Bleeding Disorders in *Bothrops atrox* Envenomations in the Brazilian Amazon: Participation of Hemostatic Factors and the Impact of Tissue Factor

Sâmella S. Oliveira 1,2, Eliane C. Alves 1,2, Alessandra S. Santos 1, Elizandra F. Nascimento 1, João Pedro T. Pereira 1, Iran M. Silva 1,2, Jacqueline A. G. Sachett 1,2, Lybia Kássia S. Sarraff 1, Luciana Aparecida Freitas-de-Sousa 3, Mônica Colombini 3, Hedylamar O. Marques 4, Marcus V. G. Lacerda 1, Marco Aurélio Sartim 5, Ana Maria Moura-da-Silva 1,3, Luiz Carlos L. Ferreira 1, Ida S. Sano-Martins 6,*,† and Wuelton M. Monteiro 1,2,*,†

1 Carlos Borborema Clinical Research Institute, Dr. Heitor Vieira Dourado Tropical Medicine Foundation, Manaus 69040-000, Brazil; oliveira.samella@gmail.com (S.S.O.); aneaalves.enf@gmail.com (E.C.A.); pedro02.tavares@gmail.com (J.P.T.P.); iramend3@gmail.com (I.M.S.); jac.sachett@gmail.com (J.A.G.S.); lybsarra@gmail.com (L.K.S.S.); marcuslacerda.br@gmail.com (M.V.G.L.); ana.moura@butantan.gov.br (A.M.M.-d.-S.); ferreira.luiz@gmail.com (L.C.L.F.)

2 School of Health Sciences, Amazonas State University, Manaus 69065-001, Brazil

3 Immunopathology Laboratory, Butantan Institute, São Paulo 05503-900, Brazil; luciana.sousa@butantan.gov.br (L.A.F.d.-S.); monica.colombini@butantan.gov.br (M.C.)

4 Hemostasis Laboratory, Amazonas State Hematology and Haemotherapy Hospital Foundation, Manaus 69050-001, Brazil; hedyomarques@gmail.com

5 Institute of Biological Sciences, Federal University of Amazonas, Manaus 69070-000, Brazil; marcosartim@hotmail.com

6 Pathophysiology Laboratory, Butantan Institute, São Paulo 05503-900, Brazil

* Correspondence: ida.sano@butantan.gov.br (I.S.S.-M.); wueltonmm@gmail.com (W.M.M.); Tel.: +55-11-627-9743 (I.S.S.-M.); +55-922-127-3555 (W.M.M.)

† These authors contributed equally to this work.

Received: 14 July 2020; Accepted: 13 August 2020; Published: 29 August 2020

**Abstract:** Bleeding is a common hemostatic disorder that occurs in *Bothrops* envenomations. We evaluated the changes in coagulation, fibrinolysis components, and platelets in *Bothrops atrox* envenomations with bleeding. This is an observational study with *B. atrox* snakebite patients (*n* = 100) treated in Manaus, Brazilian Amazon. Bleeding was recorded on admission and during hospitalization. We found that the platelet count in our patients presented a weak correlation to tissue factor, factor II, and plasminogen. Tissue factor presented weak correlation to factor V, II, D-dimer, plasminogen, alpha 2-antiplasmin, and moderate correlation to fibrinogen and fibrin/fibrinogen degradation product (FDP). Patients with systemic bleeding (*n* = 20) presented low levels of factor V, II, fibrinogen, plasminogen, and alpha 2-antiplasmin, and high levels of tissue factor and FDP compared to those without bleeding. Patients with only local bleeding (*n* = 41) and without bleeding showed similar levels of hemostatic factors. Thrombocytopenia was observed mainly in patients with systemic bleeding and increased levels of serum venom. No association was found between venom levels and systemic bleeding, or between venom levels and clinical severity of envenomation. This is the first report that shows the participation of the extrinsic coagulation pathway in the consumption coagulopathy of *B. atrox* envenomations with systemic bleeding due to tissue factor release.

**Keywords:** *Bothrops atrox* envenomation; coagulation factors; systemic bleeding; thrombocytopenia; tissue factor
Key Contribution: Bleeding disorders observed in Bothrops atrox envenomations in the Brazilian Amazon are a multifactorial outcome. Systemic bleeding is accompanied by the consumption of coagulation factors and fibrinolytic components, and the participation of the tissue factor.

1. Introduction

Systemic bleeding following Bothrops envenomations is a common hemostatic disorder that has been observed in fatal envenomation cases in the Brazilian Amazon [1,2]. The lancehead (Bothrops atrox) is the most common venomous snake in the Amazon region [3] and hemostatically active toxins present in its venom are capable of converting fibrinogen into fibrin and activating coagulation factors, as well as interfering in platelet function, fibrinolysis, and endothelial maintenance [4–15]. The outcome is the installation of a consumptive coagulopathy, which is characterized by the depletion of hemostatic components. This depletion leads to unclottable blood and disruption of capillary vessels, which may be followed by bleeding [16,17].

A previous study in the Pará State, eastern Brazilian Amazon, has shown that Bothrops envenomations cause decreased levels of fibrinogen and alpha 2-antiplasmin, elevated fibrin/fibrinogen degradation product (FDP), and D-dimer concentrations [18]. Similar results were also reported in a study involving Bothrops snakebite patients in the Amazonas State, western Brazilian Amazon, in which some coagulation components recovered within 48 h after starting antivenom therapy [19]. Despite the clinical similarities of Bothrops envenomations in Brazil, thrombocytopenia is an infrequent event in Bothrops envenomation that occurs in the Amazon (caused mainly by B. atrox) compared to those in the southern region (caused by B. jararaca) [18–20]. Nevertheless, it has been shown that unclottable blood and thrombocytopenia are factors that are independently associated with the risk of systemic bleeding in Bothrops envenomations in the Brazilian Amazon [16].

Differences have been observed in the coagulant activity of venom from B. atrox snakes from distinct locations [21,22], habitats [23], and at different ontogenetic stages [24,25], as well as when compared to the venom of other Bothrops species [26]. There is little information regarding how clotting factors and other components are affected in Bothrops snakebite patients in the Amazon who evolve to systemic or local bleeding. Therefore, the aim of the present study was to evaluate the changes in coagulation factor levels, fibrinolysis components, and platelet count associated with bleeding observed in B. atrox snakebite patients in the western Brazilian Amazon. Our data show that B. atrox envenomation with systemic bleeding presents consumption of coagulation factors, such as fibrinogen, factors II and V, and fibrinolytic components, as well as the participation of tissue factor.

2. Results

2.1. Patient Characterization

A total of 61 of the 100 patients included in our study presented showed bleeding on admission (before antivenom therapy) or during hospitalization (after antivenom therapy). Background information and clinical features of patients on admission based on the presence of bleeding are shown in Table 1. Twenty patients showed systemic bleeding and, of these, fourteen (70.0%) presented concomitant local bleeding. Nineteen (95.0%) of the patients that had systemic bleeding also had unclottable blood, and five (25.0%) were thrombocytopenic, including one patient in this group who had a platelet count of below 100 \( \times 10^3/\mu L \). In four out of twenty (20.0%) patients with systemic bleeding, both unclottable blood and thrombocytopenia were observed. In patients with only local bleeding (41/61, 67.2%), twenty-one (51.2%) presented unclottable blood, and two (4.9%) thrombocytopenia, who also had unclottable blood. In addition, thirty-nine patients showed no bleeding after the B. atrox envenomation; of these, fourteen (35.9%) had unclottable blood and three
(7.7%) presented thrombocytopenia. In two out of thirty-nine (5.1%) patients without bleeding both unclottable blood and thrombocytopenia were observed.

### Table 1. Background information and clinical features of the Bothrops atrox snakebite patients (n = 100) at the time of admission, based on the presence of bleeding.

| Characteristics                        | Systemic Bleeding | Only Local Bleeding | Non-bleeding |
|----------------------------------------|-------------------|---------------------|--------------|
| Gender                                 |                   |                     |              |
| Male:female                            | 18:2 (33.3)       | 35:6 (38.8)         | 35:4 (35.0)  |
| Age (median) years (range)             | 33 (15–58)        | 42 (14–74)          | 48 (15–79)   |
| Time taken to reach to medical assistance (hours) |                     |                     |              |
| <3 (n = 46)                            | 7 (15.2)          | 20 (43.5)           | 19 (41.3)    |
| 3–6 (n = 34)                           | 5 (14.7)          | 17 (50.0)           | 12 (35.3)    |
| >6 (n = 20)                            | 8 (40.0)          | 4 (20.0)            | 8 (40.0)     |
| Anatomical location of bite            |                   |                     |              |
| Foot (n = 73)                          | 16 (21.9)         | 30 (41.1)           | 27 (37.0)    |
| Leg (n = 18)                           | 2 (11.1)          | 6 (33.3)            | 10 (55.6)    |
| Hand (n = 9)                           | 2 (25.0)          | 5 (50.0)            | 2 (25.0)     |
| Edema                                  |                   |                     |              |
| Mild (n = 46)                          | 8 (17.4)          | 16 (34.8)           | 22 (47.8)    |
| Moderate (n = 49)                      | 8 (16.3)          | 25 (51.0)           | 16 (32.7)    |
| Severe (n = 5)                         | 4 (80.0)          | 0 (0.0)             | 1 (20.0)     |
| Pre-hospital treatment                 |                   |                     |              |
| Tourniquets (n = 24)                   | 5 (20.8)          | 12 (50.0)           | 7 (29.2)     |
| Local incisions (n = 4)                | 1 (25.0)          | 2 (50.0)            | 1 (25.0)     |
| Suction (n = 1)                        | 0 (0.0)           | 1 (100.0)           | 0 (0.0)      |
| Use of topical/oral medicines (n = 55) | 14 (25.5)         | 22 (40.0)           | 19 (34.5)    |
| Comorbidities                          |                   |                     |              |
| Arterial hypertension (n = 16)         | 1 (6.3)           | 8 (50.0)            | 7 (43.7)     |
| Diabetes (n = 6)                       | 1 (25.5)          | 1 (25.5)            | 4 (50.0)     |
| Cardiopathy (n = 1)                    | 0 (0.0)           | 1 (100.0)           | 0 (0.0)      |
| Clinical severity of envenomation       |                   |                     |              |
| Mild (n = 27)                          | 1 (3.7)           | 11 (40.7)           | 15 (55.6)    |
| Moderate (n = 59)                      | 14 (23.7)         | 22 (37.3)           | 23 (39.0)    |
| Severe (n = 14)                        | 5 (35.7)          | 8 (57.1)            | 1 (7.1)      |
| Unclottable blood                      |                   |                     |              |
| Yes (n = 54)                           | 19 (35.2)         | 21 (38.8)           | 14 (25.9)    |
| No (n = 46)                            | 1 (2.2)           | 20 (43.5)           | 25 (54.3)    |
| Thrombocytopenia                       |                   |                     |              |
| Yes (n = 10)                           | 5 (50.0)          | 2 (20.0)            | 3 (30.0)     |
| No (n = 90)                            | 15 (16.7)         | 39 (43.3)           | 36 (40.0)    |

#### 2.2. Coagulation Factors Levels and Platelets Count

Figure 1 shows correlation between platelet count and coagulation factor levels and fibrinolysis components on admission (before antivenom therapy) for B. atrox snakebite patients in the Brazilian Amazon. Platelet count presented weak correlation to tissue factor concentration ($r = -0.218; p = 0.033$), factor II ($r = 0.295; p = 0.004$), and plasminogen activity ($r = 0.228; p = 0.025$).
Figure 1. Pearson's correlation ($r$) between platelet count and coagulation factor levels and fibrinolysis components obtained on admission (before antivenom therapy) for Bothrops atrox snakebite patients in the Brazilian Amazon.
Figure 2. Spearman’s correlation ($r$) between tissue factor and coagulation factor levels/ fibrinolysis components and serum venom obtained on admission (before antivenom therapy) for Bothrops atrox snakebite patients in the Brazilian Amazon.
The correlation between tissue factor and coagulation factor levels/fibrinolysis components and serum venom on admission (before antivenom therapy) for *Bothrops atrox* envenomation was also evaluated in our study. Tissue factor presented a weak correlation to factor V ($r = 0.270; p = 0.006$), factor II ($r = 0.304; p = 0.003$), D-dimer ($r = 0.317; p = 0.001$), plasminogen ($r = 0.343; p = 0.001$), alpha 2-antiplasmin ($r = 0.338; p = 0.001$), and moderate correlation to fibrinogen ($r = 0.412; p < 0.001$) and FDP ($r = 0.441; p < 0.001$) (Figure 2). Tissue factor levels were also significantly increased in patients with moderate/severe edema (median = 25.8 pg/mL (IQR: 14.50–52.20)) when compared to those with mild edema (median = 8.3 pg/mL (IQR: 0.00–29.20)) ($p = 0.003$).

### 2.3. Bleeding vs. Coagulation Factors and Platelets

Figure 3 shows the median levels of coagulation factors, fibrinolysis components, and platelet count in *B. atrox* snakebite patients with either systemic bleeding, only local bleeding, or without bleeding and data for the healthy donor (HD) control group. Lower levels of factor II, fibrinogen, plasminogen, and alpha 2-antiplasmin, as well as higher levels of tissue factor, FDP, and D-dimer were observed in *B. atrox* snakebite patients with bleeding (systemic bleeding and only local bleeding) when compared to the HD control group. Levels of factor V were significantly decreased only in the patients with systemic bleeding. Patients with systemic bleeding showed lower levels of factor V, factor II, fibrinogen, plasminogen, and alpha 2-antiplasmin, as well as higher levels of tissue factor and FDP when compared to those without bleeding. There was no statistically significant difference in median levels of coagulation factors and fibrinolysis components between patients with only local bleeding and those without bleeding. We also observed that the median levels of factor V and plasminogen were lower in patients with systemic bleeding than in those with only local bleeding. On the other hand, there was no statistically significant difference in the median levels of factor VII and factor X between all groups. Although the median platelet count was within the normal range in *B. atrox* snakebite patients, it was lower in patients with systemic bleeding when compared to those with local bleeding alone and the HD group.

### 2.4. Venom Detection and Hemostatic Parameters

In 35/100 patients, serum levels of *Bothrops* venom above the cut off value (18.87 ng/mL) were detected. There was no statistically significant difference in the serum level of venom between patients with systemic bleeding (median = 21.10 ng/mL serum (IQR, 5.02–52.35)) and those without bleeding (median = 7.50 ng/mL serum (IQR, 2.50–16.40)) ($p = 0.209$). Moreover, there was also no statistically significant difference in the serum level of venom between patients with systemic bleeding and only local bleeding (median = 19.10 ng/mL serum (IQR, 3.75–32.03)) ($p = 0.675$). Serum levels of *Bothrops* venom were significantly higher in those patients with thrombocytopenia (median = 28.20 ng/mL serum (IQR, 21.35–64.25)) than those who did not present this alteration (median = 7.75 ng/mL serum (IQR, 3.10–23.68)) ($p = 0.021$). Furthermore, there was no significant difference in the serum level of venom between mild cases (median = 22.35 ng/mL serum (IQR, 5.95–30.60)) and moderate/severe cases (median = 7.70 ng/mL serum (IQR, 3.30–22.40)) ($p = 0.339$).
2.3. Bleeding vs. Coagulation Factors and Platelets

Figure 3 shows the median levels of coagulation factors, fibrinolysis components, and platelet count in *B. atrox* snakebite patients with either systemic bleeding, only local bleeding, or without bleeding and data for the healthy donor (HD) control group. Lower levels of factor II, fibrinogen, plasminogen, and alpha 2-antiplasmin, as well as higher levels of tissue factor, FDP, and D-dimer were observed in *B. atrox* snakebite patients with bleeding (systemic bleeding and only local bleeding) when compared to the HD control group. Levels of factor V were significantly decreased only in the patients with systemic bleeding. Patients with systemic bleeding showed lower levels of factor V, factor II, fibrinogen, plasminogen, and alpha 2-antiplasmin, as well as higher levels of tissue factor and FDP when compared to those without bleeding. There was no statistically significant difference in median levels of coagulation factors and fibrinolysis components between patients with only local bleeding and those without bleeding. We also observed that the median levels of factor V and plasminogen were lower in patients with systemic bleeding than in those with only local bleeding. On the other hand, there was no statistically significant difference in the median levels of factor VII and factor X between all groups. Although the median platelet count was within the normal range in *B. atrox* snakebite patients, it was lower in patients with systemic bleeding when compared to those with local bleeding alone and the HD group.

![Figure 3](image-url)

**Figure 3.** Plasma concentration of coagulation factors and fibrinolysis components, and platelet count on admission (before antivenom therapy) in patients with systemic bleeding (SB) (*n* = 20), only local bleeding (LB) (*n* = 41) and non-bleeding patients (*n* = 39) after *Bothrops atrox* envenomation in the Brazilian Amazon. Results are expressed as medians and interquartile ranges. *** *p* < 0.001, ** *p* < 0.01 and * *p* < 0.05, significant between patients with bleeding (SB or LB) and non-bleeding patients. & & *p* < 0.01 and & *p* < 0.05, significant between patients with systemic bleeding and only local bleeding. && *p* < 0.001, ## *p* < 0.01 and # *p* < 0.05, significant between *B. atrox* snakebite patients (SB, LB, or non-bleeding patients) and healthy donors (HD; *n* = 14), who were accompanying patients during hospitalization (control group). FDP: fibrin/fibrinogen degradation product. The shaded area is the normal range for each parameter. Normal range: FDP < 5 µg/mL; D-dimer ≤ 0.5 µg/mL.

3. Discussion

Bleeding and alterations in coagulation are the most common hemostatic disorders and frequently coexist in victims of *Bothrops* snakebite [16,27]. These disorders are caused by the action of venom’s hemostatically active toxins, wherein the variability of these toxins can lead to different mechanisms of coagulopathy [22] and bleeding. While trying to understand how clotting factors and other components are affected in *B. atrox* snakebite patients who evolve to systemic or local bleeding, we found that patients with systemic bleeding presented lower levels of factor V, II, fibrinogen, plasminogen and alpha
2-antiplasmin, and higher levels of tissue factor and FDP than those without bleeding. On the other hand, patients with only local bleeding showed coagulation factor levels and fibrinolysis components similar to those without bleeding.

*B. atrox* is responsible for 80–90% of snakebites in the Brazilian Amazon [18,28]. Despite the similarities between the toxins from the *B. atrox* and *B. jararaca* venoms [6,26], in our study, we observed that 20.0% (20/100) of *B. atrox* snakebite patients in the western Brazilian Amazon present systemic bleeding, while 46.1% of *B. jararaca* snakebite patients present this manifestation [27]. In a recent study performed by our research group in the western Brazilian Amazon it was observed that unclottable blood and thrombocytopenia were independently associated with the risk of *Bothrops* snakebite patients developing systemic bleeding [16]. In our study, unclottable blood was observed in 95.0% (19/20) of patients with systemic bleeding. Unclottable blood observed in *B. atrox* envenomation can be explained by the presence of components with thrombin-like activity, which directly convert fibrinogen into fibrin [4,5,8] and pro-coagulant toxins, which in turn activate factors II, X and V [10–12], and increase the pro-coagulant activity of factor VIII [14], which, as a result, leads to intravascular thrombin generation. The intravascular thrombin generation can also result in activation and consumption of factors V, VIII, XI, XIII, and fibrinogen, as well as thrombocytopenia [29,30]. Furthermore, fibrinogenolytic components of *B. atrox* venom can also contribute to fibrinogen consumption [15].

In our study, one interesting and novel result obtained was the detection of increased levels of plasma tissue factor in *B. atrox* snakebite patients who presented systemic bleeding. Although this has never been previously described for *Bothrops* snakebite patients, this result corroborates previous findings in animal and in vitro models, in which *Bothrops* venom and its procoagulant toxins induce tissue factor expression [31–33]. Thus, tissue factor could also contribute to the development of unclottable blood and thrombocytopenia, by leading to the intravascular generation of thrombin, and systemic bleeding. In our study, plasma tissue factor presented weak correlation to factor V, factor II, D-dimer, plasminogen, and alpha 2-antiplasmin, and moderate correlation to fibrinogen and FDP. Moreover, the median levels of plasma tissue factor were increased in patients with moderate/severe edema. Tissue factor is a transmembrane protein expressed constitutively by subendothelial cells (extravascular compartment) or induced by monocytes and endothelial cells (intravascular compartment), and it is present in plasma through the release of soluble tissue factor or in microvesicles [34]. It acts as a cofactor for factor VII, whose complex activates factors X and IX, and triggers blood coagulation [35]. Our finding shows a possible role of *Bothrops* venom in inducing the expression of intravascular tissue factor, as well as extravascular tissue factor released by local trauma and damage caused by envenomation. Considering that activators of factors X and II were found in the *B. atrox* venom [10,11,22] and the tissue factor expression detected in our patients, our results indicate that these factors are triggered during *B. atrox* envenomation. However, the normal median values of factors X and VII in our patients suggest that only minute amounts are required to induce coagulopathy, or it may be that an increased hepatic synthesis of these factors may occur in stressful situations [36]. Additionally, high levels of factor VII have been reported in envenomation caused by other snakes [37].

Our results showed that patients with systemic bleeding presented increased levels of FDP, as well as decreased levels of plasminogen and alpha 2-antiplasmin, when compared to those without bleeding. *B. atrox* venom could be acting directly on fibrin and fibrinogen resulting in the generation of fibrinolysis products, and indirectly inducing the release of tissue plasminogen activator [6,15,38,39]. Also, the capacity of *B. atrox* venom to induce factor XIII activation [14], and intravascular thrombin generation [30] can contribute to the formation of intravascular cross-linked fibrin polymer and D-dimer. Similarly, the decreased levels of alpha 2-antiplasmin indicate its consumption in order to inhibit generated plasmin [40]. Consequently, the response of fibrinolysis is essential to eliminate fibrin deposition, thus avoiding microvascular thrombotic obstruction, and resulting end-organ damage or organ failure [41].
Our findings demonstrated that platelet count presented a weak correlation to plasma tissue factor levels and factor II and plasminogen activity in *B. atrox* snakebite patients. Activated platelets are found to enhance tissue factor expression in monocytes, and also expose intracellular stored tissue factor on their own surface [42]. *B. jararaca* envenomation in rats has been shown to trigger the coagulation cascade, which induces direct activation of coagulation factors and tissue factor decryption, and both factors probably contribute to the consumptive coagulopathy [43]. Moreover, platelets also serve as assembly sites for proteins of the prothrombinase complex and plasminogen activator system [44,45], which explains the correlations observed, although they are weak. On the other hand, our results did not show a correlation between fibrinogen levels and platelet count either, similar to what is observed in *B. jararaca* envenomation [27]. Although the median platelet count was within the normal range, it was significantly decreased in patients with systemic bleeding when compared to patients with only local bleeding and healthy donors. Thrombocytopenia was observed in about 68% of *B. jararaca* snakebite patients with systemic bleeding [20,27]. In contrast, only 25% of patients with systemic bleeding were thrombocytopenic in our study. *B. atrox* venom is composed of proteins, such as botrocetin [9], and thrombocytin (a serine proteinase) [14], which induce platelet agglutination or aggregation, respectively, as well as components with platelet inhibiting properties, such as batroxrhagin (a metalloproteinase) [6] and batroxostatin (an Arg-Gly-Asp-containing peptide) [13]. In addition, crude *B. atrox* venom has no aggregating activity on washed rabbit platelets [46]. Moreover, the major toxic component in the venom of *B. atrox* from the Brazilian Amazon, called batroxrhagin, inhibits collagen-induced platelet-aggregation, induces bleeding and fibrin lysis [6]. In *B. jararaca* snakebite patients, disturbances of platelet function also contribute to the development of bleeding due to the envenomation [47]. Changes in platelet function were not evaluated in our study. Therefore, the systemic bleeding observed in *B. atrox* snakebites patients from the Brazilian Amazon may be associated with the impairment of platelet function.

In our study, the levels of venom in the serum of patients were higher in patients with thrombocytopenia than those with normal platelet count. Thus, this finding could be the result of the intravascular thrombin generation induced by the coagulant action of the venom [30], since the main toxin in *B. atrox* venom is an inhibitor of platelet aggregation [6]. On the other hand, there was no association between venom levels and systemic bleeding, or between venom levels and clinical severity of envenomation. Venom has been detected in blister aspirates collected from the bite site in *Bothrops* snakebite patients [18,48]. Moreover, local manifestations such as severe edema, blister formation, and necrosis at the bite site have been observed in *Bothrops* envenomations, which were classified as moderate/severe cases [49]. The most abundant components found in venoms from the *B. atrox* of Brazilian Amazon are metalloproteinases, c-type lectin-like, phospholipases A₂ and serine proteinases, and are frequently involved in the hemostatic disorders observed in snakebite patients [26,50,51]. Among them, venom hemorrhaghisns, which degrade capillary basement membrane components and induce endothelial disruption, and have an important role in the development of the bleeding disorders since increased serum levels of this toxin have been associated with systemic and/or local bleeding in *B. jararaca* snakebites patients [52,53]. Thus, the systemic and/or local bleeding observed in our study could also be associated with increased serum levels of hemorrhaggin. However, serum hemorrhaghin levels were not measured in our study.

In conclusion, the systemic bleeding observed in *B. atrox* envenomations in this part of the Brazilian Amazon is a multifactorial outcome. A complex pathophysiological mechanism involving thrombocytopenia, unclottable blood, and endothelial disruption contributes to the development of systemic bleeding. A new and surprising finding was that plasma tissue factor is involved in the pathophysiology of *B. atrox* envenomation with systemic bleeding in the Amazon region, which shows that the extrinsic coagulation pathway also contributes to the coagulopathy in this type of envenomation.
4. Materials and Methods

4.1. Study Design

This was an observational study designed to evaluate the changes in coagulation factor levels, fibrinolysis components, and platelet count associated with bleeding in *B. atrox* snakebite patients in the western Brazilian Amazon. This study was conducted in accordance with the Declaration of Helsinki, and the study protocol was approved by the Ethics Review Committee at the Dr Heitor Vieira Dourado Tropical Medicine Foundation, Manaus, Amazonas State, Brazil (approval number: 1.302.174, approval date: 29 October 2015). All participants and their parents/guardians (for minors) provided written and informed consent.

4.2. Participants and Data Collection

We evaluated 100 patients who had received a clinical–epidemiological diagnosis of *Bothrops* envenomation and who were treated at the Dr Heitor Vieira Dourado Tropical Medicine Foundation, between January 2016 and December 2017. These patients were also included in another study performed during this same period [19]. Victims that had undergone previous antivenom therapy at other health service centers or were ≤12 years old age were not included in the study. Of the one hundred patients, thirty-six patients brought the snake that caused the snakebite to hospital. All thirty-six snakes were identified as *B. atrox*. In nineteen patients who did not bring the snake to the hospital, *Bothrops* envenomation was confirmed by venom-specific enzyme immune assays (EIA). In forty-five patients, confirmation of *Bothrops* envenomation was based only on the clinical–epidemiological diagnosis.

In addition, 14 healthy donors of either gender, with no history of snakebite and who were accompanying the patients during hospitalization, comprised the control group. For this study, patients had additional serum and citrate plasma samples taken, and these were collected on admission (before antivenom therapy). All serum and plasma samples were immediately centrifuged after the collection, then frozen and stored at −80°C.

A standard form was filled in with patient characteristics such as gender, age, time taken to reach medical assistance, anatomical location of the bite, pre-hospital treatment (use of topical or oral medicines, use of a tourniquet and other procedures), *Bothrops* snakebite confirmation by snake identification, presence of systemic and/or local bleeding (before or after of the antivenom therapy), presence of comorbidities, edema (absent, mild (affecting 1–2 limb segments), moderate (affecting 3–4 limb segments) and severe (affecting 5 limb segments) [54]) and clinical severity of envenomation (mild, moderate, or severe cases according to the Brazilian Ministry of Health guidelines) [55,56].

4.3. Laboratory Analysis

Clotting time was determined by the 20 min whole blood clotting test [57–59]. Assays of coagulation factors and fibrinolysis components were carried out on plasma samples obtained from venous blood containing sodium citrate as the anticoagulant and 2% *v/v Bothrops-Lachesis* antivenom (to neutralize *Bothrops* venom present in the sample). The plasma tissue factor was measured by the human EIA kit (Abcam, Cambridge, UK). Plasma factor VII was estimated using deficient plasma kits (Diagnostica Stago S.A.S., Asnières sur Seine, France) with a semi-automatic Start4 coagulation analyzer (Diagnostica Stago S.A.S., Asnières sur Seine, France). Levels of plasma factors II and X were determined by performing a modified prothrombin time test using deficient plasmas kits (HemosIL®️, Instrumentation Laboratory, Bedford, MA, USA), with an ACL TOP 300 CTS coagulation analyzer (Werfen Instrumentation Laboratory, Barcelona, Spain). Plasma factor V was determined using the Denson standardized method [60]. Plasma fibrinogen was measured based on the Clauss method [61], using a fibrinogen-C kit (HemosIL®️, Instrumentation Laboratory, Bedford, MA, USA) with an ACL TOP 300 CTS coagulation analyzer (Werfen Instrumentation Laboratory, Barcelona, Spain). Plasma FDP was qualitative and semi-quantitative, and determined by latex agglutination (Diagnostica Stago S.A.S.,
Asnières sur Seine, France). Plasma D-dimer was measured by immunoturbidimetry, using a D-Dimer HS 500 kit (HemosIL®, Instrumentation Laboratory, Bedford, MA, USA), with an ACL TOP 300 CTS coagulation analyzer (Werfen Instrumentation Laboratory, Barcelona, Spain). Plasma plasminogen was determined by chromogenic assay (American Diagnostica Inc., Greenwich, UK). Alpha 2-antiplasmin was determined by the synthetic chromogenic substrate method (Diagnostica Stago S.A.S., Asnières sur Seine, France). These assays were performed according to the manufacturer’s protocols at the Hemostasis Laboratory, Amazonas State Hematology and Haemotherapy Hospital Foundation (dosage of factors II and X, fibrinogen and D-dimer) and Pathophysiology Laboratory at the Butantan Institute (dosage of tissue factor, factors V and VII, plasminogen, FDP, and alpha 2-antiplasmin). Platelet counts were carried out on venous blood samples containing potassium EDTA as anticoagulant and 2% v/v Bothrops-Lachesis antivenom, and determined in an automated cell counter (Sysmex Corp., Kobe, Japan), at the Carlos Borborema Clinical Research Institute, Dr Heitor Vieira Dourado Tropical Medicine Foundation.

EIA was used for the detection and measurement of Bothrops venom circulating in serum samples. Antigens were captured with Bothrops-Lachesis antivenoms coated on ELISA plates, and the bound toxins reacted with a second antibody raised in rabbits against B. atrox venom, followed by incubation with anti-rabbit IgG-peroxidase conjugate. Reactions were revealed by the addition of the enzyme substrates (OPD plus H_{2}O_{2}) and recorded at 492 nm. Values were calculated using standard curves with known concentrations of B. atrox venom. The cut-off level corresponded to the mean plus 2 S.D. values of negative samples obtained from the HD group. This assay was performed at the Immunopathology Laboratory, Butantan Institute.

4.4. Outcomes

The laboratory outcomes consisted in the presence of (i) unclottable blood, and (ii) thrombocytopenia on admission (before antivenom therapy). Unclottable blood was defined as when the blood had not clotted within 20 min after collection. Thrombocytopenia was defined by having a platelet count below 150 × 10^3/µL. The clinical outcome was the presence of systemic and/or local bleeding on admission (before antivenom therapy), or during hospitalization (after antivenom therapy). Local bleeding was determined as the presence of local ecchymosis; bleeding from the fang punctures.

4.5. Data Analysis

A database and descriptive analyses were produced using Microsoft Excel Office 365 software, which was fed information by two independent typists. Statistical analyses were performed using the Graphpad Prism software version 5 (Graphpad Software, Inc., San Diego, CA, USA) and STATA statistical package version 13 (Stata Corp, College Station, TX, USA). The comparison of medians between two data groups were performed using the Mann–Whitney test, whereas for comparison of the variables with three or more groups, the data analysis was performed using the Kruskal–Wallis test, followed by Dunn’s post-test for multiple comparisons between groups. Differences in results were considered statistically significant when p < 0.05. Data normality tests were done using the Shapiro–Wilk test. Pearson’s correlation coefficient was calculated to measure the degree of association between the platelet count and coagulation factor levels and fibrinolysis components. Spearman’s correlation coefficient was calculated to measure the degree of association between the tissue factor and coagulation factor levels/fibrinolysis components and serum venom. The coefficient correlation factor was considered significant when p < 0.05. The correlation index (r) was used to categorize the correlation strength as being weak (r ≤ 0.35), moderate (r ≥ 0.36 to r ≤ 0.67), or strong (r ≥ 0.90), as previously described [49,62].

Author Contributions: Conceptualization, S.S.O., I.S.S.-M., and W.M.M.; data curation, S.S.O., E.C.A., A.S.S., E.F.N., J.P.T.P., L.K.S.S., and M.C.; formal analysis, S.S.O.; funding acquisition, J.A.G.S., A.M.M.-d.-S., L.C.L.F., and W.M.M.; investigation, S.S.O., E.C.A., A.S.S., E.F.N., J.P.T.P., I.M.S., L.K.S.S., L.A.F.-d.-S., M.C., and I.S.S.-M.;
Toxins 2020, 12, 554

We wish to thank all the patients and donors who participated in this study. We thank A.M.M.-d.-S., and W.M.M. are CNPq fellows. A.S.S., E.F.N., J.P.T.P., L.K.S.S., L.A.F.-d.-S., and M.C.; visualization, S.S.O., I.M.S., L.A.F.-d.-S., H.O.M., M.V.G.L., M.A.S., A.M.M.-d.-S., I.S.S.-M., and W.M.M.; writing-original draft, S.S.O.; writing–review and editing, S.S.O., I.M.S., L.A.F.-d.-S., H.O.M., M.V.G.L., M.A.S., A.M.M.-d.-S., L.C.L.F., I.S.S.-M., and W.M.M. All authors have read and agreed to the published version of the manuscript.

Funding: The research was funded by Coordination for the Improvement of Higher Level Education Personnel (Capes/PROCAD) Grant number 2992/2014; Brazilian National Council for Scientific and Technological Development (CNPQ/UNIVERSAL) Grant number 428525/2016-4; and Amazonas Research Foundation (FAPEAM/PPSUS) Grant number 287/2013 and (FAPEAM/UNIVERSAL AMAZONAS) Grant number 006/2019. L.A.F.-d.-S. has a fellowship from the São Paulo Research Foundation (FAPESP 2017/24546-3). M.V.G.L., A.M.M.-d.-S., and W.M.M. are CNPq fellows.

Acknowledgments: We wish to thank all the patients and donors who participated in this study. We thank the medical and nursing staff at Dr Heitor Vieira Dourado Tropical Medicine Foundation for the help during the research. We also thank Socorro Costa, Jorge Carlos Conrreras Bernal, and Biochelson Najibe Ibiapina for their technical support.

Conflicts of Interest: The authors state that they have no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Oliveira, S.S.; Freitas-de-Sousa, L.A.; Alves, E.C.; Ferreira, L.C.L.; Mendonça-da-Silva, I.; Lacerda, M.V.G.; Fan, H.; Moura-da-Silva, A.M.; Monteiro, W.M. Fatal stroke after Bothrops snakebite in the Amazonas state, Brazil: A case report. Toxicon 2017, 138, 102–106. [CrossRef] [PubMed]

2. Souza, A.S.; Sachett, J.A.G.; Alcântara, J.A.; Freire, M.; Alecrim, M.G.C.; Lacerda, M.; Ferreira, L.C.L.; Fan, H.W.; Sampaio, V.S.; Monteiro, W.M. Snakebites as cause of deaths in the Western Brazilian Amazon: Why and who dies? Deaths from snakebites in the Amazon. Toxicon 2018, 145, 15–24. [CrossRef] [PubMed]

3. Wen, F.; Monteiro, W.; da Silva, A.M.; Tambourgi, D.; da Silva, I.M.; Sampaio, V.; dos Santos, M.; Sachett, J.; Ferreira, L.; Kalil, J.; et al. Snakebites and Scorpion Stings in the Brazilian Amazon: Identifying Research Priorities for a Largely Neglected Problem. PLoS Negl. Trop. Dis. 2015, 9, e0003701. [CrossRef]

4. Blomback, B. Studies on the action of thrombic enzymes on bovine fibrinogen as measured by N-terminal analysis. Ark. Kemii 1958, 12, 321–335.

5. Stocker, K.; Barlow, G.H. The coagulant enzyme from Bothrops atrox venom (Batroxobin). Methods Enzymol. 1976, 45, 214–223.

6. Freitas-De-Sousa, L.A.; Amazonas, D.R.; Sousa, L.F.; Sant’Anna, S.S.; Nishiyama, M.Y.; Serrano, S.M.T.; Junqueira-De-Azevedo, I.L.M.; Chalkidis, H.M.; Moura-Da-Silva, A.M.; Mourão, R.H.V. Comparison of venoms from wild and long-term captive Bothrops atrox snakes and characterization of Batroxrhagin, the predominant class PIII metalloproteinase from the venom of this species. Biochimie 2015, 118, 60–70. [CrossRef]

7. Freitas-de-sousa, L.A.; Colombini, M.; Lopes-Ferreira, M.; Serrano, S.M.T.; Moura-da-silva, A.M. Insights into the mechanisms involved in strong hemorrhage and dermonecrosis induced by atroxysin-1a, a PI-class snake venom metalloproteinase. Toxins 2017, 9, 239. [CrossRef]

8. Petretski, J.H.; Kanashiro, M.; Silva, C.P.; Alves, E.W.; Kipnis, T.L. Two related thrombin-like enzymes present in Bothrops atrox venom. Braz. J. Med. Biol. Res. 2000, 33, 1293–1300. [CrossRef]

9. Read, M.S.; Shermer, R.W.; Brinkhous, K.M. Venom coagglutinin: An activator of platelet aggregation dependent on von Willebrand factor. Proc. Natl. Acad. Sci. USA 1978, 75, 4514–4518. [CrossRef]

10. Hofmann, H.; Bon, C. Blood Coagulation Induced by the Venom of Bothrops atrox. 1. Identification, Purification, and Properties of a Prothrombin Activator. Biochemistry 1987, 26, 772–780. [CrossRef]

11. Hofmann, H.; Bon, C. Blood Coagulation Induced by the Venom of Bothrops atrox. 2. Identification, Purification, and Properties of Two Factor X Activators. Biochemistry 1987, 26, 780–787. [CrossRef] [PubMed]

12. Rosing, J.; Govers-Riemslag, J.W.; Yukelson, L.; Tans, G. Factor V activation and inactivation by venom proteases. Haemostasis 2002, 31, 241–246. [CrossRef] [PubMed]

13. Rucinski, B.; Niewiarowski, S.; Holt, J.C.; Soszka, T.; Knudsen, K.A. Batroxostatin, an Arg-Gly-Asp-containing peptide from Bothrops atrox, is a potent inhibitor of platelet aggregation and cell interaction with fibronectin. Biochim. Biophys. Acta 1990, 1054, 257–262. [CrossRef]
14. Niewiarowski, S.; Kirby, E.P.; Brudzynski, T.M.; Stocker, K. Thrombocytin, a serine protease from Bothrops atrox venom. 2. Interaction with platelets and plasma-coagulating factors. Biochemistry 1979, 18, 3570–3577. [CrossRef]
15. Cintra, A.C.O.; De Toni, L.G.B.; Sartim, M.A.; Franco, J.J.; Caetano, R.C.; Murakami, M.T.; Sampaio, S.V. Batroxase, a new metalloproteinase from B. atrox snake venom with strong fibrinolytic activity. Toxicon 2012, 60, 70–82. [CrossRef]
16. Oliveira, S.; Alves, E.; Santos, A.; Pereira, J.; Sarraff, L.; Nascimento, E.; de-Brito-Sousa, J.; Sampaio, V.; Lacerda, M.; Sachett, J.; et al. Factors Associated with Systemic Bleeding in Bothrops Envenomation in a Tertiary Hospital in the Brazilian Amazon. Toxins 2019, 11, 22. [CrossRef]
17. Mendonça-da-Silva, I.; Tavares, A.M.; Sachett, J.; Sardinha, J.F.; Zaparolli, L.; Santos, M.F.G.; Lacerda, M.; Monteiro, W.M. Safety and efficacy of a freeze-dried trivalent antivenom for snakebites in the Brazilian Amazon: An open randomized controlled phase IIb clinical trial. PLoS Negl. Trop. Dis. 2017, 11, e0006068. [CrossRef] [PubMed]
18. Pardal, P.P.O.; Souza, S.M.; Monteiro, M.R.C.C.; França, F.O.S.; Tomy, S.C.; Sano-Martins, I.S.; Sousa-e-Silva, M.C.C.; Colombini, M.; Kodera, N.F.; Moura-da-Silva, A.M.; et al. Clinical trial of two antivenoms for the treatment of Bothrops and Lachesis bites in the north eastern Amazon region of Brazil. Trans. R. Soc. Trop. Med. Hyg. 2004, 98, 28–42. [CrossRef]
19. De Oliveira, S.S.; Alves, E.C.; dos Santos Santos, A.; Nascimento, E.F.; Pereira, J.P.T.; da Silva, I.M.; Sachett, J.; dos Santos Ibiapina, H.N.; Sarraf, L.K.S.; Bernal, J.C.C.; et al. Bothrops snakebites in the Amazon: Recovery from hemostatic disorders after Brazilian antivenom therapy. Clin. Toxicol. 2019, 58, 266–274. [CrossRef] [PubMed]
20. Santoro, M.L.; Sano-Martins, I.S.; Fan, H.W.; Cardoso, J.L.; Theakston, R.D.G.; Warrell, D.A. Haematological evaluation of patients bitten by the jararaca, Bothrops jararaca, in Brazil. Toxicon 2008, 51, 1440–1448. [CrossRef] [PubMed]
21. Salazar, A.M.; Rodriguez-Acosta, A.; Girón, M.E.; Aguilar, I.; Guerrero, B. A comparative analysis of the clotting and fibrinolytic activities of the snake venom (Bothrops atrox) from different geographical areas in Venezuela. Thromb. Res. 2007, 120, 95–104. [CrossRef] [PubMed]
22. Sousa, L.F.; Zdenek, C.N.; Dobson, J.S.; Op den Brouw, B.; Coimbra, F.C.; Gillett, A.; Del-Rei, T.H.M.; Chalkidis, H.D.M.; Sant’Anna, S.; Teixeira-da-Rocha, M.M.; et al. Coagulotoxicity of Bothrops (Lancehead Pit-Vipers) Venoms from Brazil: Differential Biochemistry and Antivenom Efficacy Resulting from Prey-Driven Venom Variation. Toxins 2018, 10, 411. [CrossRef] [PubMed]
23. Sousa, L.F.; Portes-Junior, J.A.; Nicolau, C.A.; Bernardoni, J.L.; Nishiyama, M.Y., Jr.; Amazonas, D.R.; Freitas-de-Sousa, L.A.; Mourão, R.H.; Chalkidis, H.M.; Valente, R.H.; et al. Functional proteomic analyses of Bothrops atrox venom reveals phenotypes associated with habitat variation in the Amazon. J. Proteom. 2017, 159, 32–46. [CrossRef] [PubMed]
24. López-Lozano, J.L.; De Sousa, M.V.; Ricart, C.A.O.; Chávez-Olortegui, C.; Sanchez, E.F.; Muniz, E.G.; Bührnheim, P.F.; Morhy, L. Ontogenetic variation of metalloproteinases and plasma coagulant activity in venoms of wild Bothrops atrox specimens from Amazonian rain forest. Toxicon 2002, 40, 997–1006. [CrossRef]
25. Bernal, J.C.C.; Bisneto, P.F.; Pereira, J.P.T.; Ibiapina, H.N.D.S.; Sarraf, L.K.S.; Monteiro-Júnior, C.; da Silva Pereira, H.; Santos, B.; de Moura, V.M.; de Oliveira, S.S.; et al. “Bad things come in small packages”: Predicting venom-induced coagulopathy in Bothrops atrox bites using snake ontogenetic parameters. Clin. Toxicol. 2019, 58, 388–396. [CrossRef]
26. Sousa, L.F.; Nicolau, C.A.; Peixoto, P.S.; Bernardoni, J.L.; Oliveira, S.S.; Portes-Junior, J.A.; Mourão, R.H.V.; Lima-dos-Santos, I.; Sano-Martins, I.S.; Chalkidis, H.M.; et al. Comparison of Phylogeny, Venom Composition and Neutralization by Antivenom in Diverse Species of Bothrops Complex. PLoS Negl. Trop. Dis. 2013, 7. [CrossRef]
27. Kamiguti, A.S.; Cardoso, J.L.C.; Theakston, R.D.G.; Sano-Martins, I.S.; Hutton, R.A.; Rugman, F.P.; Warrell, D.A.; Hay, C.R.M. Coagulopathy and Haemorrhage in Human Victims. Toxicon 1991, 29, 961–972. [CrossRef]
28. Da Silva, A.M.; Colombini, M.; Moura-da-Silva, A.M.; de Souza, R.M.; Monteiro, W.M.; Bernarde, P.S. Epidemiological and clinical aspects of snakebites in the Upper Juruá River Region, Western Brazilian Amazonia. Acta Amaz. 2020, 50, 90–99. [CrossRef]
29. Hoffman, M. Remodeling the blood coagulation cascade. J. Thromb. Thrombolysis 2003, 16, 17–20. [CrossRef]
30. Crawley, J.T.B.; Zanardelli, S.; Chion, C.K.; Lane, D.A. The central role of thrombin in hemostasis. *J. Thromb. Haemost.* 2007, 5, 95–101. [CrossRef]

31. Yamashita, K.M.; Alves, A.F.; Barbaro, K.C.; Santoro, M.L. Bothrops jararaca Venom Metalloproteinases Are Essential for Coagulopathy and Increase Plasma Tissue Factor Levels during Envenomation. *PLoS Negl. Trop. Dis.* 2014, 8. [CrossRef] [PubMed]

32. Pereira, A.L.M.; Fritzzen, M.; Faria, F.; da Motta, G.; Chudzinski-Tavassi, A.M. Releasing or expression modulating mediator involved in hemostasis by Berythreactivase and Jararhagin (SVMPs). *Toxicon* 2006, 47, 788–796. [CrossRef] [PubMed]

33. Sartim, M.A.; Costa, T.R.; Laure, H.J.; Espindola, M.S.; Frantz, F.G.; Sorgi, C.A.; Cintra, A.C.O.; Arantes, E.C.; Facciolli, L.H.; Rosa, J.C.; et al. Moojenactivase, a novel pro-coagulant PIIId metalloprotease isolated from Bothrops moojeni snake venom, activates coagulation factors II and X and induces tissue factor up-regulation in leukocytes. *Arch. Toxicol.* 2016, 90, 1261–1278. [CrossRef] [PubMed]

34. Key, N.S.; MacKman, N. Tissue factor and its measurement in whole blood, plasma, and microparticles. *Semin. Thromb. Hemost.* 2010, 36, 865–875. [CrossRef] [PubMed]

35. Chen, V.M.; Hogg, P.J. Encryption and decryption of tissue factor. *J. Thromb. Haemost.* 2013, 11, 277–284. [CrossRef]

36. Jern, C.; Eriksson, E.; Tengborn, L.; Risberg, B.; Wadenvik, H.; Jern, S. Changes due to increased hepatic synthesis of these factors that may occur in stressful situations. *Thromb. Haemost.* 1989, 62, 767–771. [PubMed]

37. Isbister, G.K.; Maduwaqe, K.; Scorgie, F.E.; Shahmy, S.; Mohamed, F.; Abeysinghe, C.; Karunathilake, H.; O’Leary, M.A.; Ganathasan, C.A.; Lincz, I.F. Venom Concentrations and Clotting Factor Levels in a Prospective Cohort of Russell’s Viper Bites with Coagulopathy. *PLoS Negl. Trop. Dis.* 2015, 9, e0003968. [CrossRef]

38. Del-Rei, T.H.M.; Sousa, L.F.; Rocha, M.M.T.; Freitas-de-Sousa, L.A.; Travaglia-Cardoso, S.R.; Grego, K.; Sant’Anna, S.S.; Chalkidis, H.M.; Moura-da-Silva, A.M. Functional variability of Bothrops atrox venoms from three distinct areas across the Brazilian Amazon and consequences for human envenomings. *Toxicon* 2019, 164, 61–70. [CrossRef]

39. Klöcking, H.-P.; Hoffmann, A.; Markwardt, F. Release of Plasminogen Activator by Batroxobin. *Pathophysiol. Haemost. Thromb.* 1987, 17, 235–237. [CrossRef]

40. Kolev, K.; Longstaff, C. Bleeding related to disturbed fibrinolysis. *Br. J. Haematol.* 2016, 175, 12–23. [CrossRef]

41. Isbister, G.K. Snakebite doesn’t cause disseminated intravascular coagulation: Coagulopathy and thrombotic microangiopathy in snake envenoming. *Semin. Thromb. Hemost.* 2010, 36, 444–451. [CrossRef] [PubMed]

42. Mezzano, D.; Matus, V.; Sáez, C.G.; Pereira, J.; Panes, O. Tissue factor storage, synthesis and function in normal and activated human platelets. *Thromb. Res.* 2008, 122. [CrossRef]

43. Sachetto, A.T.A.; Rosa, J.G.; Santoro, M.L. Rutin (quercetin-3-rutinoside) modulates the hemostatic disturbances and redox imbalance induced by Bothrops jararaca snake venom in mice. *PLoS Negl. Trop. Dis.* 2018, 12, e0006774. [CrossRef] [PubMed]

44. Heemskerk, J.W.M.; Mattheij, N.J.A.; Cossemans, J.M.E.M. Platelet-based coagulation: Different populations, different functions. *J. Thromb. Haemost.* 2013, 11, 2–16. [CrossRef]

45. Loscalzo, J.; Pasche, B.; Oumiet, H.; Freedman, J. Platelets and plasminogen activation. *Thromb. Haemost.* 1995, 74, 291–293. [CrossRef]

46. Francischetti, I.M.; Castro, H.C.; Zingali, R.B.; Carlini, C.R.; Guimarães, J.A. Bothrops sp. snake venoms: Comparison of some biochemical and physicochemical properties and interference in platelet functions. *Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol.* 1998, 119, 21–29. [CrossRef]

47. Sano-Martins, I.S.; Santoro, M.L.; Castro, S.C.B.; Fan, H.W.; Cardoso, J.L.C.; Theakston, R.D.G. Platelet aggregation in patients bitten by the brazilian snake Bothrops jararaca. *Thromb. Res.* 1997, 87, 183–195. [CrossRef]

48. Cardoso, J.L.C.; Fan, H.W.; França, F.O.S.; Jorge, M.T.; Leite, R.P.; Nishioka, S.A.; Avila, A.; Sano-Martins, I.S.; Tomy, S.C.; Santoro, M.L.; et al. Randomized comparative trial of three antivenomsin the treatment of envenoming by lance-headed vipers (Bothrops jararaca) in São Paulo, Brazil. *Q. J. Med.* 1993, 86, 315–325.

49. Najibe, H.; Ibiapina, S.; Costa, A.G.; Almeida, J.; Sachett, G.; Tarragô, A.M.; Costa, J.; Neves, F.; Wendell, M.; Kerr, A.; et al. An Immunological Stairway to Severe Tissue Complication Assembly in Bothrops Atrox Snakebites. *Front. Immunol.* 2019, 10, 1882. [CrossRef]
50. Calvete, J.J.; Sanz, L.; Pérez, A.; Borges, A.; Vargas, A.M.; Lomonte, B.; Angulo, Y.; Gutiérrez, J.M.; Chalkidissi, H.M.; Mourão, R.H.V.; et al. Snake population venomics and antivenomics of Bothrops atrox: Paedomorphism along its transamazonian dispersal and implications of geographic venom variability on snakebite management. *J. Proteom.* 2011, 74, 510–527. [CrossRef]

51. Lu, Q.; Clemetson, J.M.; Clemetson, K.J. Snake venoms and hemostasis. *J. Thromb. Haemost.* 2005, 3, 1791–1799. [CrossRef] [PubMed]

52. Gutiérrez, J.M.; Rucavado, A.; Escalante, T.; Díaz, C. Hemorrhage induced by snake venom metalloproteinases: Biochemical and biophysical mechanisms involved in microvessel damage. *Toxicon* 2005, 45, 997–1011. [CrossRef] [PubMed]

53. Kamiguti, A.S.; Rugman, F.P.; Theakston, R.D.G.; Franca, F.O.S.; Ishii, H.; Hay, C.R.M.; BIASG. The role of venom haemorrhagin in spontaneous bleeding in Bothrops jararaca envenoming. *Thromb. Haemost.* 1992, 67, 484–488. [CrossRef] [PubMed]

54. Sachett, J.A.G.; da Silva, I.M.; Alves, E.C.; Oliveira, S.S.; Sampaio, V.S.; do Vale, F.F.; Romero, G.A.S.; dos Santos, M.C.; Marques, H.O.; Colombini, M.; et al. Poor efficacy of preemptive amoxicillin clavulanate for preventing secondary infection from Bothrops snakebites in the Brazilian Amazon: A randomized controlled clinical trial. *PLoS Negl. Trop. Dis.* 2017, 11, e0005745. [CrossRef] [PubMed]

55. Brazil; Ministry of Health; Secretariat of Health Surveillance; Department of Epidemiological Surveillance. Snakebite. In *Manual of Diagnosis and Treatment of Accidents by Venomous Animals*; FUNASA, Ed.; National Health Foundation: Brasilia, Spain, 2001; p. 120. ISBN 85-7346-014-8.

56. Brazil; Ministry of Health; Secretariat of Health Surveillance; General Coordination of Epidemiology Development in Services. Accidents for venomous animals. In *Guide to Health Surveillance*; Ministry of Health: Brasilia, Spain, 2019; pp. 1–740. ISBN 978-85-3342706-8.

57. Sano-Martins, I.S.; Fan, H.W.; Castro, S.C.B.; Tomy, S.C.; Franca, F.O.S.; Jorge, M.T.; Kamiguti, A.S.; Warrell, D.A.; Theakston, R.D.G. Biasg Reliability of the simple 20 minute whole blood clotting test (WBCT20) as an indicator of low plasma fibrinogen concentration in patients envenomed by Bothrops snakes. *Toxicon* 1994, 32, 1045–1050. [CrossRef]

58. Warrell, D.A.; Davidson, N.M.; Greenwood, B.M.; Ormerod, L.D.; Pope, H.M.; Watkins, B.J.; Prentice, C.R.M. Poisoning by bites of the saw-scaled or carpet viper (Echis carinata) in Nigeria. *Q. J. Med.* 1977, 46, 33–62.

59. Warrell, D.A.; Looareesuwan, S.; Theakston, R.D.G.; Phillips, R.E.; Chanthavanich, P.; Virivan, C.; Supanaranond, W.; Karbwang, J.; Ho, M.; Hutton, R.A.; et al. Randomized comparative trial of three monospecific antivenoms for bites by the Malayan pitviper (Calloselasma rhodostoma) in southern Thailand: Clinical and laboratory correlations. *Am. J. Trop. Med. Hyg.* 1986, 35, 1235–1247. [CrossRef]

60. Denson, K.W.E. The preparation of general reagents and coagulation factors. In *Human Blood Coagulation, Haemostasis and Thrombosis*; Biggs, R., Ed.; Blackwell Scientific: Oxford, UK, 1976; pp. 657–669.

61. Clauss, A. Gerinnungsphysiologische Schnellmethode zur Bestimmung des Fibrinogens. *Acta Haematol.* 1957, 17, 237–246. [CrossRef]

62. Taylor, R.; RDCS, E. Interpretation of the correlation coefficient: A basic review. *J. Diagn. Med. Sonogr.* 1990, 6, 35–39. [CrossRef]