally representative Million Death Study. Using data from verbal autopsies, modelling population changes and incorporating additional data from a systematic review of literature, Suraweera et al estimate that there are 58, 000 deaths/year in India from snakebite (2). Risk of death from snakebite varies significantly within India with approximately 70% of deaths occurring in 8 states containing 55% of the population. One third of the population of India live in areas whether there is greater than 1% risk of death from snakebite before the age of 70 years old (Figure 1) (2). Snakebite associated death in India is greatest in regions of high rural agrarian populations (2, 3). Cultivated land typically supports large rodent and snake populations resulting in high number of occupational and environmental injuries (4).

Figure 1



Spatial distribution of snakebite mortality risk for 2004-2013, reproduced from Suraweera et al (2). Colours demonstrate absolute risk (%) of death from snakebite before the age of 70 years old. Greatest risk is reported in Odisha, Andhra Pradesh, Bihar, Telangana and Rajasthan.

Across the Indian subcontinent, medically significant snake fauna varies considerably. Traditionally, India is thought to have four medically significant snake species, two elapid snakes; Indian cobra (*Naja naja*) and Common krait (*Bungarus caeruleus*) and two viperid species; Russell's viper (*Daboia russelii*) and saw scaled viper (*Echis carinatus*) (figure 2). In more recent years, an evolving body of literature supports the role of medically significant snake species in geographically discrete areas. It is now well reported that *Echis carinatus sochureki* is a cause of considerable snakebite morbidity and mortality in west India (5). Envenoming sequelae is undoubtedly exacerbated by poor para-

specificity of Indian antivenom against *E.sochurecki* venom (6, 7). Other species of pit vipers are now reported as causing medically significant envenoming in India. These include *Hypnale hypnale* from the Western Ghats (8), *Ovophis monitcola* in the north and northeast India (9), *Trimeresurus spp* in Western Ghats (10) and northeastern states and *Protobothrops spp* in northeast India (11). In many of these cases, the species of snake is only sought after the apparent ineffectiveness of antivenom suggesting that the clinical significance of these snakes is likely to be under-estimated (9). On a national level, the burden of disease secondary to these newly described species remains unclear. Although snakebite associated death is well studied, less is known about snakebite incidence and long-term morbidity. Given the rapid changes in demography, rural to urban migration and changes to climate and land use, it remains unclear how this may affect future human snake human interaction and injury (12, 13).

Figure 2.



Snakes starting from top left, working clockwise. *Daboia russelii, Echis carinatus, Naja naja and Bungarus caeruleus. Credit Professor Sir David Warrell, used with permission*.

Snake venom evolution

Snake venom is a complex mixture of peptides and proteins, unique for each species of snake and is predominantly used for predation (14, 15). Venom is thought to have evolved because of gene duplication during venom gland expression and under adaptive selection (14, 15). This has resulted in the evolutionary expansion of different toxin-encoding gene families (Table 2) (1, 16). The venom proteome, undergoes further adaptation due to post genomic mechanisms, resulting in a plethora of toxin isoforms, often with discrete pathological effects (16, 17). A potential consequence of this intraspecies variation in venom is a varying antivenomic neutralizing capacity of antivenom meaning that clinical effectiveness of antivenom can vary across a snake's geographical range (6, 7).

Furthermore, the diversity of venom make-up and clinical effect make the production of broadly neutralizing antibodies and monoclonal antibodies challenging and expensive (18).

Table 1. Venom protein families and their associated toxicity

Main Protein families	**Clinical effect**
Phospholipase A2 (PLA2)	Local and systemic myotoxicity, pain, damage to the lymphatic vessels, oedema, neurotoxicity, nephrotoxicity and haemolysis
Snake venom metalloproteinases (SVMP)	Haemorrhage, myonecrosis, extracellular matrix degradation, blistering, pain, oedema, cardiovascular shock, nephrotoxicity and coagulopathy
Hyaluronidases	Extracellular matrix degradation
Three finger toxin	Cytotoxicity, necrosis and neurotoxicity
Dendrotoxins	Neurotoxicity
Snake venom serine proteinases (SVSP)	Coagulopathy, oedema and hypotension
Vascular endothelial growth factor (VEGF)	Hypotension, vascular permeability
Disintegrins	Inhibition of platelet aggregation
Snake C-Type lectin-like proteins (snaclec)	Inhibition or promotion of platelet aggregation and thrombocytopenia
Cysteine rich secretory proteins	Smooth muscle paralysis
Small basic myotoxic peptides	Muscle contracture
Natriuretic peptides	Hypotension
Sarafotoxins	Cardiotoxicity

This is a non-exhaustive list of the major protein families found in snake venom and their associated toxicity. Adapted from Gutierrez et al (1).

Daboia russelii and *Echis carinatus* venom

In a systematic review of the literature, *Daboia russelii* (Russell's viper) was the most reported snake species causing envenoming in India and was responsible for an estimated 53.1% of positively identified venomous snakes (2). Using a combination of reverse phase high performance liquid chromatography (RP HPLC), label free mass spectrometry and sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS PAGE), it is possible to qualitatively and quantitively assess the proportion of toxins in snake venom. *Daboia russelii* snake venom exhibits astonishing intraspecies variation across it geographic range from Pakistan in the West, Bangladesh in the East and Sri Lanka in the South (19, 20). Across the geographic range, PLA2s are the most abundant protein family constituting 47.5-70.6% of venom (19). The PLA2 proteins and isoforms play a key role in the clinical manifestation of cytotoxicity of tissue at the wound site, presynaptic neurotoxicity, intravascular haemolysis and nephrotoxicity. The next abundant protein family were SVSPs constituting between 8.5-14.2% of venom (19). These include factor V activating proteinases and thrombin like fibrinogenases which are potent procoagulants causing consumption coagulopathy and defibrinogenation (21). The venom induced consumption coagulopathy (VICC) is further enhanced by factor X activating isoenzymes (19). These non haemorrhagic SVMPs form the 3rd most abundant protein family ranging between 3.4-12.6% of venom (22). Other key protein families include CRISP, VEGF and CTL (19, 20).

Although *Echis carinatus* is responsible for considerably less systemic envenoming than *Daboia russelii*, they are widely distributed across the Indian subcontinent and can cause a clinically indistinguishable toxidrome. *Echis carinatus* are estimated to be responsible for <10% of clinical envenoming in India (2). The most abundant protein families found in *Echis carinatus* venom are SVMPs followed by SNACLECs (23, 24). SVMPs cause damage to microvasculature through direct toxicity to the basement membrane and extracellular matrix (25). The abundant SVMPs and coexisting SVSPs contribute to prothrombin activation and fibrinogenolytic activity resulting in consumption coagulopathy. Echicetin is a key SNACLEC found in *Echis carinatus* venom (26). It is

responsible for blocking von Willebrand factor whilst simultaneously causing classical platelet activation (26).

Clinical toxidrome

In most regions of India, *Daboia russelii* and *Echis carinatus* co-exist (27). Both vipers share a toxidrome consisting of local cytotoxicity and haematotoxicity. However, *Daboia russelii* envenoming can cause additional complications such as neurotoxicity, capillary leak syndrome (CLS) and pituitary failure.

In India, viperid envenoming is characterized by severe pain and swelling affecting the envenomed limb. Clinical features of systemic envenoming can vary from no signs or symptoms to severe haemodynamic instability. In severe envenoming, swelling commonly progresses to involve the entire limb and is occasionally accompanied by painful lymphadenitis and spontaneous haemorrhage at the bite site. Envenomed patients may describe abdominal pain and have pronounced prostration. Patients can present with hypotension with or without evidence of systemic haemorrhage such as haematemesis, haematuria or intracranial haemorrhage (28).

An additional complication of *Daboia russelii* envenoming includes neurotoxicity which is most commonly characterized by bilateral ptosis, ophthalmoplegia, neck weakness followed by dyspnoea, reduced tidal volume and peripheral nerve weakness. Level of consciousness is typically unaffected. Additional clinical manifestations of complications include oliguria, haematuria and myoglobinuria reflecting acute kidney injury and rhabdomyolysis respectively (29). A delayed complication include CLS, which is characterized by periorbital swelling, bilateral parotid gland swelling, serositis and pulmonary oedema (30). This is a much-feared complication and has a case fatality of greater than 50% (30). Rare complications include myocardial infarction, cardiac arrhythmia, congestive cardiac failure and delayed pituitary gland failure (28, 31, 32).

Venom-induced consumption coagulopathy (VICC)

The hallmark of systemic viperid envenoming in India is VICC. This may be present in the absence of any other clinical features. *Daboia russelii* venom contains factor V and factor X activators which convert these factors to their active form (Va and Xa) (19, 33, 34). This activates prothrombinase complex (VaXa) which in turn coverts prothrombin to thrombin and thus activates the clotting cascade (34-36). The activated clotting cascade results in rapid consumption of clotting factors and fibrinogen. In practice this is demonstrated by elevated INR (commonly above the limit of detection), prolonged APTT and low/undetectable fibrinogen (33, 34, 37). Antivenom therapy, containing animal derived, toxin mediated antibodies, is believed to shorten the duration of VICC and prevent complications. Despite a lack of high-quality evidence, this belief is sufficiently held thus preventing the necessary equipoise to conduct a randomized controlled clinical trial (RCT). A recent study compared the haemostatic abnormalities in patients administered antivenom for VICC and those not administered antivenom with subclinical (false negative 20-minute whole blood clotting test (20-WBCT) VICC (38). This study confirmed that patients administered antivenom had a quicker return of haemostasis. This is due to biding of antivenom to toxins, thus allowing the production of new clotting factors.

Microangiopathic haemolytic anaemia (MAHA)

Schistocytes are fragmented red blood cells (RBC) and commonly observed in syndromes characterized by damaged blood vessels (microangiopathic), such as disseminated intravascular coagulation. In snakebite, endothelial damage can occur secondary to toxins such as SVMPs. The impaired endothelial wall is susceptible to intravascular clot formation secondary to VICC. The development of fibrin strands within the blood vessel can cause fragmentation of RBCs (39). Thrombotic microangiopathy (TMA) is a pathologic term used to describe occlusive micro or macro -vasculature and is defined by MAHA and thrombocytopenia (40). Features on blood film include fragmented schistocytes, polychromasia, anaemia and thrombocytopenia. This is commonly associated with elevated lactate dehydrogenase (LDH), low haptoglobin, negative direct antiglobulin test and reticulocytosis (40). Histological changes found in snakebite TMA are common to all causes

of TMA and include renal arteriole occlusion, endotheliosis and lumen/vessel wall fibrin (39, 41, 42). Similar to toxin mediated haemolytic uraemic syndrome (HUS), TMA in snakebite has a strong affinity with causing acute kidney injury (AKI) and is reported in approximately 90% of patients, commonly requiring renal dialysis (43-45). Although there is no clearly accepted definition of TMA, in a review of snakebite associated TMA, Noutsos et al define TMA as the presence of schistocytes (> or equal to 1% of RBCs) and thrombocytopenia (<150 $x10^9$ /L) or >25% drop in their baseline platelet count (46). The authors recommend serial blood film assessment and schistocyte quantification, acknowledging a modest intra-observer disagreement in blood film interpretation. Snakebite TMA has been described following envenoming from a wide variety of snakes that cause VICC (46). These include the major Viperidae species found in South Asia: *Echis carinatus sochureki*, *Echis carinatus*, *Hypnale* species and *Daboia russelii* (7, 43, 45).

Myotoxicity

Myotoxicity, characterized by rhabdomyolysis and elevated creatine phosphokinase (CPK), is widely described following snakebite in South Asia but less frequently quantified as a proportion of patients envenomed. This may reflect the relative lack of diagnostic equipment for detecting CPK outside of research facilities. In a prospective study in Australia, myotoxicity defined as CPK >1000 U/L was observed in 16% of all envenoming (47). In a retrospective study of AKI following envenoming in Shimla (India), 68.2% of patients had myotoxicity defined as CPK >5x upper limit of normal (48); this was considerably greater than reported in patients with confirmed *Daboia russelii* envenoming from Sri Lanka 37/245 (15.1%) in which myotoxicity was defined as CPK >300 U/L (49). Further evidence of the clinical significance of myotoxicity and rhabdomyolysis is supported by the finding of pigment nephropathy in kidney histology samples. Pigment nephropathy is a typical histological finding following rhabdomyolysis and/or haemolysis and has been observed in renal histological samples from patients with snakebite envenoming in India (29, 50).

Indian snakebite renal disease and pathophysiology

Snakebite associated acute kidney injury (SAKI) is common in India and a leading cause of mortality and morbidity from snakebite. Although the KDIGO definition of AKI is widely reported in snakebite studies, there is no accepted definition of SAKI. In a collaborative statement of core outcome measurement in snakebite, Abouyannis and colleagues state that clinically meaningful AKI should be assessed by means of (1) proportion of patients with a new onset requirement for RRT within 6 weeks of enrollment and (2) the proportion of participants with ≥ 30% reduction in estimated glomerular filtration rate from baseline at 6 weeks after randomisation (51). In JIPMER, a large tertiary hospital in Puducherry, south India, snakebite was responsible for 26.3% of all community onset AKI from medical causes (second only to pyelonephritis) and had the greatest fatality rate (52). Although SAKI is described following envenoming from *Echis* spp and *Hypnale* spp, it is more commonly observed following *Daboia russelii* envenoming (53). In a study by Priyamvada and colleagues performed in Puducherry, India, SAKI developed in 184 of 420 (43.8%) with haemotoxic snakebite (54). Of those 184 patients who developed SAKI, the AKI was evident at the time of presentation in 164 (164/184, 89.1%) (14 (7.6%) KDIGO AKI stage 1, 35 (19.0%) KDIGO stage 2 and 115 (62.5%) KDIGO stage 3). For the remaining 20 participants (10.9%), the AKI was evident on day 2 (54). Due to variation in reporting practice and incidence, an abbreviated literature search was performed to identify studies reporting on SAKI in India in the last 12 years. The data are summarized in Table 2.

Table 2: A summary of literature from tertiary hospitals reporting SAKI incidence, need for renal replacement therapy (RRT) and death from snakebite in India between January 2013 to September 2025.

First author/ Year of pub	Study site	Study design	Snake I.D	Exclusion criteria	Definition of AKI	Total patients	AKI (%)	RRT (%)	Death (%)
Adults									
Gantait 2019 (55)	Midnapore, WB	Retro	Viper	Renal disease	KDIGO guidelines	660	180*	180 (27.3)	38 (5.8%)
George (56) 2019	Vellore, TM and PC	Retro	NR	None	Creat ≥1.5mg/dL	886	184 (20.8)	70 (7.9)	14 (1.6)
Harshvadhan 2013 (57)	Hassan, KA	Pros	All	Renal disease	KDIGO guidelines	210	36 (17.1)	16 (17.6)	8 (3.8)
Kumar 2018 (58)	Malabar, KL	Pros	Viper	None	Not reported	912	281 (30.8)	83 (9.1)	82 (9.0)
Kumar 2022 (59)	Ranchi, JK	Pros	All	None	Not reported	60	15 (25.0)	11 (18.3)	8 (13.3)
Kumar 2020 (59)	Chennai, TN	Retro	All	Renal disease	KDIGO guidelines	769	159 (20.7)	114 (14.8)	15 (2.0)
Melit 2021 (60)	Thrissur, KL	Retro	All	None	Not reported	451	165 (36.6)	37 (8.2)	13 (2.9)
Menon 2016 (28)	Angamaly, KL	Retro	All	None	KDIGO guidelines	1051	220 (20.1)	110 (10.5)	38 (3.6)
Priyamvada 2019 (54)	PC	Pros	Viper	None	KDIGO guidelines	420	184 (43.8)	114 (27.1)	44 (10.5)
Sagar 2020 (61)	PC	Pros	Viper	None	KDIGO guidelines	140	70 (50)	45 (32.1)	20 (14.3)
Aggregated adults						**5559**	**1314 (23.6)**	**780 (14.0)**	**280 (5.0)**
Paediatrics									
Das 2023 (62)	Cuttack, OR	Pros	All	None	KDIGO guidelines	197	54 (27.4)	9 (4.6)	2 (1.0)
Islam 2019 (63)	Burdwan, WB	Pros	All	None	KDIGO guidelines	364	169 (46.4)	36 (9.9)	16 (4.4)

Jayakrishnan 2016 (64)	Kohzikhode , KL	Pros	All	None	KDIGO guidelines	145	36 (24.8)	8 (5.5)	15 (10.3)
Krishnamurthy 2015 (65)	PC	Pros	All	None	Not reported	61	28 (45.9)	10 (16.4)	4 (6.6)
Aggregated paediatrics						**767**	**287 (37.4)**	**63 (8.2)**	**37 (4.8)**

A table displaying published reports of SAKI, requirement of RRT and death in India since 2013 divided by adult and paediatric case series. One data field (*) did not include data on AKI but did report RRT and death. A proxy value of RRT was used for AKI. Pub (publication), Pros (prospective), Creat (creatinine), Retro (retrospective), MH (Maharashtra), WB (West Bengal), TM (Tamil Nadu), KA (Karnataka), KL (Kerala), JK (Jharkhand), OR (Odisha), PC (Puducherry). Pros (prospective), Retro (retrospective).

Studies from seven different Indian states were included. Ten papers reported data for adult patients, of which five reported prospective data, whilst five collected retrospective data. Six of ten papers reporting on adult patients defined AKI according to KDIGO guidelines, one paper defined AKI as ≥1.5mg/dL and three papers did not specify the definition of AKI. Of the aggregate included adult patients (n=5559), 1314 (23.6%) developed AKI, 780 (14.0%) required RRT and 280 (5.0%) died. Four papers were identified that reported on paediatric populations, all were conducted prospectively. Three of four papers reporting on paediatric patients defined AKI according to KDIGO guidelines, whilst one paper did not specify the definition of AKI. Of the aggregate included paediatric patients (n=767), 282 (37.4%) developed AKI, 63 (8.2%) required RRT and 37 (4.8%) died.

There was considerable heterogeneity in reporting with a mixture of prospective and retrospective observational studies included. Four studies reporting on adults limited participants to those with features consistent with viperid envenoming whilst the remaining included studies included all patients with evidence of snakebite envenoming. All included studies were conducted in tertiary referral centers offering RRT, thus likely creating positive reporting bias and an over estimation of SAKI. It is interesting to note that rates of SAKI for included studies, all performed in India, were greater than that reported in a large observational study in Sri Lanka (53/1186, 4.3%) where a similar spectrum of snake species are responsible for snakebite envenoming (66). Requirement for RRT (10/1186, 0.8%) and death (13/1186, 1.1%) was also lower in the Sri Lanka cohort than all included studies reporting from India (Table 3). To put the Indian SAKI data in perspective with other countries reporting SAKI following snakebite, AKI was reported in 557/948 (58.8%) patients with snakebite in Myanmar, 42/276 (15.2%) patients with snakebite in Brazil, 19/157 (12.1%) patients with envenoming in Australia (67-69).

There is a wide spectrum of histopathological changes described in kidney biopsy performed on patients with SAKI (29, 53, 70). These heterogenous histological findings suggest a multifactorial aetiology of renal injury. The predominant histological abnormalities can largely be divided into glomerular, tubular interstitial and vascular changes (50). Acute tubular necrosis is the most

common histological finding (59, 71). Tubular changes are further exacerbated by systemic hypotension secondary to toxin effect, haemorrhage and extravasation of fluid into the envenomed limb. Another tubular interstitial change observed in renal biopsies is AIN which is characterized by interstitial oedema with predominant lymphocytic and occasional eosinophilic infiltration (72, 73). The most commonly observed glomerular abnormalities include hyaline and pigment that result from heme and rhabdomyolysis pigment deposition (29, 74). Although AIN is less commonly reported, Golay and colleagues describe a more protracted course of SAKI, prolonged requirement of RRT and high rate of progression to chronic kidney disease (72). The aetiology of AIN is poorly understood and requires further investigation.

Acute cortical necrosis is the second most reported histological abnormality observed in patients with SAKI in India (50). ACN was generally associated with less favorable outcome when compared to acute tubular necrosis (50, 71). Chugh et al report the co-existence of ACN and fibrin thrombi in renal microvasculature in patients with MAHA (29). The authors hypothesized that SAKI developed in these patients secondary to a combined toxicity from cell-free heme causing vasoconstriction and TMA resulting in microvasculature occlusion and vessel fibrin thrombi (29, 75, 76).

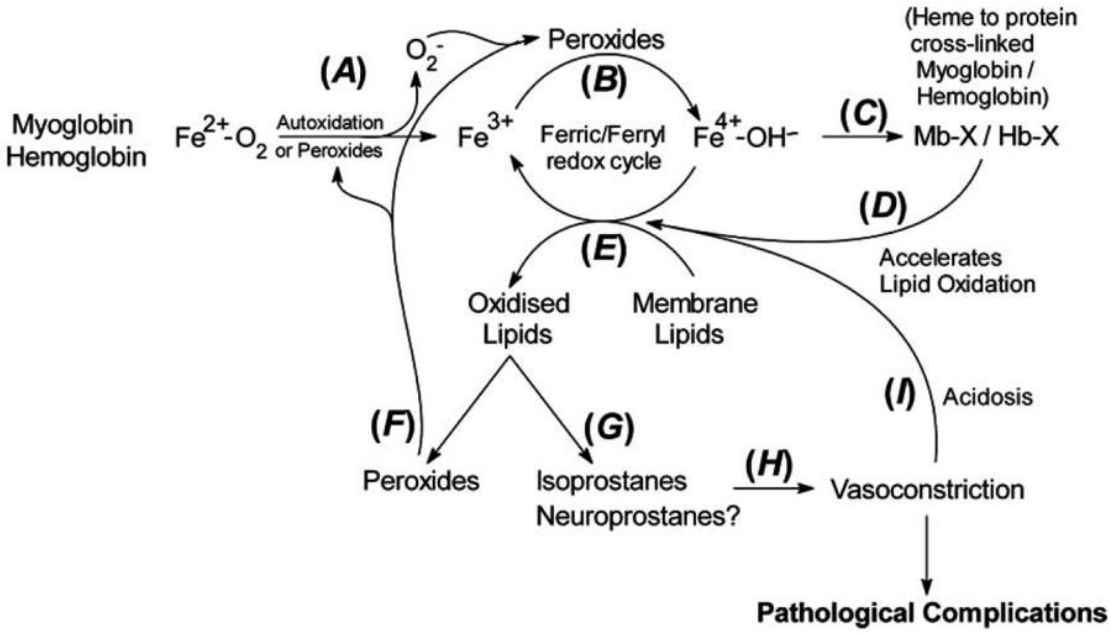
Two large observational cohort studies demonstrated that early administration of antivenom reduced incidence of SAKI (77, 78). In an observational study performed in Myanmar in patients envenomed by *Daboia siamensis*, Alfred et al demonstrated that for every hour in which antivenom was delayed after snakebite, the odds of AKI increased – OR and (95%CI) 1.7 (1.0-3.0) for 1h delay, OR 3.2 (1.5-6.8) at 2 hour delay, OR 4.2 (1.6-11.1) at 3 hour delay and 12.4 (2.5-62.9) at 4h delay, p=<0.01 (77). In the study performed in Tamil Nadu, India, the mean time to antivenom administration for patients that developed SAKI was 22.2 hours (1.0-148) compared to 5.2 hours (0.2-72) for patients that did not develop SAKI (p<0.001) (78).

The potential role of cell-free heme (CFH) in kidney injury

Myoglobin (Mb) and haemoglobin (Hb) are pro-oxidant enzymes with the shared physiological function of reversibly binding oxygen. This occurs in ferrous state [Fe(II)] and allows them to function as oxygen storage and oxygen transport proteins (79). Viperid envenoming in south India commonly causes MAHA and rhabdomyolysis that can occur either independently or simultaneously. During haemolysis, there is release of Hb that is subsequently bound by haptoglobin with high affinity and catabolized in the liver (80). Haptoglobin binding has been shown to protect against Hb induced oxidation (81). The process of haptoglobin scavenging is replicated during rhabdomyolysis, with haptoglobin binding to myoglobin, albeit with reduced affinity. Once Hb/Mb exceeds the scavenging capacity of haptoglobin, there is accumulation of cell-free heme which is excreted by the kidney (79, 82). When bound to globin, oxidation of Ferrous Iron (Fe II) to Ferric Iron (Fe III) occurs in the presence of oxygen over several hours. This reaction is catalyzed by peroxides and dramatically accelerated once heme is no longer bound to the globin protein (83). The autoxidation reaction is highly pH dependent occurring at much greater rate under acidic (pH 5) conditions, observed during oxidative stress.

Ferric heme undergoes peroxide induced redox cycling to ferryl (Fe IV) form. Under acidic conditions, protonation of the ferryl species can result in the ferric form (Fe III) and a porphyrin radical (figure 3) (79, 84). The porphyrin radical then reacts with an amino acid side chain in a termination step forming a protein-porphyrin cross-linked covalent bond (Hb-X or Mb-X) (85). Ferryl heme, including cross-linked forms, can initiate oxidation of lipid alkyl radicals from lipids or free fatty acid molecules (83, 84). This triggers a series of lipid oxidation reactions resulting in the formation of lipid hydroperoxides and ferric iron. The abundant ferric iron (Fe III) further catalyzes the reaction. Therefore, Hb/Mb can function as pro-oxidant and pseudoperoxidase enzymes that both initiate and feed a cycle of lipid oxidation. Oxidation of fatty acid side groups of lipid membranes (such as arachidonic acid) can generate F_2-isoprostanes (F_2-IsoPs) (86). Morrow et al demonstrated that levels of F_2-IsoPs are markedly elevated in rat models which undergo free radical catalyzed lipid peroxidation (86). This has been replicated in disease states including rhabdomyolysis (87). The catalysed oxidative reaction is summarized simplistically in Figure 3.

Figure 3



A simplified schematic demonstrating catalyzed redox reaction, reproduced with permission from Reeder (80). Oxidation of Fe II to Fe III (A) is accelerated by peroxide once Hb/Mb is free from globin. Peroxide catalyzes a redox cycle to Fe IV (B) forming protein porphyrin X-linked heme (C). X-linked heme accelerates lipid oxidation (D and E) to form peroxides (F) and Isoprostanes (G) and facilitated redox cycling of Fe IV to Fe III.

The porphyrin radical reaction with an amino acid side chain forming a protein-porphyrin cross-linked covalent bond (Hb-X or Mb-X) is specific to peroxide induced ferric-ferryl redox cycling and thus lipid oxidative reactions (84, 85). As the X-linked Hb/Mb is stable both in vitro and in vivo, identification of these compounds can be used for demonstrating selective oxidative processes (84). Using RP HPLC, with acidic solvent, Hb/Mb elutes separately to the protein. The X-linked heme is covalently bound to the protein and therefore cannot separate. This creates an optically distinct elution spike on the RP HPLC chromatogram (84, 88). Typically, the X-Hb/Mb elutes later in a broader band like pattern, markedly different from the discrete spikes observed for unmodified Hb/Mb and apoprotein (80).

The toxic effects of CFH and by-products of lipid peroxidation such as F_2-IsoPs have been described in a variety of medical conditions including haemolysis following cardiopulmonary surgery, sepsis and malaria (82, 89-91). In the kidney, F_2-IsoPs cause potent vasoconstriction of the renal arterioles

and a reduced glomerular filtration rate. In an observational study of patients with severe malaria, plasma CFH and F_2-IsoPs were strongly associated with a rise in creatinine and requirement for haemodialysis (82). In rhabdomyolysis, the pathophysiology of kidney injury is attributed to intense vasoconstriction and acute tubular necrosis (92). In mouse models with induced rhabdomyolysis, urinary F_2-IsoP levels were elevated by a magnitude of 7 when compared to non-exposed mice (87). These observations were later confirmed in human patients with rhabdomyolysis (93). Patients with rhabdomyolysis due to a variety of different aetiologies had elevated urinary F_2-IsoPs and detectable X-linked Mb. Human control participants with either chronic kidney diseases (CKD) or healthy controls had markedly lower rates of F_2-IsoPs and no X-linking (93). I hypothesize that in patients with snakebite and evidence of MAHA and/or severe myotoxicity, there is abundant cell-free heme which undergoes ferric-ferryl cycling within the kidney causing lipid peroxidation and acute kidney injury.

Diagnosis of systemic haemotoxic snakebite envenoming

Distinguishing asymptomatic systemic envenoming from a dry bite or mild local toxicity is vital to ensure early and judicious use of antivenom. This is particularly pertinent given the importance of early administration of antivenom (77, 94), high cost of antivenom (95), high frequency of serious adverse reactions with poor quality antivenoms (96) and the global shortage of antivenom (95). A range of laboratory diagnostic tests have been developed and assessed in patients with snakebite envenoming including immune assays (most commonly enzyme-linked immunosorbent assay (ELISAs)), enzymatic activity assays and molecular methods (97). Although many of these tests show excellent sensitivity and specificity (98, 99), limitations including time to reading, high cost, cross reactivity and requirement for technical skills have meant that just one, the Seqirus SVDK in Australia, has made it into clinical practice (100). Furthermore, detection of venom at a bite site or venom antigenaemia does not necessarily equate to systemic envenoming.

In the absence of clinical manifestations of systemic envenoming e.g. gastrointestinal haemorrhage or bilateral ptosis, clinicians in India rely upon the detection of coagulopathy to determine whether

systemic haemotoxic envenoming has occurred and therefore antivenom is warranted. Despite the wide spectrum of snakebite pathophysiology resulting in disorders of haemostasis (101), the standardized laboratory assays of prothrombin time/INR and fibrinogen have been found to be sensitive at detecting coagulopathy from a variety of different snake genera (102, 103). Unfortunately, as most snakebites occur in remote geographical locations, conventional clotting assays are not commonly available in a timely manner nor affordable. As a result, several bedside tests are used to detect clotting abnormalities - these include the 20-minute whole blood clotting test (20WBCT) (104), 30-minute whole blood clotting test (105), capillary blood clotting time (106), Lee-White clotting test (107) and Vellore manual activated clotting time (VeMac) (108). The 20WBCT is the most widely used bedside clotting test in snakebite envenoming and is recommended by the WHO regional office for South-East Asia and the Indian national snakebite management guidelines (109).

The 20WBCT was first described in 1977 by Warrell and colleagues and involves placing 'a few millilitres of freshly collected blood into a clean dry glass test tube that is then left un-disturbed for 20-minutes and then tipped to discover whether the blood has clotted' (104). Blood that fails to clot after 20-minutes is considered positive and indicative of coagulopathy and thus systemic envenoming, whilst blood that has clotted is considered negative and not indicative of coagulopathy. The 20WBCT was specifically designed with consideration of cost, speed, limited laboratory resources and reproducibility in locations with limited antivenom supply that needed to be reserved for patients at highest risk of complications. In the original paper, the test was described as an 'all or nothing' test that is an 'easy and sensitive sign of systemic poisoning in which spontaneous haemorrhage poses the greatest threat to life' (104). Despite this, there has been no systematic validation of this method compared to conventional laboratory clotting assays such as INR or fibrinogen concentration or consensus on the utility of the 20WBCT in snakebite management and its utility in snakebite management has been questioned (110). Over time, the use of the 20WBCT has evolved from its initial design to identify patients most at risk of severe bleeding. Increasingly,

guidelines have incorporated the 20WBCT as a test of detecting systemic haemotoxic envenoming (109).

Snakebite management

Antivenom remains the cornerstone of snakebite envenoming management across the world and has undergone remarkably little evolution since its discovery by Pasteur institute in 1895 (1, 111). Antivenoms comprise of whole or fragmented immunoglobulins concentrated from the plasma of domesticated animals that have been hyper-immunized with venom from one or more snake species. Antivenoms are highly specific and neutralize venoms (and venom from a few related species) used in their production. In India, polyspecific antivenom is raised against a mixture of venoms from the 'Big 4' snake species; *Daboia russelii*, *Echis carinatus*, *Bungarus caeruleus* and *Naja naja*.

Although there are different manufacturers of Indian polyspecific antivenom, the venom is sourced from one site, Irrula Snake Catchers Industrial Co-operative Society, Tamil Nadu, south India. Researchers have demonstrated that antivenom raised against snakes from southern India exhibit poor anti-venomic neutralization of *Echis carinatus* and *Daboia russelii* venom from northern and eastern India respectively (6). Atarde et al have shown that existing antivenom production techniques can be significantly improved with the adoption of modern techniques including thermocoagulation, caprylic acid precipitation and purification (112). This '2nd generation' Indian antivenom has shown significantly improved neutralization efficacy in mouse models using the widely accepted median effective dose (ED50) testing technique. Unfortunately, it has not yet received regulatory approval for use in human envenoming.

Although antivenom is used across the world and is considered standard of care for systemic envenoming, the evidence basis for their use is limited. A lack of perceived clinical equipoise has resulted in challenges conducting placebo-controlled trials. A large cohort study that compared outcomes for patients with and without antivenom (due to supply problems) demonstrated an odds

ratio of death in patients administered antivenom of 0.49 (CI 0.30-0.83) compared to patients not given antivenom (113). Antivenom has also been shown to restore haemostasis sooner in patients with systemic envenoming compared to patients not administered antivenom (38). Dosing of antivenom is still based on poorly representative in-vivo animal models and estimated venom depots (1, 114). There is a need for modern dose-finding studies using clinically relevant end points to optimise efficacy, minimise adverse reactions and cost. (115, 116).

Within the last 10 years, there has been an increased interest in the use of broadly neutralizing small molecule toxin inhibitors for snakebite management. There is mounting preclinical evidence for the neutralizing potential of PLA2 inhibitors (such as Varespladib), SVMP inhibitors (such as Marimastat) and metal chelators (such as Unithiol) (117-119). As yet, only Varespladib has been tested in a placebo controlled randomised controlled trial (120). The study, that recruited patients with systemic envenoming from USA and India, failed to meet its prespecified clinical end point of an improved modified snakebite severity score SSS at 6 and 9 hours. Varespladib was well tolerated and adverse events were comparable with placebo. Although assessed, renal outcomes were not reported. Post-hoc analyses showed a trend of improved modified-SSS in participants administered Varespladib within 5-hours of envenoming (120). The authors of this study postulate the potential role for Varespladib as a 'field therapy', adjuvant to antivenom, that can be administered soon after the bite. However, the mandated requirements of this type of intervention, namely efficacy, low cost, wide availability, and favourable safety profile have yet to be proven.

Research objectives

1. What is the diagnostic test accuracy of 20WBCT at detecting systemic haemotoxic envenoming?
2. Is X-linked heme detectable in urine from patients with snakebite envenoming?
3. Is X-linked myoglobin detectable in urine from patients with snakebite envenoming?
4. Is there correlation between X-linked Hb/Mb and

    (i) AKI

(ii) MAHA

(iii) Myotoxicity

Chapter 2. A systematic review and meta-analysis of diagnostic test accuracy of the 20-minute whole blood clotting test (20WBCT)

This section has been published and subsequently adapted from Lamb et al 2021) (37).

Permission received from all co-authors for use of published data within this thesis.

Methods

Due to the widespread use of the 20WBCT in India and around the world, low cost and binary outcome, I sought to understand the diagnostic test accuracy (DTA) of this test against a known standard (International normalized ratio (INR) >1.4 and Fibrinogen <100mg/dL) by performing a systematic review of literature and meta-analysis. The review was conducted in accordance with Preferred Reporting Items for a Systematic Review and Meta-analysis of Diagnostic Test Accuracy Studies (PRISMA-DTA) statement (121) and was prospectively registered on PROSPERO database (122). The following databases were searched; Ovid Medline; Ovid Embase; Ovid Global Health; Scopus; Web of Science Core Collection; and WHO Global Index Medicus. The search strategies applied the SIGN diagnostics search filter to text words and relevant index terms to retrieve studies about diagnostic tests which detect a coagulopathy following snakebite. No limits or language restrictions were applied to the search results (37).

Studies that reported five or more human subjects that had undergone 20WBCT and an INR or fibrinogen concentration clotting assay at the same time were included in the review (37). In all studies, the 20WBCT was considered the index test, and the paired clotting assay, the reference test. Additional information was collected from each study including year of publication, country of origin, biting snake species, method of snake identification, time from bite to index test, comparator test threshold, number of paired clotting tests, source of funding and conflict of interest (37).

Titles and abstracts were screened by me. Studies that were selected for full text review were independently reviewed in duplicate by two reviewers using a pre-designed data collection table.

Discrepancies in findings were reviewed by a separate reviewer who adjudicated using consensus to reach a decision. Most studies used an INR threshold of >1.4 and a fibrinogen threshold of <100 mg/dL to define snakebite induced coagulopathy (37). For this reason, these thresholds were used in the meta-analysis to define coagulopathy. For studies that assessed the accuracy of 20WBCT using a different threshold of INR or fibrinogen, or where incomplete data were reported, authors were contacted and asked to provide true positive (TP), false negative (FN), true negative (TN) and false positive (FP) data for the 20WBCT against an INR threshold of >1.4 and fibrinogen of <100 mg/dL. The same eligibility criteria were applied for data not presented in publication. Sub-analyses were conducted to identify the sensitivity and specificity of the 20WBCT at detecting severe coagulopathy (defined as INR >5.0 or fibrinogen <50 mg/dL) and the sensitivity and specificity of the 20WBCT at detecting resolution of coagulopathy after antivenom (37).

I and one other reviewer conducted an independent assessment of bias using a Quality Assessment of Diagnostic Accuracy Studies (QUADAS2) checklist (123). For published studies, the QUADAS2 assessment was completed using the methodology described in the published manuscripts. If unspecified, the assessment was completed by contacting the primary author (37).

A random effects meta-analysis was used to calculate aggregate sensitivity, specificity and confidence intervals using Stata-IC version 14. Simple descriptive statistics were used to describe individual patient data and a Mann-Whitney U test was used to compare distributions of skewed continuous data between two groups using Graphpad Prism version 9 and R version 4.0.3 (37).

Descriptive results of the systematic review

The search identified 3,599 studies, of which 1,580 were duplicates. Two further studies (124, 125) were identified through screening references and two datasets were considered following recommendation from experts. One thousand, eight hundred and eighty-seven studies were excluded based on review of the title and abstract. Of the 132 studies included for full-text review, 11 met the inclusion criteria. Authors from a further 29 studies that reported methodology

suggesting synchronous 20WBCT and clotting sample collection were contacted, and a further four studies (from three papers) were included (43, 102, 126). Thus, 15 studies were included in the systematic review (37). The data from the 15 studies, with 2,270 patients, included in the systematic review are summarized in Table 3. These studies report data from Thailand (n=4), India (3), Sri Lanka (3), Brazil (2), Nepal (2) and Papua New Guinea (1), on a variety of different snake genera: *Daboia* (n=7), *Trimeresurus* (5), *Hypnale* (3)*, Echis* (2)*, Bothrops* (2)*, Calloselasma* (1)*, Ovophis* (1), *Bungarus* (1) and *Oxyuranus* (1). In several studies, the identification of snake genus and species was unknown (37).

Table 3. A summary of literature comparing 20WBCT with pre-determined gold standard clotting assays

Reproduced with permission from Lamb et al (37).

First author, year	Country	Funding source/ Declaration	Snake species	Method of snake identification	Median (IQR) time to hospital	Reference test	Number of 20WBCT	True positive	False negative	True negative	False positive	Sensitivity (Confidence interval)	Specificity (Confidence interval)
Thongtonyong, 2020 (127)	Thailand	NR / No conflict of interest	*Calloselasma rhodostoma*	Dead snake and photographs	1.2 h (IQR NR	fibrinogen <100 mg/dL	296	98	23	158	17	0·81 (0·73-0·88)	0·90 (0·85-0·94)
Wijewickrama , * 2020 (43)	Sri Lanka	NMHRC/ No conflict of interest	*Hypnale* spp and *Daboia russelii*	Enzyme immunoassay (EIA)	1.0 h (0.5-2.5)	INR > 1·4	157	5	11	130	11	0·31 (0·11-0·59)	0·92 (0·86-0·96)
Tongpoo, 2020 (128)	Thailand	RHMU/ No conflicts of interest	*Trimeresurus* spp	Dead snake and syndrome	1.3 h (IQR NR)	fibrinogen < 100 mg/dL	38	4	8	26	0	0·33 (0·10-0·65)	1·00 (0·87-1·00)
Bhatt, * 2020 (126)	Nepal	NR/NR	*Ovophis monticola* and *Trimeresurus* spp	Dead snakes and photographs	NR	INR >1·4	97	20	1	55	21	0·95 (0·76-1·00)	0.72 (0·61-0·82)

Sharma, * 2020 (126)	Nepal	NR/NR	*Daboia russelii and Trimeresurus* spp	Dead snakes and photographs	NR	INR >1·4	33	8	1	24	0	0·89 (0·52-1·00)	1·00 (0·86-1·00)
Shenoy, ‡ 2020 (108)	India	CMC Vellore/ No conflicts of interest	*Daboia russelii and Echis carinatus*	Not specified	NR	INR >1·4	66	24	1	32	9	0·96 (0·80-1·00)	0·78 (0·62-0·89)
Dsilva, 2019 (129)	India	NR/ No conflicts of interest	*Daboia russelii* and *Echis carinatus*	Not specified	NR	INR >1·4	60	8	3	35	14	0·73 (0·39-0·94)	0·71 (0·57-0·83)
Saengnoi, 2019 (130)	Thailand	RHMU/ No conflicts of interest	*Trimeresurus* spp and *Daboia siamensis*	Dead snakes and photographs	NR	fibrinogen <100 mg/dL	48	0	1	43	4	0 (0-0·98)	0.91 (0·80-0·98)
Oliveira, * 2019 (102)	Brazil	CAPES, CNPQ and FAPEAM/ No conflicts of interest	*Bothrops* spp	Dead snakes and EIA	NR	fibrinogen <100 mg/dL	100	54	26	20	0	0·68 (0·56-0·78)	1·00 (0·83-1·00)

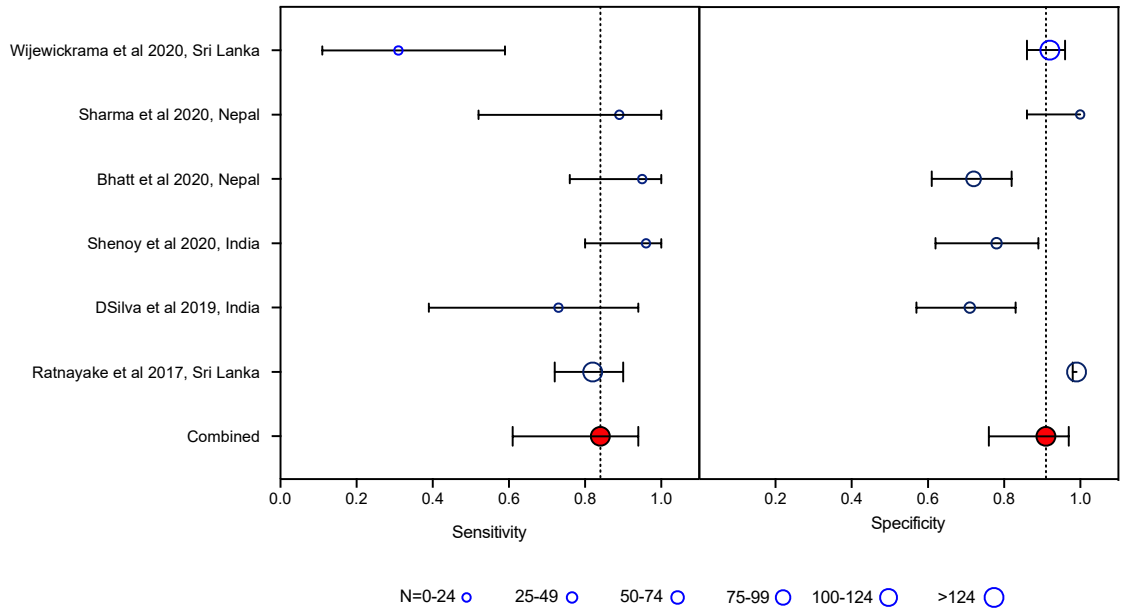
Silva, † 2018 (106)	Sri Lanka	NR/NR	*Daboia russelii, Hypnale hypnale,* and *Bungarus caeruleus*	Dead snakes	NR	INR >1·5	92	14	12	62	4	0·54 (0·33-0·73)	0.94 (0·85-0·98)
Ratnayake, 2017 (131)	Sri Lanka	NMHRC/ No conflicts of interest	*Daboia russelii* and *Hypnale* spp	Dead snakes and EIA	NR	INR >1·4	987	65	14	895	13	0·82 (0·72-0·90)	0·99 (0·98-0·99)
Biradar, 2015 (124)	India	None/ No conflicts of interest	Not specified	Dead snakes, photographs or toxidrome	NR	INR >1·5	112	33	33	41	5	0·50 (0·37-0·63)	0·89 (0·76-0·96)
Paiva, † 2015 (125)	Papua New Guinea	NR/NR	*Oxyuranus scuttelatus*	Not specified	NR	fibrinogen <50 mg/dL	60	NR	NR	NR	NR	0·93	0·91
Pongpit, 2012 (132)	Thailand	TRF and CHEMET/ No conflicts of interest	*Trimeresus albolabris* and *T. macrops*	Dead snake or visualisation of green snake	NR	fibrinogen <100 mg/dL	55	6	1	46	2	0·86 (0·42-1·00)	0·96 (0·86-0·99)
Sano-martins, 1994 (133)	Brazil	STDEPC/ NR	*Bothrops* spp	Dead snake and EIA	NR	fibrinogen <100 mg/dL	69	37	7	23	3	0·84 (0·70-0·93)	0.88 (0·70-0·98)

Key: * Data included from study that did not assess the validity of 20WBCT as primary objective, † Conference abstract, ‡ Published MD thesis in repository. NR= Not reported, NHMRC= Australian National Health and Medical Research Council, RHMU= Ramathibodi Hospital Mahidol University, CMC = Christian Medical College, CAPES = Coordenção de Aperfeiçoamento de Pessoal de Vível Superior, CNPQ = Conselho Nacional de Desenvolvimento Científico e Tecnológico, FAPEAM = Fundação de Amparo à Pesquisa do Estado do Amozonas, TRF = Thailand Research Fund, CHEMET = Commission on Higher Education, Ministry of Education, Thailand and STDEPC = Science and Technology for Development Programme of the European Community.

Diagnostic test accuracy (DTA)

Three studies were not included in the meta-analysis due to use of different reference test thresholds for defining coagulopathy with further data not being available from the study authors (106, 124, 125). These studies (n=264) were performed in Sri Lanka, Papua New Guinea and India. The remaining 12 studies (n= 2006) were included in a meta-analysis (37). Six studies (n= 1,400) used INR > 1.4 as the reference test. The aggregate weighted sensitivity and specificity of the 20WBCT at detecting INR >1.4 was 0.84 (95% confidence interval (CI) 0.61 to 0.94) and 0.91 (CI 0.76 to 0.97) respectively, with a positive likelihood ratio of 9.1 (CI 3.3 to 25.7) and negative likelihood ratio 0.18 (CI 0.07 to 0.47) (Figure 4). The summary receiver operating characteristic (SROC) curve has an area under the curve (AUC) of 0.94 (CI 0.91 to 0.96)

Figure 4



Forest plot of sensitivity and specificity of the 20WBCT when compared to a gold standard clotting assay of INR>1.4. Blue dot size represents the number of participants in each study, red dots represent pooled analyses and stems signify 95% CI. Reproduced with permission from Lamb et al (37).

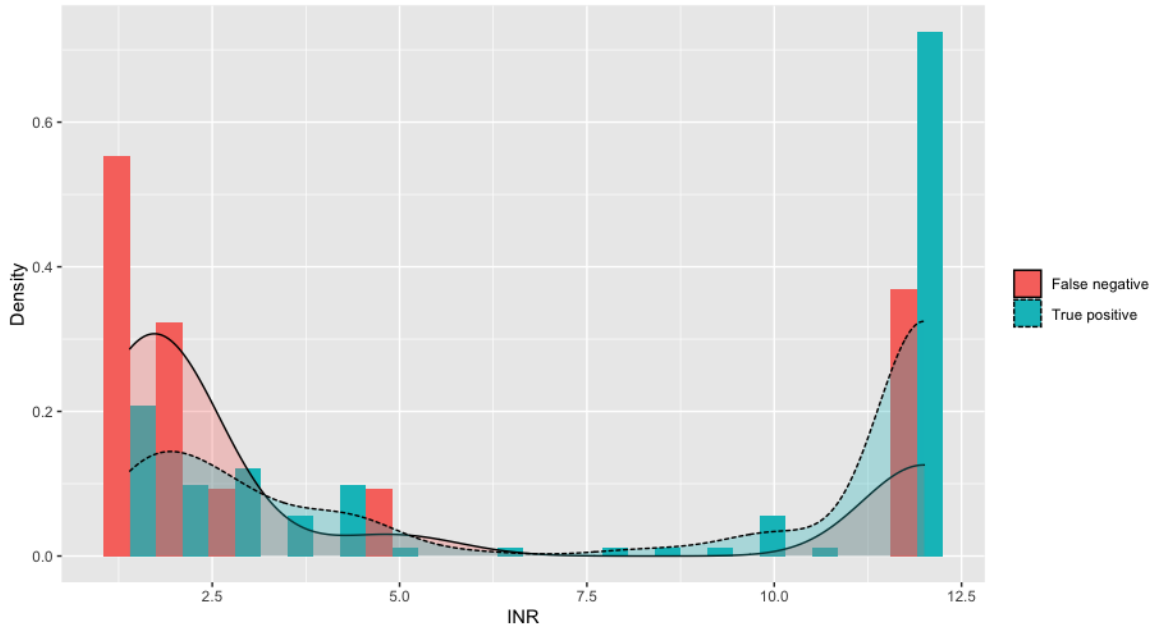
Six studies (n=606) used fibrinogen concentration as the reference test. The aggregate weighted sensitivity and specificity of the 20WBCT at detecting fibrinogen <100 mg/dL was 0.72 (0.58 to 0.83) and 0.94 (0.88 to 0.98) respectively with a positive likelihood ratio of 12.9 (6.1 to 27.5) and negative likelihood ratio of 0.30 (0.19 to 0.46). The SROC AUC is 0.93 (0.91 to 0.95). There was considerable heterogeneity in data for studies that used INR as the reference test (I^2 = 83.4 (CI 71.0 to 95.7) and 96.2 (CI 94.3 to 98.0) for sensitivity and specificity, respectively) and fibrinogen as the reference test (I^2 = 76.5 (CI 57.5 to 94.0) and 21.0 (CI 0.0 to 86.5) for sensitivity and specificity, respectively) (37).

A comparison of true positive and false negative 20WBCT

Individual patient data (n= 1,865, 82.2%), were either reported, displayed in figures or provided upon request in nine studies (37). In these studies, the median INR for patients with a false negative

20WBCT (n= 33) was 1.9 (IQR 1.6 to 12.0, skewness of 1.06 and kurtosis of -0.83). This was lower than for patients with a true positive 20WBCT (n=130) (median 12.0 (IQR 12.0 to 12.0, skewness -0.34 and kurtosis -1.78) p value <0.001 (Figure 5). The median fibrinogen (n= 56) for patients with false negative 20WBCT was 51 mg/dL (IQR 35 to 75, skewness 0.49 and kurtosis of -1.29) which was greater than for patients with a true positive 20WBCT (n= 189) (median 31 mg/dL (IQR 31 to 35, skewness 3.3, kurtosis 10.7) p value <0.001 (37).

Figure 5



A density plot displaying the distribution of false negative and true positive 20WBCT results using a reference test of INR. Reproduced with permission from Lamb et al (37)

Sub-analyses – Accuracy of the 20WBCT to detect severe coagulopathy

Using the individual patient data values described above (n= 1,865), a sub-analysis was performed to assess the ability of the 20WBCT to detect severe coagulopathy, defined as INR >5.0 or fibrinogen <50 mg/dL (37). The aggregate weighted sensitivity and specificity of the 20WBCT at detecting severe coagulopathy were 0.91 (CI 0.64 to 0.98) and 0.90 (CI 0.74 to 0.96), respectively. The 20WBCT SROC AUC for detecting severe coagulopathy is 0.96 (CI 0.94 to 0.97) (37).

20WBCT after antivenom, a test of coagulopathy resolution

Only four studies, with a total of 238 patients, provided paired data for the 20WBCT and reference test at a time interval of 6 hours or 12 hours after antivenom administration (37). The sensitivity of the 20WBCT at identifying persistent coagulopathy (defined as INR > 1.4 or fibrinogen < 100 mg/dL) at 6 or 12 h ranged from 0.05 (CI 0.01 to 0.17) to 0.67 (CI 0.09 to 0.99). The specificity of the repeat 20WBCT at 6 or 12 h ranged from 0.80 (CI 0.68 to 0.90) to 1.00 (CI 0.89 to 1.00) (37).

QUADAS2 - Test of bias assessment

The 15 included studies were subjected to a QUADAS2 assessment of bias (Table 4). Study methodology lacked details concerning patient recruitment in 6/15 studies (37). Four studies did not specify whether patients with pre-existing coagulopathy were excluded. In the absence of a published standard operating procedure for conducting the 20WBCT, 9/15 studies either referenced or adopted the methodology by Warrell et al or WHO guidelines (104, 109), three studies put 1 mL blood into a 5 mL glass tube, but otherwise followed the methodology specified by Warrell et al and three studies did not specify the 20WBCT methodology (37). All participants in each study received the same reference test. The methodology of the reference test was deemed to be suitable in the 13 studies in which it was described, and one paper reported laboratory accreditation. One paper provided no methodological detail relating to the index and reference tests. Three studies

performed the reference test on frozen samples and in two studies it was unclear how the final reported statistics were derived (37).

Table 4: An assessment of bias for included studies in the systematic review using the QUADAS-2 tool. (Reproduced with permission from Lamb et al (37)).

Studies designed with objective to assess the validity of the 20WBCT									
First author, year of Publication	Was a consecutive or random sample of patients enrolled?	Was a case control design avoided?	Did the study use appropriate exclusion criteria?	Was the 20WBCT interpreted without knowledge of reference standard?	Was the 20WBCT conducted using standardized technique?	Was the reference standard, its conduct, or its interpretation performed in a manner to avoid bias?	Did all patients receive the same reference standard?	Was the reference standard performed on site and not require freezing?	Were all patients accounted for in the analysis?
Thongtonyong, 2020	Unknown	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Tongpoo, 2020	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
DSilva, 2019	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

Saengnoi, 2019	Unknown	Yes	Yes	Yes	Yes	Yes	Yes	Unknown	No
Silva, 2018	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes
Ratnayake, 2017	Yes	Yes	Unknown	Yes	Yes	Yes	Yes	Yes	Yes
Paiva, 2015	Unknown	Unknown	Unknown	Yes	Yes	Unknown	Yes	Yes	Unknown
Biradar, 2015	Yes	Yes	Yes	Unknown	Unknown	Unknown	Yes	Unknown	Yes
Pongpit, 2012	Unknown	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes
Sano-martins, 1994	Unknown	Yes	Unknown	Yes	Yes	Yes	Yes	Yes	Yes
Studies that were not specifically designed with the objective of assessing the validity of 20WBCT									
Bhatt, 2020	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Wijewickrama, 2020	Unknown	Yes	No	Yes	Unknown	Yes	Yes	Yes	Yes

Sharma, 2020	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
Shenoy, 2020	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Oliveira, 2019	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes

Discussion

For almost 50 years, the 20WBCT has been used for the assessment and management of snakebite in a wide variety of locations across the world that lack access to more sophisticated laboratory facilities (109, 134). Contrary to its initial intended use, which was to detect patients at most risk of imminent haemorrhage, the studies reported here were mostly designed to determine whether the 20WBCT could detect mild coagulopathy (37). In snakebite patients, the sensitivity of 0.84 and 0.72 (using INR and fibrinogen as reference test respectively), specificity of 0.91 and 0.94 (using INR and fibrinogen as reference test respectively) and SROC AUC of 0.94 and 0.93 (using INR and fibrinogen as reference test respectively) of the 20WBCT at detecting coagulopathy, support its continued use in the absence of any alternative, but highlight the need to improve diagnostic tests for VICC (37).

The sensitivity of the 20WBCT improved when it was used to detect severe coagulopathy. This is an important consideration for the many countries that lack sufficient antivenom supply to treat all systemically envenomed patients (94). However, the 10% of patients with severe coagulopathy that were not identified by 20WBCT remain a concern (37). It is important to stress that the 20WBCT should be used in addition to clinical assessment. For example, some patients with a false negative 20WBCT following *Daboia russelii* envenoming may be identified as having systemic envenoming through careful clinical assessment for evidence of neurotoxicity (131).

The comparison of admission clotting assays for patients with correctly identified coagulopathy by 20WBCT (TP) and coagulopathy missed by 20WBCT (FN) (Figure 5) demonstrates that the 20WBCT is less effective at identifying less severe coagulopathy (37). This concern has been raised by authors in Sri Lanka that have reported that the mild coagulopathy, common to systemic envenoming by the Hump-nosed pit viper (*Hypnale hypnale*), is commonly not detected by 20WBCT (135). The clinical implications of missing systemic envenoming with mild coagulopathy are uncertain.

Despite the lack of sophisticated equipment and comparative ease with which the 20WBCT can be performed, the importance of using a clean, dry, glass test tube cannot be overstated (37). There is

no clinical evidence indicating the validity of plastic containers for 20WBCT. In an assessment of commercially available plastic containers in Australia, only polyethylene terephthalate plus a clot activator 'Vacuette Z/serum separator' was able to identify true negatives (the identification of negative (clotted) 20WBCT in normal controls) (136). In Benin, Benjamin et al assessed plastic syringes as a vessel for 20WBCT and found them unable to adequately detect true negatives (105). In Myanmar, imported glass test tubes were found to be coated with silicon which impeded clot activation producing false positive results (positive (unclotted) 20WBCT in normal controls) (Dr. Myat Thet Nwe, unpublished communication 2020). In a pre-clinical study performed by Paiva et al, soda lime glass test tubes compared favourably at detecting true negative (normal clotting) in comparison to borosilicate glass test tubes and BD vacutainer glass tubes over a range of ambient air temperatures (125).

The importance of a standardised technique, equipment and operator training for the 20WBCT was illustrated by a pair of studies from Sri Lanka (110, 131). The authors identified markedly improved sensitivity of the 20WBCT at detecting coagulopathy (from 40% to 82%) following the introduction of training, a standard operating procedure and implementation of single-use glass test tubes (131). Whilst the discrepancy in results underlines the importance of external validation, considerable differences may be expected if the index test (20WBCT) and reference test (INR) are collected non-synchronously, particularly given the dynamic process of haemostasis (37).

Although repeat paired sampling of the 20WBCT and clotting assays at 6 and 12 h after antivenom was conducted in far fewer patients, the comparatively poor sensitivity of the 20WBCT at detecting snakebite coagulopathy at these time points is concerning (37). Given the widespread use and recommendation in international guidelines of the 20WBCT to assess response to antivenom, it is surprising that few studies have sought to determine the diagnostic accuracy of the 20WBCT at detecting coagulopathy following antivenom (37). Work is required to understand the pharmacodynamic response to antivenom and how this may be used to assess antivenom effectiveness. Several studies have looked at the time taken for clotting assays to normalize after

antivenom in patients envenomed from snakes that predominantly cause a venom induced consumption coagulopathy (VICC). The median time in these studies ranged from 10-24 h for INR and 6-24 h for fibrinogen (37, 137). In a study of 18 patients administered supranormal antivenom dosing for systemic *Daboia siamensis* envenoming, the median time to negative (clotted) 20WBCT was 4 h (138). In contrast to the more commonly encountered VICC, anticoagulant coagulopathy following snakebite (such as coagulopathy arising from *Pseudechis australis* envenoming) may be expected to rapidly reverse following adequate dosing of antivenom (139).

Alternative strategies for detecting systemic snakebite envenoming require further investigation. Given the epidemiology of snakebite and continued scarcity of sophisticated diagnostic facilities in remote locations, the requirements of any diagnostic test for snakebite identified by Warrell et al more than 50 years ago, remain true. Tests for detecting VICC are required to be quick, cheap, require limited equipment and training and provide reliable and repeatable results. Handheld point-of-care devices for detecting snakebite coagulopathy meet many of the requirements for determining systemic envenoming. Unfortunately, the published literature, which includes three devices (i-STAT, Alere INRatio2 and Coagucheck S) reported in two studies, has found all these devices to underestimate coagulopathy and provide false negative results (140). It is hypothesised that the reason for this underestimation of coagulopathy is due to the use of a thrombin substrate within two of these devices. Providing there is sufficient prothrombin, the thrombin substrate will produce an electrochemical signal even in cases with severely depleted fibrinogen (140). Abouyannis et al hypothesise that alternative POC devices, such as those that detect fibrin clot formation, may provide more reliable results for the detection of snakebite coagulopathy (140). Further work is required for determining in vitro validity, both in VICC diagnosis and in assessing antivenom response. Whilst the up-front infrastructure costs ($500-$5000) are likely to remain prohibitive to most healthcare providers treating patients with snakebite, they represent and appealing tool for determining VICC and antivenom efficacy in snakebite research.

An alternative family of tests that warrant further investigation for the diagnosis of snakebite coagulopathy, are activated clotting tests. Activated clotting tests measure the time taken for whole blood to clot in the presence of a factor VII activator, such as kaolin. In a study performed in Vellore (not peer reviewed), the Vellore manual activated clotting time test (VeMAC) performed favourably when compared to 20WBCT (108). Using a cut-off of 240 seconds, the VeMAC had a 93.8% sensitivity and 96.6% specificity, which corresponded to an AUC ROC of 97.7%. Although these tests were performed in a laboratory, the requirement for technical skills was low and the procedures required only basic laboratory equipment including pipette, glass test tube and kaolin (a naturally occurring clay mineral).

A limitation of this systematic review of diagnostic test accuracy was the lack of confirmed snake species. Venom composition and therefore mechanism and extent of VICC, varies greatly from one snake species to the next (141). It is likely that the range of snakes responsible for envenoming in this study will have significantly contributed to the heterogeneity observed in meta-analyses. Other potential confounding factors that may have influenced the DTA in the reported studies include the time from bite to index test, type of test tube glassware, ambient air temperature, operator training and prior antivenom treatment.

Conclusion

This systematic review provides important insights into the diagnostic test accuracy of the 20WBCT and its limitations for detecting systemic envenoming. In the absence of available laboratory clotting assays, particularly in low and middle-income country (LMIC) settings, the 20WBCT remains the best available method for detecting systemic haemotoxic envenoming and a good method for detecting severe coagulopathy following snakebite (37). For this and future research projects in snakebite envenoming, the 20WBCT remains a suitable test for screening asymptomatic patients presenting with snakebite, which can later be confirmed using formal laboratory clotting assays. Further research is required to better define the role of activated clotting tests such as the VeMAC test and point of care coagulations tests in snakebite envenoming.

Chapter 3. Materials and methods of a prospective observational study of urine for cross-linked heme in patients with systemic haemotoxic envenoming, in India

Herein chapter 3, I describe the study design, sampling procedures, endpoint definitions, RP-HPLC method, internal validation approach and statistical analysis. Chapter 4 then presents the resulting clinical and chromatographic findings from the enrolled participants and relates the RP-HPL findings to myotoxicity, MAHA and AKI.

Site selection and study design

To address the study objectives 2-4, namely, to determine whether there is cross-linked heme in the urine of patients with systemic viper envenoming and its association with a snakebite pathology, a proof-of-concept observational study was planned. All participants and healthy controls were recruited from the medical wards of CMC Vellore, Tamil Nadu, India. The site was selected due to its experience in conducting snakebite research, its modern sophisticated diagnostic laboratory and adjacent research biochemistry department.

Sample size estimation was performed prospectively with the goal of recruiting five participants with severe myotoxicity and five participants with TMA. The following assumptions were made based on literature presented in chapter 1 and opinions of experienced clinicians at CMC Vellore.

- 20% of systemically envenomed snakebite patients at CMC Vellore develop severe myotoxicity defined as CPK greater than or equal to 5000 U/L.
- 40% of systemically envenomed snakebite patients at CMC Vellore develop AKI.
- 50% of systemically envenomed snakebite patients at CMC Vellore with AKI have evidence of TMA.

Based on these assumptions, in a sample of 25 systemically envenomed participants, one might expect 10 participants to develop AKI, five participants to develop TMA and five participants to have severe myotoxicity.

Efforts were made to exclude or monitor potential confounding factors that may influence the pathophysiology of envenoming. This included exclusion of participants with prior history of CKD, disorders of coagulopathy or anticoagulant medication. Study procedures also specified close monitoring of vital signs to assess for periods of hypo or hypertension.

It was expected that some patients will present to the study site having had prior treatment in an earlier healthcare facility. This is likely to affect the clotting test results by the time of presentation to the study site. For this reason, it was deemed acceptable that evidence of VICC from the referring hospital (either positive 20WBCT or an INR >1.4) would be considered sufficient for evidence of systemic envenoming. In addition to 20WBCT, blood was collected for INR, Fibrinogen and D-Dimer on admission. Given the anticipated variation in time from bite to informed consent and first blood draw, poor evidence basis for initial antivenom dosing and uncertain pharmacokinetic and pharmacodynamic of snake venom and antivenom, it was expected that some patients would have ongoing VICC beyond the first 24 hours of presentation. For this reason and the expected laboratory variation in the reading of blood films, FBC and schistocyte counts were collected at four different time points (day 1, 3, 5 and 7). Similarly, CPK values are known to peak between 24-72h after muscle injury and therefore CPK was measured on Day 1 and Day 3.

The kinetics of creatinine rise in SAKI is not well documented. Although AKI is typically present within 12 hours of snakebite bite, (54) serial measurement of creatinine was performed on days 1, 2, 3, 5 and 7 to gain greater understanding of creatinine peak and timing of renal replacement therapy Table 5).

There is limited literature on the optimal timing of measuring CFH in diseases states. Given the proposed pathophysiology outlined in chapter 1, I expected CFH to correlate closely with periods of maximal haemolysis and myotoxicity. Given pragmatic trade-offs in cost and time of analysis, urine was collected for analysis at three time points (day 1, day 3 and day 5). A full outline of blood and urine test analyses are outlined in Table 5.

Table 5. Daily procedural list for envenomed patients

	Admission	D2	D3	D4	D5	D6	D7
Daily review in CRF	**X**	**X**	**X**	**X**	**X**	**X**	**X**
FBC	**SOC**	**X**	**x**	**X**	**x**	**x**	**x**
Blood film	**SOC**	**X**	**x**	**X**	**x**	**x**	**x**
20WBCT	**SOC**						
INR	**SOC**						
APTT	**SOC**						
Fibrinogen	**SOC**						
D-dimer	**SOC**						
Creatinine	**SOC**	**X**	**x**		**x**		**x**
LFT	**SOC**		**x**				
CPK	**SOC**		**x**				
Urine RE/ME	**SOC**	**X**	**x**		**x**		**x**
Urine for storage	**X**		**x**		**x**		

Procedures were stopped if a participant was discharged prior to completing the period of observation.

Eligibility criteria

Inclusion criteria:

- History of snakebite in the preceding 24 hours
- Venom induced consumption coagulopathy (determined by either a positive 20WBCT or INR > 1.4) from CMC Vellore or a referring hospital prior to antivenom therapy.
- Aged >17 years old
- Written informed consent from the patient or caregiver where the patient lacked capacity.

Exclusion criteria:

- Known chronic kidney disease
- Known pre-existing haematological disorder
- Prior history of consuming pro-coagulant medication
- Inability of patient or caregiver to provide informed consent

Procedures

All new snakebite patients with suspected viper envenoming presenting to CMC Vellore Emergency Department were notified to the research team within 2 hours of admission. Once a patient was deemed medically stable by the attending medical team, the assigned researcher screened for eligibility criteria and provided a patient information sheet (PIS). The participant was then given opportunities to discuss the study and procedures. The PIS and informed consent form was available in the most commonly spoken languages in Vellore (Tamil, Telagu and Hindi). A copy of the consent form is attached as Appendix 1. Participants were informed that there was no financial incentive to participate however, all mandated study procedures that were not considered standard of care were

provided free of charge. Participation in the study did not alter the clinical management which was determined by an independent attending medical team.

After informed consent was obtained, the participant was provided a copy and assigned a study ID. The participant was added to a linking document retained by Medicine Unit 1 and stored separately to the informed consent form. An assigned research team member then completed the case report form (CRF) admission document and ensured that all Day 1 procedures were completed (Appendix 2). A document outlining the participation in the study and daily procedures was left in the patient's hospital record. A research team member then attended daily to complete the CRF and facilitate the collection of blood and urine samples. A research nurse completed the password protected database on Microsoft Excel and a second investigator cross checked all CRF/database entries against the source document (lab results and medical notes). The completed CRF was stored in the locked research office with access limited to delegated study staff only. The CRF was stored in a separate secured filing cabinet to the Informed consent and linking document.

Urine samples were inspected and the colour was documented in the CRF. A 5-point colour gradient description was provided as reference (although this did not include the colors themselves). Samples for microscopy were labelled with name and hospital number and sent to the clinical pathology laboratory and processed according to normal laboratory procedures. On days 1, 3 and 5, an additional urine sample was collected in a universal specimen container for ferryl-heme derivatives (FHD) at transported to the neurochemistry laboratory. Samples for FHD were labelled with study ID, date of collection and day of study enrollment**.** On receipt of the sample, a 500 µl aliquot was separated. Both the 500 µl aliquot and remaining urine were labelled and stored at -20^{o}C. At a later date, aliquot samples were transferred to the Biochemistry department in CMC Vellore in batches to enable reverse phase HPLC analysis for cross-linked heme.

Clinical endpoints

MAHA is defined as the presence of schistocytes on blood film greater or equal to 1% of all RBCs (46).

TMA is defined by MAHA ***and*** a platelet count of < 150 x10^9 ***or*** a 25% drop from the baseline recorded reading (46).

Myotoxicity was subdivided to

(i) mild myotoxicity, defined by maximum serum CPK value of greater than or equal to 500 U/L but less than 5000 U/L

and

(ii) severe myotoxicity defined by maximum serum CPK value of greater than or equal to 5000 U/L

AKI was defined according to the Kidney Disease Improving Global Outcomes (KDIGO) criteria, and graded using the definitions below, (142)

(i) AKI stage 1 was defined as an increase in serum creatinine by greater than or equal to 0.3 mg/dL within 48 hours or an increase in serum creatinine to 1.5-1.9 times baseline.

(ii) AKI stage 2 was defined as increase in serum creatinine greater than equal to 2.0 to 2.9 times baseline.

(iii) AKI stage 3 was defined as greater than or equal to 3.0 times baseline or increase in serum creatinine to greater than or equal to 4.0 mg/dL or initiation of RRT.

The baseline serum creatinine was defined as the lowest serum creatinine greater than 0.4 mg/dL recorded during follow up.

Reverse phase high performance liquid chromatography (RP HPLC) for detection of cross-linked heme

A protocol was adapted from Reeder et al (83, 84), to identify X-linked heme, which is specific to peroxide induced ferric-ferryl redox cycling, and indicative of lipid oxidative reactions. RP HPLC is the preferred method of demonstrating X-linked heme as it allows for the separation of Hb/Mb-X from apomyoglobin, unmodified 'free' heme and oxidatively modified heme (84). In experiments performed by Reeder et al, the X-Hb/Mb elutes between 19 and 24.5 minutes in a broader band like pattern, markedly different from the unmodified Hb/Mb that elutes as a single peak at 18 minutes and apoprotein that elutes as a single peak at 22 minutes (83). Procedures were fine-tuned and discussed with Prof Reeder from the University of Essex and the co-investigators based at CMC Vellore. All procedures were performed by Dr Soosaimanickam, a post-doctoral biochemist at CMC Vellore Biochemistry department.

All HPLC procedures were performed using Shimadzu CMB-20A HPLC system controller, Shimadzu LC-20 AC pump and Shimadzu SPD-20A UV/VIS detector. An Agilent Zorbax 300SB-C3 reverse phase chromatography column (particle size 5 µm, dimension 4.6x250 mm) was used for analytical column and analytical guard column.

Acid Butanone extractions of X-linked heme-protein using hydrogen peroxide (H202)

The acid butanone extraction of X-linked Hb/Mb was developed as an internal control. X-linked Mb or Hb is extracted in vitro from the addition of H202 under acidic conditions (84). For the Mb extraction, 50 µM ferric myoglobin (equine heart M1882-250MG from Sigma Aldrich) and 150 µM H_2O_2 were dissolved in 20 mM sodium acetate buffer (pH 4.9), at room temperature. For the Hb extraction, 15 µM Haemoglobin (Human haemoglobin lyophilized powder (H7379-1G) from Sigma Aldrich) and 180 µM H_2O_2 were dissolved in 20 mM sodium acetate buffer (pH 4.9), at room temperature. The mobile phase solvent (a) was MQ water (Milli-Q Water- EQ 7000) with 0.1% trifluoro acetic acid and the mobile phase solvent (b) acetonitrile with 0.1% trifluoro acetic acid. The elution profile (solvent B concentration) was set at 35% for 10 minutes followed by 37% for 5 minutes. This was increased to 40% (solvent B) for one minute and then 43% for 10 minutes and 35% for 14 minutes. The mobile phase flow rate was 0.8mL per minute at 25 °C and the total running

time was 40 minutes. The Zorbax 300SB-C3 analytical column was connected with the guard column and washed with 90% solvent B for 20 minutes and conditioned with 35% solvent B for 20 minutes. 20 µL of the Mb/Hb and H_2O_2 2 solution was filtered and injected into the port using a Hamilton syringe. The profile observed in UV-detector was observed at 280 nm, 400 nm, 630 nm and 720 nm. The separation of HPLC profile were analysed using Shimadzu LabSolutions software.

Urine spiked with myoglobin and haemoglobin

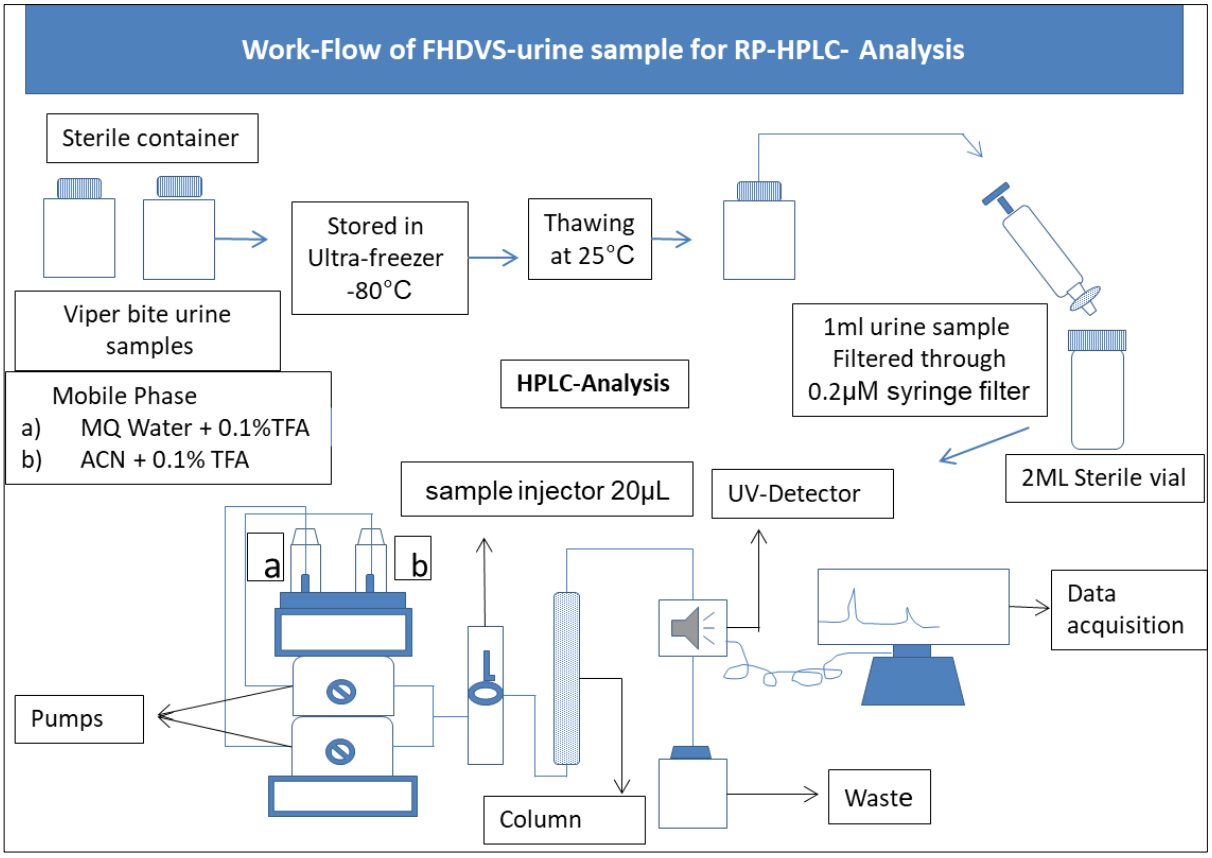
Additional control samples were prepared using urine from human healthy controls spiked with Mb and Hb. Urine samples were thawed to 25 °C and filtered through 0.22 µm syringe filter (Spin-Pure disposal nylon syringe filter - Spincotech). Urine was spiked using 15 µM Hb (human haemoglobin lyophilized powder (H7379-1G)) and a range of 1-5 µM ferric Mb. 20 µL of spiked urine was injected in the HPLC system and the profile observed using the same elution profile detailed earlier.

Study samples

Samples were thawed from -20°C to 25°C. One mL of urine was taken in 2 mL syringe, filtered using 0.22 µm syringe filter and transferred to a sterile 2ml Cryo chill vial. The mobile phase solvents (A & B) and elution gradients were performed as outlined in the acid butanone extraction. The mobile phase solvent (a) was MQ water (Milli-Q Water- EQ 7000) with 0.1% trifluoro acetic acid and the mobile phase solvent (b) acetonitrile with 0.1% trifluoro acetic acid. The elution profile (solvent B concentration) was set at 35% for 10 minutes followed by 37% for 5 minutes. This was increased to 40% (solvent B) for one minute and then 43% for 10 minutes and 35% for 14 minutes. The mobile phase flow rate was 0.8mL per minute at 25 °C and the total running time was 40 minutes. The Zorbax 300SB-C3 analytical column was connected with the guard column and washed with 90% solvent B for 20 minutes and conditioned with 35% solvent B for 20 minutes. 20µL urine sample was taken and injected into the HPLC system. The profile observed in UV-detector was observed at 280 nm, 400 nm, 630 nm and 720 nm. The separation of HPLC profile were analysed using Shimadzu

LabSolutions software. A schematic of procedures is illustrated in Figure 6. The same method was performed for healthy control urine samples.

Figure 6.



This schematic illustrates the workflow of samples from collection, storage, filtration, HPLC injection and analysis. MQ (Milli-Q Water), ACN (acetonitrile), TFA (trifluoro acetic acid).

Statistical analysis

Median and IQR was used to describe descriptive statistics and categorical data. For longitudinal variables, for example schistocyte count, CPK and creatinine, peak value was used. Unpaired categorical data was assumed to be nonparametric and was compared using Mann-Whitney test. Contingency data was assumed to be non-parametric and data was compared using relative risk with 95% confidence intervals and Fishers exact test of significance. An assessment of correlation was performed using Spearman's rank coefficient, where X and Y variables were both independent observations and assumed to be nonparametric. A two-tailed p value of <0.05 was considered statistically significant. Chromatographs were produced by Shimadzu Labsolutions software. AUC was calculated automatically by the software using a manual integration line covering the area of elution. As the data was skewed it was then log-transformed.

Ethics

Ethical approval for the study was prospectively granted by CMC Vellore Institutional Review Board on 21/06/2023 and Oxford Tropical Research Ethics Committee minimal risk (reference 513-23) on 11/03/2023.

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(i) Professor Elizabeth Ashley's principal investigator fund, amounting to 4000 GBP.

(ii) CMC Vellore Institutional Review Board Fluid grant allocation of 300,000 Indian rupees 2568 GBP.

Chapter 4. Results of a prospective observational study of urine for X-linked heme in patients with systemic haemotoxic envenoming, in India

Baseline characteristics

Recruitment of participants commenced on 31st December 2023 and was completed on 13th May 2025. During this period, 24 participants met eligibility criteria, provided informed consent, and were enrolled into the study. A further 3 participants were recruited as healthy controls from staff members at CMC Vellore on a voluntary basis. Posters were placed to promote the recruitment of healthy controls and informed consent was obtained before any study procedures were completed. The median age of participants was 45.5 years (IQR 36.5-61.5) and 17/24 (70.8%) were male table 6). All participants were identified as having 'Viper' envenoming based on a history of snakebite and compatible toxidrome. No dead snakes were brought with participants for identification. Seventeen participants (70.8%) received antivenom, median dose 10 vials, prior to presentation at CMC Vellore. The median time from bite to presentation to CMC Vellore was 4h 30m (IQR 2.30-6.40). Baseline characteristics of participants is summarised in table 6. Twenty (83.3%) participants had evidence of coagulopathy defined as INR>1.4 or fibrinogen <100 g/dL at presentation. Of the remaining four participants, three had received antivenom prior to CMC Vellore. One participant, who attended CMC Vellore directly following the snakebite and had not received antivenom prior to enrolment, had no other features of systemic envenoming aside from a positive 20WBCT. With review of all available baseline investigations, this participant was not systemically envenomed and therefore the positive 20WBCT was assessed as falsely positive by the treating clinicians.

Table 6. Baseline characteristics of study participants.

	Snakebite (n=24)	Control (n=3)
Sex	17 male, 7 female	1 male, 2 female
Median age (IQR) [years]	45.5 (36.5-61.5)	29.0 (28.0-34.0)
Medical history		
Nil significant	19	3
Diabetes mellitus	4	0
Hypertension	2	0
Ischaemic Heart Disease	1	0
Stroke	1	0
Median (IQR) time from bite[hh:mm]	04:30 (02:30 – 06:40)	N/a
Number of participants administered antivenom prior to arrival (median dose)	Antivenom: 17 (10 vials) No antivenom: 5 Unknown: 1	N/a
Mean arterial blood pressure (mmHg)	77.6 (SD 16.5)	85
MAP <65mm/Hg (n=)	5	0
Median Fibrinogen (IQR) [mg/dL]	63.5 (50.0-137.5)	227 (185.0-298.0)
Median INR (IQR)	2.6 (1.4-9.3)	1.1 (1.0-1.1)
D Dimer (IQR) [ng/ml]	11,279 (5013-55345) Log 4.1 (3.7-4.7)	134 (107-358) Log 2.1 (2.0-2.6)
Systemic bleeding	11	0
Bleeding gums	7	0
Haematuria	3	0
GI bleeding	2	0
Neurotoxicity	15	0
Ptosis	15	0
Ophthalmoplegia	1	0
Dysarthria	1	0

Baseline characteristics of envenomed and control participants. One participant had systemic bleeding at more than one site, two participants had more two features of neurotoxicity.

Outcome

Participants remained in hospital for a median duration of 6 days (IQR 5-13). One participant died on day 71 due to multiorgan failure, following a hospital acquired pneumonia caused by multidrug resistant *Acinetobacter baumannii*. Additional complications not reported in the pre-specified endpoints include four participants who were intubated and ventilated due to neurotoxicity, one anaphylactic shock following antivenom, one deep vein thrombosis on day one of admission, one cerebral infarct whilst intubated and ventilated and one patient who developed panhypopituitarism.

Complications of special interest

A summary of prespecified complications is listed in Table 7 and a trend of laboratory parameters are displayed in Figure 7. Ten participants developed AKI, of which six were graded AKI stage 3 and required RRT. Three participants required RRT within 72h of enrolment, the remaining three participants required RRT by day six of admission. The other four participants developed AKI stage 1 which had peaked within 72h of enrolment. In 4/10 participants with AKI, the AKI was present at the time of admission, the remaining six participants developed AKI on day two. In 8/10 participants, AKI progressed over the initial 48 h of admission (Figure 7). Four participants were discharged with impaired renal function. In two participants renal function had normalised at 30 days of follow up, one participant developed end stage renal failure dependent on dialysis and one participant did not attend follow up. Duration of hospital admission for participants with AKI (median 16.5 days (IQR 6-26)) was significantly greater than those without AKI (median 5 days (IQR 4.8-6.5), p=0.01). Two participants underwent renal biopsy which showed ATN in one patient and ATN plus evidence of diabetic glomerulopathy and moderate arteriolar hyalinosis in the other.

Schistocytes were seen in the blood film of 16/24 participants (66.7%). The median maximum schistocyte count was 0.5% (IQR 0-0.8) with 4 participants recording a maximum schistocyte count of 1% or greater, meeting the definition of MAHA. One additional patient developed a schistocyte count of 2.7% on day 15 but is not considered a case of MAHA during the study as the blood sampling

occurred outside of the planned study procedures (appendix 2). In the four participants with MAHA, serum Hb fell from a median of 16.1 g/dL (IQR 13.5-18.2, normal range 11-17 g/dL) on Day 1 to 8.3 g/dL (6.9-12.9) on Day 7. All four participants with MAHA developed thrombocytopenia (median nadir platelet count of 25.5 $x10^9$/L (IQR 6-62.5, normal range 150-450 $x10^9$/L), consistent with a diagnosis of TMA. Platelet count of participants with TMA was significantly lower than those without TMA (140.5 $x10^9$/L (IQR 83.3-196), p=0.01). Myotoxicity was common, occurring in 19/24 (79.2%) participants, 14 (58.3%) were classified as mild myotoxicity and five (20.8%) were classified as severe myotoxicity. The median peak CPK reading for participants with severe myotoxicity was 5472U/L (IQR 5244-21203, (normal range 28-171 U/L)), with maximum CPK reading recorded on Day 3.

Figure 7

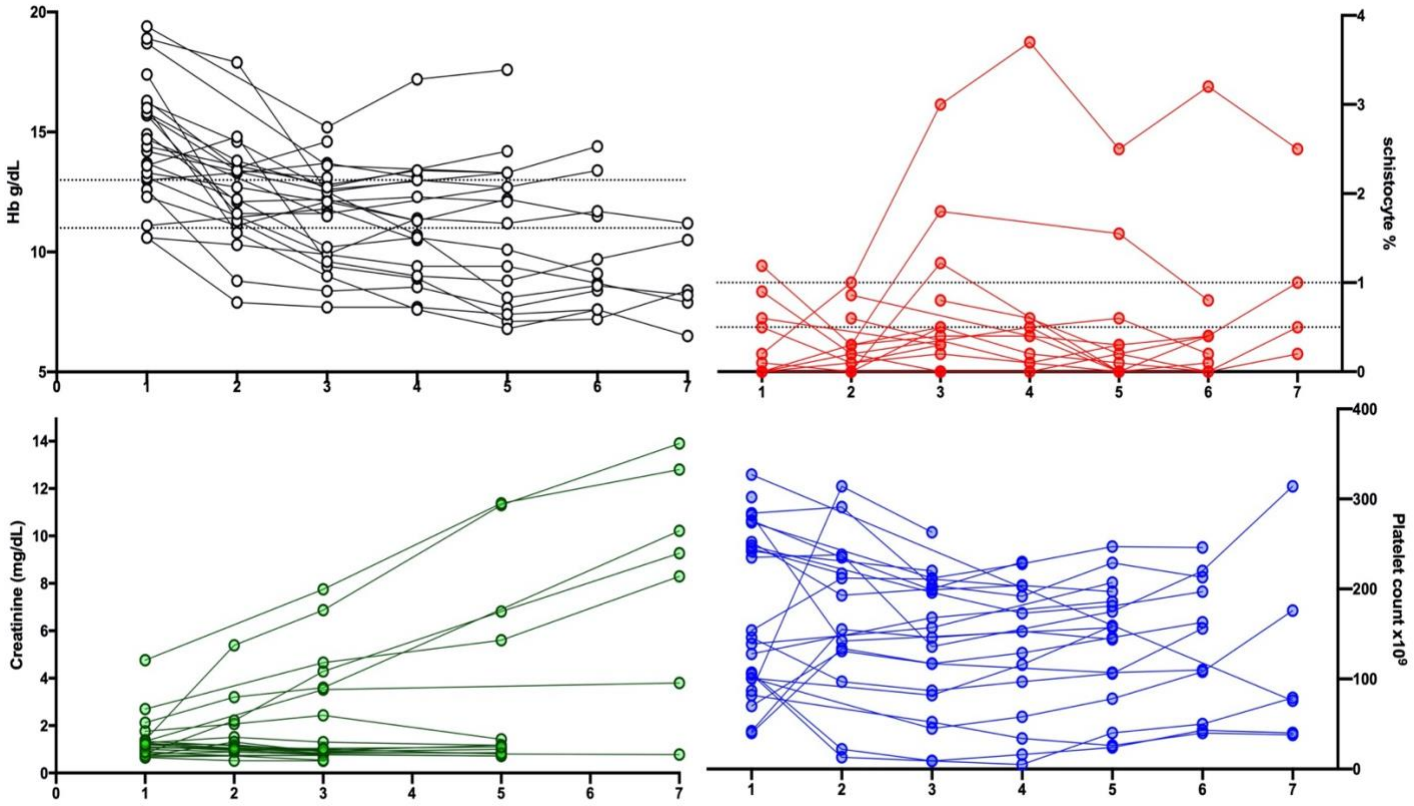


Figure 7. Graphical illustration of individual patient values over the period of observation.

Starting with top left, then rotating clockwise, trend in haemoglobin (g/dL), trend in schistocyte count (%), trend in platelet count (x10^9/L) and trend in creatinine (ug/dL) over 7 days.

Table 7. Laboratory investigations and complications of participants with snakebite divided by presence of X-linked heme.

	All (24)	X-linked heme (n=15)	No X-linked heme (n=7)	p-value
Features of Neurotoxicity	15 (63%)	9 (60%)	5 (71%)	>0.99
Intubated and ventilated	4 (16.7%)	3 (20%)	1 (14%)	>0.99
MAP on admission (IQR) [mmHg]	75 (67-93)	77 (67-100)	80 (63-93)	0.59
Admission D-Dimer (ng/mL)	11,300 (5000-55300)	17500 (8300-60200)	7400 (3900-11900)	0.08
Complications				
Peak CPK (U/L)	1279 (388-4302)	1896 (188-5240)	834 (506-1223)	0.33
Nadir platelet count ($x10^9$/L)	132 (51-193)	136 (45-173)	128 (42-217)	0.53
Peak schistocyte (%)	0.5 (0-0.8)	0.6 (0.2-0.9)	0.4 (0-0.5)	0.20
Peak Creatinine (mg/dL)	1.2 (0.9-3.5)	1.3 (1.1-8.3)	1.0 (0.7-1.1)	0.01
AKI	10	8	1	0.16

Two participants excluded from X-linked heme columns due to FP 20WBCT (1) and urine sample not available (1). Values expressed as median and IQR. Categorical data compared using Mann Whitney test, contingency data compared using Fishers' exact test.

RP HPLC Internal validation experiments

Following procedures outlined in chapter 3, a series of experiments were performed to demonstrate internal validation of the RP HPLC methodology. To begin with, urine was spiked with 5µM of equine heart ferric myoglobin (Figure 8(a)) and 15µM of standard human ferric haemoglobin (Figure 8(b)).

Figures 8(a)

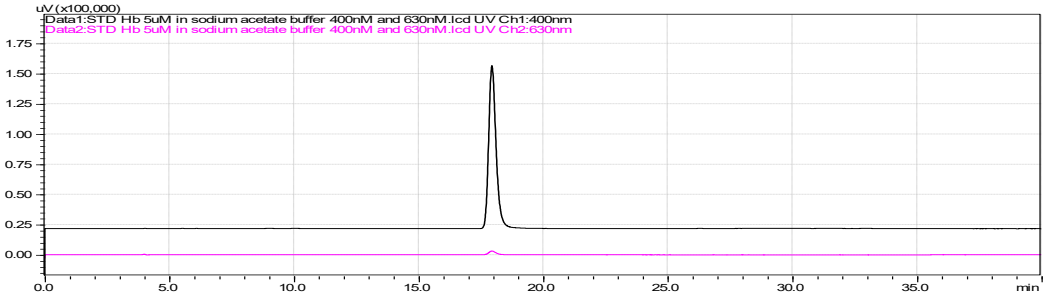
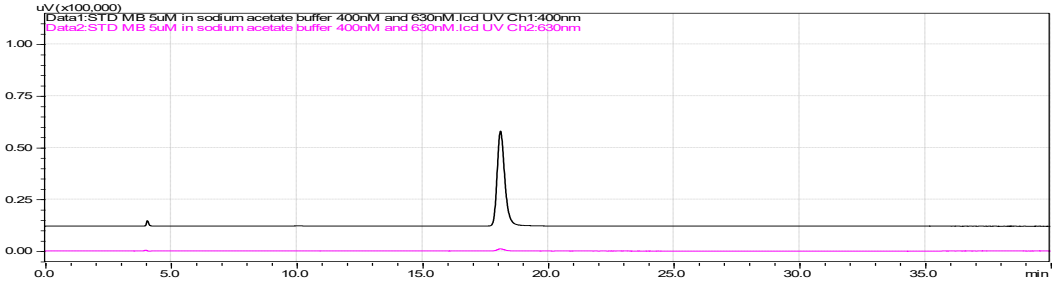


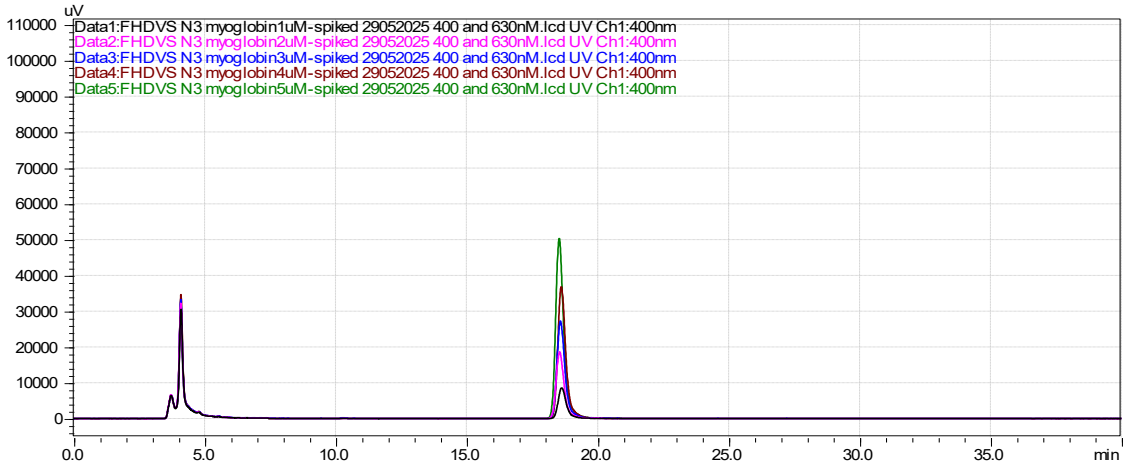
Figure 8(b)



A single peak was observed in both experiments at 18 minutes using the optical spectrum UV 400nm, demonstrating that Mb and Hb are isomeric and optically indistinguishable.

The next experiments were used to demonstrate the relationship of elution spike seen on chromatogram with the concentration of myoglobin. Five samples were run with increasing concentration of Mb in normal control urine sample. Figures 9 demonstrates urine spiked with 1µM, 2µM, 3µM, 4µM and 5µM of myoglobin.

Figure 9



The elution heme spike visible at 18 minutes was proportional to the concentration of myoglobin in each sample. There is no free Mb seen within the eluting time of 22.5-27 minutes.

The acid butanone H_2O_2 extraction of X-linked myoglobin and haemoglobin using a PDA detector is demonstrated in Figure 10a and 10b respectively. In these experiments, the Mb sample (50µM ferric myoglobin and 150µM H2O2) and the Hb sample (15µM Hb and 180µM H2O2) were dissolved in 20mM sodium acetate buffer (pH 4.9), at room temperature.

Figure 10a

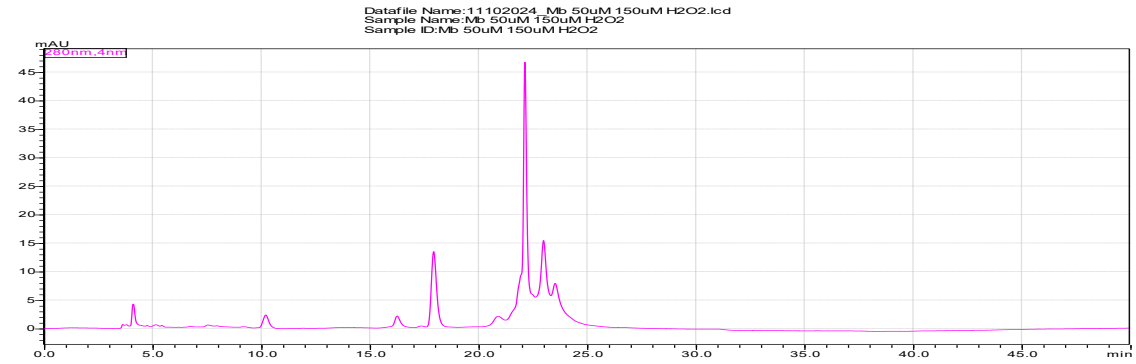


Figure 10b

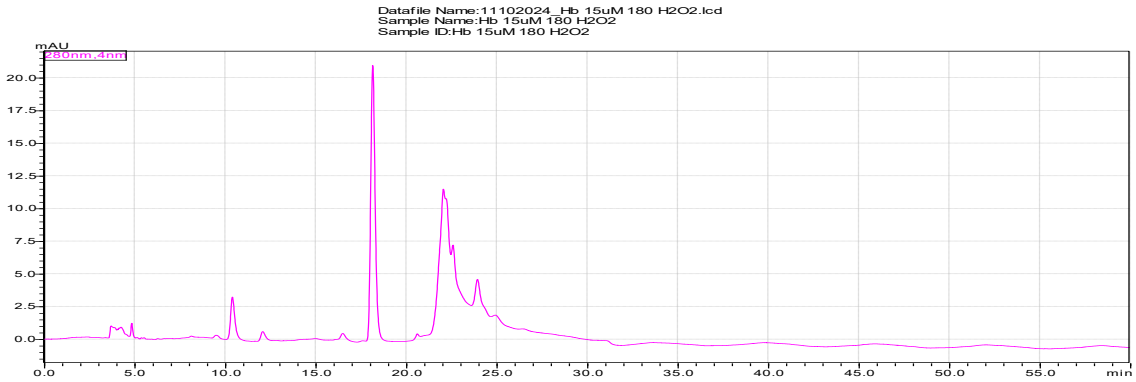


Figure 10a shows the RP-HPLC chromatogram at wavelength 280nm following acid butanone/H_2O_2 extraction of cross-linked myoglobin, while figure 10b displays the corresponding extraction of cross-linked haemoglobin. In both panels, the discrete peak at approximately 18 minutes is consistent with unmodified heme-associated Mb/Hb-derived material, while the broader elution region between approximately 22.5 and 27 minutes is consistent with cross-linked heme-associated species. Smaller early peaks, including those around 4 and 10.5 minutes, are consistent with oxidatively modified heme species.

In these experiments, the free Mb/Hb peak elutes at 18 minutes, as demonstrated in figure 8. There is broad peak that elutes in the range between 22.5-27 minutes. This is consistent with X-linked Hb and Mb as demonstrated in vitro and in vivo experiments published by Reeder et al (84, 87). The chromatogram profile for acid butanone extraction of X-linked myoglobin (Fig 10a) demonstrates greater AUC associated to the X-linked peak when compared to the extraction of X-linked haemoglobin (Fig 10b). This is due to the Hb-X being distributed between alpha and beta chains.

RP HPLC of study participants

The next series of experiments included normal control participants with no history of envenoming. Figure 11a displays the chromatogram of 3 healthy control participants and Figure 11b displays the chromatogram for one study participant who had a false positive 20WBCT and displayed no features of systemic envenoming.

Figure 11a

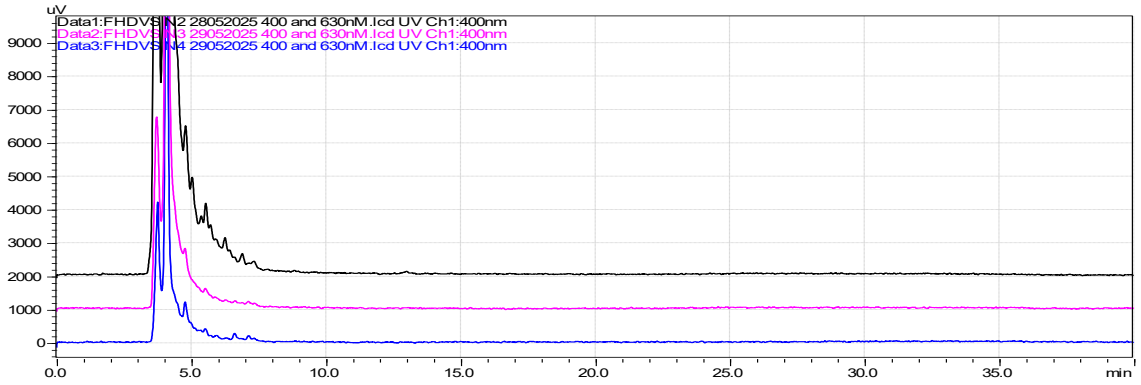
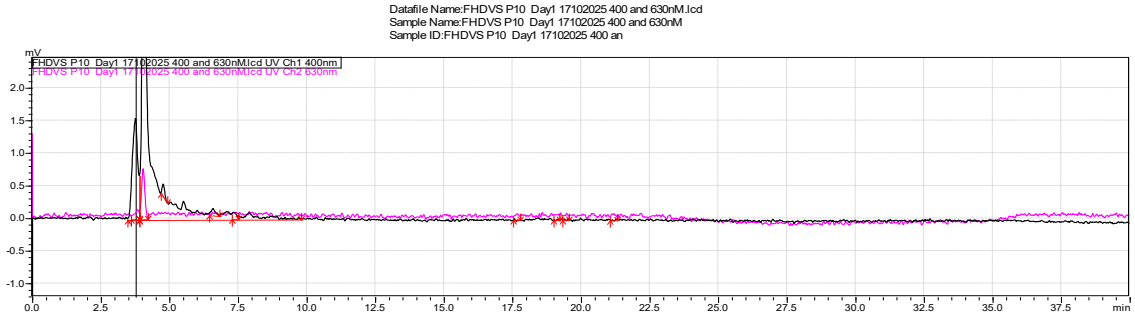


Figure 11b



In the chromatograms displayed in in Figure 11a and 11b, there are no visible peaks eluting at 18 minutes or 22.5-27 minutes, consistent with free Hb/Mb and X-linked heme respectively.

One of the remaining 23 participants had no saved urine sample for analysis after samples were accidently destroyed. Four participants (4/22, 18.2%) had normal chromatograms with no evidence of free Mb/Hb or X-linked heme. Three participants (3/22, 13.6%) had a visible elution spike at 18 minutes compatible with free Hb/Mb, without evidence of X-linking. The remaining 15 participants (15/22, 68.2%) had a visible elution spike at 18 minutes compatible with free Mb/Hb and a broad peak within the region of 22.5-27 minutes. An example of each of these three scenarios is displayed in Figure 12.

Figure 12a

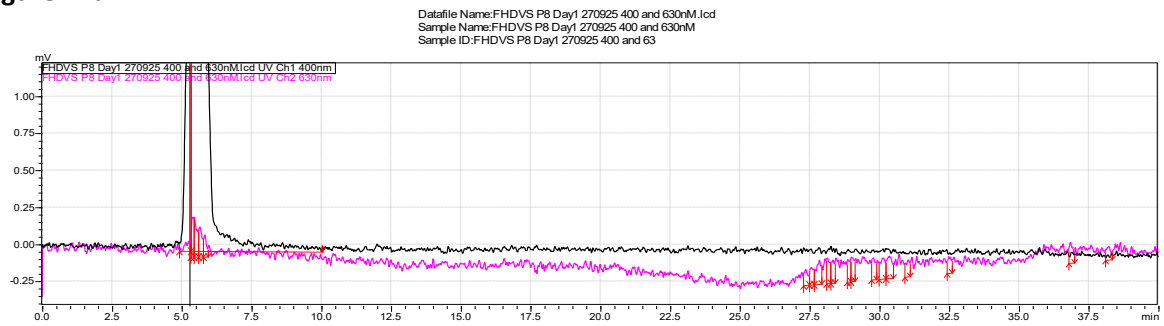


Figure 12b

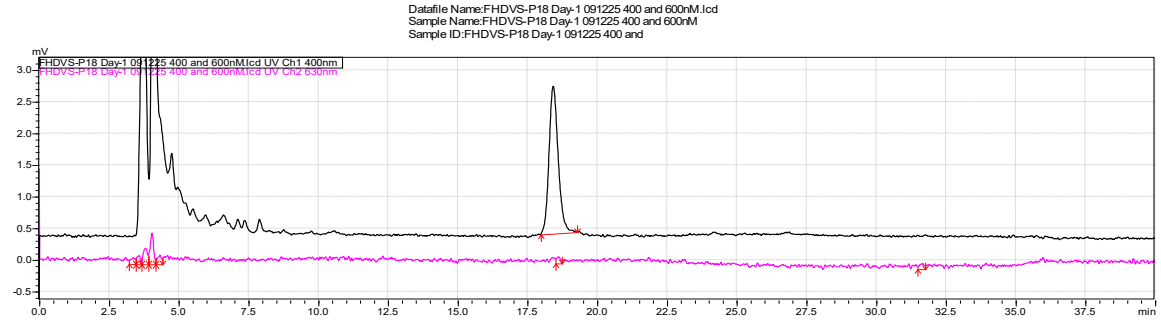


Figure 12c

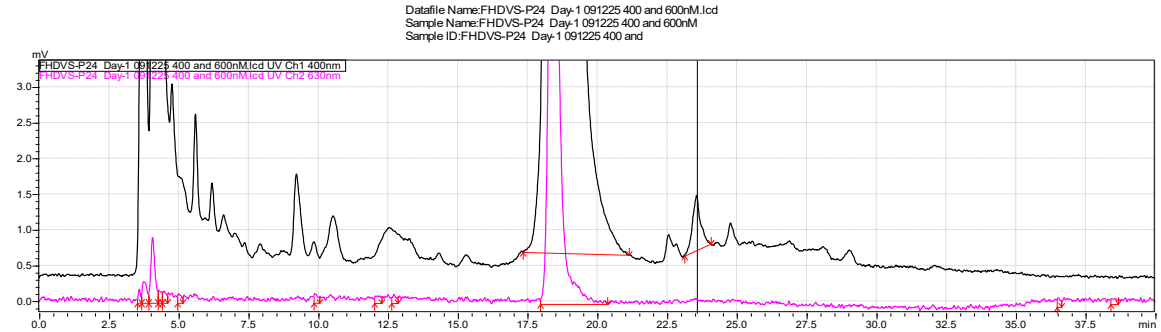


Figure 12a demonstrates no elution peak in the regions compatible with free Mb/Hb/Hb or X-linked heme. Figures 12b demonstrates an elution peak at 400 nm visible at 18 minutes and Figure 12c demonstrates elution peak in both 400 nm and 600 nm at 18 minutes with a flattened irregular elution signal at 400 nm visible within the region of 22-27.5 minutes.

Of the 15 participants with detectable X-linked heme, only one participant had no X-linked heme visible on Day 1 before it became visible on subsequent urine analysis. The chromatograms on urine collected on Day 1 urine were similar to those collected on Day 3 or Day 5 on the same participant. Although the number of participants that had detectable X-linked heme in one of two or more urine samples was small (n=11), the AUC compatible with X-linked heme did not differ significantly (Day 1 median AUC log 4.27 versus Day 3/Day 5 median AUC log 4.05, p=0.60).

RP HPLC elution peaks compatible with X-linked Mb/Hb were visible for participants that had myotoxicity without MAHA, participants that had MAHA without myotoxicity and participants that had a combination of myotoxicity and MAHA (Figure 13).

Figure 13a

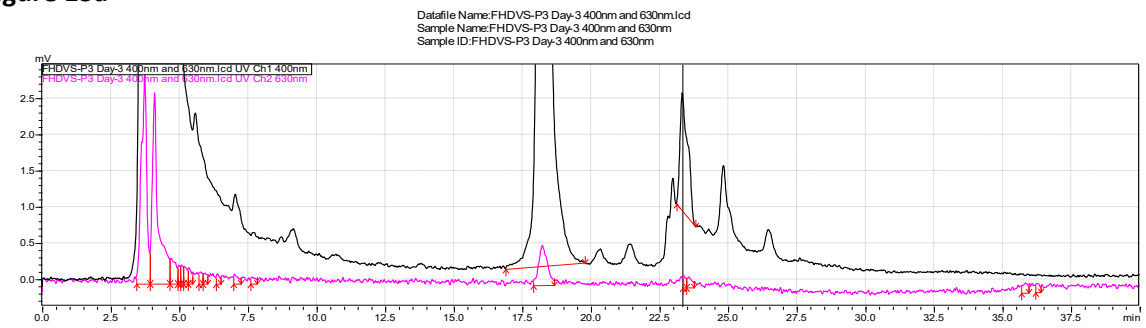


Figure 13b

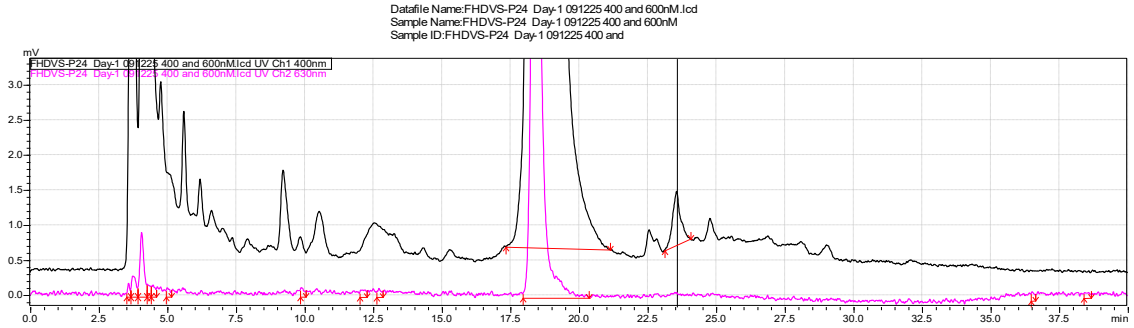


Figure 13c

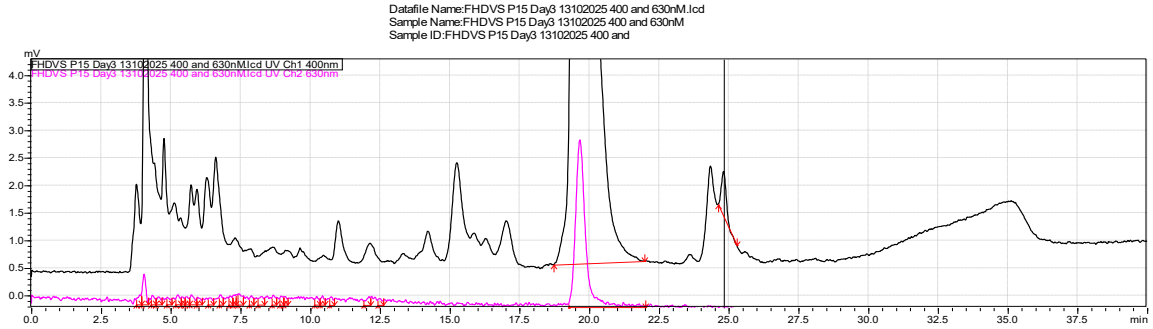


Figure 13: RP HPLC chromatograms for participants exhibiting elution profiles consistent with X-linked heme. Participant 3 in Fig 13a developed severe myotoxicity without MAHA (peak CPK 5248 IU, peak schistocyte count 0.8%), participant 24 in fig 13b developed MAHA without myotoxicity (peak CPK 182 IU, peak schistocyte 3.7%) and participant 15 in fig 13c developed severe myotoxicity and MAHA (peak CPK 5240 IU, peak schistocyte count 1.0%).

Of the 6 individual participants that had evidence of either MAHA and or severe myotoxicity, 1 participant had no evidence of X-linked heme in chromatograms of urine collected on day 1 and day 3 (Figure 14). Due to the low numbers of participants in each group, comparative statistics were not performed. The three largest recorded values for AUC associated with X-linked heme were observed in patients with severe myotoxicity (either alone or combined myotoxicity and haemolysis). This is consistent with the in-vitro acid butanone extraction of X-linked heme experiments (Figure 10) that showed greater AUC for Mb-X than for Hb-X. Nine participants had RP HPLC chromatographs consistent with X-linked heme that did not meet the definition of severe myotoxicity or MAHA. Of these, four participants had mild myotoxicity (peak CPK ≥500 but <5000IU), three had mild haemolysis (peak schistocyte count ≥0.5% but <1.0%) and two participants had combined mild myotoxicity and mild haemolysis. The remaining participant that had an RP HPLC chromatogram elution spike consistent with X-linked without haemolysis or myotoxicity had the lowest recorded AUC value (log 3.89) of all recorded (Figure 14).

Figure 14

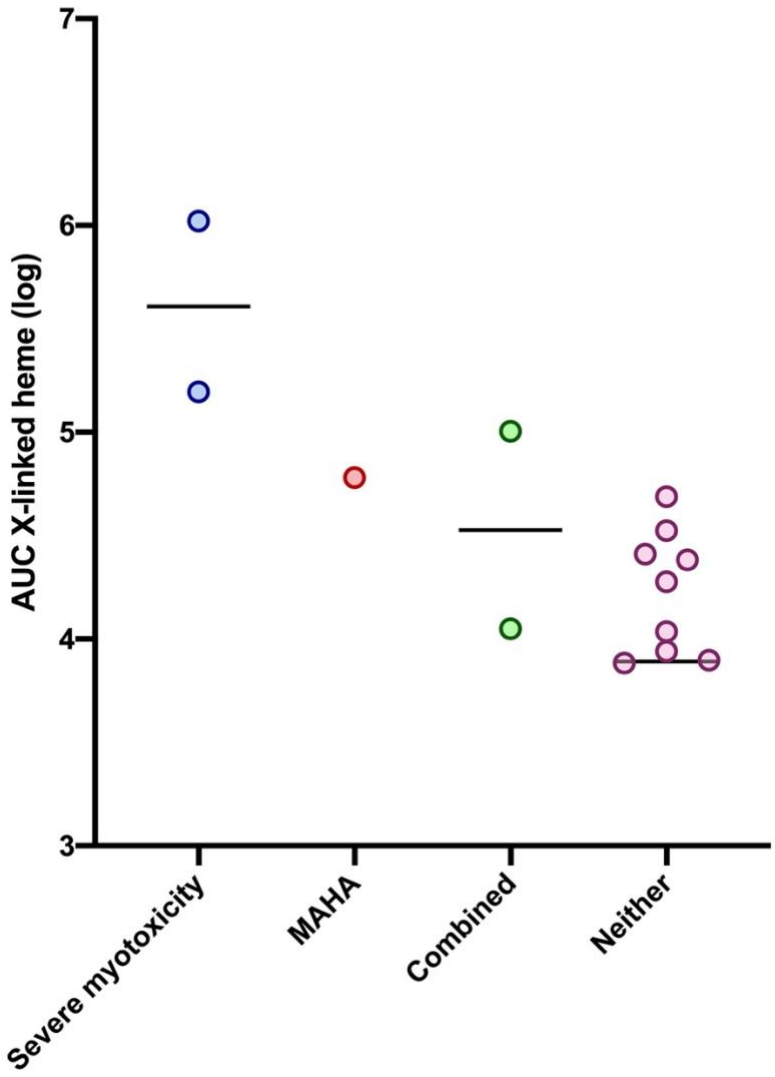


Figure 14 demonstrates the maximum recorded AUC consistent with X-linked heme on RP HPLC chromatogram, divided by clinical complications. There were eight participants with no recorded AUC consistent with X-linked heme, one in column 2 (MAHA) and seven in column four (representing participants with neither severe myotoxicity nor MAHA).

Three discrete analyses were performed to assess correlation between X-linked heme (AUC log) with myotoxicity, haemolysis and acute kidney injury (figure 15).

Figure 15a

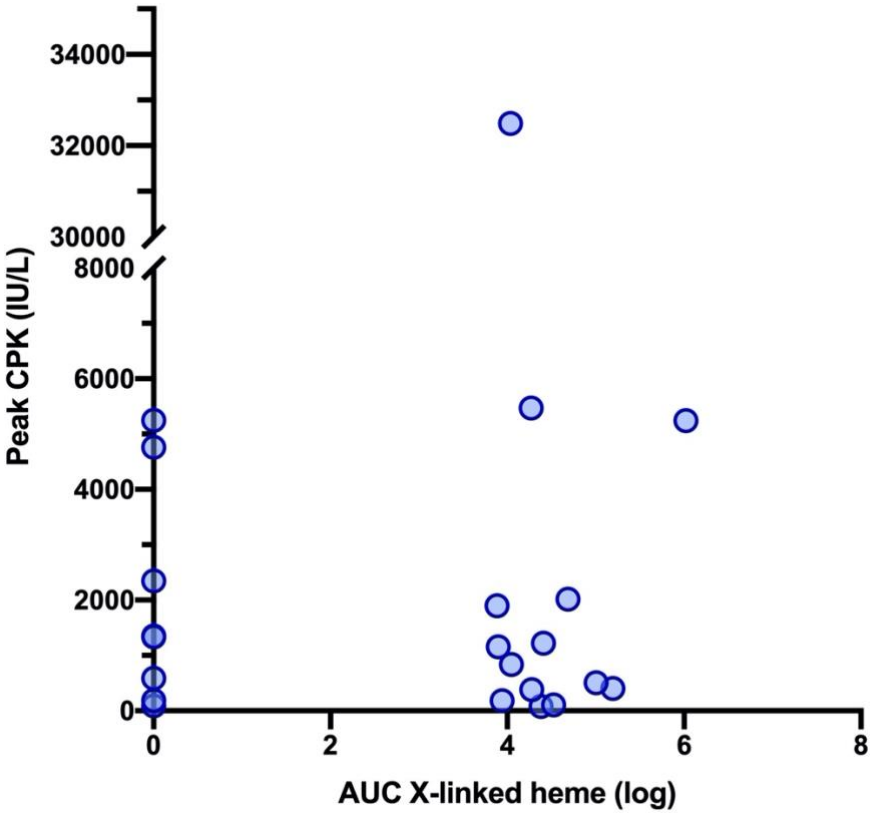


Figure 15b

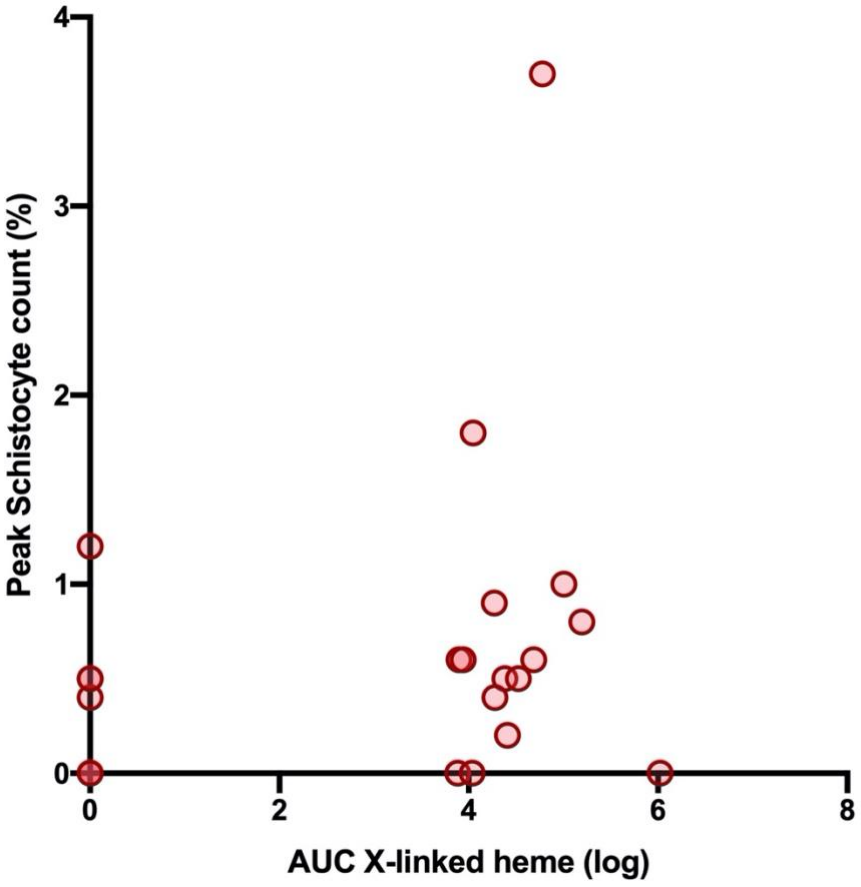


Figure 15c

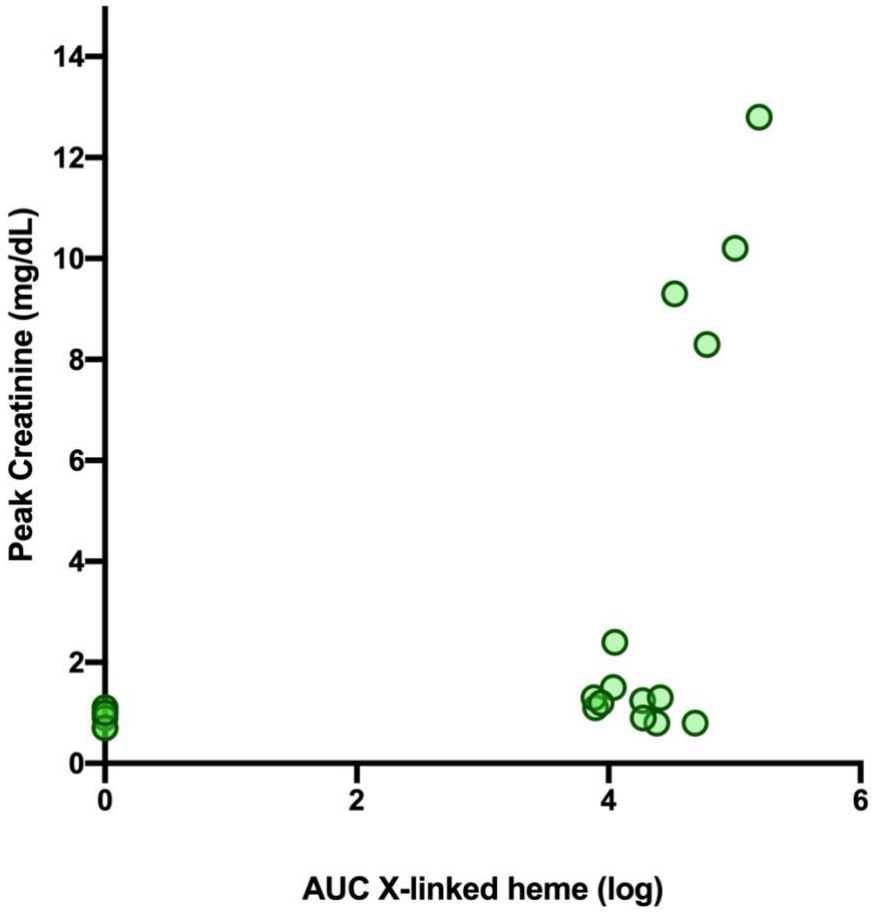


Figure 15a displays a scatterplot panel depicting individual participant values for maximum recorded CPK (IU/L) with X-linked heme AUC (log), r= 0.39 (p=0.07). Y-axis is cut to accommodate one participant with a maximum CPK value of 32,500 IU/L. Figure 15b displays a scatterplot panel depicting individual participant values for maximum recorded schistocyte count (%) with X-linked heme AUC (log), r= 0.37 (p=0.07). Figure 15c displays a scatterplot panel depicting individual participant values for maximum recorded creatinine (mg/dL) with X-linked heme AUC (log), r=0.66 (p<0.01).

Maximum recorded CPK, which is used as a marker of myotoxicity and therefore free Mb, correlated with X-linked heme AUC (log) with a Spearman's coefficient of 0.39 (p=0.07). Maximum recorded schistocyte count (%), used as a marker of MAHA and therefore free Hb, correlated with X-linked heme AUC (log) with a Spearman's coefficient of 0.37 (p=0.07). Maximum recorded creatinine, which is used as a marker of acute kidney injury, correlated with X-linked heme AUC (log) with a Spearman's coefficient of 0.66 (p<0.01).

When dividing participants into categories based on their RP HPLC chromatograms, participants that have chromatograms consistent with X-linked heme had greater peak creatinine values (median 1.3 mg/dL (IQR 1.1-8.3)) than those with chromatograms consistent with free heme without X-linked heme (0.9 mg/dL (IQR 0.7-1.1)) and participants with neither free heme nor X-linked heme (median 1.0 mg/dL (IQR 0.7-1.1)) ($p< 0.001$) (figure 16).

Figure 16

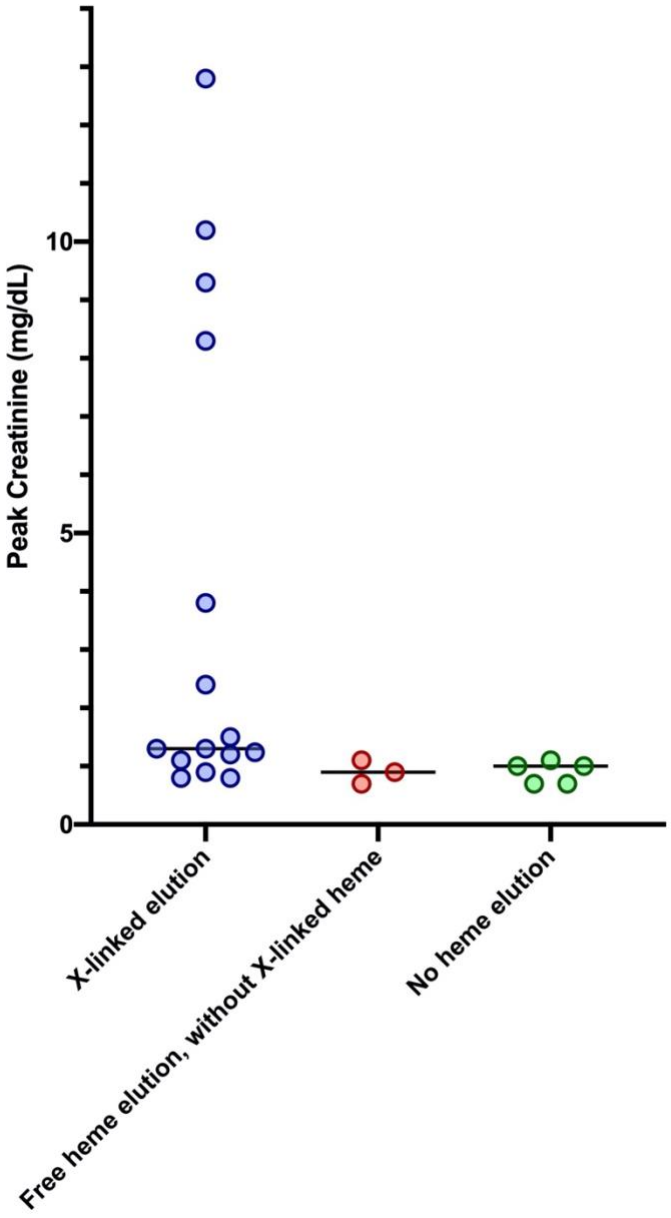


Figure 16: Blue dots represent individual peak creatinine values for patients with X-linked heme on RP HPLC chromatogram, red dots represent individual peak creatinine values for patients with visible free Hb/Mb but no X-linking on RP HPLC chromatogram and green dots represent individual peak creatinine values for no visible heme or X-linking on RP HPLC chromatogram.

As a potential confounder in the development of SAKI and presence of X-linked heme, MAP was measured and its association with AKI and X-linked heme were assessed. There were five participants with hypotension on admission defined as a MAP <65 mmHg, 3/5 developed AKI (2 AKI stage 1 and 1 AKI stage 3). This compared to 7/20 (2 AKI stage 1, 5 AKI stage 3) for participants without hypotension (MAP >64 mmHg) on admission (relative risk 1.7 (CI 0.6-3.9). Presence of X-

linked heme in urine was the same for participants with hypotension (2/4) than participants without hypotension (10/19) (relative risk 1.0 (CI 0.3-2.1). Although patient numbers were too small to explore the relationship of systemic blood pressure on admission and the development of X-linked heme and AKI in detail, the data suggests that systemic hypotension on admission is associated with AKI on whilst X-linked heme is not.

Chapter 5. Discussion

In this pilot study of patients with history of snakebite and evidence of systemic haemotoxic envenoming, 15/22 (68.2%) of participants had detectable cross-linked heme in urine, which may indicate the presence of oxidative nephropathy.

Serum biomarkers of myotoxicity (CPK) and MAHA (schistocyte count %) independently correlated with increased concentration of X-linked heme (AUC log) in urine with a Spearman's correlation coefficient of 0.39 (p=0.07) and 0.37 (p=0.09), respectively. Although neither of these values met statistical significance in this small cohort, the observation of the four largest independent readings of X-linked heme (AUC log) were observed in patients with severe myotoxicity (n=2), MAHA (n=1) or both (n=1) further supports this relationship. Participants with either severe myotoxicity and/or MAHA (n=6, two participants had both) had a median X-linked AUC log of 4.9 (IQR 3.0-5.4) compared to 3.9 (IQR 0-4.4.0) in those without severe myotoxicity or MAHA, p=0.02. The modest correlation coefficients observed in this study are similar to those observed in malaria in which PfHRP2 (a biomarker of total parasite burden) correlated with plasma CFH and F_2-IsoPs at r_s = 0.55, p<0.001 and r_s = 0.34, p=0.08, respectively and CFH correlated with F_2-IsoPs at r_s = 0.30, p<0.018) (82). It is likely that the correlation of CPK and MAHA with X-linked heme observed in this study was negatively impacted by the confounding effect of each variable. For example, 1 participant had severe myotoxicity and high X-linked heme AUC but with low to normal schistocyte count. The correlation of schistocyte count to X-linked heme in this participant was confounded by the presence of severe myotoxicity. The same is true for participants with MAHA confounding the relationship between CPK and X-linked heme AUC.

Peak X-linked heme (AUC log) positively correlated with serum peak creatinine with a Spearman's correlation coefficient of 0.66 (p<0.01). Participants with detectable X-linked heme had a higher median peak creatinine (1.3 mg/dL, IQR 1.1-8.3) than patients without detectable X-linked heme (1.0 mg/dL, IQR 0.7-1.1). Of the six participants that developed AKI stage 3 requiring renal

replacement therapy, three had severe myotoxicity, one participant had MAHA and one participant had both severe myotoxicity and MAHA. The remaining participant developed MAHA and TMA by Day 8 of admission and recorded a peak schistocyte count of 2.7% on Day 15 of admission. The correlation of urinary X-linked heme and serum creatinine in patients with snakebite is supportive of its role in within the multifactorial aetiology of SAKI.

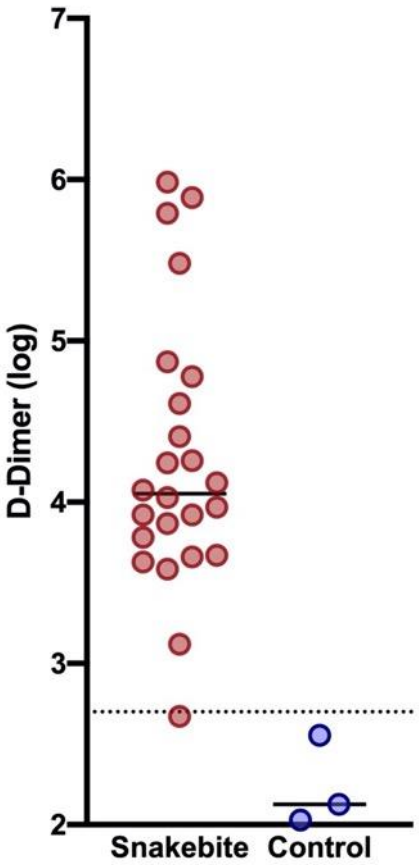
Detection of systemic envenoming

The data reported in chapter 2, in which I systematically reviewed the literature to determine the diagnostic test accuracy of the 20WBCT, was used to guide the inclusion criteria of the observational study. In practice, the 20WBCT remains ubiquitous in the assessment and management of snakebite patients in India, which is reflected in national and regional guidelines (109). The 20WBCT is performed at the bedside and gives the attending clinician a result within 20-minutes. Most healthcare facilities in India are unable to provide the 24/7 laboratory support which is available at CMC Vellore, meaning there are typically delays of 12 hours or more before laboratory clotting assays are available. Through understanding the diagnostic test accuracy of the 20WBCT, and therefore expecting some incidences of FN and FP, the 20WBCT was used as a screening test for evidence of VICC before confirming so in subsequent laboratory clotting assays (INR, fibrinogen and D-Dimer).

Another challenge faced by clinicians both in clinical practice and in research is interpreting clotting assays collected after the administration of antivenom. Pujo and colleagues have demonstrated in patients with suspected *Bothrops* envenoming, antivenom administered 6-12 hours after a snakebite restored normal INR and fibrinogen within 12 hours (137). For haemostasis to be achieved, there is a requirement for toxin reversal and production of new clotting factors by the liver. In this study, 17/24 (70.8%) of patients had received antivenom prior to presenting to CMC Vellore. Three patients had normal INR and fibrinogen collected on admission at CMC Vellore, despite a reported positive 20WBCT at the referring healthcare facility. In each of these three participants, D-Dimer was above the upper limit of normal (median 4251 ng/mL (IQR 1316-9313), normal range <500 ng/mL).

Overall, using 500 ng/mL as a cut off, D-Dimer had 100% sensitivity and 100% specificity at diagnosing haemotoxic envenoming in this cohort, accurately identifying 23/23 participants with VICC, 3/3 healthy control participants and one participant with a false positive 20WBCT (figure 17). This is consistent with a large study in Australia in which quantitative D-Dimer collected 2-6h after snakebite had a sensitivity of 97.1% (95% CI 95.0-98.3%) and specificity of 99.0% (95% CI 97.6-99.6%) (143). Although D-Dimer is not practical in most clinical settings in which patients are presenting with snakebite, the results from this study indicate that it remains a useful adjunctive test, particularly in research and in patients that have received antivenom where clotting assays such as INR and fibrinogen may have returned to normal range.

Figure 17



Scatterplot of D-Dimer readings (reported in log transformation) collected on admission for participants with history of snakebite (red) and control (blue). Dotted line represents upper limit of normal range. One participant with history of snakebite (false positive 20WBCT) recorded D-Dimer below the upper limit of normal range.

Myotoxicity and microangiopathic haemolytic anaemia

The complexity of viperid snakebite envenoming in southern India and resulting multisystem effect provides a rare occurrence in which rhabdomyolysis and haemolysis can coexist or occur independently in a single disease state. Silva and colleagues identified two PLA2 toxins (U1-viperitoxin-Dr1a and U1-viperitoxin-Dr1b) in Sri Lankan *Daboia russelii* venom that, whilst predominantly neurotoxic, are also the major myotoxins found in Sri Lanka *Daboia russelii* venom (49). Although these PLA2 toxins are abundant (comprising 42% of whole venom), they exhibited only weak myotoxic properties *in-vitro* (49). More broadly speaking, systemic myotoxic PLA2 toxins, such as those found in *Bothrops spp*, can bind and destroy the integrity of the plasma membrane of

muscle cells (1, 144). Calcium influx into the muscle cells causes myofilament hypercontraction and mitochondrial dysfunction resulting in irreversible muscle cell damage (144). Muscle injury is further exacerbated by massive extravasation of fluid into the compartments of envenomed limbs causing increased compartmental pressures (145). The resulting rhabdomyolysis causes release of muscle cell contents, including myoglobin into the circulation (92).

Outside of snakebite, AKI secondary to rhabomyolysis is typically observed in patients with peak CPK values greater than 15,000 U/L (92). However, AKI can occur in readings as low as 5,000 U/L during periods of systemic illness such as sepsis, hypotension and acidosis (146). In a study of 43 patients with snakebite induced rhabdomyolysis in China, where the most commonly identified snake were identified as *Protobothrops mangshanensis* (39.5%), the mean peak CPK was recorded as 3047 U/L (145). Of these, 15 (34.9%) developed AKI and six (14.0%) required renal replacement therapy (145). It should be noted that the authors did not adequately exclude other potential causes of AKI such as haemolysis (145). In a large prospective study of confirmed *Daboia russelii* envenoming in Sri Lanka, clinically significant myotoxicity was uncommon. In a sample of 219 envenomed patients, 37 (16.9%) had elevated CPK recorded on a blood sample collected 24h after the snakebite (49). Median CPK was 110 U/L and the highest recorded CPK was 1019 U/L meaning no patients met the definition of severe myotoxicity (>5000 U/L) in this study. In my study, five participants recorded peak CPK values between 4,700 - 8,000 U/L and one patient recorded peak CPK of 32,500 U/L (all collected prior to the onset of RRT). Of these, five (83.3%) developed AKI and four (66.7%) required renal replacement therapy. Although the venom proteome of *Daboia russelii* venom found in Sri Lanka and south India are similar, it is possible that PLA2 isoforms or other venom constituents contribute to the apparent divergence in toxidrome (19). Given a paucity of literature with systematic collection and reporting of rhabdomyolysis in India, it is difficult to know if the data on the incidence of rhabdomyolysis reported in this thesis is representative of other regions of India and beyond.

Snake venom proteins, such as SVMPs and hyaluronidases found in *Daboia russelii* venom, hydrolyse the basement membrane of capillaries resulting in microvascular damage (1). The impaired

endothelial wall is susceptible to microvascular clot formation due to toxin mediated VICC. This combination of microvascular damage and fibrin deposition within the capillary lumen causes fragmentation of RBCs and haemolytic anaemia (39). In this study, four participants (16.7%) were found to have schistocyte count greater 1%, consistent with a diagnosis of MAHA. One further participant developed MAHA on Day 8 of admission. All five of these participants developed thrombocytopenia of < 150 $x10^9$/L consistent with a diagnosis of TMA. Four patients (80%) developed AKI (one patient with AKI stage 1, three patients with AKI stage 3 all of whom required RRT) and one patient did not develop any kidney injury. Of the ten participants that developed AKI, four (40.0%) had evidence of MAHA and TMA.

Data concerning the incidence of MAHA or TMA following snakebite is sparse. The incidence reported in our study is similar to that of a retrospective observational study performed in Karnataka, south India in which 36/150 (24.0%) of patients with systemic haemotoxic snakebite developed TMA (45). All of the patients with TMA developed AKI and 25/36 (69.4%) required RRT. In a prospective study in Sri Lanka of patients presenting with haemotoxic snakebite (*Daboia russelii* and *Hypnale spp*) and AKI, 45/59 (76.3%) had evidence of TMA and 47/59 (79.7%) had evidence of MAHA (46). Although it is difficult to directly compare the relative incidence of MAHA and TMA across the three studies due to differing definitions of MAHA and TMA, the incidence rates are broadly similar. Due to a common pathophysiological process (VICC), similar reported rates of SAKI across India (Table 2) and similar incidence of MAHA and TMA in snakebite reported from Karnataka, Tamil Nadu (this study) and Sri Lanka, I argue that the data reported in this study concerning MAHA and TMA are likely to be representative of other tertiary hospital providers in south India and Sri Lanka managing viperid envenoming.

Mechanism of X-linked heme causing AKI

Once free Hb or Mb, released during MAHA and rhabdomyolysis respectively, exceeds the scavenging capacity of haptoglobin, concentrations of free Hb/Mb rise. Hb/Mb that is free from

globin protein rapidly oxidizes to ferric form, which is accelerated in acidic conditions (79). The ferric heme resulting from myotoxicity and MAHA undergoes peroxide induced redox cycling in the kidney to ferryl form (figure 3) (83). The importance of acidic condition underpinning the redox cycling and lipid peroxidation was demonstrated by Moore and colleagues who were able to show the attenuation of renal injury through alkalization of urine (87). Under acidic conditions, protonation of the ferryl species can result in the ferric form (Fe III) and a porphyrin radical (84, 85). The porphyrin radical reacts with an amino acid side chain forming a protein-porphyrin X-linked covalent bond (Hb-X or Mb-X). This protein-porphyrin covalent bond is specific to peroxide induced ferric-ferryl redox cycling, meaning detection is indicative of lipid oxidative reactions (79, 83). During separation with RP HPLC, the X-linked heme-protein compound is optically distinct from heme but indistinguishable between Hb-X and Mb-X.

The clinical significance of demonstrating this reaction is due to the pathological consequences of lipid peroxidation. Abundant ferric iron (Fe III), formed during redox cycling, catalyzes the oxidation of fatty acid side groups of lipid membranes (such as arachidonic acid) which generates F_2-IsoPs and isofurans, implicated in oxidative stress and known to cause potent renal vasoconstriction (86, 147-149). Moore et al have demonstrated markedly increased excretion of urinary F_2-IsoPs and increased F_2-IsoPs esterified in renal lipids, from rats with rhabdomyolysis, thus localizing the oxidative process to the kidney (87). The process of hemoprotein associated oxidative nephropathy has been demonstrated in number of diseases involving Mb-X and Hb-X. Holt et al demonstrated the presence of X-linked heme in urine and detected markedly elevated levels of F_2-IsoPs in patients with rhabdomyolysis (93). Similarly, Plewes et al demonstrated that parasitic burden in falciparum malaria infection correlated with increased plasma cell-free heaemoglobin. In turn, plasma cell-free heamoglobin was strongly associated with F_2-IsoPs, Isofurans and AKI (82).

Potential therapeutic interventions

Inhibiting the hemoprotein mediated lipid peroxidation reaction represents a potential for therapeutic target. Due to its natural physiological function binding to Hb-dimers, there has been

considerable interest in the role of haptoglobin at attenuating the effects of hemoprotein oxidative stress (150). In *in-vitro* studies, administration of supraphysiological concentrations of haptoglobin was able to prevent haemolysis induced renal impairment in guinea pigs (151). Several small clinical studies in Japan have shown promising results of human plasma derived haptoglobin in preventing renal injury in conditions of acute haemolysis, including cardiac bypass surgery and HELLP syndrome (152). In a systematic review, exogenous haptoglobin for management of haemolytic anaemia was associated with lower levels of AKI than placebo (OR 0.64 95% CI 0.44-0.93) (153). Whilst efforts continue to refine a potential therapeutic role of haptoglobin and other scavenger proteins, high cost is likely to limit its role in snakebite management in LMICs (152).

Desferrioxamine is an iron-chelator used in the management of iron overload. Reeder et al have demonstrated that desferrioxamine can reduce ferryl heme by donation of an electron, thus preventing the production of X-linked heme and lipid oxidation (88). Desferrioxamine and a newer orally administered iron chelator, Deferiprone have be used in a series of small clinical trials of patients undergoing cardiac pulmonary bypass surgery. Although patients were found to have lower superoxide radicals and lower levels of lipid peroxidation compared with placebo, there was no effect on markers of AKI (154). It is interesting to consider the potential role of iron chelators in attenuating heme protein mediated lipid peroxidation in snakebite in the context of another metal chelator, unithiol. Unithiol has shown promising *in-vitro* activity against the SVMPs, undergone a phase-1 trial in healthy controls and is planned for further clinical trials in envenomed patients (118, 119, 155).

The most promising potential therapeutic intervention to consider in attenuating snakebite associated heme mediated lipid peroxidation is paracetamol. Boutaud and colleagues demonstrated that paracetamol can reduce ferryl heme to its ferric form and prevent the formation of X-linked heme (147). In addition, paracetamol is capable of quenching globin radicals which further limits haemoprotein lipid peroxidation (147). The reduction of ferryl heme was enhanced at lower pH. The authors then demonstrated that rats pre-treated with paracetamol prevented rhabdomyolysis

induced lipid peroxidation with lower recorded levels of urinary and plasma F_2-IsoPs (147). In demonstrating the ability to reduce ferryl heme to ferric form and therefore prevent AKI in models of rhabdomyolysis, the authors have further cemented the causative role of free heme in oxidative nephropathy. The findings of this *in-vitro* study were supported by a retrospective observation study of 1065 patients with rhabdomyolysis that found a significant association between paracetamol exposure and a reduced risk of requiring RRT (absolute risk difference of -1.22% (95% CI -2.33 to -0.12) (156).

Following the publication of the Boutaud study, several clinical studies have been performed to assess paracetamol as a renoprotective agent against hemoprotein induced oxidative nephropathy. In a randomized, controlled, open label trial of patients with severe and moderately severe falciparum malaria, paracetamol led to a greater reduction in serum creatinine and lower risk of developing AKI, particularly in patients with high cell-free haemoglobin and F_2IsoP at enrollment. (157). The effect was greatest in patients with high rates of haemolysis and markers of oxidative stress (F_2-IsoPs and isofurans). In a similar study performed in patients with *Plasmodium knowlesi* malaria infection, paracetamol was not associated with a reduction in creatinine overall (n=396) (158). A renoprotective effect, demonstrated by a reduction in creatinine after three days and one week, was observed in patients with severe disease and in patients with haemolysis.

In a retrospective observational study of patients admitted to ICU with sepsis, Janz and colleagues identified that higher plasma cell-free heamoglobin was associated with increased risk of death (91). In addition, paracetamol exposure was associated with lower plasma concentration of F_2-IsoPs and lower risk of death (91). However, this finding was not replicated in intervention studies (159, 160). In a large Phase 2b, randomized, double blind, placebo-controlled trial (n=447), intravenous paracetamol administered for five days did not demonstrate a significant reduction in mortality, organ support or requirement for RRT(160). The authors noted comparatively low levels of cell-free haemoglobin levels compared to other studies of patients with sepsis (91).

Notwithstanding the mixed results of clinical studies assessing the role of paracetamol in conditions of increased cell-free heamoglobin, snakebite warrants special consideration for several reasons. In this study, X-linked heme was observed in a majority of patients (15/22, 68.2%) presenting with haemotoxic envenoming. Severe myotoxicity and MAHA, complications that result in increased cell-free heme, were common and occurred in 5/24, 20.8% and 4/24, 16.7% respectively. In a review of literature relating to SAKI in India (Table 2), 22.0% of adults with history of snakebite developed AKI, 13.0% required RRT and 5.0% died, highlighting the considerable morbidity and mortality associated with this complication. Furthermore, availability of RRT is often limited to tertiary medical services and is associated with considerable increased cost (161). Unlike other disease states, such as malaria or sepsis where there may be a delay of several days before seeking medical care, patients with snakebite can accurately state a time of injury and typically seek medical attention promptly. In this study the median time to presentation was 4h30m (IQR 2.30-6.40) from the time of bite, whilst 20/24, 83.3% had attended another healthcare facility prior to attending CMC Vellore.

Research is continuing to examine the role of broadly neutralizing anti-toxin agents as adjunctive therapy to antivenom for the management of snakebite (118, 120). One of the desires of these medications is that interventions could be administered 'in the field' possibly before a participant reaches healthcare facilities that can safely deliver antivenom. Paracetamol meets many of the desirable qualities required of these interventions including widespread availability, low cost, oral bioavailability, known PK/PD distribution and established safety profile.

External validity and further work

As highlighted in Chapter 1, snake venom proteome exhibits remarkable inter and intraspecies variation resulting in strikingly varied toxidrome. As such, the results from this study cannot be extrapolated to other snake species. However, the complications of MAHA and myotoxicity and associated risk of AKI are by no means unique to South Asian vipers and have been described in a variety of snake genera and species across the world. These include *Daboia siamensis* in Southeast Asia, *Protobothrops spp* in East Asia, *Bothrops spp* in Central and Southern America, *Echis spp* in Sub

Saharan Africa and the Middle East and elapids of Australasia (104, 137, 145, 162, 163). Future work leading from this thesis should attempt to distinguish the relative effect of haemolysis and rhabdomyolysis on CFH and products of lipid peroxidation including F_2-IsoPs and isofurans. Snakebite is the neglected tropical disease with greatest morbidity and mortality and urgent attention is required to identify safe, cheap and widely available adjunctive therapy to antivenom (164). I strongly believe that there is sufficient scientific basis, ethical justification, and scientific equipoise to perform a pragmatic, multicenter RCT to determine the clinical effect of paracetamol in snakebite. Clinical studies could be supported by an in vitro model of MAHA, whereby candidate therapeutics are assessed in their ability to prevent renal injury.

Limitations

There are several limitations of this work. A lack of confirmed snake species causing the envenoming has resulted in generalizations of clinical manifestations and complications across the range of snake fauna that reside within the hospital catchment area. To further understand the pathological process, confirmation of biting snake through EIA is required. The sample size of this study was small which underpowered the study at assessing correlation of X-linked heme with biomarkers of MAHA and myotoxicity and limited assessment of confounding. Recruiting patients to snakebite trials is challenging with injuries typically occurring in rural areas meaning patients present to tertiary facilities late. Expanding recruitment to more centres including government health facilities would dramatically increase the pool of patients from which to recruit. The budget of this study was low, this limited the number pf participants and range of tests that could be performed. The budget was unable to cover the costs of performing plasma CFH or F_2-IsoP measurement. Another limitation of this study was that the RP HPLC chromatogram was unable to distinguish between X-linked Hb and Mb. Although indicators could be ascertained from serum markers indicating disease process, it is unclear whether MAHA or myotoxicity had caused the elution observed on RP HPLC.

Conclusion

In this thesis, I report the results of a novel experiment, in which the urine of patients with systemic haemotoxic envenoming in India was assessed for the presence of cross-linked heme-associated species. Using RP HPLC to separate the organic compounds of urine, I have demonstrated that cross-linked heme-associated species was evident in patients with systemic haemotoxic envenoming and absent in urine of normal control participants and the snakebite patient with a false positive 20WBCT. The presence of cross-linked heme-associated species may reflect ferryl-hemoprotein mediated oxidation and contribute to lipid oxidative nephropathy. Markers of MAHA and myotoxicity weakly correlated with the quantity of cross-linked heme which in turn, correlated with AKI.

Snakebite envenoming remains a neglected tropical disease with huge associated morbidity and mortality (164). Envenoming most commonly occurs in rural areas, often as an occupational hazard (2, 4). Urgent efforts are required to identify novel therapeutics that are safe, affordable, and effective at reducing this burden.

Appendix 1

Informed consent form for

An observational study to assess for ferryl-heme derivatives, indicative of oxidative nephrotoxicity, in the urine of patients with viper envenoming

Christian Medical college, Vellore

This consent form is for patients aged 18 years and older presenting following a viper bite.

Name of Principal Investigator : Dr. Jambugulam Mohan

Name of Organization : Christian Medical College, Vellore

Name of Sponsor : Christian Medical College, Vellore

Short title of the Study : FHD Viper study

Certificate of Consent

I have been invited to participate in research study entitled, *An observational study to assess for ferryl-heme derivatives, indicative of oxidative nephrotoxicity, in the urine of patients with viper envenoming*. I am aware that participation will include the collection of additional blood and urine samples but will not affect the normal care for my condition. I am aware that I have been informed about the risks and benefits to participating and that I will not be compensated.

I have read the patient information sheet and have had the opportunity to ask questions. I consent voluntarily to take part as a participant in this research and understand that I have the right to withdraw from the research at any time without affecting my medical care.

Name of Participant_____

Signature of Participant _____

Date _____ (Day/month/year)

If illiterate

A literate witness must sign (if possible, this person should be selected by the participant). Participants who are illiterate should include their thumbprint as well.

Thumbprint of participant/Parent



I have witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Name of witness _____

Relationship of witness _____

Signature of witness _____

Date _____ (Day/month/year)

Study Doctor

I have accurately read or witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Name of Researcher_____

Signature of Researcher _____

Date _____ (Day/month/year)

A copy of this Informed Consent Form has been provided to participant (initialed by the researcher/assistant).

Appendix 2

APPLICATION FOR IRB APPROVAL OF OBSERVATIONAL
(CASE-CONTROL / COHORT/ CROSS-SECTIONAL) STUDIES

CHRISTIAN MEDICAL COLLEGE, VELLORE
(Please complete Sections **I to III** and submit with all supporting documents)

SECTION I

Fluid Research Funding

If for external funding, please provide name of funding agency and the application for submission in the funding agency's format, in addition to this application.

1. **Title of Research:**

   An observational study to assess for ferryl-heme derivatives, indicative of oxidative nephrotoxicity, in the urine of patients with viper envenoming.

2. **Title of Study (for lay public):**

   A study to identify causes of kidney damage in snakebite.

3. **Acronym if any:**

   FHD Viper study

4. **Unique Protocol ID, if any**

5. **(i) Is this project related to "Artificial intelligence or Machine Learning"? (No)**

   (**Note:** If yes, is approval letter from the CMC internal IT committee obtained? Please attach the copy along with the IRB application)

   (ii) Is the MoU with the collaborating institutions approved by the:

   a. Internal IT committee? No
   b. CMC Legal cell? No

   (**Note:** Please attach the approval copy along with the IRB application)

6. **Name of the Principal Investigator:**

   Jambugulam Mohan

   Designation / Department / Unit / of Principal Investigator:

   Assistant Professor

   Department of Medicine Unit 1 & Toxicology

   Christian Medical College, Vellore

   Employment Number: 29240

   Address for communication (including telephone and fax numbers and email id):

   Department of Medicine Unit 1 & Toxicology

   Christian Medical College, Vellore

   Tamil Nadu-632004

   Email address: jambugulam@gmail.com

Phone number (mobile): +91 9952816427

Phone number (office): +91 416 2282089, +91 416 4213513

Fax number (institutional): +91 416 2232035

If Post Graduate Registrar / Fellowship:

Enrollment date of PG Course: mm/yyyy

Completion date of PG Course: mm/yyyy

7. **Name of Guide (for Student Thesis):**

   Employment Number:

   Address for communication (including telephone and fax numbers and email id):

8. **Name of Supervisor (for Student Projects):**

   Employment Number:

   Address for communication (including telephone and fax numbers and email id):

8. **Name and Designation of Co-Investigator(s), Employment Number and Address:**

Name	Designation	Employment numb	Address
Anand Zachariah	Professor	11791	Department of Medicine-1 & Clinical Toxicology Unit, Christian Medical College, Vellore.
Ravikar Ralph	Professor	28852	Department of Medicine-1 & Clinical Toxicology Unit, Christian Medical College, Vellore.
Krupa George	Assistant Professor	29262	Department of Medicine-1 & Clinical Toxicology Unit, Christian Medical College, Vellore.
Amith Balachandran	Assistant Professor	29632	Department of Medicine-1 & Clinical Toxicology Unit, Christian Medical College, Vellore.
Prabhakaran	Professor	31055	Department of Neurological Sciences, Neurochemistry Laboratory, CMC, Vellore
Soosaimanickam	Associate Research Officer	31603	Department of Physiology, CMC, Vellore
Suceena Alexander	Professor	51288	Department of Nephrology, Christian Medical College, Vellore
Sathya Subramani	Professor	14123	Department of Physiology, CMC, Vellore
J.V. Peter	Professor	13328	Department of Critical Care, CMC, Vellore
Amita Jacob	Assistant Professor	29553	Department of Medicine-1, Christian Medical College, Vellore
Samuel George	Professor	30829	Department of Medicine-4, Christian

Hansdak			Medical College, Vellore
Tina George	Associate Professor	29141	Department of Medicine-1, Christian Medical College, Vellore
Marimuthu	Associate Research Officer		Department of Biostatistics, Christian Medical College, Vellore
Nalini Sarah Newbigging	Assistant Professor	29569	Department of Medicine-3, Christian Medical College, Vellore
Gina Mariam Chandy	Associate Professor	29122	Department of Emergency Medicine Christian Medical College, Vellore
Thomas Lamb	Specialist registrar (UK) and Clinical scientist	(GMC 7039762)	Nuffield Department of Clinical Medicine. University of Oxford.
Brandon Reeder	Associate Professor of Biochemistry		School of Life Sciences, University of Essex

9. Department of Institution where the research will be carried out:

Christian Medical College, Vellore

10. Names and addresses of other institutions where research will be carried out:

N/a

11. Duration of the Scheme:

12 months

12. Source/s of Monetary or Material Support

Major Research Grant (Associate Professor Below Gr. II Rs. 2,00,000/- per Year): Rs. 3,83,003

The HPLC column (ZORBAX StableBond 300 C3, 4.6 x 250 mm, 5 µm and accessories) will be provided by the University of Oxford. A deed of gift outlining this transfer has been received and is attached as appendix 1.

13. Permission letter from the HOU & HOD of each unit/department involved in the study. If Medical students, Nursing students & Allied Health students, nurses are involved in the study a permission letter from the appointing authority has to be enclosed. N/A

14. If this is a laboratory study if you have out-sourced genetic test to an external laboratory. Please provide evidence of the laboratory credentials. N/a

15. Is this an invention or idea that you plan to register as a patent: Please mention. N/a

16. In case there is an invention what are the benefits for the CMC as an institution in terms of patenting and royalties received? N/a

17. Aims and objectives of the study (*including any hypotheses):*

We hypothesize that cell-free heme causes oxidative nephrotoxicity in patients with viper envenoming in South India.

Objectives:

1. Standardization of detection of ferry heme derivatives in urine by HPLC.

2. To assess for ferryl-heme derivatives in the urine of patients with viper envenoming and to correlate with markers of hemolysis and kidney function.

18. Summary of the proposed research scheme (250 words).

The etiology of acute kidney injury (AKI) following viper envenoming is multifactorial and results in considerable morbidity, mortality and healthcare cost in south India. A subset of patients with venom induced consumption coagulopathy (VICC) following viper envenoming develop microangiopathic hemolytic anemia (MAHA) and subsequently, thrombotic microangiopathy (TMA). TMA in snakebite envenoming is strongly associated with acute kidney injury.

We propose conducting a pilot study to assess for the presence of highly oxidized heme (ferryl-heme derivatives) in the urine of patients with VICC following viper envenoming. The detection of ferryl-heme in the urine is a clear indicator of oxidative stress in the kidney, a consequence of which is restriction of blood flow and impaired kidney function.

In this study, we will analyse urine for ferryl-heme derivatives using high performance liquid chromatography (HPLC) in patients with VICC and a control group of non-envenomed patients. Urine will be collected at three time points and the results will be correlated with clinical markers of haemolysis (presence of schistocytes and platelet count) suggestive of TMA and renal function.

19. Present Knowledge and relevant bibliography *(Is there a justification for this study based on a detailed literature review or other sources of evidence? Please provide details)*

India is a country of high snakebite burden with an estimated 1.1-1.8 million snakebites occurring each year, resulting in approximately 58,000 deaths (2). Across India, Russell's viper (*Daboia russelli*) is the snake species responsible for the greatest number of envenoming (estimated at 43%). Envenoming commonly occurs during farming activities in rural areas (2). In addition to the substantial risk of death, snakebite causes a variety of sequelae including coagulopathy, neuroparalysis and acute kidney injury that contributes to high healthcare cost and chronic disease (165).

In a study of 420 patients conducted in Puducherry, south India, 43.8% of patients with evidence of systemic snakebite envenoming developed acute kidney injury (54). Of these patients, 114/184 (61.8%) required renal replacement therapy, and 44/184 (23.9%) died. In a subset of patients (n=73) that were followed up for a median of 15 months, 21/73 (28.8%) had adverse long-term renal outcomes including one patient that was dialysis dependent.

The aetiology of acute kidney injury following viper envenoming is complex and incompletely understood. In a case series from northern India, renal biopsies from 22 patients with snakebite induced AKI (SAKI) revealed prominent acute tubular necrosis (ATN) and to a lesser extent, cortical necrosis (166). Histological examination of kidneys revealed tubules that were often dilated with flattened epithelium, localized necrosis and desquamation of necrotic cells from the basement membrane (41, 167). Prominent features of snakebite ATN include prominent vascular lesions, increased apoptosis in the distal tubules and the presence of eosinophils, mast cells and hyperplastic fibroblasts within the interstitium (167, 168). The mechanisms underpinning these pathological processes are likely to be multifactorial including systemic hypotension and toxin mediated renal vascular damage resulting in impaired renal perfusion (41). Fibrin microthrombi deposition resulting from impaired coagulopathy and thrombotic microangiopathy (TMA) and direct cytotoxic effect of venom are also postulated to contribute to SAKI (166).

In recent studies of severe malaria, researchers have identified that cell-free heme, a degradation product of intravascular haemolysis, was strongly associated with renal oxidative stress and AKI (82). Researchers identified that clinical markers of haemolysis such as LDH and bilirubin positively correlated with serum cell-free heme. In turn, cell-free heme positively correlated with the presence of F_2-isoprostanes and isofurans (in vivo measures of oxidative stress) and acute kidney injury (8). Microangiopathic haemolytic anaemia (MAHA) is commonly observed in patients with systemic viper envenoming in south Asia (167, 169) due to indirect lytic activities on red blood cells from viperid venom (170). MAHA typically occurs alongside thrombocytopenia forming a TMA. In a systematic review of snakebite induced TMA, 94% of patients developed AKI (44).

We hypothesize that snakebite induced MAHA and TMA results in circulating cell-free heme. This circulating cell-free heme undergoes redox cycling from ferric to ferryl forms within the kidney causing oxidative nephrotoxicity and subsequently AKI (87). The highly oxidised ferryl form of heme is only produced in urine during redox cycling and thus detection is indicative of oxidative stress (84). Ferryl forms of heme are optically distinct from unbound heme, such as haemoglobin and myoglobin using high performance liquid chromatography in the presence of acid-acetonitrile solvent (93).

Co-investigators have standardized a HPLC method for detecting ferryl derivatives of myoglobin in urine in rhabdomyolysis (83). The method will be optimized to detect ferryl derivatives of hemoglobin using HPLC in this study.

Even though there are several approaches were available to detect ferryl derivative, HPLC might be an appropriate to detect the labile heme from different biological media and which also be a fast and sensitive quantification method.

References

(See bibliography)

20. Preliminary work already done by the investigator in this problem –

The investigators have conducted the following recent studies:

a. A multicentric study to describe geographic variation in snake bite species, clinical syndrome, complication rates and antivenom requirements in India.
b. Validating a bedside kaolin activated clotting test for hemotoxic envenoming
c. Variation in batch-to-batch effectiveness of Indian polyspecific antivenom
d. Thrombotic microangiopathy in hemotoxic envenoming in South India
e. Standardization of detection of ferryl derivatives of myoglobin using HPLC

21. List of publications of the investigator in the field –

1. Ralph R, Faiz MA, Sharma SK, Ribeiro I and Chappuis F. Managing Snakebite. *BMJ* 2022; 376.
2. Lamb T, Abouyannis M, Oliveira SS de, K RS, Geevar T, Zachariah A, et al. The 20-minute whole blood clotting test (20WBCT) for snakebite coagulopathy—A systematic review and meta-analysis of diagnostic test accuracy. *PLoS Negl Trop Dis.* 2021 Aug 10;15(8): e0009657.

3. Armstrong LJ, Cynthia S, George M, Zachariah A. Comparing community and hospital data of snakebite in North Bihar: a community incidence survey and a parallel hospital-based clinical study. *Trop Doct.* 2019 Oct 1;49(4):285–92.
4. Ralph R, Sharma SK, Faiz MA, Ribeiro I, Rijal S, Chappuis F, et al. The timing is right to end snakebite deaths in South Asia. *BMJ*. 2019 Jan 22;364: k5317.
5. Boutaud, Olivier, et al. "Acetaminophen Inhibits Hemoprotein-Catalyzed Lipid Peroxidation and Attenuates Rhabdomyolysis-Induced Renal Failure." *Proceedings of the National Academy of Sciences*, vol. 107, no. 6, Feb. 2010, pp. 2699–704. *DOI.org (Crossref)*, https://doi.org/10.1073/pnas.0910174107.

22. Structured abstract (Structured abstract should be in future tense)

Background

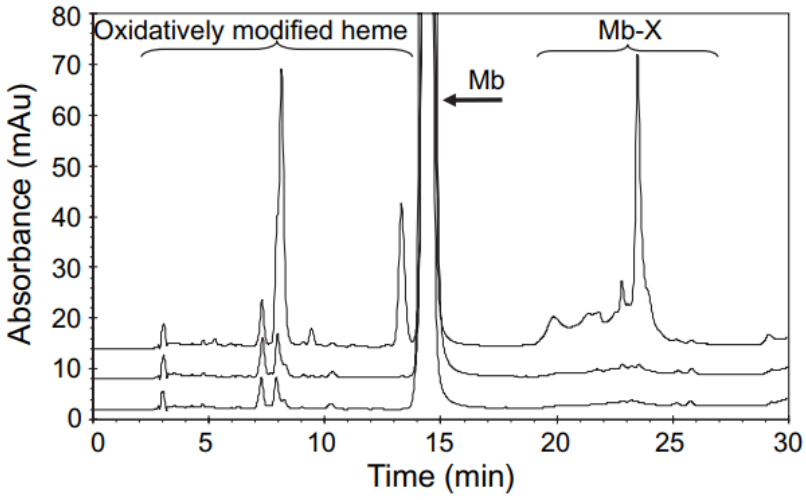
Acute kidney injury is a common and severe sequela of snakebite in India that results in considerable morbidity, mortality and healthcare cost. This observational study will seek to determine whether cell-free heme, present because of haemolysis, contributes to nephrotoxicity through oxidative stress.

Methods

We will conduct a prospective observational study to assess for the presence of ferryl heme derivatives in the urine of patients with snakebite. Patients aged >17 years that present within 24 hours of snakebite with evidence of systemic haemotoxic envenoming and no history of renal impairment will be approached to participate. A further five healthy control patients, without history of snakebite envenoming, will be recruited for control purposes. Participants with envenoming will undergo seven venipunctures and urine collection across seven days. Participation will be voluntary and informed consent will be obtained before any study procedures are performed. Blood analyses will be performed in real time using the hospital laboratory. The results will be accessible to the attending doctors to guide clinical management. Urine samples will be collected and stored at -20C and analyzed in a single batch once recruitment and data collection is complete. Anonymized data will be recorded detailing patients' demographic details, time from bite to antivenom and volume of antivenom. Daily outcome will be recorded on a simple case report form that will be completed by the responsible ward team.

HPLC for detection of ferryl heme derivatives in urine will be standardized by trial with the following method as preliminary guide-line. The ferryl heme is expected to be cross-linked to the protein in which exists, (in this case, the protein of hemoglobin), as the phenomenon happens in myoglobin. Ferryl heme cross-linked to its protein (say Hb-X) is expected to be optically distinct from normal hemoglobin, and unbound heme.

25 µL of urine sample will be loaded on a C3 reverse phase column of 5 µM pore size (300 Å, column size 250 mm X 4.6 mm). The mobile phases will be (A) 0.1% TFA and (B) acetonitrile with 0.1% TFA. Gradient elution with different proportions of mobile phases A and B will be tried to get good peak resolution (absorbance) of ferryl heme, cross-linked to the protein of Hb (Hb-X?) using a UV-vis detector set at 400 nm. Given below is a figure from reference 15, (Figure S3, supplementary information (co-investigator's publication)) which demonstrates HPLC of ferryl heme derivatives of myoglobin, cross-linked to the protein, (Mb-X, peak between 20 and 25 minutes)) using the method outlined above.



Results

Primary outcome

The proportion of patients with detectable ferryl-heme derivatives in urine measured by high performance liquid chromatography (HPLC). This will be quantified as a percentage of urinary heme.

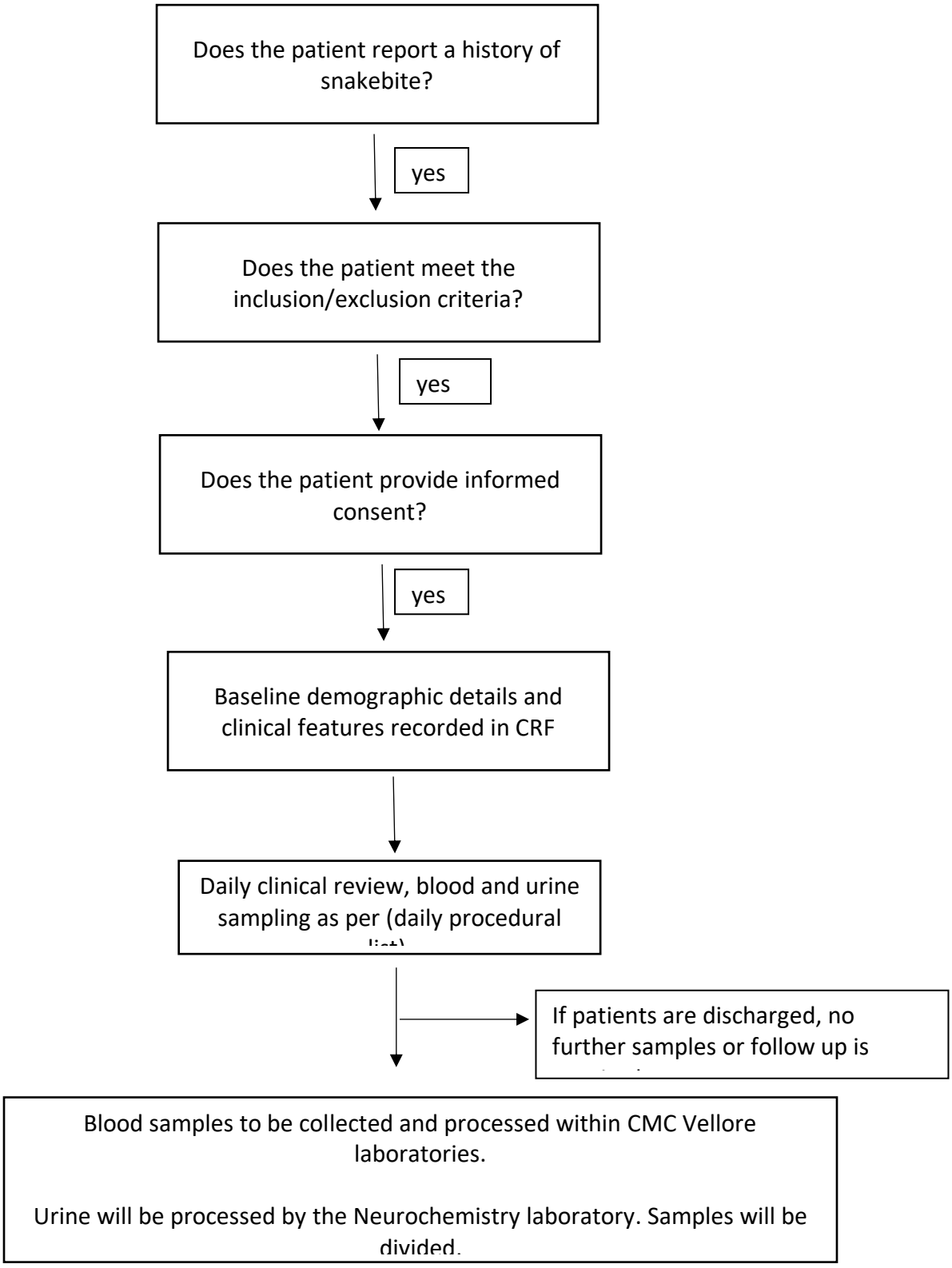
Secondary outcomes

- To determine whether detectable ferryl-heme derivatives in urine, correlate with markers of haemolysis on days 1, 3 and 5 following systemic viper envenoming. The presence of haemolysis will be determined according to the presence of schistocyte count of >1%
- To determine whether detectable ferryl-heme derivatives in urine correlate with the presence of thrombotic microangiopathy on days 1, 3 and 5 following systemic viper envenoming. The presence of TMA will be determined by the presence of haemolysis and thrombocytopenia (platelet count <150 $x10^9$).
- To determine whether detectable ferryl-heme derivatives in urine correlates with the presence of severe myotoxicity defined by a peak creatinine phosphokinase of >5000 IU.
- To determine whether detectable ferryl-heme derivatives in urine correlate with acute kidney injury (AKI) on days 1, 3 and 5 following systemic viper envenoming. Acute kidney injury will be defined using the KDIGO classification system, graded 0-3. It is likely that pre-admission levels of creatinine will not be known and hence expected baseline creatinine values will be calculated using the modification of Diet and Renal disease formula assuming a glomerular filtration rate of 75mL/min.

Results of this pilot study will not be extrapolated to infer causality.

Simple descriptive statistics using median and IQR will be used describe non-normally distributed data. Spearman's correlation will be used to assess correlation. Logistic regression will be used to assess for correlation.

23. Detailed diagrammatic Algorithm of the study


Does the patient report a history of snakebite?
yes
Does the patient meet the inclusion/exclusion criteria?
yes
Does the patient provide informed consent?
yes
Baseline demographic details and clinical features recorded in CRF
Daily clinical review, blood and urine sampling as per (daily procedural
If patients are discharged, no further samples or follow up is
Blood samples to be collected and processed within CMC Vellore laboratories.
Urine will be processed by the Neurochemistry laboratory. Samples will be divided.

Daily procedural list for envenomed participants

	Admission	D2	D3	D4	D5	D6	D7
Daily review in CRF	**X**	**X**	**X**	**X**	**X**	**X**	**X**
FBC	**SOC**	**X**	**x**	**X**	**x**	**x**	**x**
Blood film	**SOC**	**X**	**x**	**X**	**x**	**x**	**x**
20WBCT	**SOC**						
INR	**SOC**						
APTT	**SOC**						
Fibrinogen	**SOC**						
D-dimer	**SOC**						
Creatinine	**SOC**	**X**	**x**		**x**		**x**
LFT	**SOC**		**x**				
CPK	**SOC**		**x**				
Urine RE/ME	**SOC**	**X**	**x**		**x**		**x**
Urine for storage	**X**		**x**		**x**		

SOC: Standard of Care

Daily procedural list for control participants

	Admission	D2	D3	D4	D5	D6	D7
Daily review in CRF	**X**		**X**		**X**		**X**
FBC	**X**		**x**		**x**		**x**
Blood film	**X**		**x**		**x**		**x**
20WBCT	**X**						
INR	**X**						
APTT	**X**						
Fibrinogen	**X**						
D-dimer	**X**						
Creatinine	**X**		**x**		**x**		**x**
LFT	**X**		**x**				
CPK	**X**		**x**				

Urine RE/ME	**X**		**x**		**x**		**x**
Urine for storage	**X**		**x**		**x**		

Total volume of blood drawn from envenomed participants is 29 mL.

Total volume of blood drawn from envenomed participants is 21 mL.

24. Detailed research plan:

a. **Setting:** Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection

Participants will be recruited from the medical wards at CMC Vellore. The study is projected to commence recruitment from 1st May 2023 and will run until April 2024. Patients presenting with a history of snakebite will be considered for eligibility using the inclusion and exclusion criteria. All data points will be collected during routine hospital care with no additional follow up visits planned. Data will be collected on CRF's by the attending teams and kept within the medical notes until discharge.

b. **Participants:** Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases (and controls, if applicable). For matched studies, give matching criteria and the number of controls per case

Inclusion criteria

- History of snakebite in the preceding 24 hours
- Venom induced consumption coagulopathy (determined by either a positive 20-minute whole blood clotting test or INR > 1.4 or results) from CMC Vellore or a referring hospital prior to antivenom therapy.
- Aged >17 years old.

Exclusion criteria

- Known chronic kidney disease
- Known pre-existing haematological disorder

As a pilot study, we would like a spectrum of systemic viper envenoming to allow comparison of haemolysis/TMA and acute kidney impairment with the primary outcome of detectable ferryl-heme derivatives in urine. It is not apparent on admission which patients with systemic envenoming develop TMA and thus all patients with systemic envenoming (VICC) will be eligible. Delaying recruitment until haemolysis is present (a median of 3 days after envenoming) will result in the omission of blood and urine sampling from the time of admission.

Healthy control subjects will be recruited from hospital staff to enable serial blood and urine collection. The purpose of control subjects is to internally validate the HPLC analysis and demonstrate that the ferryl-heme derivatives are not present in healthy state.

c. **Variables:** Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable.

- Haemolysis will be defined as a schistocyte count of >1%.
- Thrombotic Microangiopathy will be defined as schistocyte count of >1% and a platelet count of < 150 x10^9 or a >25% fall from admission reading.
- Acute kidney injury will be defined using the KDIGO classification system, graded from 0-3. It is likely that pre-admission creatinine levels will not be known and hence expected baseline creatinine values will be calculated using the Modification of Diet and Renal disease formula assuming a glomerular filtration rate of 75 mL/min (KDIGO ref).
- Ferryl-heme derivatives will be detected using high performance liquid chromatography. Optical density will be displayed in graphical format.

HPLC Protocol:

Standard: Myoglobin (Sigma - Cat. No: M1882), dissolved in a slightly acidic buffer (e.g. pH5) with approximately 1:3 protein to H_2O_2 (higher ratios are needed as pH increases). For HPLC the injection volume ~25 µl of ~50 µM concentration is optimum but can be lower.

Sample preparation: Urine sample (200 µl) will be filtered through 0.22 µm syringe filter.

Reverse-Phase HPLC protocol: Normal urine samples spiked and not spiked with standard and urine samples from snake bite patients and normal controls will be analysed for ferryl haem derivatives using a Shimadzu Analytical HPLC system with a Zorbax StableBond C3 column (300 Å, column size 250 mm X 4.6 mm) fitted with a spectrophotometer detector. Samples will be separated over an increasing gradient of 0.1% Trifluoroacetic acid (TFA) and 0.1% TFA/Acetonitrile for a run period of 60 minutes at a flow rate of 1 mL/min at 25 °C (14,15). Crosslinked, covalently bound heme (which can only arise from a highly oxidized form of heme) does not separate and is optically distinct.

- Using large pore size (300 Å) HPLC and spectrophotometer, optically distinct peaks are present for (a) heme, (b) protein and (c) covalently attached heme/protein. (Some oxidatively modified hemes (with 720 nm peaks) that are not covalently bound to the protein can also be observed)

- Venom induced consumption coagulopathy. VICC will be diagnosed using the 20-minte whole blood clotting test and if concurrent clotting assays are available will be confirmed using concurrent INR of >1.4.

d. **Data Sources/measurement:** For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group

- Blood films will be examined by the haematology laboratory at CMC Vellore. Schistocyte count will be expressed as a percentage of red cells.

- Platelet count will be performed on an automated platform in the haematology laboratory at CMC Vellore and expressed at $x10^9$/L.
- Creatinine will be performed on an automated platform in the biochemistry laboratory at CMC Vellore and expressed as mg/dL.
-

e. **Bias:** Describe any efforts to address potential sources of bias.

The principal objective of this pilot study is to determine whether ferryl-heme derivatives are detectable in the urine from patients with systemic viper envenoming. As a pilot study, we will recruit snakebite patients at greatest risk, hence including only patients with evidence of systemic envenoming. All eligible patients will be approached sequentially. We acknowledge this inclusion criteria introduces a selection bias but have adopted this approach to maximise the scope of the pilot study. Accordingly, we will not extrapolate results beyond the eligibility criteria.

We will perform pre-study internal control (using positive and negative samples) on the HPLC analysis to ameliorate measurement bias. Urine will be analysed from all participants (including control subjects) at the study's conclusion. This will reduce operator bias and enable adherence with the standard operating procedure.

f. Sample size:(It may be suitable to have a statistician as a co-investigator)

Explain how the study size was calculated

This is an exploratory study and hence no sample size calculations have been used. Based on prior clinical experience, we anticipate the following distribution of patients.

- Systemic envenoming = 25
- AKI (40% of systemic envenoming) = 10
- TMA (50% of AKI) = 5
- Myotoxicity = 5

This should allow a minimum of 5 patients with TMA, and 5 patients with myotoxicity therefore potential for ferryl-heme in urine.

As an internal control for the study, we will recruit 5 healthy volunteers as control.

Quantitative variables: Explain how quantitative variables will be handled in the analyses. If applicable, describe which groupings were chosen and why

STROBE reporting guidelines will be followed for handling categorical data.

The quantifiable variable for creatinine will be categorised into the internationally agreed KDIGO scoring for AKI from 0 (< 1.5 times expected baseline), 1 (1.5-1.9 times expected baseline), 2 (2.0-2.9 times expected baseline) to 3 (3.0 or greater than times expected baseline).

TMA will be categorised into binary groups (present/absent) using the definition mentioned previously.

Urinary ferryl-heme derivatives will be divide both in binary terms (present/absent) and as a continuous variable (percentage of detectable urinary heme).

g. **Statistical methods:** Describe all statistical methods, including those used to control for confounding and examine subgroups and interactions. How will missing data be handled? If applicable, how will matching of cases and controls be handled? Describe any proposed sensitivity analyses.

Demographic data will be described using simple descriptive statistics, median and interquartile range for non-normally distributed data, mean and standard deviation for normally distributed data.

Simple descriptive statistics will be used to describe the primary outcome of whether urinary ferryl heme derivatives were present or not. Categorical data (AKI and TMA) for primary outcome will be compared using Chi squared or Fisher's exact test, as appropriate.

h. **Name & designation of the statistician involved in your project for Statistical Analyses:**

Marimuthu S
Associate Research Officer
Department of Biostatistics
CMC, Vellore

25. Complete budget plan

*For **FLUID research grant money cannot be allocated for travel of the investigators nor can job outsourcing** be covered with FLUID grants. Funding out of the institution can be given only for the special mission hospital grant*

*(From Fluid Research Fund, there are no grants for personnel except in a major grant application, funding is limited **Rs. 1, 50,000/- per year** for two years for standard applications,*

***Rs. 2,00,000/- per year for two years for major applications**). Website link: http://172.16.23.136/Research/index.html. > Rules for Major Fluid Research Grants. Do not exceed the budget allocated to you. In case the budget is exceeded, the amount will have to be deducted from one of your departmental special funds. Stationary, printing material and paper should not exceed should not exceed Rs. 8000/- (Rs. 10,000/- for Major Fluid Grant) of the allocated fluid grant irrespective of the study duration.*

*Please mention below the **breakdown of budget requested:** (The budgets that are drawn up should be comprehensive and should mention all subject in detail (For example – laboratory investigation should mention the specific category without generalization.)*

26. In case of major equipment purchase what is the agreement with the funding agency in terms of institutional ownership of the equipment? Not applicable

27. ***Enclose proforma for Data collection:***

Enclosed

28. If this is an application for Fluid Research Funding, please provide name and account number of any other Fluid

S. No	Study Title	IRB Min. No. and date	Grant Sanctioned amount/ Account Head	Duration / Year	Study ongoing/ completed
1.	Study of association between physical activity and BMI in different levels of staff at a tertiary care Center in South India:A case-control study	10265/ 05.09.2016	INR 50,000 only	One year	Completed

Research grant held by the PI.

29. Informed Consent Documents (patient information sheet, investigator's brochure, drug information etc and informed consent document) please submit all translations with the proposal**.**

30. Publication Plans: (List all potential authors and their likely contributions)

(Please tick √ appropriate box)

Note: Minimum 4 roles (1 role in each category I-IV) must be given to each author.

Key Note: (as per ICMJE guidelines (version Dec 2019):

Section II

APPLICATION FOR APPROVAL FROM ETHICS COMMITTEE OF THE INSTITUTIONAL REVIEW BOARD OF CMC VELLORE FOR ALL OBSERVATIONAL (CASE CONTROL, COHORT & OBSERVATIONAL) STUDIES IN HUMAN SUBJECTS

1. **Please provide a brief summary of the justification, objectives and methods in lay language, avoiding technical terms.**

   Some patients that suffer viper snakebite develop kidney failure. At present, it is not clear why some patients develop kidney failure, whilst other patients do not. This study is designed to identify whether fragments of blood cells, that have been damaged by snake venom, contribute to kidney failure in viper snakebite. As this has never previously been assessed before, we will conduct a small 'proof of concept study' study to determine whether fragmented blood cells cause damage in the kidney. This will be assessed by analyzing blood and urine samples collected during the first week of treatment.

2. **Please describe if the study uses procedures already being performed on patients for diagnosis or treatment or if modified or novel procedures are to be used?**

   All blood analyses will be tests that are frequently ordered in patients with snakebite. Urine samples will be stored and analysed in bulk using a novel High-performance liquid chromatography analysis. Whilst this analysis has been described and verified in the medical literature for detecting ferryl heme derivatives, it is not part of routine care and has never been use in snakebite envenoming.

3. **Please describe what benefits might be reasonably be expected by the participant as an outcome of participation**

   Patients may benefit from enhanced observation as a product of being a study participant. This may be reflected in more frequent blood and urine sampling. Any sampling that is perceived to be beyond 'standard of care' will be provided free of charge.

4. **Please describe what benefits to others or new knowledge might be expected as a result of this study**

   This study will add to the literature concerning the aetiology of acute kidney injury in snakebite envenoming. If the hypothesis is upheld, this will result in further studies that will assess low cost interventions that may prevent the development of oxidative renal toxicity in snakebite patients, thus reducing morbidity, mortality and cost of snakebite envenoming.

5. **Who are to be enrolled?**

The study will seek to recruit 25 patients that provide informed consent. Inclusion criteria will consist of (a) a history of snakebite with the preceding 24 hours, (b) evidence of systemic haemotoxic envenoming and (c) patients aged greater or equal to 18 years old with be recruited. 5 healthy participants (staff) will be consented and enrolled as controls subjects.

6. **If any vulnerable groups (e.g., pregnant women, children, unemployed, impoverished/below poverty line, illiterate) are to be enrolled, please provide a justification for their inclusion.**

Children will be excluded from this study. Other vulnerable groups including pregnant woman, unemployed, impoverished and patients living below the poverty line will recruited if presenting within the study period and meeting the inclusion/exclusion criteria. As an observational study, risks will be limited. It is not expected that their vulnerable status will affect their ability to provide informed consent and no undue inducement will be used to encourage participation.

7. **Mention how you will ensure that there is no undue inducement for participation of economically disadvantaged persons among the likely participants in this study.**

There will be no financial renumeration to patients that participate. Only blood and urine tests listed in the study procedures, that are provided above standard of care, will be offered free of charge.

8. **What are the potential risks to participants in this study?**

Potential risks for inclusion include the pain and discomfort of undergoing venepuncture. Other potential risks include the mismanagement of personal data resulting in breach in confidentiality.

9. **Are the risks to participants reasonable in relation to the benefits that might reasonably be expected as an outcome to the participant or to others, or the importance of the knowledge that may reasonably be expected to result? Please provide a detailed description of the above.**

The risks of this observational study are small. Where possible, blood tests will be collected at the same time thus limiting the total number of venepunctures. We anticipate that only 2-3 additional venepunctures (beyond standard of care) per patients will occur during the study period.

The risk of data breach will be mitigated using anonymised data. Where personal information is retained (e.g. on the consent form) this will be stored in a separate locked filing cabinet from the case reports forms so that personal data cannot be identified.

The relatively minor risk to participants is justifiable in relation to the final analyses that will help determine the role of oxidative renal toxicity in the development of snakebite induced AKI.

10. **Regarding informed consent to obtained from research participants or their legally authorized representative(s):**

a. **Does the informed consent document include all the required elements?**

b. **Are the participant information sheet and the consent document in language understandable to participants? (PLEASE PROVIDE WITH THIS SUBMISSION TRANSLATIONS IN ALL LOCAL LANGUAGES ANTICIPATED TO BE USED).**

Please find the attached patient information sheet and consent form in English, Tamil, Telugu and Hindi.

c. **Who will obtain informed consent (PI, nurse, other?) and in what setting?**
Patients will be recruited at the Emergency Department of the study center. The investigator will, obtain consent, and collect data. Monitoring will continue in the wards and ICU. Participants will be reviewed at least twice daily for the duration of their stay.

d. **If appropriate, is there a children's assent? If yes, please submit a copy of this form.**

N/a

e. **Is the EC requested to waive or alter any informed consent requirement?**

11. Is there provision of free treatment for research related injury? If yes, who will provide it?

Partial treatment costs (of blood tests that are not part of routine patient care, see list in study procedures) will be provided free of cost. The ordering team will include the patient unique study Id and the laboratory will invoice the study team for the costs.

12. Is there provision for compensation of participants for disability or death resulting from research related injury. If yes, who will provide it?

N/a – this is an observational study so no adverse outcome will arise from research related injury.

13. Is the study covered by insurance? If yes, please provide insurance documents from an Indian insurance company.

No

14. In addition to the overall budget in Section I, please provide details of the following

i) Justification, timing and amount of payments to study participants - Nil

ii) Justification, timing and amount of payments to investigators/departments - Nil

iii) Any other study related financial or in-kind incentives to participants or study staff - Nil

15. Please describe the plan for maintaining confidentiality of study participant information.

The only record that will document the participants name will be the informed consent form. Once consented, participants will be allocated a unique identification number and the consent form will be stored in a locked cabinet. Other person identifiable information, such as address, or telephone number will not be recorded. One data collection is complete, the CRF will be stored in a separate filling cabinet. No patient identifiable information will be recorded in the database.

The raw data will be stored in computer and back up in Medicine unit 1. The patient data base will not include patient identifiers. The analysis will be done by Biostatistics department in CMC.

16. Please describe the plans for monitoring the safety of participants, reporting and managing adverse events. If this is an externally funded study with a Data Safety Monitoring Board, please provide the name and contact information of the DSMB chairperson.

As an observational study, a data safety monitoring board will not be necessary.

17. If an internal DSMB is being conducted kindly nominate the person from your specialty act as a neutral referee (This person should not be an investigator in this study)

N/A

18. If applicable; please provide all significant previous decisions (e.g., those leading to a negative decision or modified protocol) by other ECs or regulatory authorities for the proposed study (whether in the same location or elsewhere) and an indication of the modification(s) to the protocol.

N/A

19. If appropriate, has permission from the Drug Controller General of India been obtained?

N/A

20. If this is international collaborative research, has permission from the Health Ministry's Screening Committee been obtained?

N/A

Role of Dr. Thomas Lamb, University of Oxford: Intellectual contribution in design, planning, data collection, analysis and publication. He will visit CMC Vellore for data collection. The results of the study will be used for submission for part

requirement of a Masters of Research (MRes) course in the University of Oxford. There will be no transfer of materials and raw data outside the country. The University of Oxford will directly provide HPLC column for the study.

21. For exchange of biological material in international collaborative studies, please provide a Memorandum of Understanding (MOU)/ Material Transfer Agreement (MTA) between the collaborating partners.

N/A

Title of Research Project: An observational study to assess for ferryl-heme derivatives, indicative of oxidative nephrotoxicity, in the urine of patients with viper envenoming.

22. Undertaking (to be signed by all investigators)

By signing this form, we give our consent to provide our expertise to the project. In addition:

a. We confirm that all investigators have approved this version of the protocol and have contributed substantially to its development.
b. We confirm that all potential authors are included in this protocol.
c. We confirm that we shall submit any protocol amendments, significant deviations from protocols, progress reports (if required) and a final report and also participate in any audit of this study, if required.
d. We confirm that we shall conduct this study in accordance with the Declaration of Helsinki; the ICMR National Ethical Guidelines for Biomedical and Health Research involving Human Participants 2017, with any subsequent amendments in the New Drug and Clinical Trials Rules, 2019 under the aging of the Schedule Y Drugs and Cosmetics Act, 1940; GCP guidelines; and all applicable laws of the Republic of India.
e. We also agree to submit for publication to a peer reviewed journal the complete results of this study within two years of completion of this study.
f. We declare that we have no conflicts of interest that may affect the conduct or reporting of this study (OR) we declare the following conflicts of interest below.
g. We are aware of the institution's policies regarding scientific misconduct (Falsification/fabrication/plagiarism) and agree to abide by them.

23. Signature of Principal Investigator:

24. Signature of Guide/Supervisor:

Title of Research Project: An observational study to assess for ferryl-heme derivatives, indicative of oxidative nephrotoxicity, in the urine of patients with viper envenoming.

25. Signature of Head of the Department/ Unit:

(the HOU/HOD need not be a co-investigator in the study)

Title of Research Project: An observational study to assess for ferryl-heme derivatives, indicative of oxidative nephrotoxicity, in the urine of patients with viper envenoming.

26. Co-Investigator's Consent (all co-investigators have to sign this form or supply separate letters of consent)

Name	Department	Signature	Date
Anand Zachariah	Medicine-I	Anandzachariah	4.1.23
Ravikar Ralph	Medicine-I	Ravikar Ralph	6/1/23
Krupa George	Medicine-I		6/1/23
Amith Balachandran	Medicine-I		6.1.2023
Prabhakaran	Neurology	V. Prabh.	7/1/2023
Soosai Manickam A	Physiology		7/1/2023
Suceena Alexander	Nephrology-III	Suceena	6/1/2023
Sathya Subramani	Physiology		7 Jan 2023
J.V. Peter	Critical Care Medicine		7/1/2023

Title of Research Project: An observational study to assess for ferryl-heme derivatives, indicative of oxidative nephrotoxicity, in the urine of patients with viper envenoming.

Amita Jacob	**Medicine-V**		6/1/23
Samuel George Hansdak	**Medicine-IV**		6/1/23
Tina George	**Medicine-II**	for	6/1/23
Nalini Sarah Newbigging	**Medicine-III**		6/1/23
Gina Chandy	**Emergency Medicine**		4.1.23
Marimuthu S	**Biostatistics**		6.1.23
Thomas Lamb	**University of Oxford**		6/1/23
Brandon Reeder	**University of Essex**		

Section III

Note: If the project is a resubmission a fresh copy of signatures needs to be obtained for IRB submission

N/A

CHECKLIST FOR PROTOCOLS SUBMITTED TO IRB OF CMC VELLORE FOR OBSERVATIONAL (CASE CONTROL, COHORT & CROSS SECTIONAL) STUDIES

Conflicts of interest if any: None

Please tick the appropriate boxes below to indicate that the following have been submitted and if not, please explain why:

1. Form for protocols of Observational Studies with all sections (I, and II) completed [Yes]
2. Informed consent sheet *and participant information sheet* in all relevant local languages (PDF Format) [Yes]
3. Names, affiliations and signatures of all investigators/co-investigators for the declaration [Yes]
4. Signature of the Head of the department or unit as applicable (for interdepartmental studies, an agreement letter from concerned departmental heads is desirable, especially if they are not co-investigators). [Yes]
5. Recent curriculum vitae of all the investigators indicating qualification and experience and relevant publications in the past five years. [Yes]
6. If applicable, proposed compensation and reimbursement of incidental expenses and management of research related and unrelated injury/ illness during and after research period. [No]
7. If applicable (in study-related injuries), a description of the arrangements for insurance coverage for research participants and copy of insurance documents from an India insurance agency. [No]
8. If applicable; all significant previous decisions (e.g., those leading to a negative decision or modified [No]

   protocol) by other ECs or regulatory authorities for the proposed study and an indication of the modification(s) to the protocol made on that account. The reasons for negative decisions should be provided. [No]
9. Plans for publication of results - positive or negative - while maintaining the privacy and confidentiality of the study participants, with names of proposed authors and their expected contributions. [Yes]
10. All other relevant documents related to the study protocol like product information and statement of relevant regulatory clearances. [Yes]
11. If applicable, any material used for advertisement to recruit participants to the study - this may include flyers, brochures, posters, radio and TV advertisements. [No]
12. For externally funded studies, details of Funding agency/ Sponsors and breakdown of fund allocation. [No]
13. **One hard copy (No need of CV's) and a soft copy (All documents including CV's) on CD of all the above should be submitted to Research Office.**

Bibliography

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2. Suraweera W, Warrell D, Whitaker R, et al.; Trends in snakebite deaths in India from 2000 to 2019 in a nationally representative mortality study. *Elife* 2020;**9**. doi: 10.7554/eLife.54076.

3. Majumder D, Sinha A, Bhattacharya SK, et al.; Epidemiological profile of snake bite in south 24 Parganas district of West Bengal with focus on underreporting of snake bite deaths. *Indian J Public Health* 2014;**58**(1):17-21. doi: 10.4103/0019-557x.128158.

4. Stephen S, Mohanty CR, Radhakrishnan RV, et al.; Clinico-Epidemiological Profile, Trends, and Health-Related Outcomes of Snakebite Victims: A One-Year Prospective Study from Eastern India. *Wilderness Environ Med* 2024;**35**(2):155-165. doi: 10.1177/10806032241239628.

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