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Bothrops atrox, the most important snake involved in human envenomings in the amazon: How venomics contributes to the knowledge of snake biology and clinical toxinology

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ABSTRACT

Bothrops atrox snakes are mostly endemic of the Amazon rainforest and is certainly the South American pit viper responsible for most of the snakebites in the region. The composition of *B. atrox* venom is significantly known and has been used to trace the relevance of the venom phenotype for snake biology and for the impacts in the clinics of human patients involved in accidents by *B. atrox*. However, in spite of the wide distribution and the great medical relevance of *B. atrox* snakes, *B. atrox* taxonomy is not fully resolved and the impacts of the lack of taxonomic resolution on the studies focused on venom or envenoming are currently unknown. *B. atrox* venom presents different degrees of compositional variability and is generally coagulotoxic, inducing systemic hematological disturbances and local tissue damage in snakebite patients. Antivenoms are the effective therapy for attenuating the clinical signs. This review brings a comprehensive discussion of the literature concerning *B. atrox* snakes encompassing from snake taxonomy, diet and venom composition, towards clinical aspects of snakebite patients and efficacy of the antivenoms. This discussion is highly supported by the contributions that venomics and antivenomics added for the advancement of knowledge of *B. atrox* snakes, their venoms and the treatment of accidents they evoke.

1. *Bothops atrox:* the snake

Bothrops atrox, the common lancehead, is a South American pitviper responsible for most of the snakebites in the Amazon region (Wen et al., 2015). Although this species was described by Linnaeus in 1758, no integrative systematic review is available so far (Werman, 2005) and its taxonomic status remains controversial (Alencar et al., 2016; Campbell and Lamar, 2004; Pyron et al., 2013; Wüster et al., 1997; Zaher et al., 2019). Wüster et al. (1999), based on a limited sampling, provided genetic evidences that *B. atrox* probably represents a complex of several species by showing the existence of at least four distinct mitochondrial DNA (mtDNA) lineages across the species distribution. The study also shows that *B. moojeni*, *B. leucurus*, *B. isabelae* and *B. marajoensis* are phylogenetically nested inside the mtDNA diversity *of B. atrox* (Wüster

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et al., 1999). The non-monophyly of *B. atrox* mtDNA lineages was recently confirmed by other studies based on a more comprehensive geographic sampling (Nascimento, 2014; Silva-de-Oliveira, 2014). Gibbs et al. (2018), studying nuclear DNA through RADseq data in samples from four close sites in Brazilian Amazon, also indicate the presence of different lineages and high levels of genetic variability among *B. atrox* lineages. Some ecological studies on *B. atrox* (Martins et al., 2001) even considered these lineages as different evolutionary units showing contrasting results among them. The combined scenario provided by the genetic studies strongly suggests caution when considering *B. atrox* taxonomy—as it stands—as a backbone for evolutionary hypotheses. The impacts of the lack of taxonomic resolution on the studies focused on venom or envenoming are currently unknown. Although *B. atrox* might not represent an exclusive evolutionary lineage, much of the conclusions about venom variability and clinical aspects of snakebites are derived from local studies, focused on very delimited population sampling (Amazonas et al., 2018; Calvete et al., 2009, 2011; Moretto Del-Rei et al., 2019; Núñez et al., 2009) and the main conclusions provided by such studies probably will not be affected by future taxonomic decisions. Currently, there is no strong published evidence indicating the hybridization between *B. atrox* and other species of *Bothrops*, neither in captivity nor in the field. The suggestions of possible natural hybridization are derived from collected specimens identified as belonging to the *B. atrox* complex but showing intermediate morphology (Wüster et al., 1997). Supporting evidence for hybridization was reported among several other species of *Bothrops* (Balestrin et al., 2002; Prudente et al., 1995; Santoro et al., 2015; Sazima, 1992; Vellard, 1929).

Considering the current taxonomy, *B. atrox* is mostly endemic of the Amazon rainforest, but it is also registered in the Bolivian Yungas, Colombian Llanos (Nogueira et al., 2020) and likely in some Caatinga enclaves of moist forests (Brejos de Altitude) in northestern Brazil (Loebmann and Haddad, 2010). Tipically, the species is found in the tropical lowlands of South America in the east of the Andes, including southeastern Colombia, southern and eastern Venezuela, Guyana, Suriname, French Guiana, eastern Ecuador and Peru, Panama, northern Bolivia and the northern region of Brazil (Nogueira et al., 2020). *B. atrox* is generally one of the most abundant species in studies on snake communities in the Amazon (Frazão et al., 2020; Martins and Oliveira, 1998; Masseli et al., 2019; Oliveira and Martins, 2001). It inhabits most exclusively forested areas, although it may be occasionally found in disturbed habitats around human settlements, including pastures and crops, and urban areas (Bernarde, 2014; Campbell and Lamar, 2004; Doan and Arriaga, 2002; Martins and Oliveira, 1998).

The adult size of *B. atrox*, measure by the snout-vent length (SVL), ranges from 537 mm to 1532 mm (Oliveira and Martins, 2003; Silva et al., 2019), with males attaining maturity with smaller sizes than females (Bisneto and Kaefer, 2019; Silva et al., 2017, 2019Silva et al., 2019). SVL sexual dimorphism is frequently documented in species of *Bothrops*, and females presenting larger size than males have been registered in all species studied so far (Hartmann et al., 2004; Monteiro et al., 2006; Nunes et al., 2010; Sazima, 1992; Silva et al., 2017). However, *B. atrox* males reach relatively larger body sizes when compared with other species of *Bothrops*, being similar in size only to *B. moojeni* and *B. leucurus* (Almeida-Santos et al., 2017; Nogueira et al., 2003). These are the species of *Bothrops* in which male–male combat has been reported (Almeida-Santos et al., 2017; Almeida-Santos and Salomão, 2002). Silva et al. (2019) suggested that male-male combat probably favored the evolution of larger male body size in *B. atrox* and associated species.

Bothrops atrox presents high level of morphological polymorphism throughout its distribution (Silva-de-Oliveira, 2014). The color pattern is highly variable, including a ground color that can be olive, brown, tan, gray, yellow, or rarely rusty with darker blotches forming trapezoid shape in lateral view (Campbell and Lamar, 2004; Martins and Oliveira, 1998). The species is predominantly nocturnal, presenting higher encounter rate at night but may also be active during the day (Oliveira and Martins, 2001). At night, adults are found mainly on the ground, coiled in a typical ambush hunting posture (sit-and-wait), but juveniles can be also found on vegetation, up to 1.5 m height (Oliveira and Martins, 2001; Turci et al., 2009). Observations of mating have been recorded in the wild in the months of January, April, May and November, and an additional mating observed in captivity in June (Martins and Oliveira, 1998; Sanaiotti et al., 2005; Silva et al., 2019). Based on these observations, no general pattern of seasonal mating behavior can be identified, although there is no mating record during the wettest months (February and May) and the driest months (July and August) in most of the Amazonian region. This is true for Manaus area (Central Amazon), where most of the data comes from; however, the rainy seasons in Amazonia vary geographically, due to the enormous size of the region, and because Amazon occurs both south and north of the equator line, thus resulting in differences in *B. atrox* abundance across the region. Although pregnant females are found throughout the year, births were only registered from June to February, occurring mainly at the end of the dry season in the months of September and October (Silva et al., 2019). Females of *B. atrox* can store sperm in the uterus and in the posterior infundibulum (Silva et al., 2019) allowing the asynchrony between mating and ovulation (Almeida-Santos and Salomão, 1997, 2002). Similar to other median size viviparous snakes, the litter size of *B. atrox* is correlated with female SVL and can vary between 3 and 43 cm in young individuals (Martins and Oliveira, 1998; Silva et al., 2019). Although Martins and Oliveira (1998) indicate that newborns present total body size varying from 280 mm to 350 mm, their observation is based on presumed age categories of individuals preserved in scientific collections. So far, the largest offspring that was born in captivity presented 254 mm SVL and the smallest juvenile of *B. atrox* already measured from scientific collections had 156 mm SVL (Silva et al., 2019). Silva et al. (2017) suggests that males and females are born with similar SVL, but already showing some level of sexual dimorphism, with males presenting relatively larger tails and female presenting larger heads, both in length and width.

A limitation of most studies regarding the reproductive cycle of *B. atrox* is the lack of histological analysis in males (i.e., a direct evaluation of the presence of sperm) and whole comparative analysis of the reproductive tract for females (i.e., presence of secondary ovarian follicles, oviductal embryos, corpora lutea, sperm storage, and folded oviducts) (Almeida-Santos et al., 2014). Instead of that, most studies use only the simple evaluation of the presence of convoluted ductus deferentia for males and measurements of the follicles size for females. Pregnant females were found throughout the year (except May), but births occurred mainly between August and October (end of dry season in most of the biome), whereas males exhibited kidney hypertrophy and sperm production from November to April (rainy season in most of the biome) (Silva et al., 2019). Sperm storage occurs in the posterior infundibulum and nonglandular uterus in vitellogenic females (Silva et al., 2019). Silva et al. (2019) found that sexual segment of the kidney is a better proxy than ductus deferentia to sperm production. However, in some studies with large numbers of specimens, simpler approaches are used to estimate the reproductive stage of *B. atrox* snakes considering just the snake size. It has been considered 800 mm SVL a threshold between mature and immature females, and 470 mm SVL the threshold for males (Bisneto and Kaefer, 2019; Oliveira and Martins, 2003; Silva et al., 2017). However, it has already been reported the presence of embryos in the oviduct or females smaller than 800 mm SVL as well as large females and males that were not sexually mature (Silva et al., 2019).

One important aspect to be evaluated is whether the snake reproductive stage or differential activity due to seasonal changes could interfere in accidental encounters with humans, with consequent envenoming. Even aware of the limitations discussed above, we estimated the reproductive stage of the snakes involved in human accidents in Manaus using the SVL size of a collection composed of 612 specimens of *B. atrox* snakes brought to a reference health service in the Brazilian

Amazon, by the envenomed patients. We observed seasonal differences in the snakes: in the rainy season, more snakes are causing snakebites, while in the dry season there is a drop in the number of snakes brought to the hospital, which suggests a decreasing chance of an encounter with humans (Fig. 1). This difference in the activity among seasons can be explained by the reproductive cycle of the species, associated with the rainy season. This synchronization could mean that the new-borns appear in a time when prey, mostly anurans, are more abundant, making it easier the foraging to acquire energy to grow, increasing their chances of surviving (Sasa et al., 2009; Sazima, 1992). Indeed, this relationship among anuran availability and *B. atrox* frequency was already suggested for Western Amazon (Turci et al., 2009). Alternatively, other factors, such as a general reduction in ecosystem (overall biomass) productivity in the dry season and even the dry weather per se, may simultaneously affect the activity of most animals, including snakes and their prey (Oliveira and Martins, 2001).

2. *Bothrops atrox*: habitat partitioning and diet

Adults of *B. atrox* are found predominantly on the ground, while juveniles are mostly found on the vegetation (Duellman, 1978; Martins and Oliveira, 1998; Oliveira and Martins, 2001). A similar ontogenetic shift in microhabitat use occurs in other pit vipers as *B. asper* (Campbell, 1998) and *B. jararaca* (Sazima, 1992), and also in snakes belonging to other families (Martins et al., 2002). Fig. 2 shows *B. atrox* individuals presenting different ontogenetic stages, resting or foraging in different substrates.

Predation of snakes by terrestrial arthropods, like ants and tarantulas, may be responsible for most of the predator pressure on the ground, driving snakes to sleep on vegetation (Martins and Gordo, 1993). Juveniles of *B. atrox* are probably more susceptible to be preyed upon by terrestrial arthropods than adults. Such predator pressure could explain the prevalence of juveniles found on the vegetation (Martins and Gordo, 1993). Indirectly, intraspecific habitat partitioning, and diet variation may help to avoid competition for resources in different ontogenetic stages of *B. atrox* (Martins et al., 2001).

B. atrox has a generalist diet, preying on centipedes, fish, frogs, lizards, snakes, birds and small mammals (Bernarde and Abe, 2010; Bisneto and Kaefer, 2019; Macedo-Bernarde and Bernarde, 2005; Martins and Gordo, 1993; Martins and Oliveira, 1998; Nascimento et al., 2008; Oliveira and Martins, 2003). Ontogenetic shift in diet has been described for *B. atrox* (Bisneto and Kaefer, 2019; Martins and Gordo, 1993; Martins and Oliveira, 1998), with juveniles consuming almost exclusively ectothermic prey, whereas adults being more generalists with a tendency to consume endothermic prey (Figs. 3 and 4). This shift in diet can be explained by the increase in the absolute size of the snake over the course of the individual development, accompanied by its mouth gap and ability to swallow larger items (Silva et al., 2017). Mammals represent the main prey item of an adult *B. atrox* and although they are probably more profitable energetically than an ectothermic prey, preying on mammals usually requires a larger and stouter body for snakes (Martins et al., 2002), and this morphology is not present in juveniles of *B. atrox*. Additionally, juveniles of *B. atrox* seem to be adapted to prey on ectotherms. They usually bear a distinctly colored tail tip, paler than the remainder of the tail, which can be used to attract prey through caudal luring, which is believed to be more effective in amphibians and arthropods than in rodents (Martins et al., 2002). These observations were confirmed when we analyzed the stomach content of 612 specimens of *B. atrox* involved in human accidents in Manaus and conserved in FMT-HVD snake collection. Adults' diet was composed mostly of mammals (56.3%) while juveniles present a higher variability of prey, with predominance of lizards (44.0%). Inferring that insect remains found in the hindgut of pit vipers result from secondary digestion mostly from frogs (Martins and Gordo, 1993), anurans (26.7%) represent the second most common prey found in juveniles. Neonates fed mostly on anurans (72.8%) and lizards (18.2%). Mammals were not found in the diet of neonates.

It has been accepted that venom-induced hemostatic disturbances are fundamental features of pit viper venoms for the purpose of prey capture by snakes, in which hemorrhage, plasma extravasation and the rapid formation of endogenous thrombin could result in prey incapacitation through circulatory shock induction (Chacón et al., 2015; Sousa et al., 2018). *B. atrox* belongs to a genus in which an ontogenetic shift of diet from ectotherms to endotherms is well established (Martins and Gordo, 1993; Martins et al., 2002; Oliveira and Martins, 2001). Like in other snakes, venom variation in *B. atrox* is argued to represent an adaptation that has evolved to facilitate the capture and digestion of prey. Venom properties vary ontogenetically in *B. atrox* and in some other species of the same genus, likely caused by differences in the feeding habits of juveniles and adults (Andrade and Abe, 1999; Furtado et al., 1991). For adults, it is admitted that one additional role of the venom is the initiation of digestion as the increase in prey size offering digestive resistance would be accompanied by a concurrent increase in venom proteolytic activity (Furtado et al., 1991; López-Lozano et al., 2002). The greater volume of venom injected by adults would compensate the venom activity that may improve prey capture (Furtado et al., 1991; Puorto et al., 1996). In juveniles, otherwise, the primary role of venom would correlate to prey immobilization (Andrade et al., 1996; Andrade and Abe, 1999).

Venoms of juvenile specimens of *B. jararaca* and *B. moojeni* are

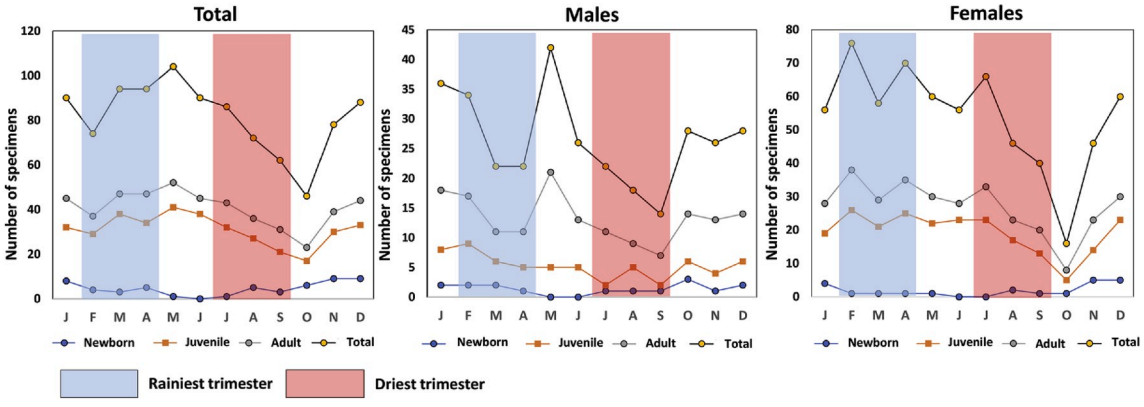

Fig. 1. Seasonal characteristics of 612 *Bothrops atrox* specimens causing envenomations in Manaus, Western Brazilian Amazon. Above) Frequency of *B. atrox* responsible for snakebites brought seasonally to the hospital, separated by size and sex. Colors show the rainiest (blue) and driest (red) trimesters of the year. The specimens were grouped by age as follows: neonate males and females (snout-vent length, SVL<300 mm), juvenile males (SVL between 300 and 470 mm) and juvenile females (SVL between 300 and 850 mm), adult males (SVL>470 mm) and adult females (SVL>850 mm) according to Silva et al. (2017). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

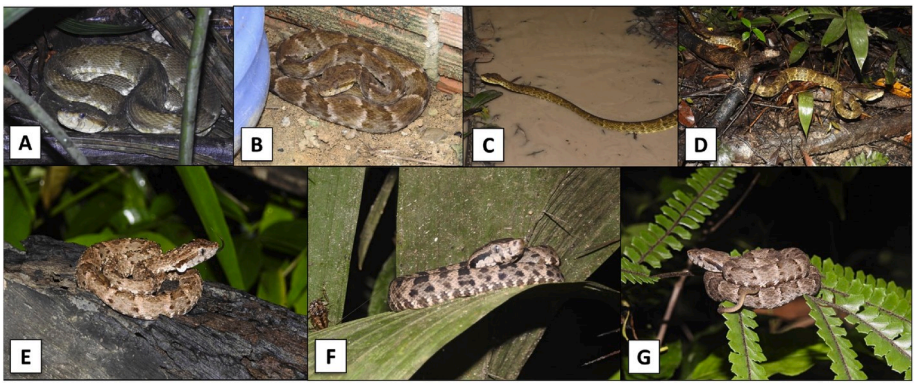

Fig. 2. *Bothrops atrox* specimens resting or foraging in different substrates. A-D pictures show adult specimens on the ground: A) ambush hunting in a forest environment; B) resting in an urban place, between a wall and a container for water storage. C) moving in a forest environment on a small body of water. D) moving on the leaf-litter. E-G pictures show juvenile specimens in ambush hunting on different aerial substrates. Photos: Paulo S. Bernarde.

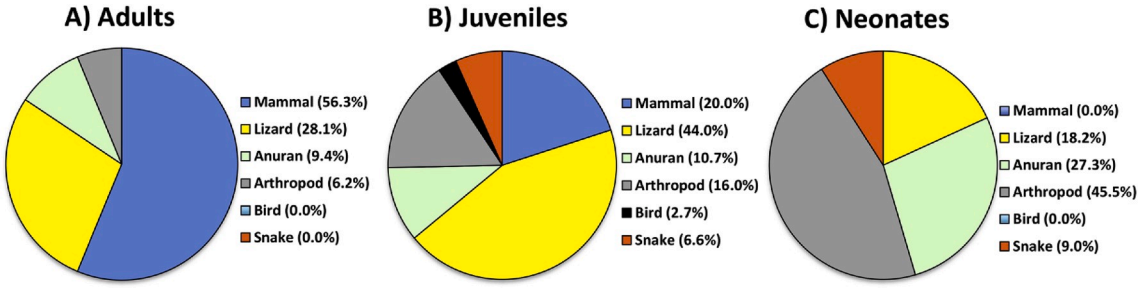

Fig. 3. Proportion of prey found in the diet of *Bothrops atrox* specimens from the region of Manaus, according to sizes. The stomach content was analyzed in 612 specimens of *B. atrox* snakes brought to the health service by the envenomed patients and classified according to the reproductive stage classified as described in Fig. 1: A) Adults, B) Juveniles, C) Neonates.

especially lethal on anurans (Andrade et al., 1996; Andrade and Abe, 1999) and on adult phase, lose 70 and 86% of their toxicity, respectively, upon the preferred prey of the juvenile phase. In mice, however, lethality rates for adult and juvenile venoms are similar. The synergistic interaction of tail luring, food availability, time of birth, ability to hold preys, and high venom lethality on anurans seems to maximize the prey capture, immobilization and death by juveniles (Martins et al., 2002). Interestingly, in *Bothrops* species with no diet shift during the lifetime, which feed preferably on mammals at any age, the venom of juveniles lacks an increased toxicity on ectothermic organisms (Andrade and Abe, 1999).

Regarding the effect of the venom composition on its biological role in different preys, information is scant. In *B. asper*, there are marked differences between newborn and adult venoms from both regions in electrophoretic and immunoelectrophoretic patterns (Alape-Girón et al., 2008; Gutiérrez et al., 1980). The loss of high molecular weight fractions of the venom during ontogeny (Furtado et al., 1991; Meier, 1986) with further loss of specific high lethality of venom on anurans was reported for *Bothrops* species (Andrade et al., 1996). Venoms from neonate *B. asper* specimens are more hemorrhagic and lethal, whereas those of adult specimens are more hemolytic and induce a stronger myonecrotic action (Gutiérrez et al., 1980). In comparison with adult female *B. alternatus* and *B. cotiara*, their offspring have an extremely high procoagulant activity in mice (Furtado et al., 1991). A comparative study of venoms from wild juvenile, sub-adult and adult *B. atrox* specimens from Manaus region, Brazil, shows that human plasma coagulant activity was higher in venoms from juvenile and sub-adult specimens than in adults (López-Lozano et al., 2002).

The higher procoagulant activity in juvenile specimens of *B. atrox* could be explained by a selective pressure of prey availability and prey escape driving juveniles to have a more coagulotoxic venom for reptile and amphibian preys, which have blood coagulation mechanisms with several peculiarities in relation to mammals. Actually, this hypothesis was firstly raised for *B. atrox* taking into consideration a geographic rather than an ontogenetic variation. Thromboelastographic analysis revealed a convergence between speed and productions of strong clots in amphibian plasma for *B. atrox* inhabiting floodplains in Santarém (Pará State, Brazil), which preys heavily upon amphibians. Otherwise, this toxin specialization was not observed from snake venoms collected in drier Amazonian ecotopes, where the snakes are less dependent on anurans and their venoms unable to clot amphibian plasma (Sousa et al., 2018). Unfortunately, there is no information from studies comparing the coagulotoxicity of juvenile and adult venoms in plasma from different prey. Additionally, studies on the coagulotoxic potential of the *B. atrox* venom on the plasma of reptiles are lacking in the literature. For *B. neuwiedi* complex, it is known that distinct coagulotoxins appear to be more specialized to mammals or chicken blood, strengthening the current hypothesis that toxin diversity enhances the possibilities of the snakes for hunting different prey or evading different predators (Bernardoni et al., 2014).

Regarding reptiles, the absence of factor XII and low levels of factors VIII and IX have been reported in some species, slowing down the intrinsic coagulation pathway (Lavras et al., 1979). Some Australian species present poor intrinsic thromboplastin activity, then with a very prominent effect of natural antithrombin compared to mammals (Hackett and Hann, 1967). Slow clotting times were also observed in snakes, iguanas and turtles (Lavras et al., 1979; Nahas et al., 1981; Soslau et al., 2004; Vieira, 2014). In addition to the absence or low levels of some clotting factors, the presence of inhibitors is a well-documented fact in several species of venomous (de Morais et al., 2008; Nahas et al., 1973; Tanaka-Azevedo et al., 2004) and non-venomous reptiles (Arocha-Piñango and Gorzula, 1975; Arocha-Piñango et al., 1982). A few

Fig. 4. Prey of *Bothrops atrox*, according to literature records (Bernarde and Abe, 2010; Bisneto and Kaefer, 2019; Macedo-Bernarde and Bernarde, 2005; Martins and Gordo, 1993; Martins and Oliveira, 1998; Nascimento et al., 2008). A-K pictures represent preys associated to neonate or juvenile specimens of *B. atrox*. A) Centipede climbing a tree trunk; B-D) pictures of anurans (*Leptodactylus fuscus*, *Adenomera andreae*, *Pristimantis fenestratus*, respectively), preys of neonate or juvenile specimens of *B. atrox*; E) A tropical house gecko (*Hemidactylus mabouia*, an exotic species) preyed on by a juvenile *B. atrox*; F and G) Two species of lizards (*Kentropyx pelviceps* and *Ameiva ameiva*, respectively), preys of all ontogenetic stages of *B. atrox*; H–K) *Epictia tenella*, *Leptodeira annulata*, *Tantilla melanocephala* and *Imantodes cenchoa*, respectively, preys of neonate or juvenile *B. atrox*; L) *Taraba major*, a prey rarely reported for *B. atrox*; M-P) Rodents on the forest ground. Taxonomic identification of rodents is rarely permorfed in snake dietary studies. Photos: A = Rafael Marllus Negreiros de Almeida; B – K and M - P = Paulo S. Bernarde; L = Vanderley Pereira dos Santos.

studies carried out in amphibians show that some hemostasis-related genes lack in this group, such as the factor XI in toads (Doolittle, 2009). Other coagulation factors, such as the factor V, are found in lower concentrations compared to mammals (Hackett and Le-Page, 1961). Conversely, fibrinolysis time is longer in frogs compared to several species of mammals (Niewiarowski and Latallo, 1959), possibly by the increased concentration of antifibrinolytic agents in toad plasma (Tentoni et al., 2010).

3. *Bothrops atrox*: the venom

As currently accepted, evolutionary mechanisms for generation of snake venom toxic arsenals include recruitment of genes coding for endogenous digestive enzymes that eventually evolved to toxins (Fry et al., 2009a, 2009b). In agreement, *B. atrox* venom might also been originated from digestive enzymes. During this process, ancestor genes suffered duplications and the copies recruited for venom generation underwent several genetic diversifying mechanisms resulting in neofunctionalization which enabled the new toxic properties to the descendent molecules comparing to the endogenous functions coded by the ancestor genes (Casewell et al., 2013; Fry et al., 2009b; Moura-da-Silva et al., 1996). In some species of rattle snakes, some enzymes, particularly PLA2s, evolved to neurotoxins and those venoms became extremely potent to kill a variety of prey (Boldrini-França et al., 2010). However, in the case of *Bothrops* snakes, particularly *B. atrox*, no neurotoxin has ever been described and the selection of genes coding for venom components apparently occurred when new proteins displayed certain degrees of hemotoxicity, or enhancement of the tissue-damaging effect of the ancestor digestive enzymes. As result of the process, *B. atrox* venom is weakly lethal with LD_{50}s above 100 μg/20g mice in experimental models (Sousa et al., 2017). In human accidents, the lethality rates are also very low, but the venom induces severe coagulopathies and extensive tissue damage. Several components have been isolated from *B. atrox* venom (Table 1) and their structural and functional studies allowed a great understanding on the mechanisms involved in the evolution of this complex phenotype and consequently, to understand the pathophysiology of *B. atrox* human envenomings.

3.1. Components involved in hemostatic disturbances

As many venoms of viper snakes, the lethality of prey by *B. atrox* venom has been greatly attributed to chock due to hemostatic disturbances (Chacón et al., 2015). Moreover, patients bitten by *B. atrox* present unclottable blood accompanied by low levels of fibrinogen and alpha 2-antiplasmin, and high levels of fibrin/fibrinogen degradation products and D-dimers (Pardal et al., 2004; Silva de Oliveira et al., 2019).

Since early studies on biochemical characterization of venom

Table 1
Toxins isolated and characterized from *Bothrops atrox* venom.

Toxin	Geographical origin of the venom	Effect	References
Snake Venom Metalloproteinases (SVMPs)			
Metalloenzyme	Stock of the Pasteur Institute	Activator of Prothrombin	Hofmann and Bon (1987a)
Metalloenzyme	Stock of the Pasteur Institute	Activator of coagulation Factor X	Hofmann and Bon (1987b)
Batroxhragin	Santarém PA (Brazil)	Hemorrhagic Fibrinolytic Inhibition of platelet aggregation	Freitas-de-Sousa et al. (2015)
Atroxlysin III	Alto Marañon (Perú)	Hydrolysis of fibrinogen and ECM proteins Inhibition of platelet aggregation	Oliveira et al. (2019)
HI-5	Butantan Institute (Brazil)	Hemorrhagic (low)	Petretski et al. (2001)
Batx-I	Meta (Colombia)	Hemorrhagic low) Myotoxic (low) Fibrin (ogen) olytic	Patiño et al. (2010)
Atroxlysin I	Ucayali (Perú)	Hemorrhagic Fibrin (ogen) olytic Inhibition of platelet aggregation	Sanchez et al. (2010)
Atroxlysin Ia	Butantan Institute (Brazil)	Hemorrhagic Dermonecrotic Hydrolysis of fibrinogen and ECM proteins Pro-inflammatory	(Almeida et al., 2020; Freitas-de-Sousa et al., 2017)
Batroxase	Pará State (Brazil)	Hemorrhagic Fibrin (ogen) olytic Pro-inflammatory Antithrombotic Activation of Complement system	(Cintra et al., 2012; Jacob-Ferreira et al., 2017; Menaldo et al., 2016, 2017)
Batroxostatin	Sigma (USA)	RGD-disintegrin	Rucinski et al. (1990)
Snake Venom Serine Proteinases (SVSPs)			
Batroxobin	Unknown	Thrombin-like activity Anticoagulant drug – Reptilase Form fibrin films – Plateltex	(Funk et al., 1971; Mazzucco et al., 2008; Stocker and Barlow, 1976)
BA III-4	Lima (Perú)	Thrombin-like activity	Ponce-Soto et al. (2007)
SVSPs	Manaus AM (Brazil) Tucuruí PA (Brazil)	Thrombin-like activity	Cavinato et al. (1998)
Thrombocytin	Pentapharm Laboratories (Switzerland)	Thrombin-like activity (low) Activation of platelet-aggregation Activation of factors XIII and VIII	(Kirby et al., 1979; Niewiarowski et al., 1979)
Phospholipases A_2 (PLA_2s)			
$BaPLA_2$-I	Butantan Institute (Brazil)	Basic – K49 Myotoxic Pro-inflammatory	Kanashiro et al. (2002)
$BaPLA_2$-III	Butantan Institute (Brazil)	Neutral - Enzymatically active Myotoxic Pro-inflammatory	Kanashiro et al. (2002)
B. atrox PLA_2	Peri Mirim MA (Brazil)	Neutral - Enzymatically active	Menaldo et al. (2015)
Mytoxin I	Meta (Colombia)	Basic – K49 Myotoxic Pro-inflammatory Bactericidal	Núñez et al. (2004)
BaTX-I	Porto Velho RO (Brazil)	Basic – K49 Pro-inflammatory	Furtado et al. (2014)
BaTX-II	Porto Velho RO (Brazil)	Basic – D49 Pro-inflammatory	Furtado et al. (2014)
Ba PLA_2	Porto Velho RO (Brazil)	Acidic – D49 Pro-inflammatory	Furtado et al. (2014)
Others			
Galatrox	Sanmaru Serpentarium (Brazil) Butantan Institute (Brazil)	C-type lectin Hemagglutinin Pro-inflammatory	(Mendonça-Franqueiro et al., 2011; Sartim et al., 2014)
B. atrox LAAO	Sanmaru Serpentarium (Brazil)	L-Amino Acid Oxidase Induces apoptosis Cytotoxic to cancer cells	Alves et al. (2008)
B. atrox LAAO	Oswaldo Meneses Serpentarium (Perú)	L-Amino Acid Oxidase Induces apoptosis, necrosis and autophagy	Costal-Oliveira et al. (2019)
Hyal-Ba	Oswaldo Meneses Serpentarium (Perú)	Hyaluronidase activity Enhances venom-induced hemorrhage	Vivas-Ruiz et al. (2019)
BPP-BAX12	Porto Velho RO (Brazil)	Bradikinin Potentiating Peptide	Coutinho-Neto et al. (2013)
Nucleotidase	?	Release of purines Hypotension potentiation	(Aird, 2002; Sulkowski et al., 1963)
Phosphodiesterases	Sigma (USA)	Release of purines Hypotension potentiation	(Aird, 2002; Bjork, 1963; Castrop, 2007; Frischauf and Eckstein, 1973; Philipps, 1976)

components, *B. atrox* venom is known as a rich source of thrombin-like enzymes, classified as Snake Venom Serine Proteinases (SVSPs) which directly hydrolyzes fibrinogen in fibrin. One of the most famous thrombin-like toxin isolated from snake venoms is certainly Batroxobin, a serine proteinase which functions as anti-coagulant by specific cleavage the alpha-chain of fibrinogen which spontaneously converts into

loose fibrin clots. Batroxobin was first described as a proteinase from *Bothrops* snakes (von-Klobusitzky and König, 1939) and its isolation and biochemical characterization was reported later by Stocker and Barlow (1976). Since then, several therapeutic applications have been attributed to Batroxobin, which is one of the widely used therapeutic agents derived from snake venoms for distinct applications. Reptilase® is used both as anticoagulant drug and as a diagnosis reagent for the detection of fibrinogen deficiency or abnormalities (Funk et al., 1971). Plateltex® found application in treating chronic wounds to allow the formation of platelet rich fibrin films, and has various surgical and medical uses (Mazzucco et al., 2008). Homologous serine proteinases from other snake venoms have also encountered therapeutic uses as Hemocoagulase Agkistrodon (HCA) or Ancrod (Viprinex®), which are following the same road as Batroxobin for human therapeutic uses (Waheed et al., 2017). Other coagulotoxic serine proteinase isoforms have been isolated from *B. atrox*. Ponce-Soto et al. (2007) isolated the BA III-4 SVSP from the venom of snakes originally from Peru. BA III-4 also showed thrombin-like activity but only 72% sequence identity to Batroxobin, evidencing the variability of this toxin group in *B. atrox* venom. Structural and functional variability was also reported for two thrombin-like SVSPs isolated from Manaus and Tucuruí (Brazilian Amazon) venoms, which shared only 80% identity with Batroxobin N-terminal and differences in physicochemical and enzymatic activities, highlighting the geographical variation of venom composition (Cavinato et al., 1998; Petretski et al., 2000).

Adding to thrombin-like enzymes, disturbances in hemostasis are also evoked by some toxins included in the Snake Venom Metalloproteinase group (SVMPs). The pro-coagulant mechanism of this group of toxins is the direct activation of factors II and X, resulting in the formation of endogenous thrombin (Hofmann and Bon, 1987a, b). It was also reported the activation of factors XIII and VIII by Thrombocytin, a SVSP isolated from *B. atrox* venom (Niewiarowski et al., 1979). Unfortunately, we found no information about the primary structure of those activators that would allow structure-function inferences and comparisons to more recent data.

B. atrox venom components also interfere on platelet-aggregation. The SVSP Thrombocytin induces platelet-aggregation and secretion of its mediators (Niewiarowski et al., 1975). On the other hand, SVMP inhibitors of platelet-aggregation in *B. atrox* venom include Batroxostatin, an RGD-disintegrin targeting the integrin $\alpha_{2b}\beta_3$, the platelet fibrinogen receptor (Rucinski et al., 1990), and the PIII-class SVMPs Batroxrhagin, that inhibits platelet-aggregation targeting the collagen receptor integrin $\alpha_2\beta_1$ (Freitas-de-Sousa et al., 2015), and Atroxlysin III that Induces shedding of glycoprotein VI and impairs platelet function (Oliveira et al., 2019). However, the effects of these components are not apparently relevant for the pathophysiology of the envenomings. *B. atrox* crude venom does not induce aggregation of washed platelets from rabbits, and presented a low inhibitory effect (Francischetti et al., 1998). Moreover, only 8–12% of patients bitten by *B. atrox* show thrombocytopenia (Oliveira et al., 2019b; Pardal et al., 2004).

B. atrox venom hemorrhagic toxins are well characterized and are involved in the local and systemic effects of human victims of snakebite (Oliveira et al., 2017) and it is very likely that they contribute to prey killing. Hemorrhage is primarily attributed to PIII-class SVMPs that induce hemorrhagic lesions in the dorsal skin of mice in low doses. In this group, Batroxrhagin is the most abundant hemorrhagin in venoms of *B. atrox* specimens from Brazilian Amazon (Amazonas et al., 2018). This toxin was isolated and characterized in our lab as a PIII-class SVMP with hemorrhagic doses of approximately 2 μg/mice, and exhibited very similar biological properties as Jararhagin, the archetype of PIII-class hemorrhagin from *B. jararaca* venom (Freitas-de-Sousa et al., 2015). Recently, another isoform of PIII-class SVMPs, Atroxlysin-III, was isolated from venoms of Peruvian *B. atrox*. Atroxlysin-III is homologous to Jararhagin and Batroxrhagin displaying great structural and functional similarity (Oliveira et al., 2019). In the venom of *B. atrox*, PI-class SVMPs also play important roles in venom-induced hemorrhage. At least four distinct PI-SVMPs have been isolated: HI-5 (Petretski et al., 2001), Atroxlysin –I (Sanchez et al., 2010), BATXI (Patiño et al., 2010) and Batroxase (Cintra et al., 2012) are able to hydrolyze fibrinogen and fibrin and induce hemorrhage in mice in doses from 10 to 50 μg. Sequence information is available only for Atroxlysin-I and Batroxase revealing that they are distinct isoforms sharing 89% identity. Interestingly, we have recently isolated Atroxlysin-Ia in our lab, from venoms of specimens originally from the Brazilian Amazon. Atroxlysin-Ia sequence showed only one amino acid substitution M135K was found comparing to Atroxlysin-I sequence, which was previously isolated from Peruvian venoms. However, the hemorrhagic activity of Atroxlysin-Ia was higher, in levels comparable to PIII-class SVMPs, and this toxin induced a fast onset dermonecrosis in the dorsum of mice, in doses from 10 to 20 μg, not observed previously with isolated SVMPs from viper venoms (Freitas-de-Sousa et al., 2017). The different degrees of hemorrhagic activity of SVMPs have been attributed to their hydrolytic activity of extracellular matrix components such as laminin, type IV collagen and fibronectin (Cintra et al., 2012; Freitas-de-Sousa et al., 2017; Jacob-Ferreira et al., 2017) and also to the fact that PIII-class SVMPs bind to collagens present on basal membranes allowing the concentration of the enzyme adjacent to the capillaries and venules, making more productive the hydrolysis and rupture of the vessel (Baldo et al., 2010). Moreover, SVMPs are highly glycosylated and it has already been reported the relevance of sugar moieties for the onset of hemorrhagic activities (Andrade-Silva et al., 2016; Oliveira et al., 2010). Thus, differences in glycosylation patterns could also respond for differences in functional activities of SVMPs, and explain the functional differences observed between Atroxlysin-I and Atroxlysin-Ia.

3.2. Tissue-damaging components

Local tissue damage is one of the most important effects in human victims of snake bites. Intravascular coagulation, rupture of blood capillary vessels and digestion of ECM proteins described above greatly contribute to impair the correct oxygen flow to tissues adjacent to the bite, favoring the development of the severe tissue-damaged pictures frequently observed in snakebite patients with delays to reach the hospital. Together with pro-coagulant and hemorrhagic enzymes, *B. atrox* venom Phospholipases A_2 (PLA_2s) also contribute to local tissue damage due to their myotoxic activity, provoking lysis of muscle cells and release of intracellular proteins that also enhance the reactive pro-inflammatory status of the tissues close to the bite. $BaPLA_2I$, $BaPLA_2III$ are toxins from PLA_2 group isolated from *B. atrox* venom (Kanashiro et al., 2002). $BaPLA_2I$ is a basic K49, enzymatically inactive isoform, and $BaPLA_2III$ is catalytically active, both capable of inducing myonecrosis. Myotoxin-I is also a myotoxic K49 homologue isolated from Colombian venom (Núñez et al., 2004), and there is still a neutral PLA_2 isolated from venoms from the eastern Amazon that although displaying catalytic activity, is not myotoxic (Menaldo et al., 2015). Not sufficiently, *B. atrox* venom contains toxins that directly induce inflammatory effects, leading to plasma exudation and accumulation of polymorphonuclear and mononuclear leukocytes to the injury site (Almeida et al., 2020; Barros et al., 1998; Magalhães et al., 2011; Moreira et al., 2012). Furtado and collaborators (2014) described the isolation of three PLA_2s from venom of *B. atrox* snakes collected around the city of Porto Velho, State of Rondônia, Brazil. BaTX-I, a basic catalytically inactive K49 variant, BaTX-II, a basic catalytically active D49, and $BaPLA_2$, an acidic catalytically active D49. The action of these toxins on J774A.1 macrophages was evaluated in vitro showing that, in spite of differences in enzymatic activity or isoelectric point, the three toxins induced inflammatory events on this cell line. SVMPs, such as Atroxlysin-Ia and Batroxrhagin are capable of inducing edema and leukocyte accumulation at the injury site, as well as to generate fragments by hydrolysis of basement membrane components that amplify the direct action of SVMPs through activation of endogenous signaling pathways (Almeida et al., 2020). Also, C-type Lectins (CTLs) as Galatrox (Mendonça-Franqueiro et al.,

2011) add to *B. atrox* venom pro-inflammatory effect by binding to leukocytes expressing glycans containing N-acetyllactosamine and inducing their activation (Sartim et al., 2014). The establishment of inflammatory effects induced by the venom or isolated components is accompanied by the release of the eicosanoids derived from COX-1 and COX-2, chemokine, and cytokines (Almeida et al., 2020; Barros et al., 1998; Magalhães et al., 2011; Moreira et al., 2012). In human victims, serum levels of CXCL-9, CXCL-10, IL-6, and IL-10 immunological soluble molecules were increased compared to healthy controls; the concentration of chemokines (CXCL-8, CXCL-9, and CCL-2), anaphylatoxins (C3a, C4a, and C5a), and cytokines (IL6, TNF, and IL-10) were increased in the blister compared with circulating serum profile (Ibiapina et al., 2019). Interestingly, the same study has shown that patients presenting severe envenoming, with systemic manifestations and local signs, such as ecchymosis, severe edema, blister formation and necrosis at the bite site, presented a more polarized profile for Th1 response. Severe patients presented also a more intense local immune response, with a Th1/Th2/Th17 uncoordinated response (Ibiapina et al., 2019). Fig. 5 shows a proposal for local tissue damage in individuals of *B. atrox* snakebites.

3.3. Complementary toxins or digestive proteins?

Other components have been isolated from *B. atrox* venom but, interestingly, although present in reasonable quantities on snake venoms, their toxic activities are not very well understood or limited to marginal effects on venom-induced lesions. In this group, we include nucleotidases (NUC), phosphodiesterases (PDE) and L-amino acid oxidases (LAAO) and hyaluronidases (HYAL) previously isolated from *B. atrox* venom.

Nucleotidases (Sulkowski et al., 1963) and phosphodiesterases (Bjork, 1963; Frischauf and Eckstein, 1973; Philipps, 1976) were isolated from *B. atrox* venom in a period when big efforts were given in the search of enzymes to act as tools to study the structure of nucleic acids. However, the results obtained were very faint concerning their role in the venom toxicity. In a recent review Dhananjaya and D'Souza (2011) discuss that the role of these enzymes in venom toxicity is related to endogenous release of purines, which could act potentiating several venom-induced processes as hypotension, renal failure and cardiac arrest (Aird, 2002; Castrop, 2007). Recent findings have also proposed

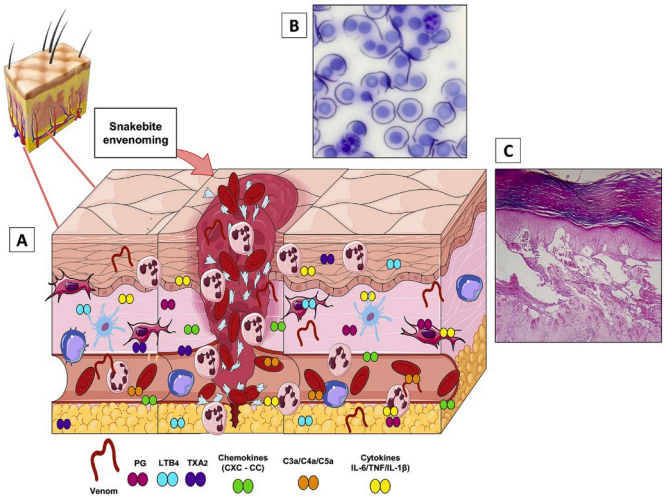

Fig. 5. Local tissue damage in *B. atrox* envenoming. A) schematic presentation of the intense inflammatory process in local of *B. atrox* snakebites. B) presence of polymorphonuclear cells with phagocytic action and cytoplasmic vacuoles in blister exudates. C) picture demonstrating intense cell migration and detachment of the upper layer of the skin in sample biopsy after blister formation, resulting from the inflammatory process in *B. atrox* envenoming.

that venom DNases could modulate local toxicity, preventing toxins accumulation in the site of injection by disrupting neutrophil extracellular traps (Katkar et al., 2016). Moreover, snake venom PDE has been reported to inhibit ADP-induced platelet aggregation (Uzair et al., 2018). However, their function in snake venoms are still under evaluation.

Another intriguing component isolated from *B. atrox* venom is the L-amino acid oxidase (LAAO). LAAOs are abundant on venoms from different *Bothrops* species. *B. atrox* LAAO was isolated (Alves et al., 2008) and the purified enzyme induced caspase-mediated apoptosis, necrosis and autophagy (Costal-Oliveira et al., 2019), suggesting its contribution to the tissue-damage induced by *B. atrox* venom. Further, BatroxLAAO also induced platelet aggregation (Alves et al., 2008). A Hyaluronidase, Hyal-Ba, was also isolated and characterized from *B. atrox* venom from Peruvian specimens (Vivas-Ruiz et al., 2019). The toxin did not induce detectable local effects as edema or hemorrhage in mice, but enhanced the hemorrhagic activity of the whole venom and reduced the edema induced by a LAAO isolated from the same venom.

Bradykinin-potentiating peptides (BPPs) are also abundant in venoms of viper snakes. Among these peptides, BPP-BAX12 was isolated from the venom of snakes collected at Brazilian Amazon. Peptide sequence was ZKWPRPGPEIPP, homologous to other BPP from Bothrops moojeni venom, suggesting similar biological activity (Coutinho-Neto et al., 2013).

3.4. Overall composition of B. atrox venom

A great advance in understanding the overall venom composition came from the establishment of proteomics methodology on the 1990s. Proteomics was introduced in venom research at early 2000s in a series of different reports. Fry et al. (2002), reported a characterization of *Acanthophis* venoms (dead adders) using LC/MS fingerprinting. More detailed data was presented by Serrano et al. (2005) who submitted venom spots separated by 2-dimension electrophoesis to LC/MS/MS technology for the visualization of venom proteomes, demonstrating the great diversity of snake venom components. Venom proteomics evolved to an association of mass spectrometry with distinct methods of previous fractionation of venom components as RP-HPLC and SDS-PAGE, and complementary characterization of N-terminal sequences of proteins contained in the separated bands (Juárez et al., 2004). This platform currently associated as 'venomics' has been used for the characterization of composition of venoms from different taxa, allowing the interpretation of relationships between venom composition with snake ecology, evolution of clinics of snakebites (Calvete, 2017).

The early proteomics methods were powerful for the annotation of a number of proteins present on the venom mixtures, but the quantitative estimations of the abundance of each protein family was still uncertain. To overcome this problem, the Venomics platform estimates the quantification of each protein group by the densitometry of their corresponding bands stained after separation by SDS-PAGE (Calvete, 2017). However, this approach is time-consuming and might present some limitations underestimating the presence of some venom proteins, as CTLs, that are poorly detected by conventional SDS-PAGE dyes. Nevertheless, venomics is still a powerfull platform even for CTLs since analysis under non-reduced and reduced conditions represents a convenient strategy to identify and distinguish them from other components of similar native molecular mass, such as SVSPs or CRISPs (Eichberg et al., 2015). Other possibilities are used for protein quantification in mass spectrometry. Isotope-based methods are used for the comparative analysis of differential isotope-tagged proteomes (Elliott et al., 2009); however, the method is of little applicability for quantification of multiple proteins present in complex mixtures as snake venoms.

The other possibility that has been used as estimative of protein abundance is the use of label-free methods in which spectral counting of proteolytically derived peptides represent a measure of the presence of

the parental protein in a complex mixture (Navarro et al., 2016). However, this strategy was developed for label-free quantification of proteomes of model organisms for which comprehensive genomic or transcriptomic databases are available, and thus matching the MS/MS spectra does not represent a limiting factor (Calvete, 2018). Label-free methods also have disadvantages as differences in the ionization efficiency and/or detectability of the many peptides in a given sample may interfere in the spectral counting of different toxins. However, when associated to high resolution mass-spectrometry platforms, allows shotgun procedures that may be executed with multiple comparative runs of individual venom samples (Amazonas et al., 2018). New possibilities using high resolution mass spectrometry or top-down Venomics approaches are under crescent development and may be soon applied in venom research as a solution for a more precise protein quantification and proteoforms characterization (Calvete, 2018).

Proteomics has been extensively used to understand *B. atrox* venom composition and its correlation to snake ecology, evolution and clinics of the accident in the Amazonian rainforest. Using 2-DE and mass spectrometry, Guércio et al. (2006) described for the first time the proteome of *B. atrox* venom from specimens of different ontogenetic stages collected at Manaus, Brazilian Amazon. The authors suggested that venom proteome alters upon ontogenetic development and P-III class SVMPS and SVSPs are more abundant in juvenile specimens, while PI-class SVMPs are more expressed in venoms from adults. Venomics platform was then applied to characterize proteomes of *B. atrox* venom from Colombia, Brazil, Ecuador, and Peru (Núñez et al., 2009). The authors estimated the relative abundance of different components of *B.* atrox and evidenced the existence of two geographically differentiated venom phenotypes. The venom from Colombia comprised about 26 different proteins belonging to 9 different groups of toxins in which PI-class SVMPs and PLA2 molecules represent the most abundant toxins. On the other hand, the venoms from Brazilian, Ecuadorian, and Peruvian *B. atrox* specimens contain predominantly PIII-metalloproteinases (Núñez et al., 2009). Following this study, Calvete et al. (2011) analyzed *B. atrox* venom from specimens collected in 16 different areas along the Amazon river basin, from the Colombian Magdalena Medio Valley to São Bento in the Brazilian State of Maranhão. With these samples, it was possible to elucidate some important parameters related to ontogenetic and geographical variability of *B. atrox* venom. Colombian and Venezuelan venoms showed two ontogenetic phenotypes with predominance of PIII-class SVMPs in venoms of juveniles and of PI-class SVMPs and PLA2s in venoms from adult snakes whereas Brazilian venoms exhibit only the juvenile phenotype with predominance of PIII-class SVMPs in venoms of juvenile and adult snakes of all sampled regions, indicating a paedomorphic phenotype with conservation though the species dispersion along the Amazon Basin. The juvenile phenotype with predominance of PIII-class SVMPs was confirmed in Peruvian and Brazilian populations of *B. atrox* snakes by different groups and methodologies. Kohlhoff et al. (2012) analyzed by venomics *B. atrox* venom from Peruvian snakes and detected 20 to 25 proteins generating a venom composition picture similar to the previously described for Brazilian snakes, with predominance of PIII-class SVMPs. Sousa et al. (2013) compared the composition of *B. atrox* venom with venoms from other species of *Bothrops* snakes by the shotgun approach, and observed the juvenile phenotype in venoms of *B. atrox* similarly to *B. jararaca, B. alternatus, B. cotiara* and B. *neuwiedi*, with predominance of PIII-class SVMPs. The most distinct venom was of *B. jararacussu* snakes, with a phenotype consisted predominantly of PLA2s and SVSPs. Interestingly, within the group analyzed, *B. jararacussu* is the closest species to *B. atrox* by different systematic distributions (Carrasco et al., 2012; Fenwick et al., 2009; Wüster et al., 2002) suggesting that the phylogenetic position has little effects on the abundance of each protein group on venom composition (Sousa et al., 2013).

One bottleneck regarding proteomic characterization is the size and specificity of databanks used for annotation of MS peptides. Databanks are based on sequences of isolated toxins and by sequences derived from venom glands transcriptomes. In 2009, Neiva et al. (2009) reported the first transcriptome of *B. atrox* venom glands using specimens from Manaus. The results were based on 211 expressed sequence tags (ESTs), organized in 26 clusters with a high content of SVMPs, but unfortunately, lacking full-length sequences. With the advances of New Generation Sequencing (NGS), venom gland transcriptomes became more comprehensive (Brahma et al., 2015; Durban et al., 2011; Rokyta et al., 2011; Valente et al., 2009), and consequently the number and length of sequences available in the databanks improved considerably. Using the Illumina® methodology, we have recently characterized the individual transcriptomes of venom glands from five specimens of *B. atrox* from the left and write margins of the Amazon River, at the Western region of Para State, Brazil (Amazonas et al., 2018). We annotated 152 full length sequenced isoforms of toxins present in this venom. Using the set of sequences transcribed, we were able to identify the expression level of each different isoform in the venom and following this approach, new mechanisms underlying venom variability were suggested. The abundance of each toxin family was conserved in all specimens, both in transcripts and in venom protein levels, with predominance of PIII-class SVMPs. However, when expression of independent paralogues was analyzed, remarkable differences were observed between individuals and between populations. Other important evidence was that some transcripts coding for functionally essential venom proteins ("keystone" toxins) are highly expressed in all specimens while other paralogues show lower or variable expression levels and the toxins they code for may be related to the adaptive capacity of the phenotype due to environmental changes.

The analysis of proteomes using the species transcriptome as databank is therefore a powerful tool to understand venom variability at the isoform level and may help to understand the role of certain phenotypes in defining adaptive variation or even in defining different clinical pictures of human envenomings due to snake venom phenotypic variability. Indeed, the *B. atrox* transcriptome was used as database to identify and quantify the isoform exclusive spectra in venoms of snakes from 4 different environments of the Amazonian rainforest (Sousa et al., 2017). Venoms from forest, pasture or recently degraded area showed a similar venom composition concerning protein families. However, venom from snakes collected at the floodplain presented a higher expression of certain SVSP, SVMP and CTL isoforms that conferred a higher procoagulant activity on those venoms (Sousa et al., 2017), which could represent an adaptive advantage for snakes that live in the most variable seasonal differences in their environment with flood and dry long periods.

Analyzing proteomes at isoform levels is also very useful to understand eventual differences in the clinics of snakebites. In a recent study we attempted to correlate the individual venom proteome of the snake brought to the hospital by the patient seeking for medical assistance with the clinical signs observed in the same patient. The abundance of each protein family was quite similar among the venom samples, while the isoforms composition was highly variable. Some isoforms presented expression levels with statistically significant positive correlation to symptoms presented by the patients confirming previous assumptions that venom composition modulates human symptoms of snakebites (Moura-da-Silva et al., submitted). Thus, proteomics approaches are invaluable at the clinics, to indicate possible toxin candidates for the development of toxin inhibitors or to improve antivenom selectiveness, important actions for the next generation treatments of snakebites.

4. *Bothrops atrox:* human envenomings

4.1. Epidemiological aspects

B. atrox is certainly the South American pit viper responsible for most of the snakebites in the Amazon region. In Amazonas State, in Brazil, the mean incidence rate in is 52.8 cases per 100,000 person/year, reaching more than 150 cases per 100,000 person/year in some municipalities

(Feitosa et al., 2015b). *Bothrops* envenomings in the Amazon region is more incident in lowlands, with high preserved original vegetation cover and higher rainfall indexes; such landscape characteristics possibly lead to higher prey availability and further abundance of pit vipers (Alcântara et al., 2018). Underreporting to official surveillance systems certainly occurs, because reaching health centers is difficult for riverine and indigenous populations as a result of distance and low availability of transport means (Fan and Monteiro, 2018). Men aged between 16 and 50 years are the most affected group, and around a half of the cases are related to occupational activities (Magalhães et al., 2019). Harvesting of palm fruits and other forest products is a conducive activity linked to a higher risk for *B. atrox* snakebites; palm trees may attract rodents, which feed on the fruits that fall to the ground or on the tops of trees, serving as a food source for both adult and juvenile snakes, respectively (Mota-da-Silva et al., 2019). Snakebites are also associated to forestry and agriculture, especially because the lack of preventive measures, such as wearing protective footwear or protection leggings by rural workers (Feitosa et al., 2015a). This results in 85–90% of the bites occurring in the lower limbs (Feitosa et al., 2015b). Reports of urban cases are less frequent, but also occur because pit vipers are able to occupy a wide environmental gradient and maintain populations in forest fragments within very anthropized areas encrusted in sylvatic environments, as observed for *B. atrox* in the Amazon (Bernarde, 2014). Many urban environments are home to rodents, marsupials, frogs, geckos and other synanthropic animals serve as food sources. Forestry, hunting and recreational activities, expect to be risk factors for snakebites, are common in population living along urban fringes.

Bothrops bites are mostly caused by small-sized specimens, probably due to the greater abundance of *B. atrox* juveniles, and also because small snakes are more difficult for people to see (Bernal et al., 2019; Mota-da-Silva et al., 2019a). Out of 247 *B. atrox* individuals confirmed as agents of envenomation in Manaus, sex was identified in 193, being 57 males (29.5%) and 136 females (70.5%). Most of the perpetrating snakes were classified as juveniles (62.7%). Considering males, 68.5% of the snakes were adults, while females were mostly juveniles (57.9%) (Bernal et al., 2019).

In the Alto Juruá region, Western Brazilian Amazon, half of the patients performed some kind of ineffective first aid (not drinking water, use of tourniquet, incision at the site of the bite, use of traditional medicines) and also delaying antivenom therapy (Mota-da-Silva et al., 2019a). Regarding time elapsed from the bite until medical assistance, more than 30% of the patients were assisted with more than 6 h after bite (Feitosa et al., 2015b).

4.2. Clinical aspects

B. atrox envenomings cause local and, in a significant proportion, systemic manifestations, depending on the stage of the perpetrating snake, characteristics of the victim and circumstances of the injury. Importantly, clinical examination should be initiated by assessing the affected region to search for bite signs, especially fang marks that can be double or single when only one fang is introduced. *Bothrops* snakebite may result in negligible or no envenoming, even if fang marks are visible, the called 'dry-bites'.

Local envenoming ranges from a painless reddened injury to intense pain and swelling at the site of bite, starting minutes after the event (Otero et al., 1996; Pardal et al., 2004). Bleeding caused by traumatic injury due to fang introduction is observed in almost a half of the patients (Silva de Oliveira et al., 2019). Ecchymosis around fang punctures is observed in ~20% of the patients (Silva de Oliveira et al., 2019). Local manifestations may increase progressively and may affect the whole limb. Enlargement of the regional lymph nodes draining the site of bite and bruising can also be observed some hours after bite, in one third of the cases (Otero et al., 1996; Pardal et al., 2004). Blistering may appear within the first 24 h and tissue necrosis becomes evident within 1 day of the bite (Otero et al., 1996; Pardal et al., 2004; Sachett et al., 2017; Silva de Oliveira et al., 2019). Blisters may be observed around or even far from the bite site in the affected limb; blisters can contain a clear exudate, a serosanguinolent or a fully bloody content. Blisters of purulent content may be also observed in secondary infections. Cellulitis or abscess occurs mostly in the moderate or severe cases, generally as a polymicrobial infection. Gram-negative bacteria have been implicated in secondary bacterial infection, whose frequency may vary according to region. In Manaus, secondary bacterial infections were observed in around 40% of the *Bothrops* snakebites, caused mostly by *Morganella morganii* (Sachett et al., 2017). Necrosis of variable extension is more frequent when tourniquet is used, associated with initial treatment with traditional medicines, and delayed hospital admission. Although uncommon, compartment syndrome is a dangerous complication because of the potential ischemia, tissue necrosis and neuropathy (Pardal et al., 2004). Fig. 6 shows pictures of local signs and complications from *B. atrox* envenomings. A large case series of *B. atrox* snakebites confirmed that envenomings inflicted by adult snakes cause more severe local inflammatory effects, whereas venom-induced coagulopathy is more frequent in envenomings caused by juvenile specimens (Bernal et al., 2019).

Signs and symptoms of systemic envenoming result mainly from uncoagulable blood. Hemorrhage from venipunctures, other sites of trauma or healed wounds, gingival bleeding, hemoptysis, macrohematuria, and hematemesis are observed in 14–20% of *Bothrops* snakes (Pardal et al., 2004). In Manaus, unclottable blood was observed in 54% of the patients, systemic bleeding in 14% and thrombocytopenia in 10% upon hospital admission (Silva de Oliveira et al., 2019); gingival bleeding was the most frequent form of systemic bleeding, followed by macrohematuria and ecchymosis. Laboratorial investigation also showed low levels of fibrinogen and alpha 2-antiplasmin, and high levels of fibrin/fibrinogen degradation product (FDP) and D-dimers in these patients. Unclottable blood and thrombocytopenia on admission were independently associated with systemic bleeding during hospitalization (Oliveira et al., 2019b). Negative correlation was found between the number of platelets and mean platelet volume upon hospital admission (Oliveira et al., 2019b). Interestingly, the frequency of thrombocytopenia increased in the first 24 h after antivenom therapy, and decreased on hospital discharge. Ecchymosis distant from the bite site, generally in the proximal region of the bitten limb, may be observed even after antivenom therapy and recovery from hemostatic disorders such as hypofibrinogenemia and thrombocytopenia (Silva de Oliveira et al., 2019). Lethal cases associated to systemic bleeding are seen if the central nervous system (CNS) is affected causing hemorrhagic stroke (Machado et al., 2010; Oliveira et al., 2017; Pérez-Gómez et al., 2019). CNS bleeding can occur in *B. atrox* snakebites even with clottable blood and normal platelet counts (Oliveira et al., 2017, 2019b; Pérez-Gómez et al., 2019). Likewise, microhematuria was present in 41.5% of *B. atrox*-envenomed patients, being recorded even with normal values of fibrinogen (de Brito Sousa et al., 2019). Fig. 7 shows pictures of hemorrhagic manifestations from *B. atrox* envenomings.

An important systemic complication of *Bothrops* snakebites is acute kidney injury (AKI), which has a great impact on morbidity and mortality. Oliguria or anuria may develop within the first 24 h of the bite. If patient is not treated, blood pressure rises within a few days of the onset of oliguria and signs of uremia (drowsiness, irritability, vomiting, hiccups, convulsions) develop within 3–7 days after bite. AKI was observed in 12.9% of the patients in Manaus (Alves et al., 2018) and in 20.5% of the cases in Colombia (Otero et al., 1996).

A lethality rate of 0.6% is observed for snakebites in the Brazilian Amazon, which higher than in other regions of the country (Feitosa et al., 2015b). This rate is higher in other Amazonian countries, especially if antivenom is unavailable (Chippaux, 2017). The most frequent systemic complications associated to death were respiratory failure/acute lung edema (37.0%), acute renal failure (29.1%), sepsis (24.4%), circulatory shock (21.3%) and systemic bleeding (15.0%) (da Silva Souza et al., 2018). Older age (≥65 years) and time to medical

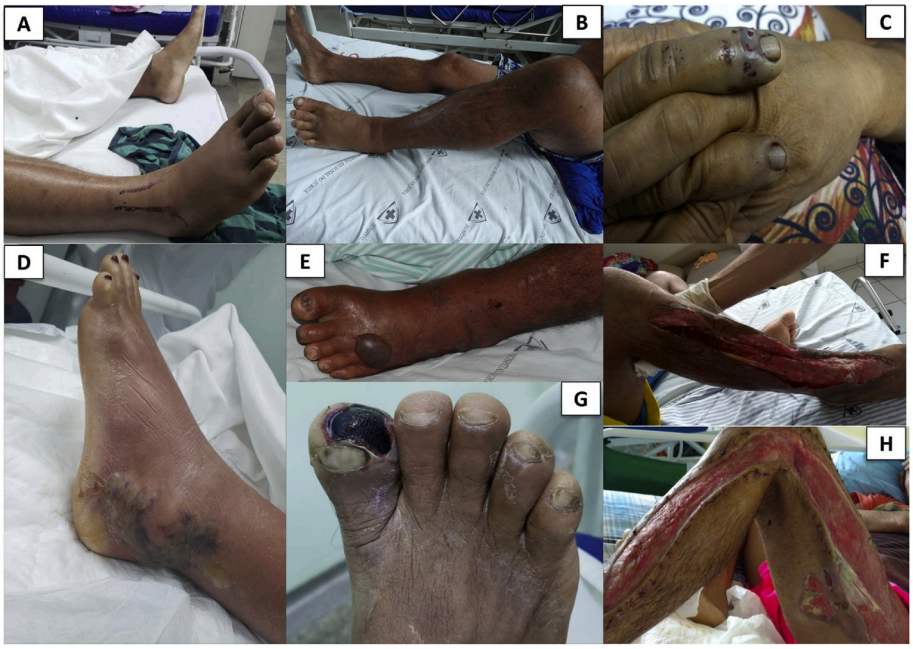

Fig. 6. Local manifestations from *Bothrops atrox* envenomings. A and B) Extensive edema in lower limb of two male patients; picture A also shows local bleeding. C) Edema, secondary infection and necrosis in fingers of the right hand of a female patient. D) Edema, secondary infection and an extensive necrotic plaque in the external side of the left foot of a female patient. E) Extensive edema, ecchymosis and blister with bloody content in the left foot of a male patient. F) An impressive necrotic area, with tissue exposure, in the right lower limb of a male patient. G) A necrotic plaque in the toe of the right foot of a male patient. H) Compartment syndrome across the left lower limb extension, post-fasciotomy. Photos: A, C, D, G and H = Lisele Brasileiro; B, E and F = Ageane Mota da Silva.

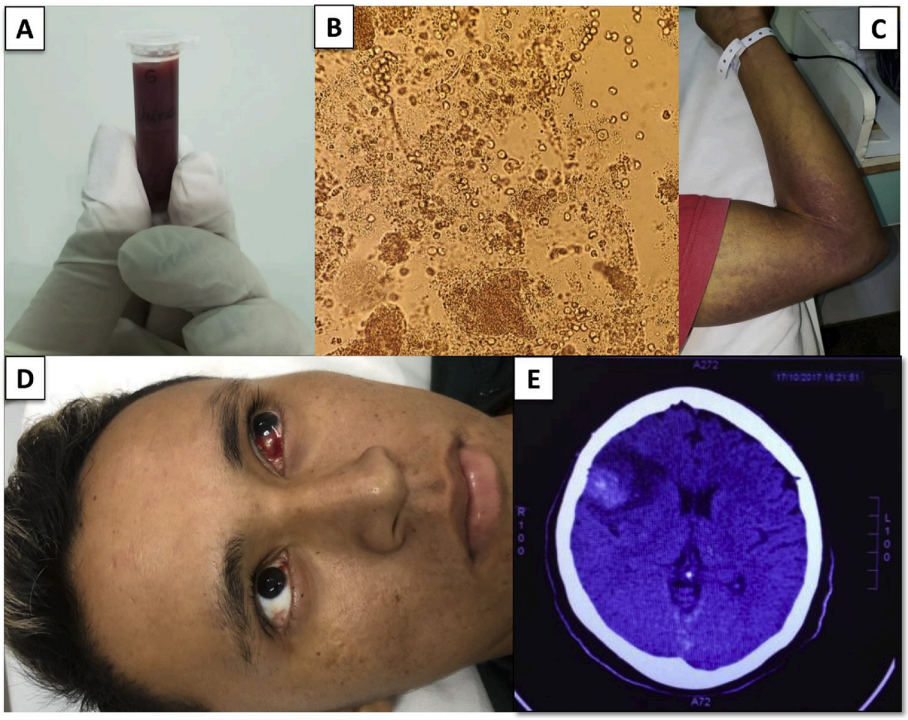

Fig. 7. Hemorrhagic manifestations from *Bothrops atrox* envenomings. A) macroscopic hematuria; B) presence of erythrocytes in urine, observed by optic microscopy; C) conjunctival bleeding; D) ecchymosis in the whole upper limb in a patient bitten in the hand; E) brain presenting hypodense lesion in the right frontal lobe and edema in cortical-subcortical, and perilesional areas on a CT scan. Photos: A, B, C and D = Lisele Brasileiro; E = Aline Pérez-Gómes.

assistance >6 h were factors independently associated with the risk of death (Feitosa et al., 2015b). In other study, distance from the reference center >300 km, Indigenous status and a lack of antivenom therapy were also independently associated with case fatality (da Silva Souza et al., 2018).

Snakebite complications may leave great physical and motor sequelae in those patients who manage to survive. These may become chronic, leading to great disability and are poorly described for *B. atrox* envenomings. Scarring, muscular atrophy and amputation affecting mobility are potential chronic outcomes (Fig. 8). The persistence of sequelae greatly impacts on quality of life and may leave life-lasting disabilities, especially in children and agricultural workers, as rehabilitation is rarely available to snakebite victims in such regions.

4.3. Antivenom therapy

Currently, antivenoms are the only specific treatment for snakebites envenomings. There are several antivenoms produced in Latin America

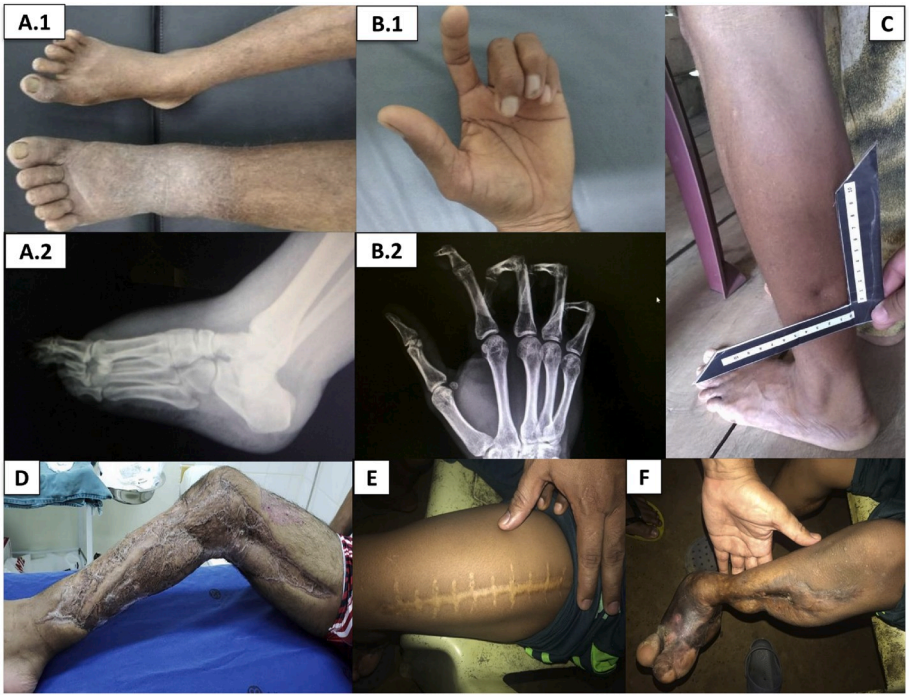

Fig. 8. Sequelae from *Bothrops atrox* envenomings. A) Chronic lymphedema in an elder male patient, after 5 years of envenomation, with extensive edema and skin thickening accompanied by pain, parestesia and itching in the left lower limb (A.1); A.2 shows a radiological image of the affected limb. B) Left hand presenting claw-like positioning of fingers with permanent atrophy and stiffness of the 1st, 2nd and 3rd fingers after 3 years of envenomation; physical examination reveals atrophy, paresthesia and immobility (B.1); hand radiography showing permanent structural alterations (B.2). C) young female presenting a reddish skin and scaring after several years of envenomation at the bitten site; D) leg aspect after 4 months of extensive skin grafting secondary to compartmental syndrome in and young adult – patient presented with extensive necrotic areas after delayed access to proper health care; E) young male presenting scarring secondary to surgical incision as management of compartment syndrome secondary to snakebite F) leg aspect after 3 years of snakebite in the left leg – delayed access to proper care lead to extensive muscular, nerve and skin tissue excision, which resulted in atrophy as patient got older; leg is not functional and patient currently waits for amputation surgery. Photos: A and B = Iran Mendonça da Silva; C = Guilherme Salazar; D = Fernando Val and Jacqueline Sachett; E and F = Altair Seabra de Farias.

which are efficient for the treatment of human accidents with *Bothrops* snakes. There are antivenom-manufacturing laboratories in Mexico, Costa Rica, Venezuela, Colombia, Peru, Bolivia, Brazil and Argentina, most are public organizations providing products for its own national demands. The antivenoms commercially available are the complete molecule or F (ab')2 fragments of immunoglobulins purified from the plasma of horses hyperimmunized with different mixtures of snake venoms. These venoms are usually selected based on the geographical distribution of the medically important snakes while studies on immunogenicity (Anderson et al., 1993). In the 1980s, a study based on cross-neutralization between specific *Bothrops* venoms from 10 different species and antivenoms guided the formulation of immunization pools in Brazil (da-Silva et al., 1989), which excluded venom from *B. atrox* snake, the main cause of snakebites in the Amazon. This issue is mitigated by the great similarity in the composition of *B. atrox* venom with venoms of other species of *Bothrops* included in the immunization pool as *B. jararaca* and *B. alternatus* (Sousa et al., 2013) and also by the phylogenetic relatedness between *B. atrox* and *B. moojeni* (Carrasco et al., 2012), also included in the immunization protocol. Indeed, clinical trials have been conducted with antivenoms produced in Brazil, Colombia, Ecuador and Costa Rica that confirmed the efficacy of such antivenoms for the treatment of *B. atrox* bitten patients (Otero et al., 1996; Pardal et al., 2004; Smalligan et al., 2004). Interestingly, two of these trials compared antivenoms obtained using the standard immunization protocols with antivenoms produced including *B. atrox* venom in the immunization protocol. Otero et al. (1996) included in the study 39 patients envenomed by *B.* atrox in Antioquia and Chocó, Colombia, to compare the efficacy and safety of standard and experimental antivenoms prepared at Instituto Clodomiro Picado and reported that both antivenoms were equally efficient in the neutralization of the most relevant signs of envenoming (hemorrhage and blood clotting time alteration). In Brazil, Pardal et al. (2004) compared the efficacy of the commercial *Bothrops*-Lachesis antivenom with a *B. atrox-Lachesis* antivenom experimentally produced to treat 74 patients attended at Belém, at Pará State of Brazilian Amazon. The results once more indicated that both antivenoms were equally effective in reversing all signs of envenoming detected both clinically and in the laboratory. However, there are still concerns if the interspecific and intraspecific venom variation associated with the ontogeny or geographical distribution of snakes may affect the effectiveness of therapeutic antivenoms against the Amazon *Bothrops* venom (Calvete et al., 2011; Guércio et al., 2006).

Currently, *B. atrox* venom is included in the mixture of venoms prepared for immunization in *Bothrops* antivenom from *Instituto Nacional de Salud*, Colombia, and *Centro Nacional de Productos Biológicos*, Peru (Fan et al., 2019). Nevertheless, antivenoms produced in various countries, using different venoms in the immunizing mixture, were efficient to neutralize the principal toxic activities of *B. atrox* venoms from distinct Latin American countries in experimental, pre-clinical tests (Bogarín et al., 2000; Segura et al., 2010), and more specific studies report the neutralization of *B. atrox* venoms from snake populations from Brazil (Furtado et al., 2010; Muniz et al., 2000; Sousa et al., 2013), Colombia (Otero et al., 1995), Ecuador (Laines et al., 2014; Theakston et al., 1995), Peru (Rojas et al., 2005) and French Guiana (Resiere et al., 2020). These studies reveal the efficacy of these antivenoms to neutralize *B. atrox* venoms from different regions, but also highlight quantitative differences in the effective doses of the various antivenoms, comparing to the doses necessary to neutralize venoms used in the immunization protocols. The in vivo potency of *Bothrops* antivenoms varies according to each national pharmacopeia, resulting in products with potency from 2 to 7 mg/mL, serum neutralization tested in mice against a given venom-reference (Fan et al., 2019). In parallel, schedules of doses vary considerably from country to country were *B. atrox* is prevalent. These observations are very important and may be even more relevant due to the intraspecific variability observed in venoms of *B. atrox* snakes from different regions or ontogenetic stages and indicate that more detailed studies on antivenoms specificity should be carried out in order to adequate the use of the available antivenoms to treat accidents in the Amazonian rainforest.

Most of intraspecific variability of *B. atrox* venom is related to components involved in hemostatic disturbances. It has been reported that hemostatic disturbances are higher in human accidents by juvenile snakes (Bernal et al., 2019). Experimentally, higher pro-coagulant activity in venom of juvenile snakes was also demonstrated and attributed to the abundance of SVMPs on venoms (López-Lozano et al., 2002). The intensity of pro-coagulant activity is also dependent on the geographical distribution of the *B. atrox* snakes (Moretto Del-Rei et al., 2019; Salazar

et al., 2007). Fortunately, despite the differences in toxicity, pro-coagulant activity is neutralized by antivenoms (Moretto Del-Rei et al., 2019). However, it has been recently reported that, in a specific situation, the standard antivenom failed to neutralize the pro-coagulant activity of venoms from the floodplain area of the Amazon river, a specific habitat subjected to marked seasonal changes between flood and dry periods (Sousa et al., 2017). The authors further demonstrated that the weak effect of the antivenom was related to neutralization of SVMPs, from which the antivenom showed a better performance in neutralizing prothrombin activation activity than neutralizing Factor X activation activity (Sousa et al., 2018). Taken together, these observations indicate that the presence and/or abundance of particular isoforms of the major toxin classes may explain the eventual lack of neutralization of certain activities by antivenoms. Several evidences support the expression of distinct isoforms of procoagulant enzymes in *B. atrox* venoms from different geographical areas. SVSPs isolated from Manaus was different than the SVSP isolated from Tucurui and both presented less than 80% identity to Batroxobin (Cavinato et al., 1998; Petretski et al., 2000). Concerning SVMPs, sequences of Batroxase (Cintra et al., 2012) and BATX-1 (Patiño et al., 2010), previously isolated from *B. atrox* venoms from other regions, were not found in the transcriptomes of venom glands from *B. atrox* snakes collected at oriental Brazilian Amazon (Amazonas et al., 2018). Similar sequences of Atroxlysin-I and Atroxlysin-III, isolated from Peruvian venoms, were found in the reported transcriptome. Even though, a remarkable difference on the hemorrhagic and dermonecrotic activity was observed in Atroxlysin-Ia, the isoform of Atroxlysin-I isolated from the Brazilian venom (Freitas-de-Sousa et al., 2017). Therefore, methods allowing the characterization of distinct isoforms should be used to have a comprehensive view of antivenom specificity. Currently, the method of choice has been the antivenomics.

Antivenomics is a proteomics-based protocol to quantify the extent of reactivity of antivenoms against fractionated venom components. It was first introduced to quantify the reactivity at toxin resolution of commercial antivenom from the *Instituto Clodomiro Picado* toward the heterologous venoms of *Bothriechis* species (Angulo et al., 2008). Since, antivenomics evolved to a second (Pla et al., 2012) and third (Pla et al., 2017) generations and became a reliable platform combining immunoaffinity techniques, venom components fractionation by RP-HPLC and identification by mass spectrometry. The method provides qualitative and quantitative information on toxins bearing antivenom-recognized epitopes and those toxins exhibiting poor immunoreactivity. The combination of antivenomics and in vivo and in vitro neutralization tests constitutes thus a powerful toolbox that provides a robust evaluation of antivenom preclinical efficacy (Calvete et al., 2018). Currently, several attempts are in progress by antivenom-manufacturing laboratories to implement antivenomics as part of in-process or quality control tests.

Antivenomics was first applied in *B. atrox* venom research to test the reactivity of *B. atrox* venoms from different regions against a Costa Rican polyvalent commercial antivenom. This antivenom was more efficient immunodepleting proteins from the venoms of *B. atrox* from Brazil, Ecuador, and Peru than venoms from Colombia and the authors correlated such behavior by the higher content of the poorly immunogenic toxins, such as PLA_2 and PI-class SVMP molecules in the venoms of Colombian snakes (Núñez et al., 2009). In further studies, antivenomics was applied to test the reactivity of antivenoms raised in Brazil and Costa Rica towards venoms from *B. atrox* snakes originally from a wide distribution range, from the Colombian Magdalena Medio Valley along the Amazon River basin up to São Bento, in the Brazilian State of Maranhão (Calvete et al., 2011). Both antivenoms immunodepleted very efficiently the PIII-class SVMPs or SVSPs, but had impaired reactivity towards PLA_2 and PI-class SVMP molecules. The low reactivity of antivenoms with PI-class SVMPs and PLA_2s was also noted by Sousa et al. (2013) using a distinct method. The authors evaluated the ELISA reactivity of Brazilian commercial antivenom with independent venom fractions obtained by RP-HPLC of venoms from different species of *Bothrops*, including *B. atrox*. Fractions rich in P-III-class SVMPs, from all venoms, were the most reactive antigens. Intermediate levels of reactivity were detected in fractions containing PI-class SVMPs or SVSPs and only a moderate reactivity with PLA_2 fractions. Interestingly, similar reactivity pattern was observed between the reactivity of *B. atrox* venom fractions and fractions from venoms included in the immunization protocol for antivenom production evidencing the poor immunogenicity of PI-class SVMPs and PLA_2 molecules.

The low immunogenicity of toxins included in PI-class SVMPs and PLA_2s is an important question regarding antivenom improvements. As discussed above, PLA_2s participate in the onset of venom-induced tissue damage by their myotoxic and proinflammatory activities (Gutiérrez and Lomonte, 2013). In parallel, *B. atrox* PI-class SVMPs contribute to local tissue-damage since they are hemorrhagic toxins, able to induce local necrosis (Freitas-de-Sousa et al., 2017). PI-class SVMPs also induce inflammation either directly or by liberating ECM fragments that activate endogenous pro-inflammatory pathways (Almeida et al., 2020). Consequently, the impaired reactivity of antivenoms with these groups of toxins could be one parameter involved in the low neutralization of local tissue-damage by antivenoms in patients bitten by *Bothrops* snakes. The evidences arising from these observations point to the necessity to discuss the immunization protocol for antivenom production aiming to improve the immunogenicity of the low immunoreactive toxins for production of an antivenom with broader specificity that could be more efficient for the treatment of snakebites. Improvements of antivenom reactivity could be achieved by several instances, including the addition of recombinant PI-class SVMPs and PLA_2s as complementary antigens in the immunization protocols, allowing production of more antibody molecules reactive to these antigens during the process of horse hyperimmunization. Progress into toxin-specific monoclonal antibodies and their use as alternatives for future treatment strategies is developing very fast and could help to drive for neutralization of the specific toxins related to the major effects of the venoms. Frauches et al. (2013) reported the neutralization of *B. atrox* venom lethality by a pool of monoclonal antibodies against a phospholipase A_2, a hemorrhagic SVMP and a SVSP isolated from *B. atrox* venom. However, the complexity of *Bothrops* venoms and the participation of different isoforms in the clinical symptoms presented by the patients raise the issue whether *Bothrops* antivenom based on monoclonal antibodies or oligoclonal antibody mixtures is a real possibility.

Complementary therapies to be administered together with antivenoms are currently one of the most promising approaches and small molecules have been suggested as complementary therapeutics for inhibition of SVMP or PLA_2 activities (Albulesco et al., 2012; Villalta-Romero et al., 2012). Batimastat and Marimastat have been shown to be efficient in neutralizing local tissue damage induced by a PI-class SVMP from *B. asper* venom (Escalante et al., 2000). Recently, peptidomimetic and thioester compounds have been designed as inhibitors of Batx-I, the PI-class SVMP from *B. atrox* venom (Henao Castañeda et al., 2019; Preciado et al., 2019). The use of PLA_2 inhibitors as varespladib and methyl-varespladib has been suggested as first-aid or adjunctive treatments for snakebites and could be hugely beneficial to victims (Lewin et al., 2016). Plant-derived components also have been tested with good possibilities to act as complementary therapies in *B. atrox* snakebites (de Moura et al., 2016, 2017, 2018; Magalhães et al., 2011). However, it is very unlike to develop synthetic or plant-derived inhibitors selective to the venom enzymes and it is reasonable expectable the developments of side-effects due to inhibition of the ancestor endogenous enzymes as MMPs, ADAMs od PLA_2s that are essential for the maintenance of mammalian homeostasis.

5. Concluding remarks

B. atrox is responsible for most of the snakebites in the Amazon region and is mostly endemic of the Amazon rainforest. The composition

of *B. atrox* venom is significantly known and has been used to trace the relevance of the venom phenotype for adaptation at different environments and for the evolution of the snake species. Understanding the effects of toxins present on the venom mixture also have impact in the clinics of human patients accidently inflicted to snakebites and alights the physiopathology of this medical grievance indicating routes for patients' treatments. However, in spite of the wide distribution and the great medical relevance of *B. atrox* snakes, there are still some issues that should be addressed when analyzing the vast literature related to this species, mostly mentioned in this review.

The combined scenario provided by the genetic studies strongly suggests caution when considering *B. atrox* taxonomy, as it stands, as a backbone for evolutionary hypotheses. The impacts of the lack of taxonomic resolution on the studies focused on venom or envenoming are currently unknown. Although *B. atrox* might not represent an exclusive evolutionary lineage, much of the conclusions about venom variability and clinical aspects of snakebites are derived from local studies, focused on very delimited population sampling and the main conclusions provided by such studies probably will not be affected by future taxonomic decisions.

The effects of the venom and venom components should also be accepted with some caution. Due to the complexity of venom effects involving multiple physiological targets, the studies on mechanisms of action are mostly conducted with isolated toxins. The general effect of whole venom is very likely to include additive or synergistic actions of different toxins and also by the activation of endogenous factors that amplify the range of physiological targets disrupted by the action of snake venoms (Almeida et al., 2020; Ibiapina et al., 2019; Rucavado et al., 2016). Another important issue is that most studies on mechanisms of action of venoms and venom toxins are carried out on mammalian experimental models. This is justified since the early studies on venom-induced effects were thought to explain pathophysiology of human envenomings and the results are, indeed, relevant for this purpose. However, how are these data applicable to understand the action of venoms on the natural prey? The major targets of *B. atrox* venom are related to the hemostatic disturbances, involved in cases of sequela and death of human patients. However, differences in the coagulation system of birds, reptiles and amphibians have been reported and the action of the venom may be different in these taxa. Also, tissue damage can be cruel to human victims of snakebites, but tissue-damage is of slow onset and it is very unlike that it may act as an advantage for hunting or killing prey. In contrast to the neurotoxic venoms, toxins from *B. atrox* and other hematotoxic venoms may still be evolving to reach more efficient killing mechanism and currently still display weak lethal power. Perhaps, the role of *B. atrox* venom in prey digestion is under estimated and certainly, many components of the venom must still be relevant for prey digestion due to the ancestral characteristics of venom toxins.

Human accidents with *B. atrox* follow similar clinical characteristics than accidents by other *Bothrops* snakes, but due to the difficult access, the severity is more pronounced. As medical emergencies, *Bothrops* snakebites require a quick response by administering antivenom, preferably within the first 6 h after the bite. However, many vulnerable groups may take days to reach health care at a time when antivenom is no longer able to counteract the envenomation effects. In addition, reports of snakebite envenomation in remote rural areas are unreliable, and the number of patients who remain deprived of antivenom therapy remains an issue to be properly investigated. Thus, the expansion of antivenom therapy to more health facilities would increase patients' access to treatment.

In conclusion, venomics is a great platform introduced in the field of venom research and allowed important contributions to the knowledge of *B. atrox* venom that have been currently applied in understanding the snake biology and the clinics of snakebites. In parallel, antivenomics has important application in pre-clinical tests assuring the efficacy of antivenoms against venoms from different geographical regions or to advise countries that do not produce antivenoms about what commercially available antivenom would be more efficient to that particular area. Venomics and antivenomics are golden-standard tools still to be better exploited to understand snake biology and to overcome the issue of venom variability for the treatment of human victims of snakebites.

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The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Wuelton Marcelo Monteiro: Conceptualization, Visualization, Funding acquisition. **Jorge Carlos Contreras-Bernal:** Writing - original draft, Writing - review & editing. **Pedro Ferreira Bisneto:** Writing - original draft, Writing - review & editing. **Jacqueline Sachett:** Writing - original draft, Writing - review & editing. **Iran Mendonça da Silva:** Writing - original draft, Writing - review & editing. **Marcus Lacerda:** Visualization, Funding acquisition. **Allyson Guimarães da Costa:** Visualization. **Lisele Brasileiro:** Writing - original draft, Writing - review & editing, Writing - original draft, Writing - review & editing. **Sâmella Silva-de-Oliveira:** Writing - original draft, Writing - review & editing. **Paulo Sérgio Bernarde:** Visualization. **Igor L. Kaefer:** Visualization. **Felipe Gobbi Grazziotin:** Conceptualization, Visualization. **Fan Hui Wen:** Conceptualization, Visualization. **Ana Maria Moura-da-Silva:** Conceptualization, Visualization, Funding acquisition.

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