UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"
FACULDADE DE MEDICINA DE BOTUCATU
PROGRAMA DE PÓS-GRADUAÇÃO EM DOENÇAS TROPICAIS
BOTUCATU, SÃO PAULO, BRASIL

Joeliton dos Santos Cavalcante

DANO TECIDUAL LOCAL CAUSADO POR VENENOS DE *Bothrops*: PERSPECTIVAS TROMBOINFLAMATÓRIAS USANDO PROTEÔMICA E BIOLOGIA DE SISTEMAS

Dissertação apresentada ao programa de Pós-Graduação em Doenças Tropicais da Faculdade de Medicina de Botucatu, Universidade Estadual Paulista "Júlio de Mesquita Filho", para obtenção do título de Mestre.

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"No final eu acabei acreditando em uma coisa que eu chamo de física da procura. A força in natura governada por leis tão verdadeiras quanto às leis da gravidade. A regra da física da procura é mais ou menos assim:

Se você for corajoso o bastante para deixar tudo para trás que é familiar e cômodo que pode ser qualquer coisa desde a sua casa até a amargura de antigos ressentimentos e planejar uma viagem de busca verdadeira tanto externa quanto interna... E se você estiver realmente disposto a considerar tudo o que acontecer com você nessa viagem como uma pista... E se você aceitar todos os que encontrar ao longo desse caminho como professores...E se estiver preparado acima de tudo a enfrentar e perdoar algumas realidades bem difíceis sobre si mesmo. A verdade não será negada a você."

— Liz Gilbert

“Se as pessoas querem que algo esteja errado com você - elas vão fazer coisas erradas sobre você. É por isso que acredito que nunca devo tentar provar nada a ninguém. Os diamantes verdadeiros pertencem a pessoas que sabem identificar um diamante real; e não a pessoas que precisam ser convencidas de que você é um diamante de verdade. São os idiotas que precisam ser convencidos de algo que eles ainda não podem ver.”

— C. JoyBell C.

Resumo

Com 5,4 milhões de picadas de serpentes em todo o mundo por ano, o envenenamento por serpentes é um sério problema de saúde que afeta 1,8-2,7 milhões de pessoas, causando 81.000-138.000 mortes e provocando mais de 400.000 casos de deficiências crônicas, como por exemplo, amputações. Proteínas, peptídeos e aminoácidos e compostos de baixa massa representam a principal composição dos venenos de serpentes, que podem causar necrose celular, hemólise, edema, inflamação, hemorragia, coagulopatia e, eventualmente, morte. A diversidade de serpentes e venenos, bem como sua variabilidade foi muito bem estudada para muitas espécies. No entanto, o mecanismo detalhado de ação do envenenamento por serpentes ainda é cheio de lacunas. Dessa forma, o objetivo desta pesquisa foi identificar proteínas do plasma sanguíneo de camundongos envenenados pelas serpentes *Bothrops atrox*, *B. erythromelas* e *B. leucurus*, as quais apresentassem abundâncias diferenciais quantitativamente quando comparadas com o plasma sanguíneo de camundongos não envenenados. Adicionalmente, este estudo também objetivou avaliar a presença de moléculas em potenciais no plasma sanguíneo de camundongos i) que possam responder pelo grau do efeito edematogênico dos venenos apontados e/ou ii) que possam servir de base para estudos futuros relacionados na distinção do agente causador do envenenamento botrópico. Assim, a estratégia investigativa adotada neste trabalho foi induzir edema na pata de camundongos Swiss com diferentes doses (1.25, 2.5 e 5 µg) dos venenos de *B. atrox*, *B. erythromelas* e *B. leucurus* e a partir do plasma sanguíneo destes animais, identificar e quantificar as proteínas utilizando a estratégia proteômica do tipo *Shotgun*, a qual é baseada na hidrólise em solução de todas as proteínas presentes nas amostras biológicas, sequenciamento dos fragmentos peptídicos por meio de um equipamento de espectrometria de massas Q-Exactive e análise dos dados por ferramentas de bioinformática. Os espectros de massas foram deconvoluídos e analisados por *softwares* de biologia de sistemas. Identificamos proteínas com diferenças na abundância (DAPs) responsáveis por descrever o grau de edema causado por esses venenos. Hemoglobina e suas subunidades (Hba, Hbb-b1/b2), peroxiredoxina 2 (Prdx2), fator IX (F9), fator de crescimento semelhante à insulina 1 (Igf1), fator de crescimento epidérmico contendo proteína de matriz extracelular tipo fibulina 1 (Efemp1) e fibulina (Fbln1) foram as principais responsáveis pela discriminação do grau do edema causado pelo veneno de *B. atrox.* As proteínas que podem servir para evidenciar o grau de edema causado pelo veneno de *B. erythromelas* foram: apolipoproteina A1 (Apoa), proteína amilóide sérica A-4 (Saa4), adiponectina (Adipoq), Fbln1, fator XII

(F12) e a proteína Z dependente de vitamina K (Proz). Serpinas, apolipoproteinas, fatores de complemento e suas subunidades, catepsinas, quinases, oxidoredutases, inibidores de proteases, proteases, colageno, fatores de crescimento explicaram os efeitos causados pelo envenenamento causado por *B. leucurus*. Assim, nota-se que as DAPs presentes nas três propostas de envenenamento experimental estão envolvidas em diversos processos e vias de sinalização tais como ativação do sistema complemento, cascata de coagulação, sistema lipídico, degranulação de plaquetas e neutrófilos e morte celular, contribuindo assim, com desfechos tromboinflamatórios. Em conclusão, os venenos das serpentes estudadas induzem alterações no padrão proteico do plasma dos camundongos envenenados. Estas alterações podem estar associadas com a variabilidade interespecífica dos venenos botrópicos e podem auxiliar a melhor compreender as manifestações fisiopatológicas do envenenamento experimental por serpentes.

Palavras-chave: Espectrometria de massas, biologia de sistemas, tromboinflamação.

Abstract

With 5.4 million snakebites worldwide each year, snake envenomation is a serious health problem that affects 1.8-2.7 million people, causing 81,000-138,000 deaths and causing over 400,000 cases of chronic disabilities, such as amputations. Proteins, peptides and amino acids and low-mass compounds represent the main composition of snake venoms, which can cause cell necrosis, hemolysis, edema, inflammation, hemorrhage, coagulopathy and, eventually, death. The diversity of snakes and venoms, as well as their variability has been very well studied for many species. However, the detailed mechanism of action of snake envenomation is still full of gaps. Thus, the objective of this research was identifying blood plasma proteins of mice envenomed by *Bothrops atrox, B. erythromelas and B. leucurus*, which presented quantitatively differential abundances when compared to the blood plasma of non-envenomed mice. Additionally, this study also aimed to evaluate the presence of potential molecules in the blood plasma of mice i) that could account for the degree of the edematogenic effect of the snake venoms indicated and/or ii) that could serve as a basis for future studies related to the distinction of the causative agent. of bothropic envenomation. Thus, the investigative strategy adopted in this work was inducing the edema in the paw of Swiss mice with different doses (1.25, 2.5 and 5 µg) of *B. atrox, B. erythromelas and B. leucurus* venoms and identify and quantify blood plasma proteins using the *Shotgun* proteomic strategy, which is based on the hydrolysis in solution of all proteins present in biological samples, sequencing of the peptide fragments using a Q-Exactive mass spectrometry equipment and data analysis by bioinformatics tools. The mass spectra were deconvoluted and analyzed by systems biology software. We identified proteins with differences in abundance (DAPs) responsible for describing the degree of edema caused by these venoms. Hemoglobin and its subunits (Hba, Hbb-b1/b2), peroxiredoxin 2 (Prdx2), factor IX (F9), insulin-like growth factor 1 (Igf1), epidermal growth factor containing fibulin-like extracellular matrix protein 1 (Efemp1) and fibulin (Fbln1) were the main responsible for describing the degree of edema caused by *B. atrox* venom. The proteins that may serve to demonstrate the degree of edema caused by *B. erythromelas* venom were: apolipoprotein A1 (Apoa), serum amyloid protein A-4 (Saa4), adiponectin (Adipoq), Fbln1, factor XII (F12) and Vitamin K-dependent protein Z (Proz). Serpins, apolipoproteins, complement factors and component subunits, cathepsins, kinases, oxidoreductases, protease inhibitors, proteases, collagens, growth factors have explained the effects caused by *B. leucurus* envenomation. Thus, it is noted that the DAPs present in the three experimental

envenomation proposals are involved in several processes and signaling pathways such as activation of the complement system, coagulation cascade, lipid system, platelet and neutrophil degranulation and cell death, thus contributing, with thromboinflammatory outcomes. In conclusion, the snake venoms have studied inducing alterations in the protein pattern of the plasma of the injured mice. These changes may be associated with the interspecific variability of bothropic venoms and may help to better understand the physiopathological manifestations of experimental snake envenomation.

Keywords: Mass spectrometry, Systems biology, Thromboinflammation.

Sumário

Introdução

Envenenamentos por serpentes

Os envenenamentos por serpentes têm representado um dos principais problemas de saúde pública negligenciadas em países tropicais, o que a caracteriza uma doença tropical dos países em desenvolvimento (Geneviève et al., 2018; Longbottom et al., 2018), especialmente em alguns países asiáticos e africanos (Alirol et al., 2010; Williams et al., 2011), latino-americanos (Chipaux, 2017) e oceânicos (Johnston et al., 2017). Aproximadamente 2,7 milhões de pessoas podem ser acometidas por serpentes anualmente, resultando em 81.000 - 138.000 casos fatais e 400.000 casos de morbidade. Estima-se que aproximadamente 6,5 bilhões de pessoas vivem dentro da faixa de risco para uma ou mais espécies de serpentes consideradas nocivas ao homem (Longbottom et al., 2018).

A subnotificação e divergências entre os sistemas de notificações brasileiros (Sistema de Informação de Agravos de Notificação – SINAN e Sistema de Informações sobre Mortalidade - SIM) frente aos acidentes ofídicos, compromete os esforços em mensurar o tamanho real desse problema (Bochner and Souza, 2019) e tomadas de decisões pelos órgãos competentes. A maior parte dos envenenamentos por serpentes no mundo são geralmente registrados em áreas rurais e geograficamente de difícil acesso que, por sua vez, faz com que a vitima venha a receber o atendimento hospitalar adequado tardio, o que pode acarretar no desfecho clínico (Kasturiratne et al., 2008; Kasturiratne et al., 2017; Longbottom et al., 2018).

Com o aumento dos acidentes ofídicos ao longo dos anos, a Organização Mundial da Saúde (*World Health Organization* - WHO) incluiu esta doença na categoria A das doenças negligenciadas em 2017 (Chippaux, 2017). A partir de 2019, estratégias ao combate do ofidismo com a proposta de redução de cerca de 50% dos acidentes por serpentes até 2030, foram elaboradas (Minghui et al., 2019). Dentre os direcionamentos estão: (i) especificar os requisitos para a produção de antivenenos regionais; (ii) educação da população em risco; (iii) acessibilidade e redução de custos dos antivenenos e (iv) treinamento de profissionais da saúde pública no acolhimento e tratamento dos pacientes acidentados por serpentes (WHO, 2019 a, b).

O maior número de envenenamentos por serpentes tem sido identificado no sul da Ásia e na África subsariana. Os sistemas de notificação de saúde africanos indicam que a incidência de envenenamento por serpentes na África é de aproximadamente de 315.000 por ano. Desse total, mais de 7000 casos resultam em amputações e entre 7.000 – 32.000 óbitos (Appiah, 2012). É válido ressaltar, que não se sabe ao certo a incidência, mortalidade e

número de amputações por envenenamentos ofídicos na África pela possibilidade da subnotificação destes acidentes e a frequente a excassez de antivenenos nas instituições de saúde (Schiermeier, 2015). A Índia tem a maior incidência de mortalidade por serpentes que vão de 13.000 a 50.000 casos por ano (Alirol et al., 2010; Warrell, 2010; Mohapatra et al., 2011). Na África subsariana a incidência anual de envenenamentos por serpentes é calculada em 75.5 e 5.2 envenenamentos por 100.000 habitantes em áreas rurais e urbanas, respectivamente. Quanto à mortalidade, em áreas urbanas está estimada em 0.02-0.1 óbitos por 100.000 pessoas e 0.96-1.75 óbitos por 100.000 pessoas em áreas rurais (Chippaux, 2011).

Nas Américas, a taxa de mortalidade é perceptivelmente menor quando comparada a outros países apresentando uma incidência anual média de 57.500 picadas de serpentes (6.2 por 100.000 habitantes) e a mortalidade próxima de 370 casos (0.04 por 100.000 habitantes). Todavia as taxas apresentam grandes variações entre e dentro desses países (Chippaux, 2017). No Brasil o número médio de picadas de cobra foi de cerca de 27.200 por ano (15 por 100.000 habitantes). O gênero *Bothrops* é o principal responsável pelos casos de envenenamentos humanos, mais de 70% dos casos estão atribuídos a essas espécies devido à ampla distribuição (Chippaux 2017 b). Atualmente, 36 espécies de *Bothrops* foram catalogadas com grande variabilidade morfológica, e também, na composição do veneno (Alencar et al., 2016 ; Carrasco et al., 2016, Ferraz et al. , 2019; Tasoulins and Isbister, 2017).

Na região Amazônia a espécie *Bothrops atrox* é responsável pela maioria dos casos de envenenamentos, perda tecidual/amputações e mortes na região (da Silva Souza et al., 2018; de Oliveira et al., 2016; Roriz et al., 2018). Por outro lado, no Nordeste brasileiro, *B. leucurus* é a espécie de viperídeo mais comum na faixa atlântica desta região até agora registrada desde os estados do Ceará até o Espírito Santo, é a principal serpente envolvida com acidentes ofídicos na região (Lira-da-Silva et al., 2009; Mise et al., 2016). Além disso, *B. erythromelas* é a outra espécie de relevância médica para o Nordeste que é endêmica da Caatinga (Lira-da-Silva et al., 2009; Albuquerque et al. al., al., 2020).

Os envenenamentos por *Bothrops* apresentam aspectos clínicos que são replicados no envenenamento causado por outras espécies do gênero, entre eles: dor, edema, formação de bolhas, mionecrose, lesão vascular, isquemia, necrose e distúrbios da coagulação (Cavalcante et al., 2021; Silva et al., 2019; da Costa et al., 2020; Oliveira et al., 2020a; Oliveira et al., 2020b). Embora seja reconhecida a ampla variabilidade no proteoma do veneno dessas espécies, esse padrão de manifestações clínicas, embora de intensidade

variável, sugere a presença de um mecanismo de degradação tecidual compartilhado resultando principalmente em síndromes compartimentais, lesão tecidual local intensa e amputações (Luciano et al., 2009; Schulz et al., 2016; Graciano and Carvalho, 2018; Valle et al., 2018).

Efeitos tóxicos causados pelos venenos de *Bothrops*

Efeitos locais

Os venenos animais são misturas complexas de proteínas, peptídeos e aminoácidos (Calvete, 2017). Os venenos de serpentes da família Viperidae, gênero *Bothrops*, apresentam majoritariamente em sua composição metaloproteases dependentes de zinco (SVMPs), fosfolipases A_2 (PLA_2s), e serinoproteases (SVSPs), responsáveis por manifestações locais e sistêmicas (Tasoulis and Isbister, 2017; Gutiérrez et al., 2017; Gutiérrez et al., 2018; Sanchez et al., 2017). O conjunto destas moléculas presentes nos venenos botrópicos são responsáveis por diversos desfechos clínicos como dano tecidual local, tais como bolhas, comprometimento da junção derme-epiderme, degradação de componentes da matriz extracelular, dano ao sistema de vasos que irrigam os tecidos locais e, por fim, a degradação e necrose do tecido muscular (Gutiérrez et al., 2018). Venenos de *Bothrops* podem causar sequelas permanentes em decorrência do dano tecidual que apresentam uma doença diversificada e complexa (Gutiérrez et al., 2018 a e b). Em parte, esse dano é causado pela ação de SVMPs, PLA_2s e ativação endógena de proteinases pela lise celular, que consequentemente, libera o conteúdo proteico citoplasmático moduladas pela ação citotóxica das toxinas presentes nesses venenos (Rucavado et al., 2016; Almeida et al., 2020; Bickler, 2020).

A formação de bolhas e dano epitelial são achados comuns nos pacientes acometidos, que é causado ação de SVMPs de classe P-I, desprovidas de efeito hemorrágico, mas que experimentalmente induzem a separação das camadas da pele (derme-epiderme) (Gutiérrez et al., 2016). Entretanto, a identificação dos fatores chaves determinantes da separação derme-epiderme ainda permanecem desconhecidas (Gutiérrez et al., 2018 b). A dor também é comumente relatada nos envenenamentos por *Bothrops*, cujo sua causas estão associadas principalmente com a produção de compostos neurogênicos e não neurogênicos no local da picada, que se dá pela ação direta e/ou indireta de toxinas dos venenos de *Bothrops*, além da ativação de celulas não neurais que contribuem para a sensibilização periférica (Cavalcante et al., 2021). Esses compostos interagem com seus respectivos receptores alterando a

transdução do sinal nociceptivo. Alguns desses componentes já foram identificados, entre elas: substância P, óxido nítrico, citocinas, histamina, 5-hidroxitriptamina, bradicinina, mediadores derivados de lipídios (prostaglandinas, leucotrienos e PAF), bem como a participação de células leucocitárias produzidos durante a resposta inflamatória (Herrera et al., 2018; Zambelli et al., 2017).

Outro alvo da ação de toxinas incluem o sistema de vasos do organismo. Artérias, veias e outros vasos do sistema circulatório sofrem alterações na permeabilidade e integridade, principalmente pela ação de SVMPs que atuam na membrana basal vascular, constituída principalmente por colágeno tipo IV, laminina, nidogênio/entactina e perlecan o que resulta na formação de lesões hemorrágicas (equimoses e patequias) (Baldo et al., 2010; Herrera et al., 2015; Gutiérrez et al., 2016 a, b). Além disso, outras toxinas também atuam nos vasos sanguíneos, como por exemplo, as hialuronidases que atuam em capilares e espaços intersticiais, e desintegrinas que atuam nos espaços interticiais afrouxando o tecido de ancoragem (Bickler, 2020).

Dessa forma, a ação hidrolítica de toxinas do veneno sobre esses componentes, comprometeria a estabilidade mecânica do endotélio resultando em sangramento. Outro mecanismo que parece contribuir para a hemorragia é a incoagulabilidade sanguínea. As toxinas do veneno catalisam a ativação de zimogênios de fatores da coagulação e precursores das integrinas ou receptores o que resulta, consequente, em uma coagulação intravascular disseminada seguida de uma coagulopatias de consumo (Kini and Koh, 2016). Entretanto, a relação entre este fenômeno e a hemorragia ainda não estão bem esclarecidos.

Adicionalmente, o tecido muscular esquelético é outro alvo da ação de toxinas que induzem um quadro de degeneração muscular e mionecrose (Gutiérrez et al., 2018), manifestações no tecido muscular que culminam em sequelas temporárias e, em casos mais graves, amputação. O quadro de dano muscular é conferido pela ação de miototoxinas, Asp49 e Lys49, que atuam de forma sinérgica alterando o influxo de íons Ca^{2+}, induzindo consequentemente, a morte celular (Mora-Obando et al., 2014; Sunitha et al., 2015).

Efeitos sistêmicos

A incoagulabilidade sanguinea é o efeito sistêmico predominante nos envenenamentos por *Bothrops* (Tavares et al., 2017; Silva et al., 2019; Oliveira et al., 2020; Silva de Oliveira et al., 2020). Esse efeito é deflagrado pelas toxinas pró-coagulantes, que catalisam a ativação de diversos zimógenos da cascata de coagulação, seguido da incoagulabilidade pelo

consumo destes fatores de forma exarcebada. Com isso, os venenos de *Bothrops* induzem sangramentos no local da picada, pelas gengivas, e em orgãos vitais (Oliveira et al., 2019). Além disso, os envenenamentos por *Bothrops* apresentam trombocitopenia, causadas provavelmente pela ação de toxinas sobre as plaquetas, bem como formar uma superfície ativa para a cascata de coagulação, o que leva ao aumento de sangramentos (Oliveira et al., 2020).

A miotoxicidade sistêmica é caracterizada por aumentos na concentração sérica de mioglobina e na atividade da creatina quinase (CK), hipercalemia, e também lesão renal aguda, principalmente devido à toxicidade da mioglobina nos túbulos renais (Albuquerque et al., 2020; Gutiérrez et al., 2018). O veneno deflagra estresse oxidativo caracterizado pelo aumento de peroxidação lipídica, atividade da catalase e da glutationa-S-transferase no fígado, bem como aumento dos níveis plasmáticos de aspartato aminotransferase (AST) e alanina aminotransferase (ALT), indicando hepatotoxicidade (Sunitha et al., 2015). Além disso, alterações pulmonares - insuficiência respiratória/edema agudo de pulmão - foram relatadas como as complicações sistêmicas associadas ao óbito em casos de envenenamento por *B. atrox* (da Silva Souza et al., 2018).

Os sistemas em cascatas (complemento, coagulação e fibrinolíse) e as plaquetas, células endoteliais e leucócitos estão envolvidos na resposta imediata a venenos de serpentes (Oliveira et al., 2020; Slagboom et al., 2020; Pidde-Queiroz et al., 2010; Teixeira et al., 2019; Zuliani et al., 2020). Classicamente vistos como dois sistemas independentes, as discussões sobre sua inter-relação tem sido relacionadas a outras doenças humanas, que assim como os venenos de *Bothrops*, disparam múltiplos eventos em cascata que contribuiem para a amplificação da doença (Foley and Conway, 2016; de Bont et al., 2019; Kohli and Isermann, 2021; Bickler, 2020).

A caracterização dos envenenamentos por *Bothrops* como um estado pró-trombótico, inflamatório com a ativação de múltiplas células do sangue é apoiado por estudos experimentais, clínicos e laboratoriais, bem por relatos de caso (Cañas, 2016; Sachett et al., 2020). Do ponto de vista experimental, diversos componentes do sistema hemostático já foram caracterizados como alvos diretos e/ou indiretos de venenos de *Bothrops* e/ou suas toxinas (Cintra et al., 2012; de Oliveira et al., 2013; Vivas-Ruiz et al., 2020; Vivas-Ruiz et al., 2013). O mesmo pode se afirmar para o sistema complemento, cujo diferentes venenos tem a capacidade de clivar efetores chaves causando a ativação do complemento pela via clássica, alternativa ou das lectinas (Pidde-Queiroz et al., 2010).

Evidências apoiam o cruzamento da inflamação com hemostasia: (i) estudos em modelos animais que relatam disturbios da hemostasia; apesar de alguma heterogeneidade dentro do modelo, e do veneno, um sistema hemostático menos eficaz está associado a um aumento de manifestações hemorrágicas (Oliveira et al., 2020); (ii) demonstrações de que componentes do sistema de coagulação, como as plaquetas, também sinalizam por vias por vias imunologicas (Teixeira et al., 2019); (iii) exemplos de toxinas e venenos que alteram a hemostasia e induzem inflamação (Malange et al., 2019; Rudresha et al., 2021; Freitas de Sousa et al., 2015; Almeida et al., 2020) e (iv) estudos mostrando que os leucócitos e plaquetas não são encontrados apenas no sitio do envenenamento, mas também em trombos arteriais e venosos (Santoro and Sano-Martins, 2004).

A formação de agregados plaquetários-leucocitários, que é considerada um evento importante em vários contextos de tromboinflamação, é mediada pela via alternativa do complemento e, especificamente, por seu regulador properdina, sugerindo que o complemento pode desempenhar um papel na trombocitopenia (Riedl et al., 2017; Blatt et al., 2016). Embora a formação de agregados plaquetários-leucocitários seja um feito já relatado experimentalmente no envenenamento por *B. jararaca* (Santoro and Sano-Martins, 2004), a reprodução desse fenomeno pelo veneno de outras espécies, mecanismos pelos quais os venenos de *Bothrops* induzem trombocitopenia e a possível relação do sistema complemento sobre plaquetas necessitam de elucidação.

Proteômica e biologia de sistemas aplicadas ao estudo do envenenamento por serpentes

Muitos são os estudos analíticos sobre a composição de toxinas dos venenos de serpentes na última década, principalmente utilizando metodologias aliadas à técnica de espectrometria de massas que, têm evidenciado as variações inter e intra-específicas destes venenos (Tasoulis and Isbister, 2017). A complexidade (diversidade qualitativa e quantitativa) dos componentes dos venenos ofídicos aliada às ferramentas investigativas convencionais utilizadas para compreender a fisiopatologia do envenenamento ofídico, representam verdadeiros parceiros em potenciais na resolução dos diversos desafios desta doença tropical.

Diferentes técnicas analíticas fazem parte da estratégia investigativa denominada Análise Proteômica, as quais podem ser utilizadas de forma isolada ou combinada, a fim de permitir melhor conhecer a composição das misturas complexas são os venenos animais (Calvete 2017, Lomonte and Calvete 2017). A inserção destas ferramentas analíticas resultou

na geração de muitos informações relevantes que podem ser utilizadas para correlacionar com os efeitos, diagnósticos e tratamentos dos acidentes ofídicos (Tasoulis and Isbister, 2017).

E como pode ser definido a estratégia investigativa Análise Proteômica? E quais são as principais ferramentas analíticas adotadas nesta abordagem? A proteômica é o estudo em larga escala de proteínas produzidas em um organismo, sistema ou contexto biológico num determinado momento para entender os mecanismos de doenças, vias celulares e dinâmica das organelas (Lundemberg and Borner, 2019). É sabido que o desenvolvimento da instrumentação analítica para a investigação de proteínas dos venenos animais, permitiram aplicá-las no campo da 'venômica' (Melani et al., 2017; Patra et al., 2017; Andrade-Silva et al., 2018; Ghezellou et al., 2019), área esta responsável por identificar e quantificar as toxinas presentes nos venenos animais. Porém, embora a utilização da aboragem proteômica seja incidente na identificação de proteinas/toxinas nos venenos de serpentes e corrrelação com as manifestações clínicas do acidente ofídico, poucos são os estudos que elucidam os procesos biológicos e vias de sinalizações envolvidas na fisiopatologia dos envenenamentos por serpentes (Paes-Leme et al, 2012).

Neste contexto, sabe-se que camundongos são modelos animais muito utilizados em pesquisas experimentais seguidos de análises proteômicas para melhor compreender as alterações patológicas que ocorrem em um tecido ou órgão ou em fluídos corpóreos (Bhavnani et al. 2015, Geyer et al., 2016; Herrera et al. 2018) seja para correlacionar os efeitos ocasionados pelos venenos ofídicos (Herrera et al. 2016, Rucavado et al. 2016 , Herrera et al. 2018, Macedo et al. 2019) seja para avaliar a eficácia neutralizante de antivenenos (Rucavado et al. 2012; Uzozie e Aebersold 2018).

A espectrometria de massa (MS) é a técnica analítica central da abordagem proteômica, a qual é representa uma balança analítica de elevada sensibilidade e resolução, que mede a razão massa/carga (m/z) das moléculas, onde m é a massa molecular em Daltons e z é a quantidade de carga dos íons das moléculas presentes em uma determinada amostra (Martins et al., 2022). MS pode ser usado para identificar com precisão analitos desconhecidos, quantificar compostos conhecidos e determinar propriedades estruturais e químicas de moléculas (Klassen et al., 2017; Haag et al., 2016).

A espectrometria de massa é a principal ferramenta que auxiliou na exploração e catalogação de toxinas de venenos de várias espécies de serpentes, devido ao fato de os venenos serem adequados para uma abordagem investigativa (Tasoulis and Isbister, 2017).

A análise proteômica baseada em MS é empregada por dois métodos: *Botton-up* e *Top-down*. De forma simplificada, a proteômica *Top-down* envolve a análise de proteínas intactas (Bharucha et al., 2019); mas, como muitas dessas moléculas associadas a doenças humanas apresentam massas semelhantes, seu uso pode ser ineficaz para subtipagens distintas (Catherman et al., 2014). Por outro lado, a proteômica *Bottom-up* não analisa a proteína intacta, mas sim, seus fragmentos peptídicos, o que requer etapas de processamentos prévios a MS como a hidrólise enzimática da mistura de proteínas nas amostras biológicas, criando uma mistura de fragmentos peptídeos a serem analisados (Dupree et al., 2020). Uma peptidase (enzima) comumente usada nos estudos *Botton-up* é a tripsina, a que cliva proteínas em sótios ativos específicos (na extremidade carboxila dos aminoácidos lisina e arginina, exceto quando são seguidos por prolina) (Dupree et al., 2020).

A individualização de fragmentos peptídos por cromatografia líquida previdamente a espectrometria de massas tem sido amplamente utilizada para uma identificação mais eficaz de proteínas em um diagnóstico, por exemplo (Neubert et al., 2020). A quantificação da abundância de proteínas tem sido uma das principais aplicações da proteômica; entretanto, a manutenção da acurácia pode ser afetada por vieses e erros que podem ocorrer em muitas etapas do processo experimental. Assim, estratégias de normalização são essenciais para tentar superar esse viés e devolver a amostra à sua condição biológica perante a problemática a ser investigada (O'Rourke et al., 2019).

Sabe-se que as proteínas estão inseridas em complexos proteicos e vias de sinalizações durante uma condição a qual o organismo está exposto. Com o avanço das estratégias de análises empregadas na biologia de sistemas, é possível identificar redes celulares e proteínas em potenciais que são alteradas de acordo com a abundância em uma amostra biológica (Safari -Alighiarloo et al., 2014). A modelagem de redes biológicas, vias e enriquecimento de processos é um dos aspectos mais interessantes e recentes da biologia de sistemas, que permite a projeção e análise de múltiplos aspectos fisiopatológicos, a partir de um banco de proteínas de interesse obtido pela espectrometria de massas (Patel et al., 2017).

Uma das principais análises que tem contribuído para a associação de proteínas (produtos gênicos) a uma determinada doença é a anotação de ontologia gênica (GO) que podem auxiliar na compreensão dos efeitos patológicos ao longo do envenenamento, conforme relatado por Montoni e colaboradores (2020). A Base de Dados para Anotação, Visualização e Descoberta Integrada (DAVID) permite a análise de longas listas de genes, e

tem como princípio o enriquecimento de genes que coopera em um (ou mais) determinado grupo(s) e/ou processo(s) biológico(s) que estão funcionalmente relacionados a um gene/proteína alterado durante uma determinada condição (Denis et al., 2003). O algoritmo GOplot permite, com base dos dados gerados, a construção de imagens que, por sua vez, possibilita o usuário examinar rapidamente grandes quantidades de dados, expondo tendências/padrões e correlações de dados. GOplot segue um padrão estrutural hierárquico para análise, começando com um quadro geral dos conjuntos de termos analisados e limitando-se a subconjuntos de genes definidos e termos selecionados que podem auxiliar na confirmação da falsificação de hipóteses (Walter et al., 2015).

Sabe-se que compreensão da estrutura da rede de interação proteína fornece uma dimensão da maquinaria bioquímica responsável pela gênese, manutenção e progressão de uma doença. Uma das ferramentas de visualização mais recentes é por meio da ferramenta Metascape, um algoritmo que trabalham com listas de genes/proteínas de interesse, combinando enriquecimento funcional, análise de interação e anotação de genes, sendo uma ótima opção para análises sob o ponto de vista da biologia de sistemas (Zhou et al., 2019).

JUSTIFICATIVA

Muitos são os desafios enfrentados no âmbito dos envenenamentos por serpentes entre eles estão: o tempo decorrido entre o acidente e a chegada da vítima nas instituições de saúde, as incertezas sobre qual a espécie que causou o envenenamento ofídico (de Castañeda et al., 2019), qual a quantidade de veneno circulante no organismo da vítima (Daswani et al., 2017), variabilidade inter e/ou intraespecífica dos venenos ofídicos (Del-Rei et al., 2019), capacitação/experiência da equipe responsável pela conduta clínica em casos dos envenenamentos ofídicos (Gutiérrez et al., 2015), disponibilidade de antivenenos específicos nas unidades de saúde, além de medidas culturais tomadas pela vítima ou por familiares e amigos como torniquete no membro acidentado e/ou uso de substâncias no local da picada etc (Fry, 2018; Mahmood et., 2019). Todos estes aspectos podem comprometer a recuperação do paciente e/ou evitar sequelas e óbito.

Paralelamente, pouco se sabe sobre as alterações do perfil proteico do plasma sanguíneo de vertebrados relacionadas aos efeitos fisiopatológicos dos envenenamentos ofídicos. Assim, conhecer a dinâmica destas proteínas alteradas no plasma pode contribuir com uma melhor compreensão das manifestações clínicas relacionadas com o grau do envenenamento ofídico. Adicionalmente, estes desdobramentos científicos podem também abrir caminhos para o desenvolvimento de futuras ferramentas diagnósticas capazes de distinguir o gênero de serpentes causadora do acidente ofidico. Uma vez que estudos proteômicos, propiciaram o monitoramento da progressão de uma determinada doença, por meio da presença ou não e/ou da quantidade de componentes proteicos plasmáticos como consequência das possíveis desordens fisiopatológicas (Ray et al., 2014), a estratégia investigativa aqui proposta apresenta um potencial valioso para as finalidades apontadas e perante venenos de serpentes brasileiras negligenciadas/não participantes no pool de imunização dos animais soroprodutores dos antivenenos comerciais brasileiros.

OBJETIVOS

Dessa forma, o objetivo primário desta pesquisa foi identificar proteínas do plasma sanguíneo de camundongos envenenados pelas serpentes *Bothrops atrox, B. erythromelas* e *B. leucurus,* as quais apresentassem abundâncias diferenciais quantitativamente quando comparadas com o plasma sanguíneo de camundongos não envenenados. De forma secundária, e não menos importante, este estudo também objetivou avaliar a presença de moléculas em potenciais (candidatos à biomarcadores) no plasma sanguíneo de

camundongos a serem validados futuramente i) que possam responder pelo grau do efeito edematogênico dos venenos apontados e/ou ii) que possam servir de base para estudos futuros relacionados na distinção do agente causador do envenenamento botrópico. Assim, a estratégia investigativa adotada neste trabalho foi induzir edema de pata em camundongos Swiss com diferentes doses (1.25, 2.5 e 5 µg) dos venenos de *B. atrox*, *B. erythromelas* e *B. leucurus* e a partir do plasma sanguíneo destes animais, identificar e quantificar as proteínas utilizando a estratégia proteômica do tipo *Shotgun*, a qual foi baseada na hidrólise em solução de todas as proteínas presentes nas amostras biológicas, sequenciamento dos fragmentos peptídicos por meio de um equipamento de espectrometria de massas Q-trap/TOF e análise dos dados por ferramentas de bioinformática.

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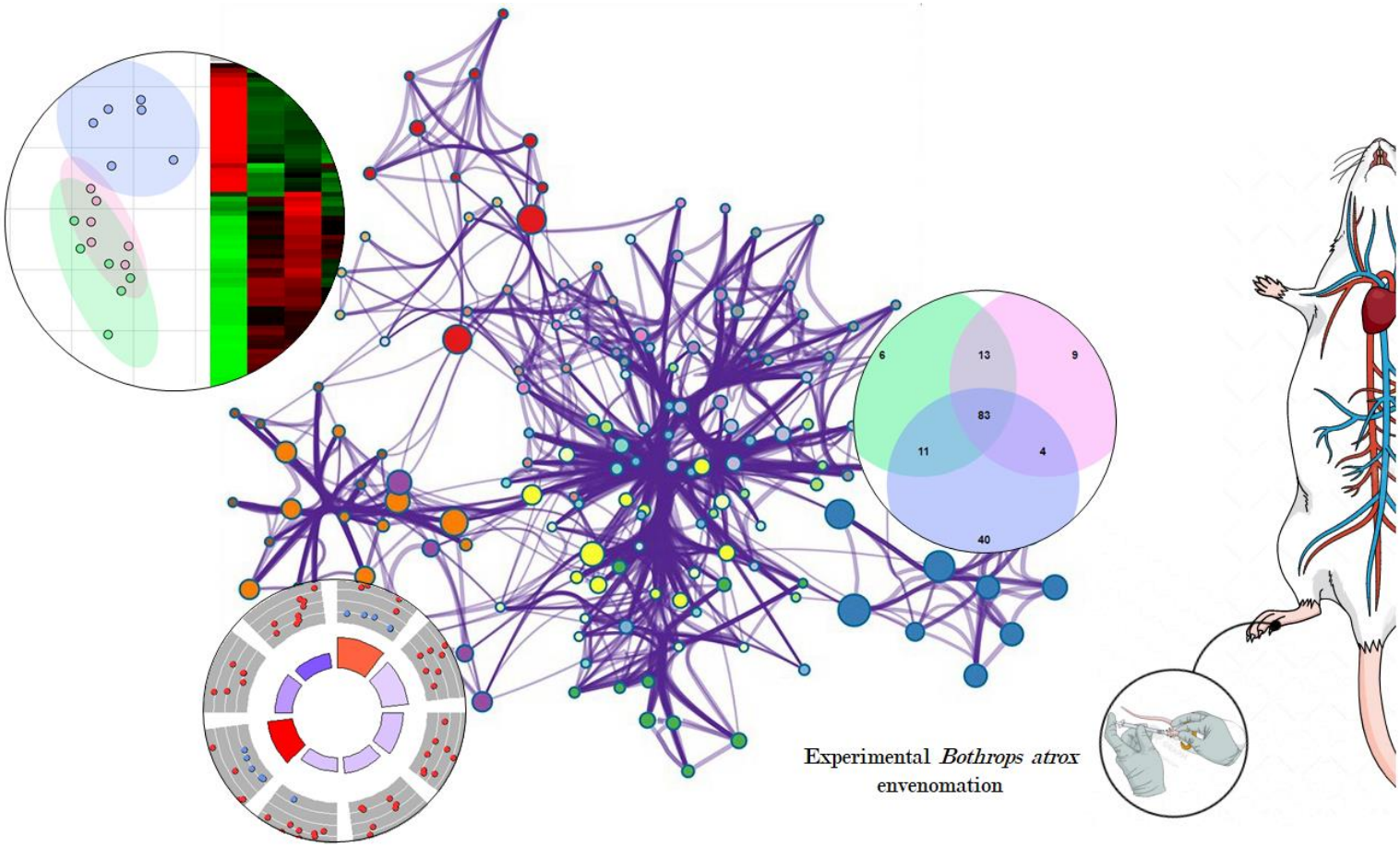
02

Bothrops atrox experimental envenomation: blood plasma proteome effects after local tissue damage and perspectives on thromboinflammation



Graphical abstract was developed by Microsoft PowerPoint (Journal requirement). This figure represents a summary of the main findings of the study.

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Bothrops atrox experimental envenomation: blood plasma proteome effects after local tissue damage and perspectives on thromboinflammation

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Highlights

- Plasma proteome profile is related to the percentage of edema caused by *B. atrox* venom.
- Prdx2, Hba and F9 may represent biomarkers for degree of edema by *B. atrox.*
- Blood plasma proteins from pathways that regulate cell survival were altered.

Abstract

The clinical manifestations of *Bothrops atrox* envenoming involve local and systemic changes, among which, edema requires substantial attention due to its ability to progress to compartmental syndromes, and sometimes, causing tissue loss and amputations. Thus, the present study aimed to explore the systemic pathological and inflammatory events that are altered by intraplantar injection of *B. atrox* venom in mice. Plasma samples collected showed differential abundance proteins related to complement, coagulation, lipid system, platelet and neutrophil degranulation and pathways related to cell death and ischemic tolerance. The plasma levels of some proteins identified in this study, including : hemoglobin and subunits (Hba, Hbb-b1/b2), peroxiredoxin 2 (Prdx2), factor IX (F9), insulin-like growth factor 1 (Igf1), epidermal growth factor containing fibulin-like extracellular matrix protein 1 (Efemp1) and fibulin (Fbln1), showed a unique tendency to increase or decrease in their abundance among the different groups, and therefore, may serve as potential indicators of the severity of edema/inflammation in mice caused by *B. atrox* venom. Thus, we emphasize the pathophysiology induced by *B. atrox* venom results from the combination of the action of toxins associated with the co-option of endogenous signaling pathways. Furthermore, *B. atrox* venom induce a different pattern of blood proteome alterations of mice according to the severity of the edema.

Keywords: Snake venom; *Bothrops atrox;* Edema; Proteomic analysis; Inflammation, Thromboinflammation.

1. Introduction

In the Amazon region, *Bothrops atrox* is responsible for most cases of human envenomation, and represents the greatest burden due to the seriousness of the cases related to the prolonged time between the accident and the arrival of patients in the hospital service with available antivenom (Silva et al., 2019; Monteiro et al., 2020). *B. atrox* snake venom has shown high variability, however snake venom metalloproteases (SVMPs), snake venom serineproteases (SVSPs) and phospholipases A2 are the main toxins of venom, corresponding to most of its composition. L-amino acid oxidase (LAAO), cysteine-rich secretory protein (CRiSP), C-type lectins and C-type lectin like (CTL/SNACLEC), disintegrin (DISI) and natriuretic peptides (NP), including vasoactive peptides, bradykinin potentiating and inhibitory peptides can also be found (Sousa et al., 2017; Tasoulis and Isbister, 2017).

Snake venoms are cocktail of enzymatic and non-enzymatic proteins, peptides and other highly complex components that trigger a wide range of local manifestations - pain, edema, hematoma, bleeding at the bite site, blisters, secondary infection, cellulitis, local lymphadenopathy, necrosis, abscess, compartment syndrome – and systemic ones, such as hemostatic disorders, hemorrhages and acute renal failure (dos Santos Cavalcante et al., 2021; Silva et al., 2019; Oliveira et al., 2020; Ibiapina et al., 2019. Silva et al., 2021).

However, the local and systemic pathophysiology by snake venom is not restricted to a condition mediated by the direct action of toxins, but also by the body's reaction to them. Based on converging biochemical and physiological information, it was possible to determine which endogenous processes allows venoms to make use of the physiological machinery of victim to amplify their toxicity (Bickler, 2020). The most important effects of the venom are based on endogenous processes in molecular cascades that undergo a disproportionate activation causing positive feedback of coagulation, inflammation and

tissue degradation/remodeling, among other processes (Ferraz et al., 2019; Teixeira et al., 2019). In this context, the term “thromboinflammation” has been used to describe the activation of the cascading blood systems as well as the activation of blood cells which can contribute to amplify the toxicity of venoms (Teixeira et al., 2019; Cavalcante et al., 2022).

Therefore, the present study aimed to investigate quantitative alterations in the plasma proteome of mice envenomated by *B. atrox*. This study was carried out aiming to explore biological pathways affected simultaneously after the access of *B. atrox* venom to the murine organism. Our studies depended on an exploration regarding the quantitative behavior of the murine plasma proteome. From the integration of the quantitative values of its components, it was possible to reveal the modification of biological pathways through proteins belonging to a category that underwent changes in their abundance. This allowed us to identify the signaling systems related to inflammation, hemostasis, multicellular blood system and other disorders simultaneously, suggesting the establishment of thromboinflammation after *B. atrox* snakebite envenoming.

2. Material and Methods

2.1 Bothrops atrox snake venom

B. atrox venom pool was obtained from 29 specimens donated to the Center for the Study of Venoms and Venomous Animals (CEVAP) at Sao Paulo State University (UNESP) under authorization from the Brazilian Institute for the Environment and Renewable Natural Resources (IBAMA 1/35/92/0044-1, Proc. No. 02001.005670/90-77). This research project is registered at National System of Genetic Heritage and Associated Traditional Knowledge (SISGEN) under nº A54E8E8. For venom extraction, the snakes were anesthetized with CO_2, and the venom prepared according to Santos et al. (2021).

100 *2.2 Animals and experimental envenomation*

101 Male Swiss mice (n=6/group) were injected with 1.2µg, 2.5µg or 5.0µg of *B. atrox* venom
102 or sterile saline solution (0.9% [m/v] NaCl). The individual thickness of the right hind paw
103 was measured before the venom injection (baseline) and 30 minutes after the edema
104 induction using a digital caliper (Digimess, São Paulo, SP, Brazil). Edema was expressed as
105 the percentage difference between the thickness of the paw after (at respective times) and
106 before (basal values) venom injection using the formula: (Te-T0) / T0 * 100. The statistical
107 analysis was performed using the T test, one Way-ANOVA, followed by the multiple
108 comparison test ($p \leq 0.05$) and expressed as mean and standard deviation.

109 *2.3 Hematologic and lipidic analysis*

110 To obtain blood, 30 minutes after the edema induction, all mice received ketamine (80 mg /
111 kg, ip) and xylazine (8 mg / kg, ip), and were bled via cardiac puncture. Blood samples
112 containing EDTA were analyzed in an automated hematology counter BC-5000
113 (Mindray®). Measured parameters included red blood cell count (RBC), hemoglobin
114 (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin
115 (HCM), mean corpuscular hemoglobin concentration (MCHC), white blood cell count
116 (WBC), neutrophils (Neu), lymphocytes (Lym) and platelets (PLT). In addition, serum
117 samples were used to measure cholesterol, triglycerides, high density lipoprotein cholesterol
118 (HDL), low density lipoprotein cholesterol (LDL) and very low-density lipoprotein
119 cholesterol (VDL). The statistical analysis was performed using the T test, one Way-
120 ANOVA, followed by the multiple comparison test ($p \leq 0.05$).

121

122

123 *2.4 Collection, selection and grouping of plasma samples*

124 The blood was stored in EDTA tubes. The samples were discarded in case of hemolysis or
125 contamination by residues from other organs. Blood was immediately placed on ice and
126 centrifuged at 1.000 g for 15 min at 4 °C with plasma stored to -80°C. Three pools per
127 experimental group were prepared from 10 uL of plasma to each two animals per pool.

128 *2.5 Proteomic Analysis*

129 Protein quantification, in solution protein digestion, LC-MS/MS analyses and Data
130 processing were executed according to Cavalcante and collaborators (2022). Briefly, plasma
131 proteins (30µg) were reduced, alkylated and digested using trypsin enzyme. Peptides were
132 desalting and submitted to a nano-ultra-performance by an Ultimate 3000 LC (Dionex,
133 Germering, Germany) accoupled to nanospray Q-Exactive mass spectrometer (Thermo
134 Scientific, Waltham, MA) with a data-dependent acquisition mode. Raw MS files were
135 obtained by Thermo Xcalibur software (version 4.0.27.19, ThermoFisher Scientific Inc.) and
136 submitted to PatternLab for Proteomics 4 for protein identification and quantification
137 (Carvalho et al., 2016). Extracted ion chromatograms (XIC) of peptides were analyzed in
138 pairs and the fold change values (Log-Fc) were calculated using T-test using MetaboAnalyst
139 (Chong et al., 2019). Then, the individual lists by group of validated proteins were analyzed
140 by Jveen (Bardou et al., 2014) to obtain the distribution of proteins between experimental
141 groups. Common proteins to all groups were analyzed by discriminant analysis by partial
142 least squares analysis (PLS-DA) and Variable Importance Projection (Vip) Score using
143 MetaboAnalyst software to identify the proteins responsible for the difference between those
144 groups (Chong and Xia, 2020). All proteins were analyzed according to ontological gene
145 annotations using DAVID and the results plotted using GoPlot (Hung et al., 2007; Walter et
146 al., 2015). Finally, proteins were also analyzed for their interactions using NetworkAnalyst

(Zhou et al., 2019a) to elucidate key proteins among the interaction's networks, and by Metascape (Zhou et al., 2019b) to analyze enriched clusters and physical interactions between proteins.

3. Results and Discussion

Edema is the most common inflammatory sign in *B. atrox* envenomation, which can progress to a compartment syndrome resulting in tissue loss and amputations (Luciano et al., 2009; Graciano and Carvalho, 2018; Valle et al., 2008). Considering the total edema effect at its maximum developmental peak time (30 min), we found that 1.2μg of *B. atrox* venom resulted in an increase in paw volume of 28.2% ± 0.48 ($p < 0.0001$). Furthermore, 2.5μg induced a response similar to 1.25μg (28.20% ± 0.58, $p < 0.0001$), characterizing a constant response between these doses. On the other hand, 5.0μg of *B. atrox* venom induced 21% ± 0.44 ($p < 0.0001$) of edema, lower when compared to previous doses (Figure 1A).

Here, we unveil an important step for understanding the mechanisms involved in the systemic response against the severity of edema caused by *Bothrops atrox* venom. For this purpose, plasma samples were inspected 30 minutes after the injection of three increasing doses of venom to assess the first events involved in the envenomation. We found that *B. atrox* venom induced edema at all doses tested following the time of 30 minutes according to studies that show the maximum development of edema in this period, followed by progressive decreases (Santos Barreto et al., 2017). During this period, we focused on understanding the direct action of the venoms toxins, avoiding the interference of the secondary effects of the endogenous components released by the local reaction or tissue repair system.

The proteome was based on the number of unique peptides (≥1) and log-Fold change generating a list with 113, 109 and 138 proteins with differentially abundance proteins

171 (DAPs) in the plasma from mice after injection with 1.2, 2.5 and 5.0μg of *B. atrox* venom,
172 respectively (Figure 1B). In general, 83 common plasma proteins (CPPs) were changed in
173 all groups regardless of the dose of venom used (Figure 1 C and D). Further comparisons
174 among experimental groups were detailed on a heat map. Compared to the control group, 27
175 proteins showed a reduction in their abundance after injection of *B. atrox* venom, while
176 another 56 increased (Figure 1D).

177 Various DAPs identified in the plasma of mice injected with *B. atrox* venom, as described
178 here and in our other study with mice injected with *B. leucurus* venom (Cavalcante et al.,
179 2021). However, we found a difference in the number of DAPs for *B. atrox* envenomation
180 when compared with the amount identified in the plasma of mice envenomed with the *B.*
181 *leucurus* venom reported by Cavalcante et al. (2021). This difference may be related to the
182 variability in the amount of edema caused by the same amount of venom using these two
183 venoms.

184 PLS-DA analysis have showed that the plasma proteomes of the three experimental groups
185 injected with different doses of venom differed from the control group, as well as in the
186 edema quantification assay. The plasma proteome content of mice injected with 1.2 and
187 2.5μg of *B. atrox* venom have showed an overlap, indicating a high degree of similarity
188 regarding changes in proteins abundance. Furthermore, the proteins profile of the plasma of
189 mice injected with 5.0μg of *B. atrox* venom (Figure 1 E) have showed a great dissimilarity
190 when compared to the groups injected with 1.2 and 2.5μg of venom. The classification of
191 the patient's clinical condition is based on signs and symptoms (Funasa, 2001). Thus, the
192 identification of proteins capable of discriminating between different classifications of
193 severity of envenomation can help the clinical team in decision making regarding the
194 management and therapeutic conduct.

We sought to determine the particularities of each group, generating a list of significant characteristics validated by VIP scores among experimental and control groups (Figure 1 F). VIP scores have evidenced a dose-response among experimental groups in relation to the control: hemoglobin and subunits (Hba, Hbb-b1/b2), peroxiredoxin 2 (Prdx2), factor IX (F9), insulin-like growth factor 1 (Igf1), epidermal growth factor containing fibulin-like extracellular matrix protein 1 (Efemp1) and fibulin (Fbln1) can act as possible biomarkers of lesion at *B. atrox* envenomation (Figure 2).

After *B. atrox* envenoming, we found an increase in the presence of extracellular Hb, suggesting erythrocyte rupture, whose cause may be related to thrombotic microangiopathy, which is reported experimentally and clinically (Senise et al., 2015; Bucaretchi et al., 2019; Malaque et al., 2019; Mota et al., 2020). In addition, ecchymoses, as well as their resolution process, also set up a mechanism for the release of extracellular Hb. Hb induce the induction of programmed necrosis in macrophages (Fortes et al., 2012) and maintenance of M1 macrophages due to formation of intracellular iron stores from erythrocytes (Krzyszczyk et al., 2018).

This scenario suggests that in an envenomation, this molecule may be a signal of a positive feedback of tissue degradation caused by the *B. atrox* venom, in addition to a likely role as a monitoring biomarker for tissue damage. However, alterations in Hb on blood counts were not found (Supporting Information: Figure 1). This discrepancy between detecting changes using these different methods is likely due to the fact that spectrometry detects and infers proteins in a sample more sensitively.

Furthermore, Prdx2 is a negative regulator of hypoxia-inducible factors (HIF) and inhibits the transcriptional activity of STAT3 through redox effects (Luo et al., 2016; Sobotta et al., 2015). Thus, the presence of increased levels of Prdx2 according to the amount of venom in

our study may be associated with edema-induced hypoxia. However, the origin of Prdx2 must be clarified, since thrombi can increase the levels of this protein, as this molecule is the primary antioxidant in erythrocytes (Lee et al., 2003). Interestingly, Prdx2 showed an increase in the proteome of mice injected with 5.0µg of *B. atrox* venom. This protein can act attenuating the activation of matrix metalloproteinases (MMPs) and reducing oxidative stress (Jeong et al., 2020; Rhe et al., 2005).

Furthermore, factor IX is a vitamin K-dependent zymogen activated by factor XIa/Ca++ (intrinsic pathway) or by factor VIIa/tissue factor/Ca++ (extrinsic pathway) associated with bleeding disorders (Chandler, 2019). Our data showed an increase of this protein after injection with *B. atrox* venom. It is known that this experimental envenomation is classically marked by bleeding disorders, in which, in cases of systemic bleeding, there is consumption of coagulation factors and fibrinolytic components, in addition to the participation of tissue factor (Oliveira et al., 2020). However, Factor IX does not seem to be related to such phenomena and, probably, it may be associated with the inflammation caused by the *B. atrox* venom or even play a role hitherto unknown in the pathophysiology of the envenomation.

It is important to note that these proteins can be an indicator of the acute phase of envenomation. Thus, it remains to be seen whether these preclinical markers identified in plasma for edema severity and thromboinflammation identified in our study are validated in larger preclinical cohorts, as well as in clinical cohorts. A potential caveat for this study is the possibility of greater variability among mice, since pools were used for MS analysis.

The analysis of regulatory trends of the main altered biological processes in which CPPs are involved was explored. Thus, there were 40 main altered biological processes classified into 4 main clusters: (i) processes related to the lipid system, (ii) to the immune systems and

coagulation, (iii) to the multicellular blood system, and (iv) to regulation of gene expression and cell cycle.

The processes related to the lipid system were up-regulated, namely: efflux, response to peptide hormone, cholesterol efflux and lipid transport. Furthermore, alterations related to positive regulation of cholesterol esterification, triglyceride homeostasis, high-density lipoprotein particle remodeling, lipoprotein metabolic process, cholesterol metabolic process and cholesterol homeostasis were also evidenced (Figure 3 A e B). In this study, we verified changes in lipid homeostasis after experimental envenoming. It is known that PLA2 can induce the formation of lipid droplets in macrophages (Leiguez et al., 2018). *B. moojeni* venom induces the release of prostaglandin E2 in pre-adipocytes (Teixeira et al., 2019), while the *B. atrox* venom induces local inflammation mediated by the synthesis and substantial release of several inflammatory mediators (Moreira et al., 2012), which supports the enrichment related to the efflux of lipids pointed here.

Our results suggest that *B. atrox* venom induces an overregulation of lipid processes that would be related to an increase in lipid levels in plasma after injection of venom. Then, we investigated possible changes in cholesterol, triglycerides, HDL, LDL and VLDL levels in mice plasma. Our findings have ratified the changes observed in cholesterol and HDL levels in mice injected with 2.5 and 5.0 µg of *B. atrox* venom, respectively (Figure 3 C and D). However, changes in LDL and VLDL triglyceride levels were not identified (Supporting Information: Figure 2). Once again, this divergence can due to the sensitivity differences between methods used. Since spectrometry detects and infers proteins in a sample more sensitively, while GO analysis classifies and frames them in the ways in which these proteins are functional and biochemical assays analyze the concentrations of lipid markers. However, it is clear that lipid mediators are potent immune response stimulators (Rogerio et al., 2014;

Esser-von Bieren, 2019) and thus cholesterol and HDL may be related to the severity of the envenomation.

The cluster of biological processes related to the immune and coagulation systems showed particular regulatory tendencies. Hemostasis, innate immune response, proteolysis and fibrinolysis were down-regulated in mouse plasma after *B. atrox* envenomation in our study. On the other hand, complement activation, classical pathway, acute-phase response, negative regulation of inflammatory response and vasodilation have presented an up-regulation. Furthermore, although DAPs were found to be related to the terms GO negative regulation of blood coagulation and negative regulation of angiogenesis, these processes remained neutral when analyzed (Figure 4). Then, we asked whether such alterations in hemostatic and immunological processes could cause significant systemic disturbances.

Regarding the categories involved with the multicellular blood system, response to peptide hormone and response to cytokine were the processes with the greatest change (upregulation), although receptor-mediated endocytosis, response to oxidative stress, negative regulation of neuron death and cell redox homeostasis were also up-regulated. In contrast, *B. atrox* venom induced an attenuation in the ratio of negative regulation of cell proliferation, positive regulation of fibroblast proliferation, positive regulation of cell proliferation and positive regulation of cell proliferation (Figure 5 A and B).

As our findings showed that *B. atrox* venom produces alterations in biological processes in the plasma of mice related to homeostasis/cellular response, we proceeded with blood cell counts in these mice to confirm the alterations in the multicellular blood system. We identified leukocytosis among all groups injected with *B. atrox* venom compared to the control group (Figure 5 C). In addition, neutrophilia (Figure 5 D) and lymphocytosis (Figure 5 E) were found in mice injected with venom. In addition, all mice had thrombocytopenia

(Figure 5 F), but changes in the red blood series were not observed (Supporting Information: Figure 2).

Thus, platelet alterations were confirmed through the circulating platelet count after envenoming. In all cases, thrombocytopenia was present, supporting the previous findings regarding the alteration in the abundance of proteins related to platelet processes. Thrombocytopenia is a phenomenon already reported in patients after snakebite envenoming by *B. atrox* (Oliveira et al., 2020), however, the role of platelets and if there is sequestration, apoptosis or other phenomenon that justifies the reduction in platelet count in the snakebite envenomation by this species stills unknown (Yamashita et al., 2011; Santoro and Sano-Martins, 2004; Monteiro et al., 2020).

Various toxins, especially PLA_2, isolated from *Bothrops* venoms have already been cataloged with the potential to inhibit or induce platelet aggregation. In the case of *B. atrox,* Batroxhragin (Freitas-de-Sousa et al., 2015), Atroxlysin I and III (Sanchez et al., 2010; Oliveira et al., 2019), thrombocytin (Niewiarowski et al., 1975) and BatroxLAAO (Alves et al., 2008) are the known toxins that act on platelets, which could justify the degranulation of platelets as an event found in this study. However, the presence of signaling proteins for platelet degranulation suggests that thrombocytopenia may be an effect induced by secondary mechanisms during envenomation, rather than by the direct action of toxins.

Our data set evidenced a heightened neutrophil degranulation, suggesting its early activation during the envenomation. We have identified an increase in the abundance of complement factor D (Cfd), catepsina b (Ctsb), granulin (Grn), Pkm, alpha-1-antitrypsin (Serpina1c) e quiescin sulfhydryl oxidase 1 (Qsox1). Three pathways can initiate the complement cascade, all of which culminate in the generation of Cfd that cleaves C3 (de Bont et al., 2019). Furthermore, activated neutrophils have been shown to express C3, Factor B and properdin,

the three components of the alternative pathway needed to produce and stabilize functional Cfd (Yuen et al., 2016). Ctsb expression can be increased by neutrophil elastase, showing that Ctsb is positively correlated with the degree of inflammation and neutrophil recruitment (Buttle et al., 1990; Taggart et al., 2003; Geraghty et al., 2007). The function of Grn in the immune system has not been clearly identified and elaborated. However, its high expression has been reported in a subpopulation of neutrophils, where its production occurs by the conversion of Pgrn into Grn by the action of elastase released by neutrophils, induces the expression of IL-8 in epithelial cells, a chemokine that recruits neutrophils and macrophages to initiate tissue repair (Zhu et al., 2002; Puga et al., 2012; Jian et al., 2013). Thus, the drop in Grn levels in the plasma of mice injected with 1.2 and 2.5 µg, followed by an increase in those injected with 5.0 µg, suggest a relationship between neutrophil activation and the severity of the envenoming.

Neutrophils are present in myonecrotic and hemorrhagic areas, or even in the inflammatory infiltrate (Zuliani et al., 2020), and once activated, they produce pro-inflammatory cytokines, phagocytize and release neutrophil extracellular traps (NETs), acting in tissue repair (Beavers and Skaar, 2016; Manda-Handzlik and Demkow, 2015; Mortaz et al., 2018; Naegelen et al., 2015; Ravindran et al., 2019; Rosales, 2018; Wang, 2018). Here, we identified that mice showed neutrophilia after experimental envenomation, as also described in clinical findings, in which neutrophilia of variable intensity has been reported in *Bothrops* envenomation, especially with greater intensity in cases with tissue loss and/or limb amputations (Luciano et al., 2009; Graciano et al., 2009; al., 2017; Pedroso Lacerda et al., 2017; Valle et al., 2018).

We also found up-regulation of Cdc42, which suggests its role in the immune response induced by *B. atrox* venom, as it is known that activation of Cdc42 is important for motility

and directionality of neutrophil migration (McCormick et al., 2019; Szczur et al., 2006), regulating ROS formation, degranulation, and neutrophil activation in a stimulus-dependent manner (Tackenberg et al., 2020). T-cell homeostasis (limitation and proliferation) restricts Th1 and Th17 cell differentiation via glycolysis repression, while inducing Th2 and iTreg differentiation and nTreg cell homeostasis and stability by cell stability iTreg and nTreg through induction of glycolysis (Guo, 2021).

On the other hand, we verified the attenuation of the down-regulation via ERK1/2 and of the up-regulation of MAPK. ERK1/2 is a member of the "generic" mitogen-activated protein kinase (MAPK) signaling pathway (Shaul and Seger, 2007). The ERK1/2 signaling pathway plays a crucial role in cell proliferation and gene expression impacting the cell cycle, cell migration, cell invasion (Kim and Choi et al., 2010), apoptosis (Kolch, 2005), and autophagy (Cagnol and Chambard, 2010; Martinez-Lopez and Singh, 2014) cell metabolism (Papa et al., 2019) and inflammation (Lu and Malemud, 2019). However, MAPK signaling regulates many cellular events, such as cell proliferation, differentiation, cell migration, controlled cell death (apoptosis) and senescence, as well as certain aspects of cell cycle progression, cell survival, metabolism and transcription (Sun et al., 2015). The suppression of down-regulation of the ERK1/2 pathway may suggest that cellular pathways and programs related to cell cycle and cell death are overexpressed. Furthermore, the attenuation of MAPK upregulation suggests an attempt to control cell death, probably those injured at the site of injection of *B. atrox* venom.

Although a serine proteinase from *B. atrox* venom induces angiogenesis through the PI3K/Akt signaling axis (Bhat et al., 2016), we found an attenuation in the PI3K/Akt and HIF-1 pathways after *B. atrox* envenomation. Since the discovery of the HIF-1 pathway, several studies have been carried out to understand the role of this pathway in various

diseases (Lee et al., 2019). However, it is the first time that a possible role of this pathway has been demonstrated in snakebite envenoming, although it is known that HIF-1 is responsible for signaling adaptive responses in the absence of oxygen that provide ischemic tolerance, causing upregulation of transcriptional cascades for tissue protection and adaptation (Lee et al., 2019; Liu et al., 2020) and thus, this pathway may be related to tissue plasticity against ischemia caused by the *B. atrox* venom.

After injection of *B. atrox* venom, processes related to coagulation, complement and tissue spatial arrangement enriched (Figure 7 A). Enrichment analysis identified clusters in which DAPs were associated with complement and coagulation cascades, blood coagulation, Intrinsic Pathway of Fibrin Clot Formation (Figure 7 B and C). Based on the association among envenoming and the set of immune-related DAPs, we further explored the immune history. Several DAPs have been linked to immune system processes: regulation of tissue remodeling, regeneration and hydrogen peroxide catabolic process (Table S5). The functions of Apolipoproteins and Serpins family members were predicted by the analysis of GO and KEGG in Metascape (Figure 8).

We found evidence of downregulation in the abundance of epidermal growth factor receptor (Egfr), a regulator of oxidative stress in macrophages involved in the maintenance of oxidative stress, macrophage infiltration and induction of proinflammatory cytokines, downstream activation of transcription factors, as the nuclear factor-κB (NF-κB) (Gao et al., 2013; Troib and Azad, 2015; Zhang et al., 2004; Chattopadhyay et al., 2015; Wang et al., 2017). These findings likely reflect a compensatory response to counteract the rise in reactive oxygen species during *B. atrox* envenomation.

4. Conclusion

Our results have revealed that the plasma proteome is modulated according to the amount of edema induced by the *B. atrox* venom. Differential abundances of some proteins (Hba, Hbb-b1/b2, Prdx2, F9, Igf1, Efemp1 and Fibulin) have suggested that these molecules can serve as potential indicators of the severity of edema/inflammation in mice caused by *B. atrox* venom to be tested in futures studies. However, monitoring the quantitative trends of these proteins based on the course-time of envenomation was beyond the scope of this study. This leaves a lack of information about certain key envenomation responses during the regulation of mouse proteins in the *B. atrox* venom response. These could be an effective future continuation of the present investigation, especially as the results continue to advance for translational research. Collectively, our findings have provided some new mechanistic insights into the evolution of *B. atrox* envenomation, and we anticipate that this will accelerate opportunities for the development of clinical trials based on the envenomed organism's proteins to monitor the severity and complexity of envenomation, not only by *B. atrox*, but also by other species of snakes and other poisonous animals.

We also underscore the view that the venom-induced pathophysiology of *B. atrox* results from a combination of the direct action of venom toxins and indirect mechanisms derived from the tissue inflammatory response to envenomation. Differences in tissue inflammatory responses of this venom may contribute to variations in the pathophysiological scenario including thromboinflammatory, alteration in lipid metabolism and disturbances in the cell state characterized by oxidative stress, as well as effects on pathways that regulate the survival and cell cycle and gene expression.

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Legend of figures

Figure 1. *B. atrox* venom induces edema and changes in the blood plasma proteome of mice.

A. Edematogenic profile of *B.* atrox venom by dose-response. *B. atrox* venom was injected i.pl. in the right hind paw of male swiss mice (n = 6). Paw edema 30 minutes after injection with venom the aid of a digital caliper and expressed in % increase in relation to the initial thickness. **B.** Number of proteins with differential abundance (DAPs) (log-FC ≥1) identified in each group. **C.** Venn diagram describing the distribution of DAPs among groups. **D.** Heatmap shows the turnover of common plasma proteins (CPP) obtained from mice injected with 1.2, 2.5 and 5.0μg of *B. atrox* venom. High protein abundance is shown in shades of red and low abundance are shown in shades of green. The abundance of each protein was normalized by the internal standard to generate a data matrix consisting of signal intensity values followed by scaling data centered on the mean and generalized logarithmic transformation. Values are presented as the mean of each experimental group. **E.** PLS-DA analysis imp. features, $p < 0.05$ of the proteome of blood plasma mice (n = 6: three biological pools/replicas × two technical replicas). **F.** The Variable Importance Projection (VIP) plot displays the top 20 most important protein resources identified by the PLS-DA. The colored boxes on the right indicate the relative abundance of the corresponding protein for plasma samples from mice injected with venom. VIP is a weighted sum of squares of the PLS-DA loads taking into account the amount of Y variable explained in each dimension.

Figure 2. Comparative analysis of hemoglobin and subunits (Hba, Hbb-b1/b2), peroxiredoxin 2 (Prdx2), factor IX (F9), insulin-like growth factor 1 (Igf1), epidermal growth factor containing fibulin-like extracellular matrix protein 1 (Efemp1) and fibulin (Fbln1) level alterations in mice 30 min after intraplantar injection with *B. atrox* venom.

Trends of a few selected differentially abundant proteins in of blood plasma proteome alterations identified by mass spectrometry. Data are represented by the total intensity of proteins captured by extracted ion chromatogram. The columns represent the mean ± SD (n = 6/group). *p < 0.05, **p < 0.01 and ***p < 0.005 and ****p < 0.0005 for the comparisons indicated (one-way ANOVA).

Figure 3. Principal biological processes related to the lipid system that were enriched blood plasma of mice 30 min after intraplantar injection with *B. atrox* venom.

A. Identification of the main differentially regulated ontological (GO) gene terms, graphically displayed according to significance, with a measure of overall up or down regulation for category after experimental envenomation using *B. atrox* venom. The outer circle shows the number of proteins whose color of each protein corresponds to the logarithmic change, with red representing

the increase in abundance and the blue representing the decrease. The inner rectangles represent the p-value of the GO term and are colored according to the z-score to represent the general direction of change for each individual term. **B.** The table lists the GO terms. **C – D.** Cholesterol and HDL values in the serum of mice after injection with 1.2, 2.5 and 5.0µg of *B. atrox*.

Figure 4. Principal biological processes related to immune systems and coagulation that were enriched in blood plasma of mice 30 min after intraplantar injection with *B. atrox* venom.

A. Identification of the main differentially regulated ontological (GO) gene terms, graphically displayed according to significance, with an overall up or down regulation measure for the category after injected with *B. atrox* venom. The outer circle shows the number of proteins whose color of each protein corresponds to the logarithmic change, with red representing the increase in abundance and the blue representing the decrease. The inner rectangles represent the *p*-value of the GO term and are colored according to the z-score to represent the general direction of change for each individual term. **B.** The table lists the GO terms.

Figure 5. Principal biological processes related to blood cells and in mouse leukocyte and platelet counts 30 min after intraplantar injection with *B. atrox* venom.

A. Identification of the main differentially regulated ontological gene (GO) terms, graphically displayed according to significance, with an overall measure of up or down regulation for the category after injection with *B. atrox* venom. The outer circle shows the number of proteins whose color of each protein corresponds to the logarithmic change, with red representing the increase in abundance and the blue representing the decrease. The inner rectangles represent the *p*-value of the GO term and are colored according to the z-score to represent the general direction of change for each individual term. **B.** The table lists the GO terms. **C – F**. Count of circulating total leukocytes (WBC), neutrophils (Neu), lymphocytes (Lym) and platelets (PLT) in the blood of mice 30 min after injection with 1.2, 2.5 and 5.0µg of *B. atrox*. The columns represent the mean ± SD (n = 6/group). *p < 0.05, **p < 0.01 and ***p < 0.005 and ****p < 0.0005 for the comparisons indicated (one-way ANOVA).

Figure 6. Principal signaling pathways related to regulation of gene expression and enriched cell cycle in blood plasma of mice 30 min after intraplantar injection with *B. atrox* venom.

A. Identification of the main differentially regulated ontological gene terms (GO), graphically displayed according to significance, with an overall up or down regulation measure for the category after injection with the *B. atrox* venom. The outer circle shows the number of proteins whose color of each protein corresponds to the logarithmic change, with red representing the increase in abundance and the blue representing the decrease. The inner rectangles represent the *p*-value of the

GO term and are colored according to the z-score to represent the general direction of change for each individual term. **B.** The table lists the GO terms.

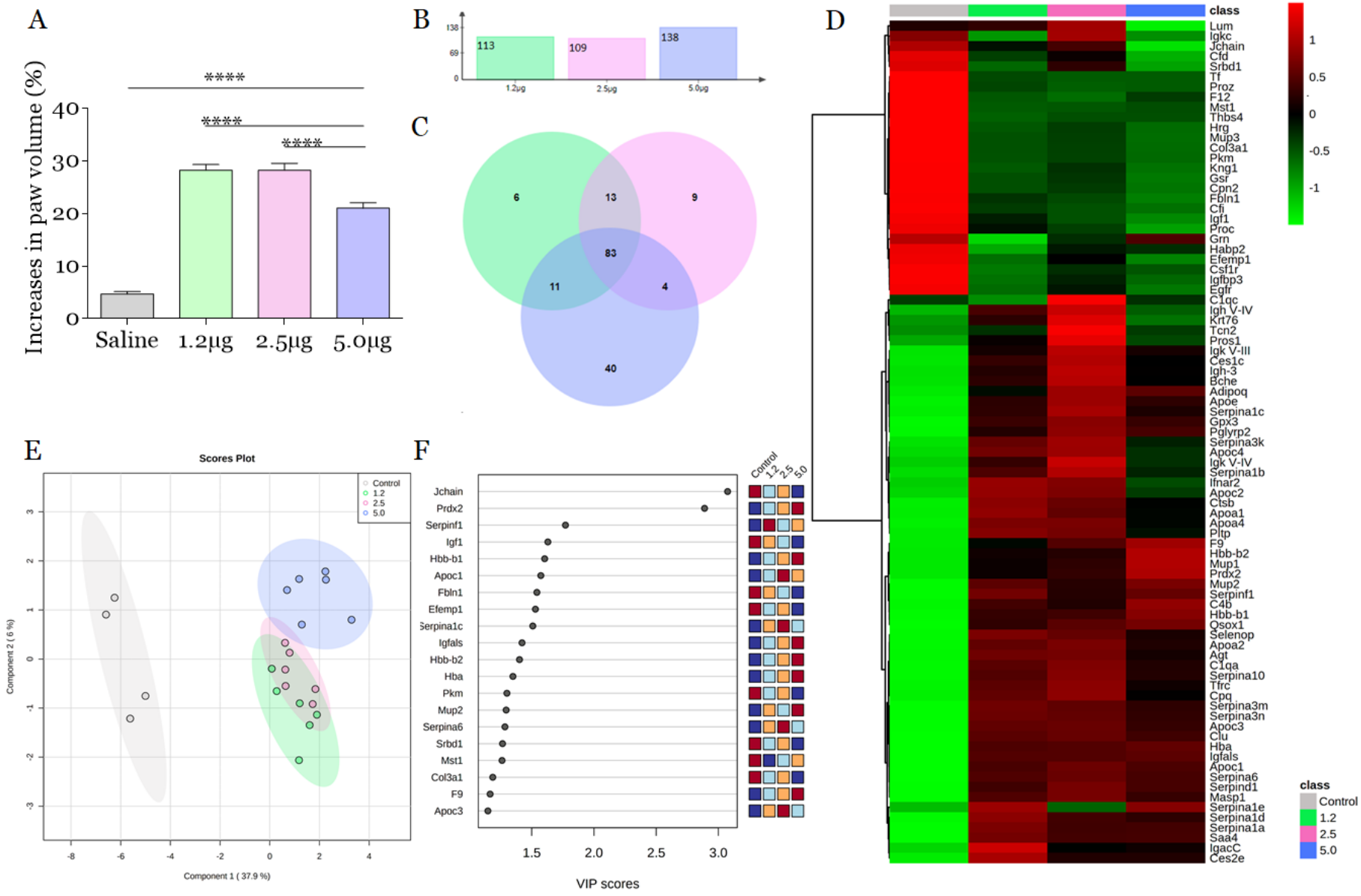
Figure 7. Network of protein-protein interactions and major cluster of altered processes blood plasma of mice 30 min after intraplantar injection with *B. atrox* venom.

A. Main proteins of the iteration network. The proteins were mapped to the interaction database (NetworkAnalyst) to produce a large sub-network ("continent") with several smaller sub-networks ("islands"). Subnets of predictive interactions with at least three nodes departing from the proteins of interest are presented. Only proteins that showed at least 3 interactions were considered. **B.** Network of enriched terms colored by cluster ID, where nodes that share the same cluster ID are typically close to each other. **C.** Network of enriched terms colored by *p*-value, where terms containing more genes tend to have a more significant *p*-value.

Figure 8. Protein-protein Interaction Enrichment analysis and altered processes blood plasma of mice 30 min after intraplantar injection with *B. atrox* venom.

For each given gene list, protein-protein interaction enrichment analysis has been carried out with the following databases: STRING, BioGrid, OmniPath, InWeb_IM. Only physical interactions in STRING (physical score > 0.132) and BioGrid are used. The resultant network contains the subset of proteins that form physical interactions with at least one other member in the list. The Molecular Complex Detection (MCODE) algorithm has been applied to identify densely connected network components.

915 **Figure 1**


A
Increases in paw volume (%)
Saline
1.2µg
2.5µg
5.0µg
****
B
113
109
138
C
6
13
9
83
11
4
40
D
class
Control
1.2
2.5
5.0
E
Scores Plot
Component 1 (37.9 %)
Component 2 (6 %)
F
VIP scores
Jchain
Prdx2
Serpinf1
Igf1
Hbb-b1
Apoc1
Fbln1
Efemp1
Serpina1c
Igfals
Hbb-b2
Hba
Pkm
Mup2
Serpina6
Srbd1
Mst1
Col3a1
F9
Apoc3

916 **Figure 2**

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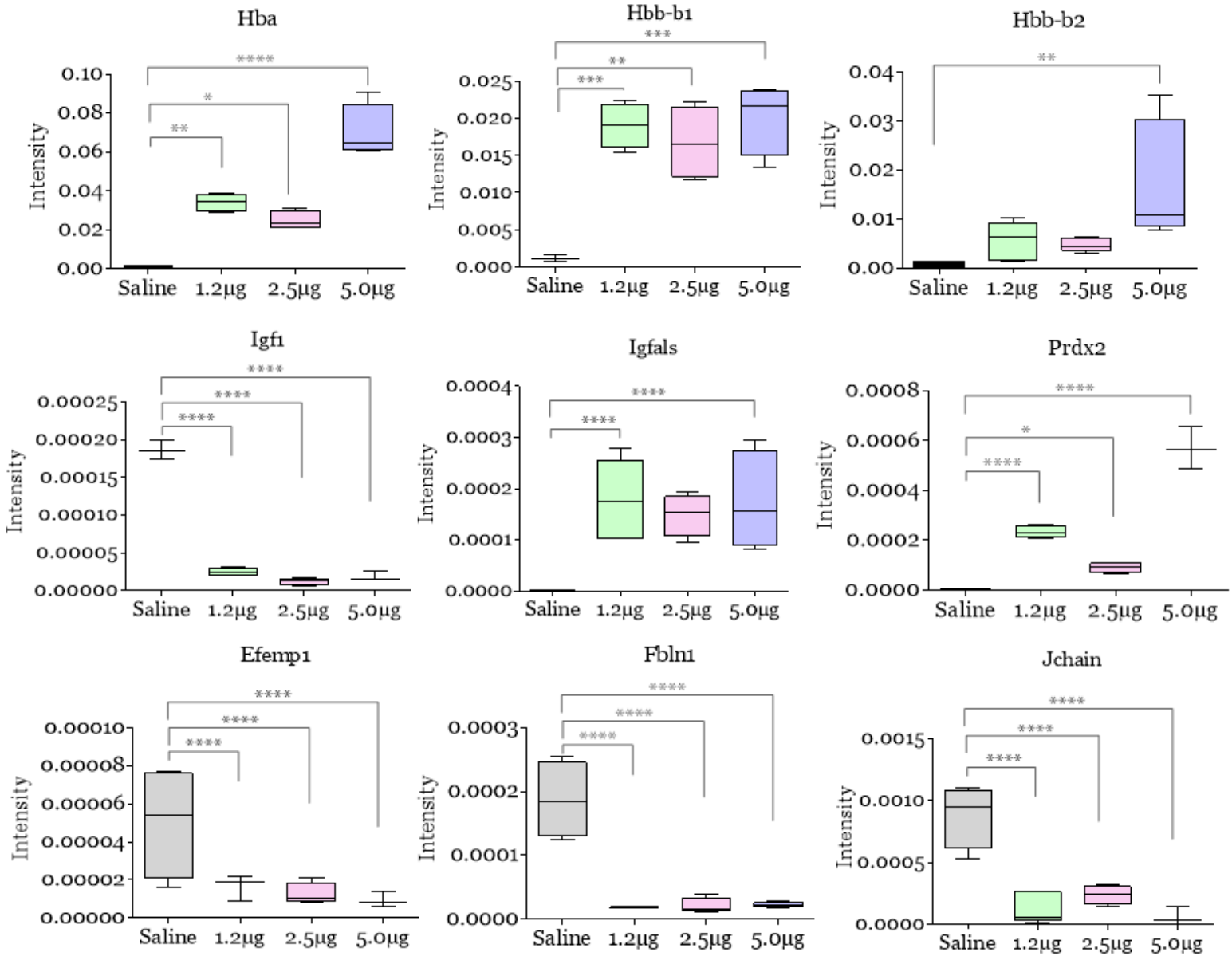
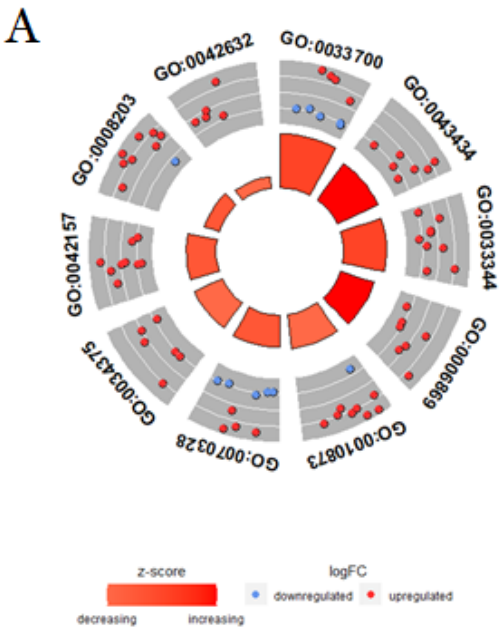
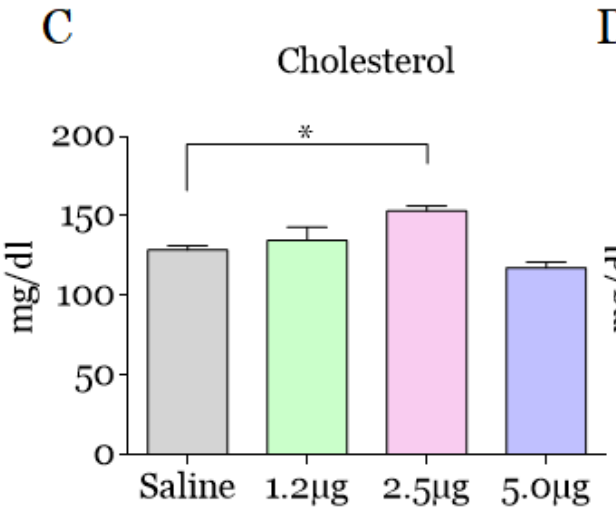


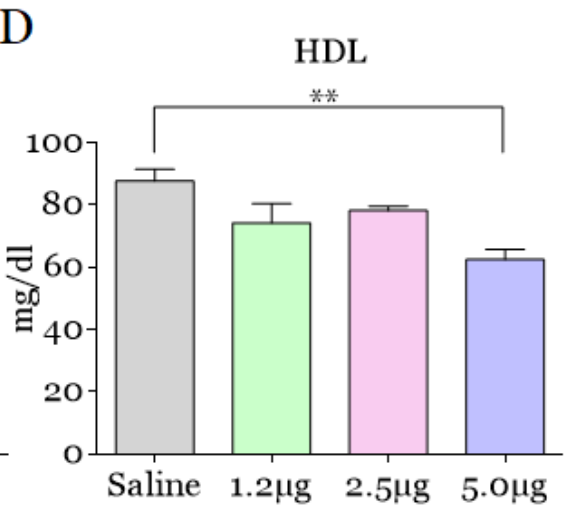
Figure 3



B

ID	Description
GO:0033700	phospholipid efflux
GO:0043434	response to peptide hormone
GO:0033344	cholesterol efflux
GO:0006869	lipid transport
GO:0010873	positive regulation of cholesterol esterification
GO:0070328	triglyceride homeostasis
GO:0034375	high-density lipoprotein particle remodeling
GO:0042157	lipoprotein metabolic process
GO:0008203	cholesterol metabolic process
GO:0042632	cholesterol homeostasis

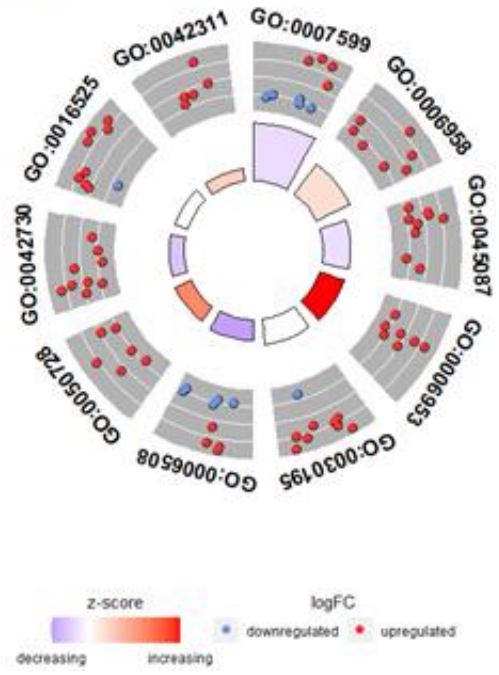




921 **Figure 4**

922

A



B

ID	Description
GO:0007599	hemostasis
GO:0006958	complement activation, classical pathway
GO:0045087	innate immune response
GO:0006953	acute-phase response
GO:0030195	negative regulation of blood coagulation
GO:0006508	proteolysis
GO:0050728	negative regulation of inflammatory response
GO:0042730	fibrinolysis
GO:0016525	negative regulation of angiogenesis
GO:0042311	vasodilation

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926 **Figure 5**

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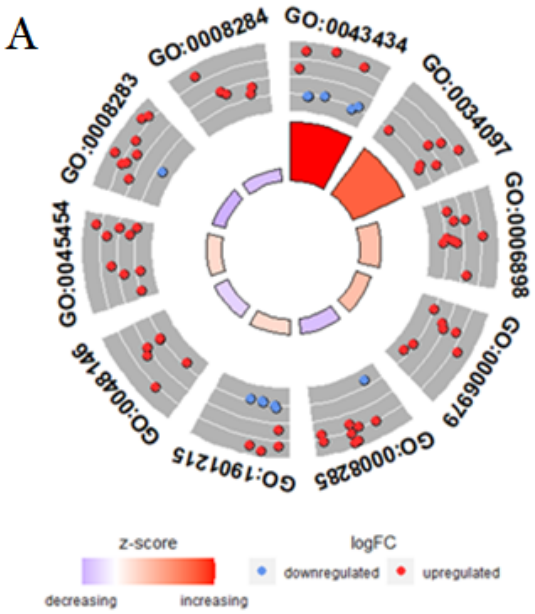
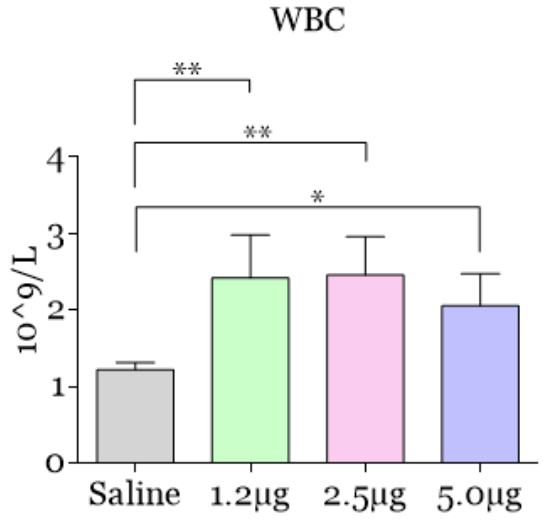
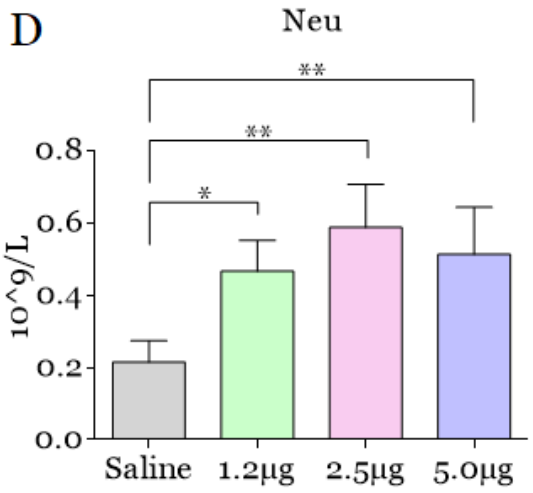
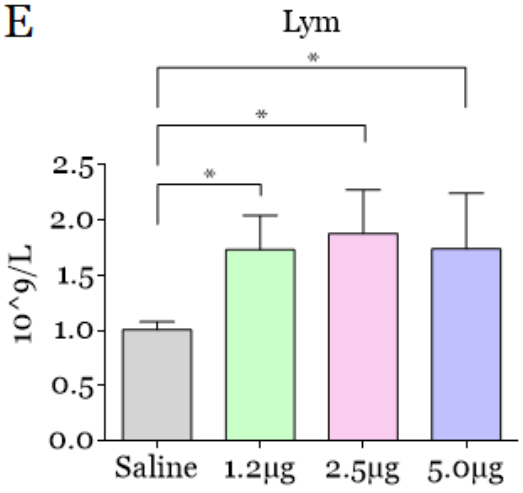


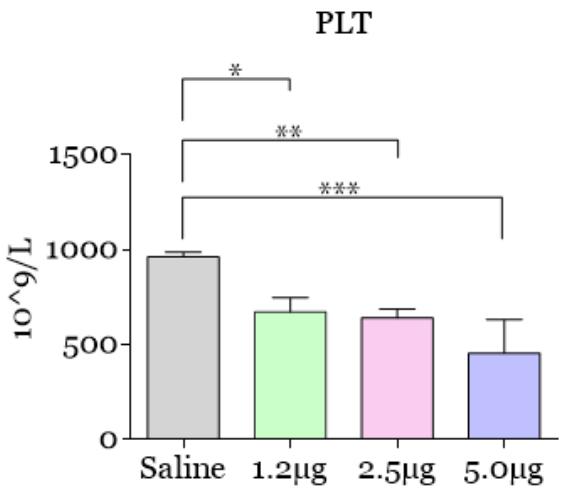
B

ID	Description
GO:0043434	response to peptide hormone
GO:0034097	response to cytokine
GO:0006898	receptor-mediated endocytosis
GO:0006979	response to oxidative stress
GO:0008285	negative regulation of cell proliferation
GO:1901215	negative regulation of neuron death
GO:0048146	positive regulation of fibroblast proliferation
GO:0045454	cell redox homeostasis
GO:0008283	cell proliferation
GO:0008284	positive regulation of cell proliferation



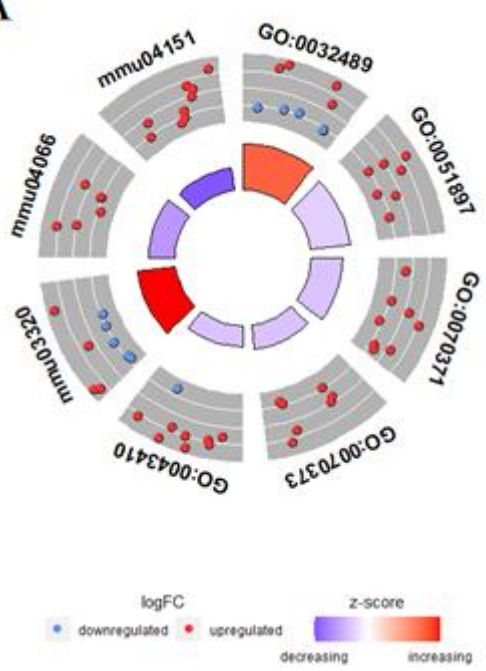






928 **Figure 6**

A

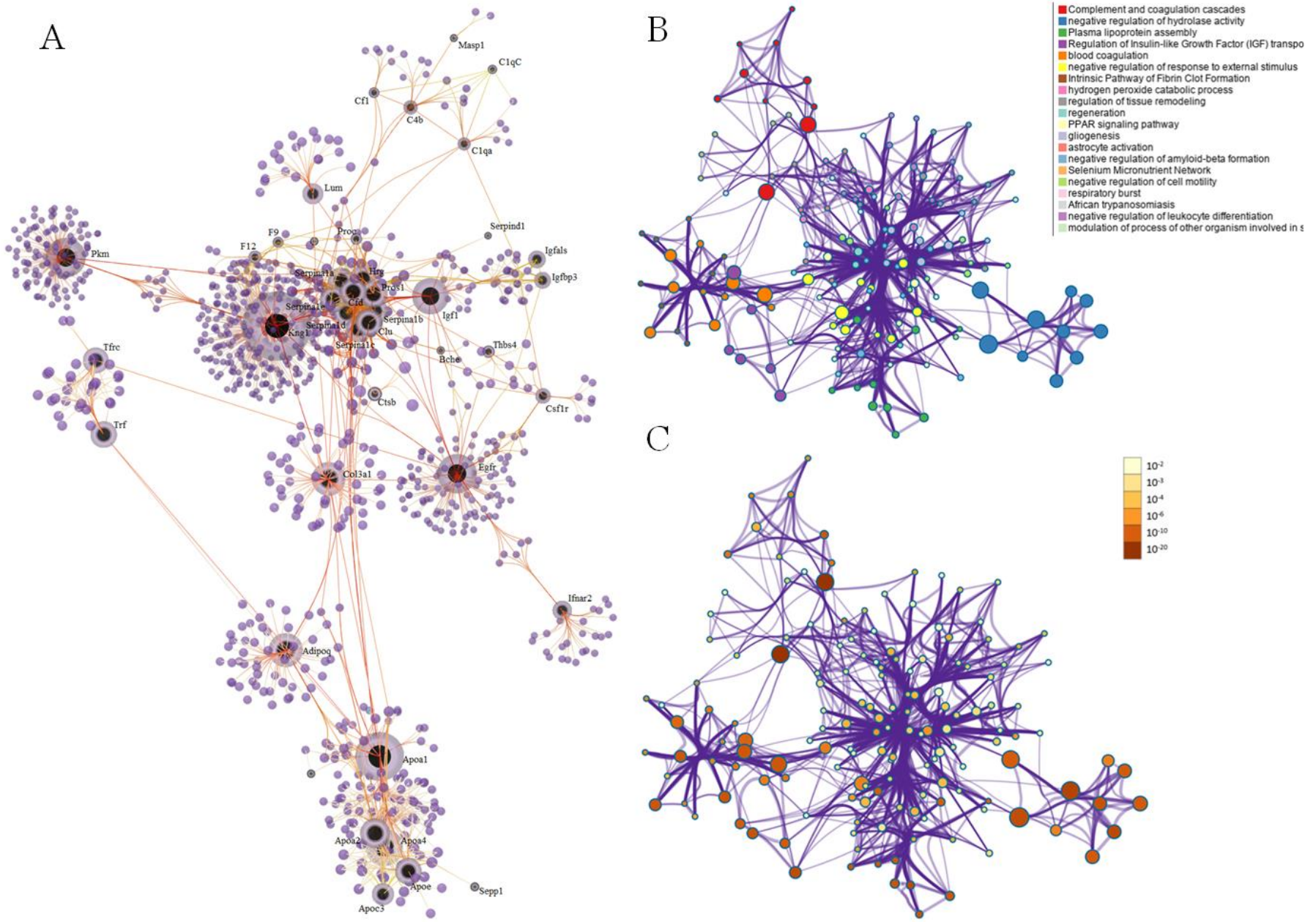


B

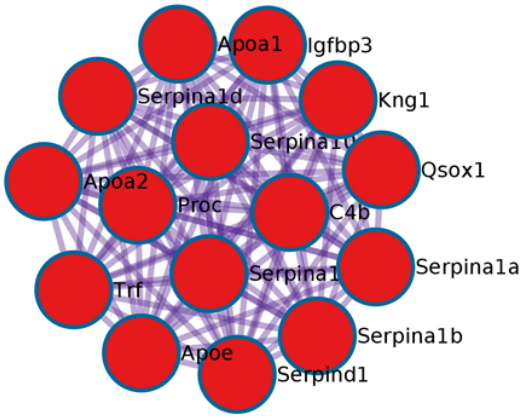
ID	Description
GO:0032489	regulation of Cdc42 protein signal transduction
GO:0051897	positive regulation of protein kinase B signaling
GO:0070371	ERK1 and ERK2 cascade
GO:0070373	negative regulation of ERK1 and ERK2 cascade
GO:0043410	positive regulation of MAPK cascade
mmu03320	PPAR signaling pathway
mmu04066	HIF-1 signaling pathway
mmu04151	PI3K-Akt signaling pathway

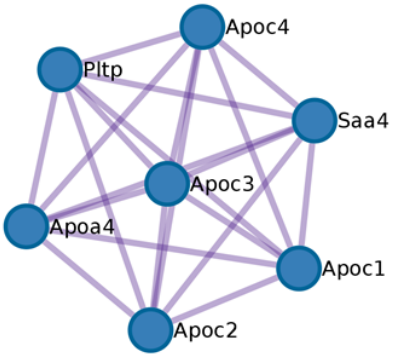
929

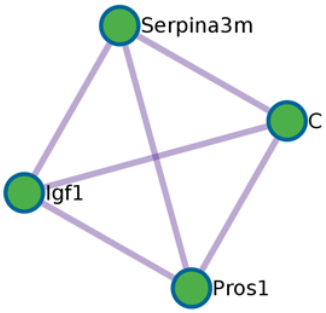
930 **Figure 7**


A
Masp1
C1qC
Cf1
C4b
C1qa
Lum
F9
Proc
F12
Pkm
Serpind1
Igfals
Igfbp3
Serpina1a
Hrg
Cfd
Pros1
Igf1
Serpina1e
Serpina1b
Serpina1d
Kng1
Clu
Serpina1c
Thbs4
Bche
Tfrc
Ctsb
Csf1r
Trf
Col3a1
Egfr
Ifnar2
Adipoq
Apoa1
Apoa2
Apoa4
Apoe
Sepp1
Apoc3
B
Complement and coagulation cascades
negative regulation of hydrolase activity
Plasma lipoprotein assembly
Regulation of Insulin-like Growth Factor (IGF) transpo
blood coagulation
negative regulation of response to external stimulus
Intrinsic Pathway of Fibrin Clot Formation
hydrogen peroxide catabolic process
regulation of tissue remodeling
regeneration
PPAR signaling pathway
gliogenesis
astrocyte activation
negative regulation of amyloid-beta formation
Selenium Micronutrient Network
negative regulation of cell motility
respiratory burst
African trypanosomiasis
negative regulation of leukocyte differentiation
modulation of process of other organism involved in s
C
10-2
10-3
10-4
10-6
10-10
10-20

931 **Figure 8**







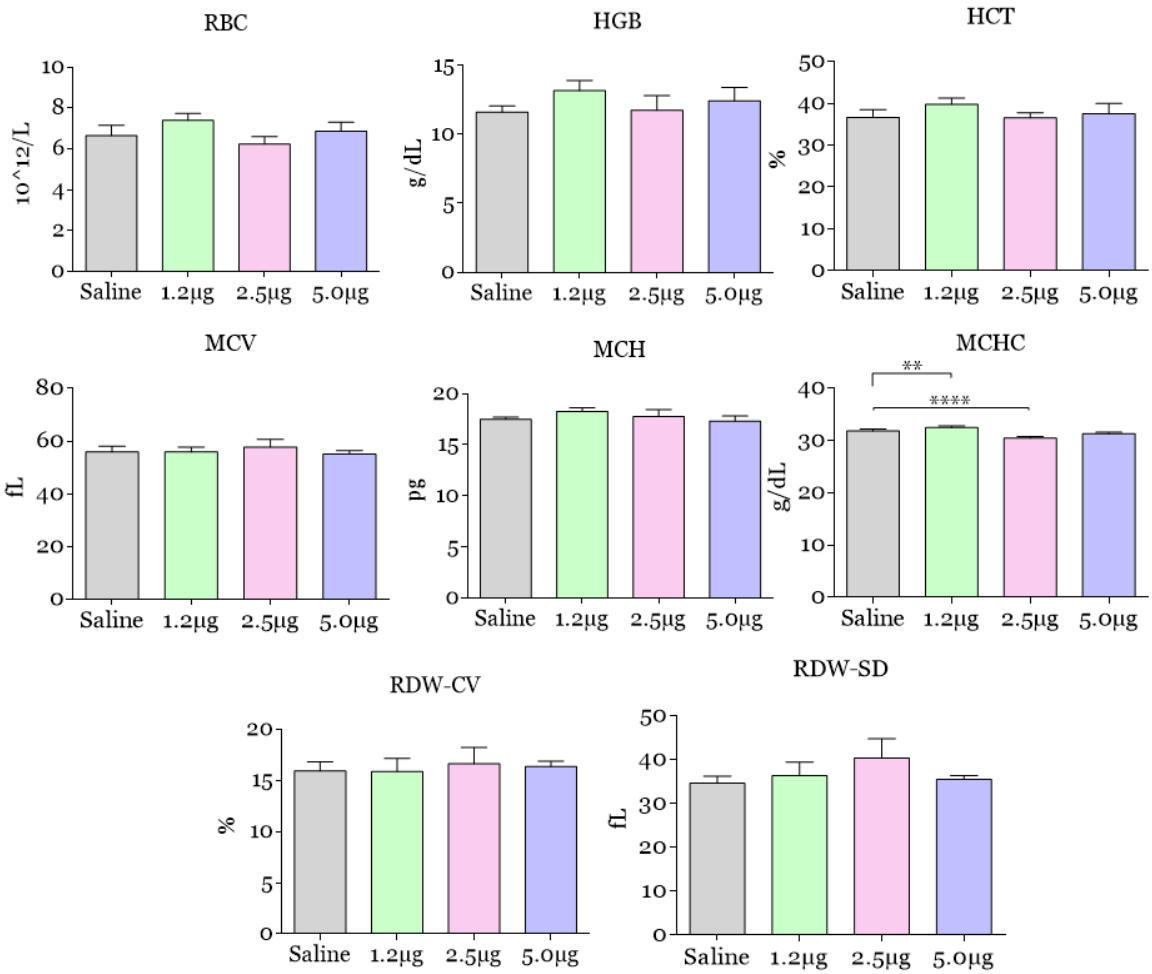
Color	MCODE	GO	Description	Log10(P)
	MCODE_1	R-MMU-8957275	Post-translational protein phosphorylation	-19.3
	MCODE_1	R-MMU-381426	Regulation of Insulin-like Growth Factor (IGF) transport and uptake by Insulin-like Growth Factor Binding Proteins (IGFBPs)	-19.1
	MCODE_1	ko04610	Complement and coagulation cascades	-15.3
	MCODE_2	R-MMU-174824	Plasma lipoprotein assembly, remodeling, and clearance	-14.5
	MCODE_2	WP1	Statin Pathway	-14.1
	MCODE_2	R-MMU-8963898	Plasma lipoprotein assembly	-14.1
	MCODE_3	R-MMU-114608	Platelet degranulation	-6.2
	MCODE_3	R-MMU-76005	Response to elevated platelet cytosolic Ca2+	-6.1
	MCODE_3	R-MMU-76002	Platelet activation, signaling and aggregation	-5.2

933

934

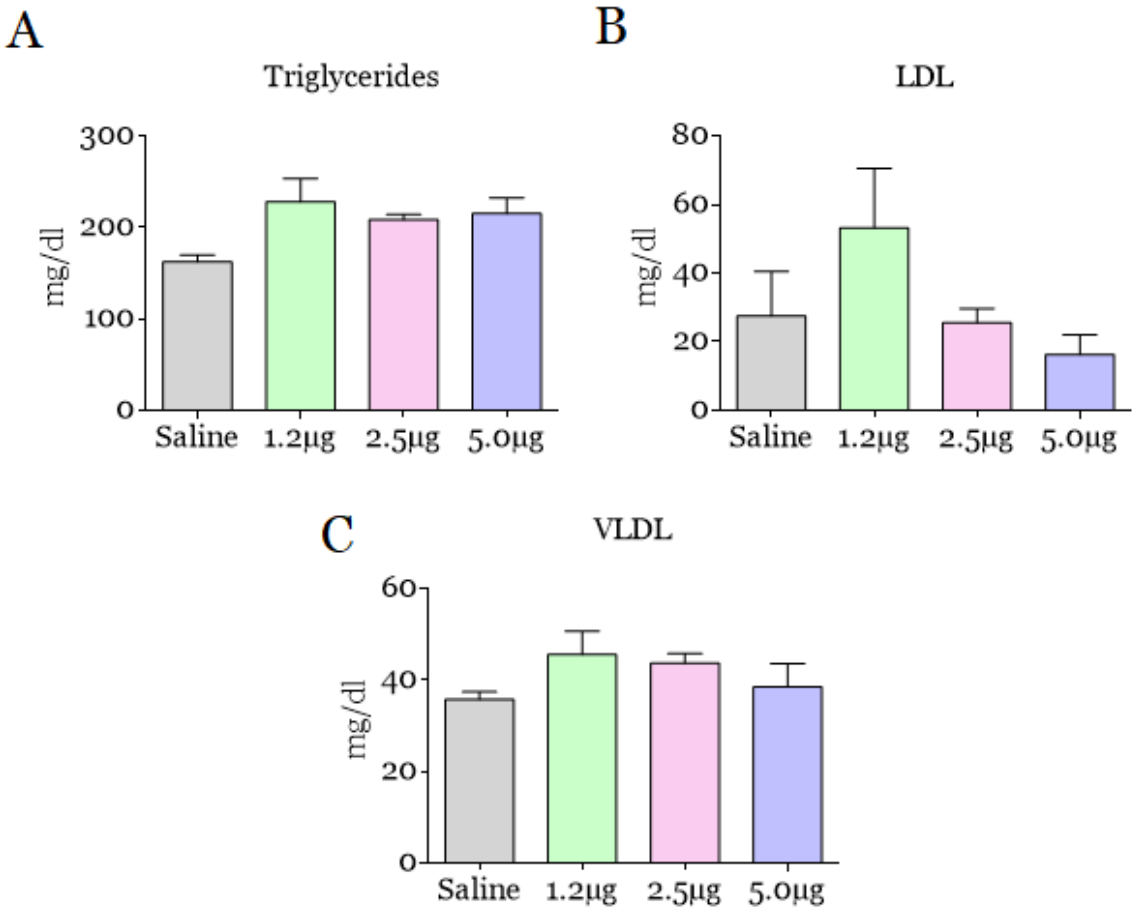
Supporting Information

Figure 1



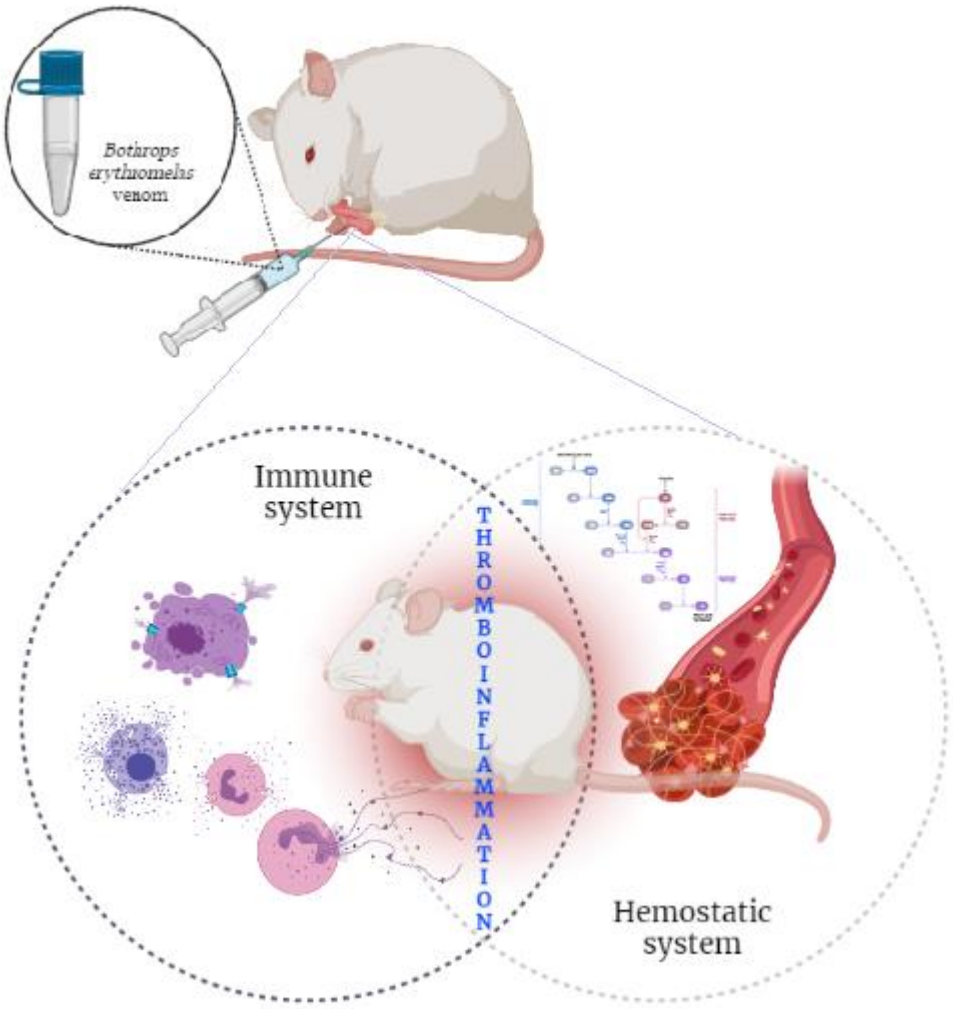
939 **Figure 2**

940



03

Thromboinflammation in mice after tissue damage induced by *Bothrops erythromelas* venom: specific signatures and changes endogenous signaling pathways



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Thromboinflammation in mice after tissue damage induced by *Bothrops erythromelas* venom: specific signatures and changes endogenous signaling pathways

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Abstract

Bothrops spp. is responsible for about 70% of snakebites in Brazil, causing a diverse and complex pathophysiological condition. *Bothrops erythromelas* is the main species of medical relevance found in the Caatinga from Brazilian Northeast region. The pathophysiological effects involved *B. erythromelas* snakebite as well as the organism's reaction in response to this envenoming are little explored. Thus, edema was induced in mice paw using 2.5, and 5.0μg of *B. erythromelas* venom, and the percentage of edema was measured 30 minutes after injection. The plasma was collected and analyzed by mass spectrometry. We identified a total of 112 common plasma proteins (CPPs) differentially abundant among experimental groups, are which involved with the complement system and coagulation cascades, oxidative stress, neutrophil degranulation, platelets degranulation and inflammatory response. Apolipoprotein A1 (Apoa), serum amyloid protein A-4 (Saa4), adiponectin (Adipoq) showed up-regulated in mice plasma after injection of venom, while fibulin (Fbln1), factor XII (F12) and vitamin K-dependent protein Z (Proz) showed dow-regulated. The results indicate a protein pattern associated to a scenario of thrombo-inflammation to the *B. erythromelas* snakebite, evidencing potential biomarkers for monitoring the severity of this snakebite, new therapeutic targets and their correlations with the degree of envenoming once showed modulations in their abundance among the different groups according to the amount of venom injected into the mice.

Keywords: Snake venom; Edema; Mass spectrometry; Inflammation, Thromboinflammation

Introduction

Snakebite envenoming is a recurrent disease that affect tropical and subtropical countries (Chippaux, 2017 a and b, 2019; Williams et al., 2019), being the lower socioeconomic segments of society affected the most (Gutiérrez et al., 2017; Kasturiratne et al., 2019; 2021; Longbottom et al., 2018). In Brazil, snake species of the *Bothrops* genus are responsible for most cases of human envenomation (Moreira and Morato, 2014), and the venom-induced pathophysiology of these species induces several complex manifestations at local and systemic level, including edema, pain, hemorrhage, necrosis and hemostatic disorders (Magalhães et al., 2017; Valle et al., 2018).

Bothrops erythromelas, commonly known as "drought jararaca", has been identified as the main causative agent of snakebite accidents in Northeastern Brazil (Oliveira et al., 2010; Albuquerque et al., 2020). Occurring mainly in Caatinga, the species has records in the states of Alagoas, Bahia, Ceará, Maranhão, Minas Gerais, Paraíba, Pernambuco, Piauí, Rio Grande do Norte and Sergipe (Lira-da-Silva et al., 2009; Albuquerque et al., al., 2020). In addition, *B. erythromelas* can be found in arid and semiarid environments that surround dry and deciduous tropical forests, rocky areas, undergrowth of terrestrial bromeliads and riverbanks (Campbell, 2004). Also, its occurrence in the Metropolitan Region of Salvador has already been registered. The expansion of its distribution is not restricted to Bahia, occurring too in Paraíba, Alagoas and Rio Grande do Norte (Lira-da-Silva et al., 2009).

B. erythromelas venom is composed mainly of snake venom metalloproteases (SVMPs), phospholipases A_2 (PLA2), snake venom serineproteases (SVSPs), type C lectins (CTL), bradykinin potentiating peptides (BPP) and disintegrins (Jorge et al., 2015). The venom doesn't have thrombin-like activity due to its fibrinogenolytic potential, but it does have toxins with a high potential for activating prothrombin (Silva et al., 2003). The toxin

responsible for this last effect, berytractivase, has been already isolated and characterized (Silva et al., 2003). Furthermore, PLA_2 Asp49 acts as an inhibitor of platelet function (de Albuquerque Modesto et al., 2006), while the function of svVEGF has not yet been characterized (Junqueira-de-Azevedo et al., 2004).

In snakebite accidents, in general, the faster the diagnosis is done, and the antivenom administered, the lower the chances of the clinical condition worsening. The scarcity of studies reporting the local and systemic changes that this venom triggers, makes the real diagnosis of the patient's clinical condition even more difficult. This increases the difficulty of early identification by the clinical signs, and if the accident was caused by a snake of the species, worsening the scenario even more. In this context, it is evident that one of the greatest difficulties in snakebite is the absence of tools for monitoring the clinical course, which until then, have not yet reached the clinic. Here, we show that plasma proteins from the injured organism can be used to monitor the severity of the envenomation.

Material and Methods

Bothrops erythromelas snake venom

B. erythromelas venom was donated by Jorge and collaborators (2015). It was obtained by collecting venom from adult specimens (size ranging from 44 to 85 cm) that were anesthetized with the aid of carbon dioxide (CO_2). Venom samples from each specimen were pooled, lyophilized and stored at -20ºC.

Edema-inducing activity

Edema were induced in mice (n = 6/group) using 2.5, and 5.0µg of *B. erythromelas* venom in 15µL of 0.9% (w/v) sterile saline injected via intraplantar (Félix-Silva et al., 2017). The individual thickness of the right hind paw was measured before the venom injection

(baseline) and 30 min, 1, 3, 6 e 12 hrs after the edema induction using a digital caliper (Digimess, São Paulo, SP, Brazil). The edema was expressed as the percentage difference between the paw thickness after and before the venom injection calculated with the following formula: (Te-T0)/T0*100. The statistical analysis was performed using the T-test, one Way-ANOVA, followed by the multiple comparison test ($p \leq 0.05$) and expressed as mean and standard deviation.

Sample preparation

Blood was collected from each animal 30 minutes after injection of *B. erythromelas* venom by cardiac puncture, transferred to tubes with EDTA, and then centrifuged at 5.000 rpm for 10 min at 4 °C. Thirty micrograms of proteins from each pool were solubilized in 60 µL of 50 mM buffer ammonium bicarbonate and 10 µL of 1% (w/v) RapiGest SF Surfactant (Waters) were added. Then, the samples were incubated for 60 min at 37°C. The samples were reduced with 2.5 µL of 200 mM dithiothreitol (Calbiochem) (37°C, 40 min) and alkylated with 2.5 µL of 375 mM iodoacetamide (30 min, light-free room temperature (Sigma-Aldrich, Munich, Germany). Then, a trypsin solution at 0,01 ug/uL (Promega, Mannheim, Germany) (1:30 enzyme: protein) was added and the samples were incubated for 12 hours at 37°C. The enzymatic reaction was stopped with the addition of 5% (v/v) trifluoroacetic acid (Roth, Mannheim, Germany). Tryptic digests were obtained by centrifuging the samples at 16000×g for 30 min and the supernatants were collected, dried, and stored at -20°C until further analysis.

LC-MS/MS

The peptides were resuspended in 50 mM ammonium bicarbonate in 50% (v/v) acetonitrile (ACN) at room temperature and desalinized using Peptide Cleanup C18 Spin Tubes (Agilent) according to the manufacturer's instructions. The peptides were dried and

resuspended in 0.1% (v/v) formic acid and subjected to nano-ultra-performance liquid chromatography by an Ultimate 3000 LC (Dionex, Germering, Germany) using 0.1% (v/v) formic acid in Mili-Q water as solvent A and 0.1% (v/v) formic acid in ACN as solvent B. The peptides were loaded onto a C18 column, 30µm x 5mm (ThermoFisher Scientific) under an isocratic gradient of 2-40% buffer B for 120 minutes at a flow of 300 nL/min and eluted by a Reprosil-Pur C18-AQ analytical column, 3µm, 120Å, 105mm (PICOCHIP, New Objective), followed by 55% to 90% B for 1 min, maintained at 90% B for 10 minutes and rebalanced to 2% B for 10 minutes. The peptide elution was coupled directly to a Q-Exactive mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) set for Data Dependent Acquisition, with the MS spectra acquired from 200 to 2000 m/z, in a resolution of 70.000 and injection time of 100 ms. The fragmentation chamber was conditioned with collision energy between 29 to 35%. MS/MS spectra were acquired with 17,500 resolution, injection time of 50 ms, 2.0 m/z isolation window and 45s dynamic exclusion time. The raw MS files were acquired using the Thermo Xcalibur software (version 4.0.27.19, ThermoFisher Scientific Inc.).

Data analysis

Raw MS files were analyzed to PatternLab for Proteomics 4 for protein identification and quantification. The files containing protein intensity, obtained by the extracted ion chromatograms (XIC) of peptides, were analyzed in pairs (control vs. envenoming) and the fold change values (Log-Fc) and *p*-values were calculated using the t test of Student using MetaboAnalyst. Then, the individual lists by group of validated proteins were analyzed by Jveen to obtain the distribution of proteins between groups. Proteins that were common to all groups were analyzed by PLS-DA and Vip Score using MetaboAnalyst to identify the proteins responsible for the difference between those groups. All proteins were analyzed for

138 their interactions using NetworkAnalyst, and by Metascape to analyze enriched clusters and
139 physical interactions between proteins.

140 **Results**

141 ***B. erythromelas* venom induces edema and local tissue changes**

142 *B. erythromelas* venom induced paw edema and nociception at the doses 2.5 and 5.0µg tested
143 between 15 min to 12 h (Figure 1 A). Mice that were injected with venom show a facial
144 grimace of pain compared to the saline group with the following characteristics: narrowing
145 of the orbital area, contracted cheek muscle, the presence of a lump on top of the nose and
146 ears and whiskers retraction. Also, hemorrhagic manifestations were noticeable at the venom
147 injection site (Figure 1 B, C and D, upper).

148 Histological analysis showed progressive tissue hemorrhage and dermis-epidermis
149 separation (Figures 1 E, F, G and H, lower), in addition to an alteration in the thickness of
150 the epidermis in the group injected with 2.5µg of venom (Figure 1 I). Finally, 5.0 µg of
151 venom caused a characteristic scenario of blister formation with bloody content,
152 characterized by the marked presence of erythrocytes inside the blister (Figure 1 G).

153 ***B. erythromelas* venom induces changes in the blood plasma proteome**

154 Overall, 4933 peptide sequences were identified corresponding to 243 proteins (maximum
155 parsimony analysis) identified with at least one unique peptide. Regarding the overlapping
156 profiles of the identified proteins, we verified 112 common plasma proteins (CCPs) in both
157 experimental groups, in addition to 8 and 17 exclusive proteins in the group injected with
158 2.5 and 5.0µg of venom, respectively. Plasma proteome of mice injected with 2.5 and 5.0µg
159 of *B. erythromelas* venom exhibited 120 and 129 differentially abundant proteins (DAPs),

respectively (Figure 2 A). We found that, compared to the control group, 42 proteins showed a reduction in their abundance after envenomation, while another 70 increased (Figure 2B).

Heatmap analysis indicates differential abundance protein profiles between experimental groups but they are dissimilar compared to the control group. These results indicated that, despite the local effect, the edema caused by *B. erythromelas* venom presents a systemic interface evidenced by alterations in the plasma proteome of mice, suggesting the involvement of "new actors" in the pathology. We then asked whether the proteins could have any potential for diagnosis or for monitoring the severity of the snakebite envenoming. PLS-DA analysis of the differentially abundant proteins was able to segregate the groups, indicating that the altered proteins can be effectively used to distinguish between injected with venom versus groups no injected with venom, and also for the degree of snakebite envenoming (Figure 2 C and D). Thus, we suggest the possibility of using these proteins as potential biomarkers for *B. erythromelas* envenomation.

Plasma proteins acting as biomarkers for snakebite envenoming

Based on VIP score values, peptides of six main proteins were analyzed according to abundance, sensitivity and specificity (Figure 3) to improve the knowledge about these potential biomarkers. Apoa, Saa4 and Adipoq have showed increasing trends in mice plasma after envenomation with high sensitivity and specificity of these proteins in predicting envenomation, as well as the severity in all cases (Figure 3 A, B and C). On the other hand, Fbln1, F12 and and Proz have showed a reduction in their abundance but with high sensitivity and specificity.

B. erythromelas **venom induces thromboinflammation**

Among the number of initial proteins identified with altered abundance, we sought to highlight the plasma proteins that may represent the key in the *B. erythromelas* envenomation, indicating the scale and complexity of process dysregulation in this snakebite envenoming (Figure 4A). We verified that these proteins are involved in multiple biological processes, which we highlighted the Top 20 (Figure 4B). These proteins are mainly involved in the coagulation and complement cascades, platelet degranulation and inflammatory response. In addition, detoxification of reactive oxygen species, complement activation via the alternative pathway, and neutrophil and platelet degranulation were also enriched (Figure 4 B and C).

These data suggest that the *B. erythromelas* venom produces a thromboinflammatory scenario, in response to the activation of the blood cascade systems (complement and coagulation) and activation of the multicellular system by signaling involving neutrophils and platelets.

B. erythromelas **venom induces hematological disturbances**

We analyzed the involvement of the multicellular blood system after verifying the involvement of multiple pathways associated with thromboinflammation. For this purpose, blood samples were collected for analysis of hematological parameters (Figure 5). *B. erythromelas* venom induced total leukocytosis characterized by neutrophilia and lymphocytosis. Interestingly, this venom also changed the parameters related to red blood cells, indicating polycythemia, microcytosis and hypochromia. We have also observed thrombocytopenia and a decrease in mean platelet volume suggesting peripheral platelet destruction.

Discussion

Bothropic envenoming is characterized by the rapid development of an inflammatory process at the site of venom injection (Félix-Silva et al., 2014; 2017; 2018). In our experimental model, the venom was able to rapidly induce edema, alternating among the amount of edema during the course of the envenomation by *B. erythromelas* venom. Probably, the maximum edematogenic effect, followed by its decrease, was associated with the direct action of this venom. However, the subsequent increase in edema may be a consequence of the formation of molecules that signal the inflammatory response. Farther, thre are a co-option of endogenous signaling pathways, which act as positive feedback, aggravating the inflammatory response according to Bickler (2020).

This response often leads to severe edema as indicated in our date, and its prolonged maintenance can to cause complication such as tissue loss and amputations (Luciano et al., 2009; Schulz et al., 2016; Graciano; Carvalho, 2018; Valle et al., 2008). We also found that the venom induced nociception and intense local haemorrhage in the experimental groups. This problematic may be associated with ischemia and neural compression, which can result in a compartment syndrome and lead to permanent tissue loss or amputation of the affected site due to necrosis (Teixeira et al., 2009). We are currently investigating the effects of these processes on nociceptive signaling, as well as the mechanisms and secondary effects of ischemia caused by this local hemorrhage through different separations compatible with MS and eluent compositions to provide: 1) altered biological processes in snakebite; 2) candidates for biomarkers; and 3) the influence of the residual venom, which can remain active, with slow release into the bloodstream. This observation could open a new way for the treatment of *B. erythromelas* envenoming and other species of the *Bothrops* genus. However, these researches are ongoing and are beyond the scope of this study.

We found histological changes at the site of venom injection, including intense hemorrhage and formation of dermo-epidermal blisters, which are common manifestations of envenoming in humans (Gimenes et al., 2021). SVMPs are one of the main toxins that compose the *B. erythromelas* venom, and these proteins act mainly in hemostasis, while other components act in the extracellular matrix and are responsible for the hemorrhagic activity (Jorge et al., 2015). So far, the role of metalloproteases from the venom of *B. erythromelas* in hemorrhage remains unclear, although the presence of berytractivase, a prothrombin activating metalloproteases, has no hemorrhagic effect.

Here, we unveil an important step for understanding the systemic response to the severity of edema caused by *B. erythromelas* venom. For this purpose, plasma samples were inspected for 30 minutes to assess the first events involved in the envenoming, as previous studies indicate that this is the time for maximum effect of edema development (Félix-Silva et al., 2014; Félix-Silva et al., 2017; Félix-Silva et al., 2018), and as observed in our study. A panel of proteins were responsible for the discrimination of the groups injected with *B. erythromelas* venom in a dose-response manner in relation to the control, suggesting a possible role of this panel of proteins as biomarkers for the lesion.

Apolipoproteins identified in our study have gained prominence in the inflammatory context analyzed by interaction networks due to the number of interactions that they can create. To illustrate the importance of these proteins in inflammation, we emphasize the contributions of some members. ApoA1, for example, is negatively regulated, exercising negative regulation at the start of and throughout inflammation, favoring an out-of-control inflammatory scenario (Sengupta and Mukhopadhyay, 2016). On the other hand, a pro-inflammatory role by the toll-like receptor (TLR) has been attributed to ApoA1, suggesting its reconversion into an inflammatory apolipoprotein. ApoA1 affects NF-κB and MAPK

254 downstream pathways and induces IκBα and JNK phosphorylation, therefore considered as
255 DAMPs (de Seny et al., 2015).

256 Serum amyloid A-4 has been used as a clinical indicator detected by immunoturbidimetry
257 or immunonephelometry to monitor inflammatory processes and efficacy of drug therapy
258 (Zhang et al., 2019). Its pro-inflammatory effects include activation of the classical
259 complement system through C1q, activation of the NRLP3 inflammasome, chemotaxis and
260 enhanced production of IL-1β and TNF-α, contributing to cytokine storm, in addition to
261 inducing atypical coagulation (Migita et al., 2012; Ying et al., 1993; Zhang et al., 2019).
262 Furthermore, recent reports suggests that the acute increase of this protein in the plasma
263 could contribute to the genesis of coagulopathies (Gonçalves and Sesterheim, 2020),
264 supporting its involvement in the coagulation process in face of *B. erythromelas*
265 envenomation.

266 Adiponectin (Adipoq) is the major/principal protein in human plasma that acts in
267 sensitization that plays a role in adipose tissue, skeletal muscle, vascular endothelium and
268 others. Adipoq promotes the polarization of macrophages to the M2 profile, while
269 suppressing the polarization to the M1 profile (Choi et al., 2020). In addition to decreasing
270 the phagocytic activity of macrophages, it inhibits their transformation into foam cells
271 (Okamoto, 2006; Ouchi et al., al., 2001) and increases the production of nitric oxide (NO)
272 (Margaritis et al., 2013). Farther, there is decreasing the production of TNF-α (Cao, 2014)
273 and the adhesion of monocytes to endothelial cells, and also attenuates the proliferation of
274 vascular smooth muscle cells (VSMCs) (Okamoto, 2006).

275 In addition, Adipoq induces tissue inhibitor protein synthesis of metalloproteinase in
276 macrophages through increased production of IL-10, also inhibiting endothelial cell
277 apoptosis (Van Gaal et al., 2006). Beyond its anti-inflammatory properties, Adipoq exhibits

pro-inflammatory functions (Geagea et al., 2018; Choi et al., 2020). Thus, increased levels of Adipoq in the of experimentally envenomated mice plasma suggest the involvement of this protein in the pathophysiology. However, whether Adipoq exhibits an anti-inflammatory or pro-inflammatory function in the envenomation is something to be investigated.

Fibulin-1 (Fbln1) is an extracellular matrix protein expressed mainly in the outermost layer of the tunica media from arterial wall (Roark et al., 1995; Cangemi et al., 2014). This protein is a classic biomarker of elastolysis (Cangemi et al., 2011, Scholze et al., 2013), heart failure (Hutchinson et al., 2010), and kidney disease (Scholze et al., 2013). The down-regulation of Fibulin1 levels is related to aortic dissection (Cheuk and Cheng, 2011; Mohamed et al., 2009).

Another protein with potential biomarker is Factor XII (FXII), an effector of the intrinsic prothrombin activation pathway (Wheeler and Gailani, 2016). It is likely that the decrease detected here is due to the exacerbated activation of the coagulation system, which would result in its consumption. Thus, the reduction in FXII may be related to the activation of the intrinsic coagulation pathway, causing the consumption of these factors and could be used for monitoring coagulation. However, this conjecture can be easily dismissed, since berytractivase acts to convert prothrombin to thrombin (Silva et al., 2013), and thus, FXII as a coagulation biomarker may not reflect the real coagulation scenario.

Our approach also identified Z protein (Proz) as a discriminator between cases of experimental envenoming. In the mice plasma proteome, Proz levels were significantly reduced depending on the amount of venom used. Proz increases factor Xa inhibition (Escobar et al., 2019), and in this case, its reduction may be associated with dysregulation of the hemostatic system observed in cases of envenoming, with systemic and localized

activation of coagulation. This may suggest a mechanistic and biomarker role for Proz in this process.

We identified changes in the quantitative rates of proteins related to the complement system, coagulation, neutrophil and platelet degranulation, inflammatory response and oxidative stress. Until then, the effect of toxins against these systems had been explored separately, generating valuable contributions to the comprehension of the pathophysiology caused by *B. erythromelas* venom. However, the study of isolated systems limits the understanding of systemic disturbances and their influence on the response of the poisoned organism to the venom. Thus, plasma proteomic analysis allowed obtaining a fingerprint of multiple affected systems, suggesting thromboinflammation.

Another point of view of our interest was to evaluate the interactions among proteins through two approaches: generic network of interactions and physical interactions. While the network of generic interactions have showed a great representation and interactions among Apolipoproteins, Serpins and Complement factors, the network of physical interactions have demonstrated their involvement in new processes relevant to *B. erythromelas* snakebite.

We also identified platelet degranulation as a clearly altered process, and this finding is in line with the effects of Phospholipase A2 (Asp49) present in the snake venoms, BE-I-PLA2, which has a high inhibitory potential on aggregation through arachidonic acid and collagen, with no action on the main platelet receptors, suggesting an increase in its potential antiplatelet activity (De Albuquerque Modesto et al., 2006). Santoro and Sano-Martins (2004) evidenced *B. jararaca* venom induced a marked decrease in platelets within 1 hour after envenomation and that this condition of thrombocytopenia continued for up to 96 hours. This fact is similar in victims of *B. erythromelas* associated with the delay in the hospital

service (Cavalcante et al., 2017), which supports the alteration in the platelet process found here.

On the other hand, the process of neutrophil degranulation and detoxification to reactive oxygen species was also present among the processes modulated by proteins, suggesting the activation of these cells. In fact, *B. erythromelas* venom induces neutrophil chemotaxis which appears to be related to its phospholipase activity and the production of arachidonic-derived lipoxygenase metabolites, such as leukotriene B4 (Flores et al., 1993). Neutrophils are producers of reactive oxygen species that, when dysregulated, disseminate a systemic response (Laforge et al., 2020) and, therefore, this may configure an indirect mechanism in which *B. erythromelas* can induce oxidative stress.

Another phenomenon associated with the severity of the envenoming was hemostasis disturbances. It is indisputable that its change during envenomation matches the severity of envenomation caused by *B. erythromelas*. Studies have indicated that the venom of this species has a large coagulant potential (Santoro et al., 2015; Furtado et al., 1991). One of the toxins responsible for the high effect on coagulation is berytractivase, a prothrombin activator that comprises approximately 5% of the crude venom, although showing a high structural similarity with other metalloproteinases, this protein not induces hemorrhagic manifestations (Ito et al., 2001; Silva et al., 2003; Moura-da-Silva et al., 2008).

Although the activation of the complement system modulated by animal toxins is well reported (Ayres et al., 2015; Delafontaine et al., 2018; Luchini et al., 2019; Menaldo et al., 2016; Pidde-Queiroz et al., 2013; Tambourgi and van den Berg, 2014), we demonstrate in an unprecedented way: the disturbances suffered by the complement system during an experimental envenoming model using an *in vivo* system. Thus, our results corroborate to Pidde-Queiroz et al. (2010) which identified the activation of the complement system for the

first time by the *in vitro* action of the *B. erythromelas* venom. In addition, we present a compilation of complement proteins that can be used to monitor their activation or attenuation.

As the inflammatory response is triggered quickly after tissue injury, we found a large number of associated pathways and our findings suggest the presence of a thromboinflammatory scenario such as observed in the *B. leurucus* experimental envenomation in our previous study (Cavalcante et al, 2022). The term "thromboinflammation" has been used to refer to the activation of cascading systems present in the blood, as well as the activation of its multicellular system, whose simultaneous interaction between inflammation and hemostatic changes has never been addressed in the investigation of snake envenomation. The cascade systems (complement, coagulation and fibrinolysis) and the multicellular blood system (comprising platelets, endothelial cells and inflammatory leukocytes) are involved in the immediate response to snake venoms. Classically seen as two independent and distinct systems, discussions about their interrelationship have been related to other human diseases, which like *Bothrops* venoms, trigger multiple cascading events that contribute to the amplification of the venom's toxicity.

Our top20 of biological processes indicated an enrichment of the complement and coagulation cascades pathways, the degranulation of platelets and neutrophils, which corroborates previous studies that indicate the involvement of these elements in snakebite caused by *B. erythromelas*. Furthermore, we evidenced an enrichment of processes related to tissue remodeling, justified by the dermis-epidermal separation found in our study, as well as other reported alterations. This may be associated with a mechanism of ischemia tolerance and/or tissue repair, as these processes are involved in wound healing. Finally, enriched oxidative stress has yet to be elucidated causes and consequences in *B. erythromelas*

envenoming and requires further exploration. It is possible that it acts as result of the activation of neutrophils, which respond to invading agents with oxidative explosion and formation of extracellular neutrophil traps (Vorobjeva; Chernyak, 2020).

To understand the turnover of hematological cells during thromboinflammation caused by *B. erythromelas* venom, we performed hematological analyses. We found leukocytosis, lymphocytosis, neutrophilia and thrombocytopenia. Neutrophils are present in myonecrotic and hemorrhagic areas, or even in the inflammatory infiltrate (Zuliani et al., 2020). Once activated, they produce pro-inflammatory cytokines, phagocytose and release neutrophil extracellular traps (NETs), acting in the repair process, or potential tissue damage (Beavers and Skaar, 2016; Manda-Handzlik and Demkow, 2015; Mortaz et al., 2018; Ravindran et al., 2019; Rosales, 2018; Wang, 2018). The evaluation of the effects of *Bothrops* venoms on neutrophils has been explored, generating a great amount of information over the decades. In vivo studies have shown that venoms have the ability to induce the migration of polymorphonuclear neutrophils to snakebite sites (Zuliani et al., 2020)

It is known that patients with severe tissue damage caused by *B. atrox* venom have a polarized immune profile for a Th1 response, and have a more intense local immune response with high levels of IL-1β, IL-6, TNF-α, MIP-1 (CXCL-1) and MIP-2 (CXCL-2) (Escocard et al., 2006; Ibiapina et al., 2019). However, the Th1 response evoked by the *B. atrox* venom is regulated by neutrophils and via the myeloid differentiation factor 88 (MyD88) pathway (Moreira et al., 2013). Leukocytosis with neutrophilia and thrombocytopenia of variable intensity has been reported in snake envenoming, however, more intense changes have been reported in cases that had tissue loss and/or limb amputations (Luciano et al., 2009; Graciano et al., 2009; al., 2017; Pedroso Lacerda et al., 2017; Valle et al., 2018).

Thus, leukocytosis, neutrophilia and thrombocytopenia, beyond being a tool to monitor general inflammation and coagulation, could also represent a way to monitor thromboinflammation and tissue damage in cases of *Bothrops* envenomations. Neutrophilia associated with high concentrations of NETs in the circulating blood and thrombocytopenia, are described as indicators of severity and poor clinical outcomes in thromboinflammatory diseases (Chaudhary et al., 2021; Veras et al., 2020; Chen et al., 2020) and thus may also be indicators for thromboinflammation and in predicting poor clinical outcomes in snake envenomations.

Analysis of the red series indicated the presence of polycythemia and microcytic and hypochromic erythrocytes. Alterations in erythrocytes are neglected in cases of envenomation by *Bothrops*, although they have great relevance due to the association of thrombotic microangiopathy (TMA) and acute kidney injury (AKI) (Albuquerque et al., 2020). Rats injected with *B. jararaca* venom manifested thrombotic microangiopathy with hemolysis and presence of poikilocytosis due to the generation of intravascular thrombin (Senise et al., 2015).

Although we have not analyzed blood smears, we were able to identify changes in erythrocyte morphology by RDW values that indicate different sizes, which may indicate defects in the shape of these cells, making them more susceptible to rupture. We also found that low MCV and MCH strengthen morphological changes in erythrocytes, as well as reducing the amount of hemoglobin present in these cells. Clinically, cases of TMA have been reported after *B. jararaca* and *B. erythromelas* envenomation, some of which have been associated with thrombocytopenia and acute kidney injury (Bucaretchi et al., 2019; Malaque et al., 2019; Mota et al., 2020). Likewise, we found laboratory abnormalities in the red blood

series after *B. erythromelas* envenoming. However, markers of microvascular hemolysis and anemia after snake envenomation are not yet specific (Noutsos et al., 2019).

Conclusions

We evidenced the importance of using proteomic approach to study thromboinflammation associated with *B. erythromelas* envenoming. For that, we applied the screening approach to the topic of inflammation induced by venom via intraplantar in mice, as this is one of the most serious and frequent pathologies observed in snakebite. We elucidate the trends of improvement and/or worsening of the edema caused by this venom. The mechanisms of action of the proinflammatory and coagulopathic toxins in the *B. erythromelas* venom are diverse and can result in anticoagulant and procoagulant effects. The methodology applied here has facilitated the rapid identification of altered plasma proteins during the envenoming and their abundance are based on the severity of the envenoming not previously known. We also report multiple altered processes during envenomation, expanding knowledge of the pathophysiology of envenomation. Thus, our future research efforts are now focusing on validating candidate biomarkers and developing platforms for screening and identifying those that are important for the selection of molecules for diagnosis.

Declaration of competing interest

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.

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691 **Legend of figures**

692 **Figure 1 -** *Bothrops erythromelas* venom induces edema and changes in the mouse plasma
693 proteome. **A.** Edematogenic profile of *B. erythromelas* venom by dose-response: samples of
694 sterile saline solution were injected into the right hind paw of male swiss mice (n = 6). Paw
695 edema was measured after injection of venom with the aid of a digital caliper and expressed
696 as a % increase in relation to the initial thickness. **B, C** and **D**. Representative images of
697 mice (face and paw) 30 minutes after saline administration (**B**) and 2.5 and 5.0µg (**C** and **D**)
698 of *B. erythromelas* venom in mice. **E**, **F**, and **G**. Representative light micrographs of mouse
699 paw tissue 60 min after intraplantar injection with 2.5 and 5.0µg of the *B. erythromelas*
700 venom. Coloration: H&E. **H** and **I**. Quantification of morphological changes in mouse paw
701 tissue 30 min after intraplantar venom injection with 2.5 and 5.0µg of the *B. erythromelas*
702 venom.

703 **Figure 2 -** Blood plasma alteration after injection with *B. erythromelas* venom. **A.** Venn
704 diagram describing the distribution of DAPs between groups and number of proteins with
705 differential abundance (DAPs) (log-FC ≥1) identified in each group. **B.** Heatmap shows the
706 turnover of common plasma proteins (CPP) obtained from mice injected with 2.5 and 5.0µg
707 of *B. erythromelas* venom. High protein abundance is shown in shades of red and low
708 abundance are shown in shades of green. The abundance of each protein was normalized by
709 the internal standard to generate a data matrix consisting of signal intensity values followed
710 by scaling data centered on the mean and generalized logarithmic transformation. Values
711 are presented as the mean of each experimental group. **C.** The PLS-DA analysis shows the
712 dissimilarity between the envenomation groups compared to the control group. **D.** The
713 Variable Importance Projection (VIP) plot displays the top 20 most important protein
714 resources identified by the PLS-DA. The colored boxes on the right indicate the relative
715 abundance of the corresponding protein for plasma samples from mice injected with venom.
716 VIP is a weighted sum of squares of the PLS-DA loads taking into account the amount of Y
717 variable explained in each dimension.

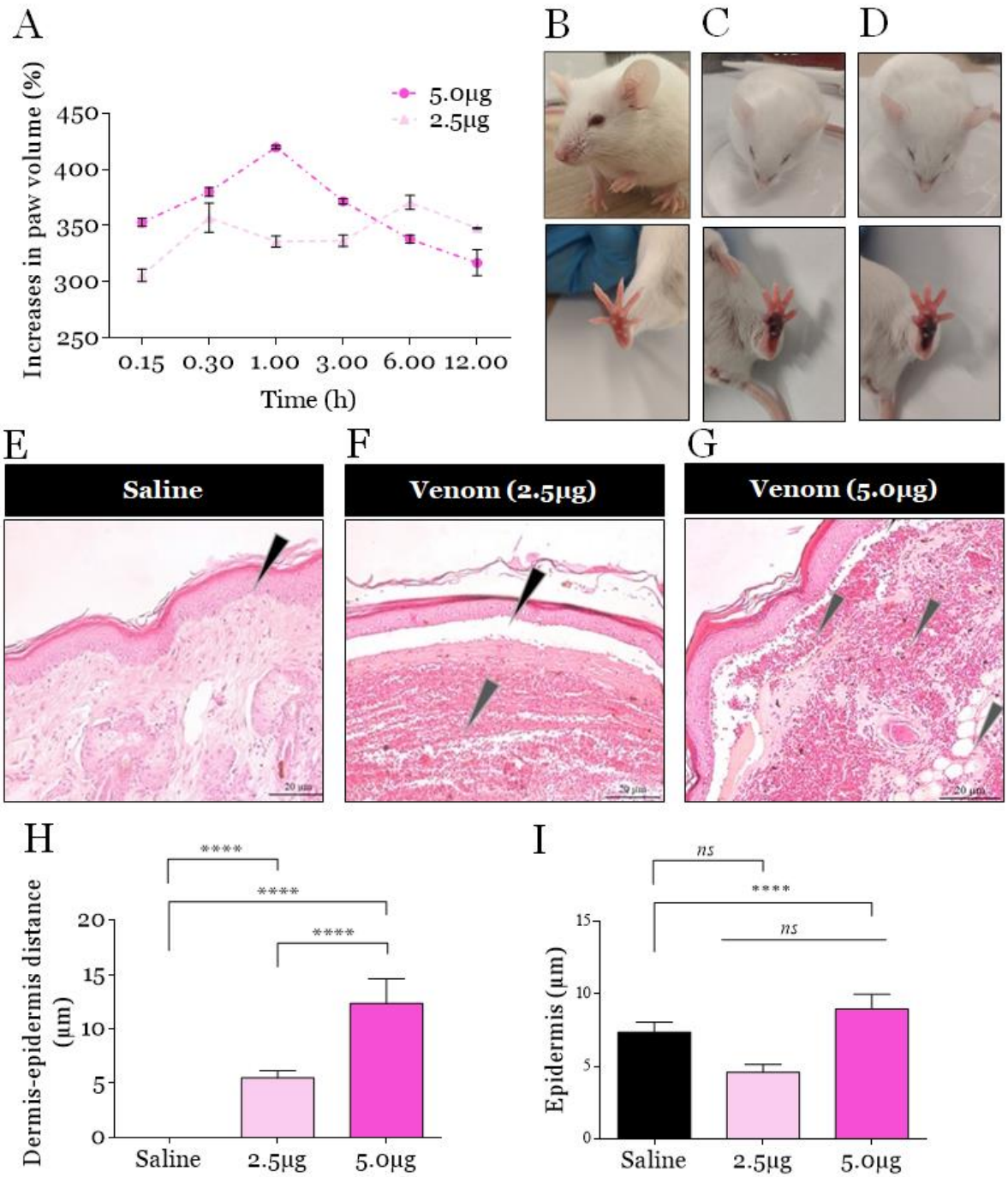
718 **Figure 3 –** Intensity and peptide sequence map of Apolipoprotein A1 (Apoa), Serum
719 amyloid protein A-4 (Saa4), Adiponectin (Adipoq), Fibulin (Fbln1), Factor XII (F12) and
720 Vitamin K-dependent protein Z (Proz) in mice plasma after injection of *B. erythromelas*
721 venom. The identified tryptic peptides were mapped to protein sequence alignments (tryptic

peptides have been identified: blue). Quantitative trends of selected differentially abundant proteins in the plasma of mice after injection with 2.5 and 5.0µg of *B. erythromelas* venom. Data are represented as the intensity of XIC's for each protein. *p < 0.05, **p < 0.01 and ***p < 0.005 and ****p < 0.0005 for the comparisons indicated (one-way ANOVA).

Figure 4 - Network of protein-protein interactions with the main common plasma proteins (CPP) differentially abundant in mouse plasma 30 min after intraplantar injection with *B. erythromelas* venom. **A.** Main proteins involved in the interactions of altered biological processes after *B. erythromelas* envenomation. The proteins were mapped to the interaction database (NetworkAnalyst) to produce a large sub-network ("continent") with several smaller sub-networks ("islands"). Subnets of predictive interactions with at least three nodes starting from the proteins of interest are presented. Only proteins that showed at least 3 interactions were considered. **B.** Network of enriched terms colored by cluster ID, where nodes that share the same cluster ID are typically close to each other. **C.** *P*-value of enriched terms. Terms containing more genes tend to have a more significant p-value.

Figure 5 - Changes in the number of circulating total blood cells. WBC (White Blood Cells), Neu (Neutrophil), Lym (Lymphocytes), RBC (Red Blood Cell), HCT (Hematocrit), HGB (hemoglobin), MCV (Mean Corpuscular Volume), MCH (Mean Corpuscular Hemoglobin), MCHC (Mean Corpuscular Hemoglobin Concentration), RDW-CV (Red Cell Distribution), RDW-SD (Red Cell Distribution), PLT (Platelets) and MPV (Mean Platelet Volume) in mice injected with *B. erythromelas* venom. The cells in blood samples were counted in an automated hematological analyzer. The columns represent the mean ± SD (n = 6/group). *p < 0.05, **p < 0.01 and ***p < 0.005 and ****p < 0.0005 for the comparisons indicated (one-way ANOVA).

745 **Figure 1**



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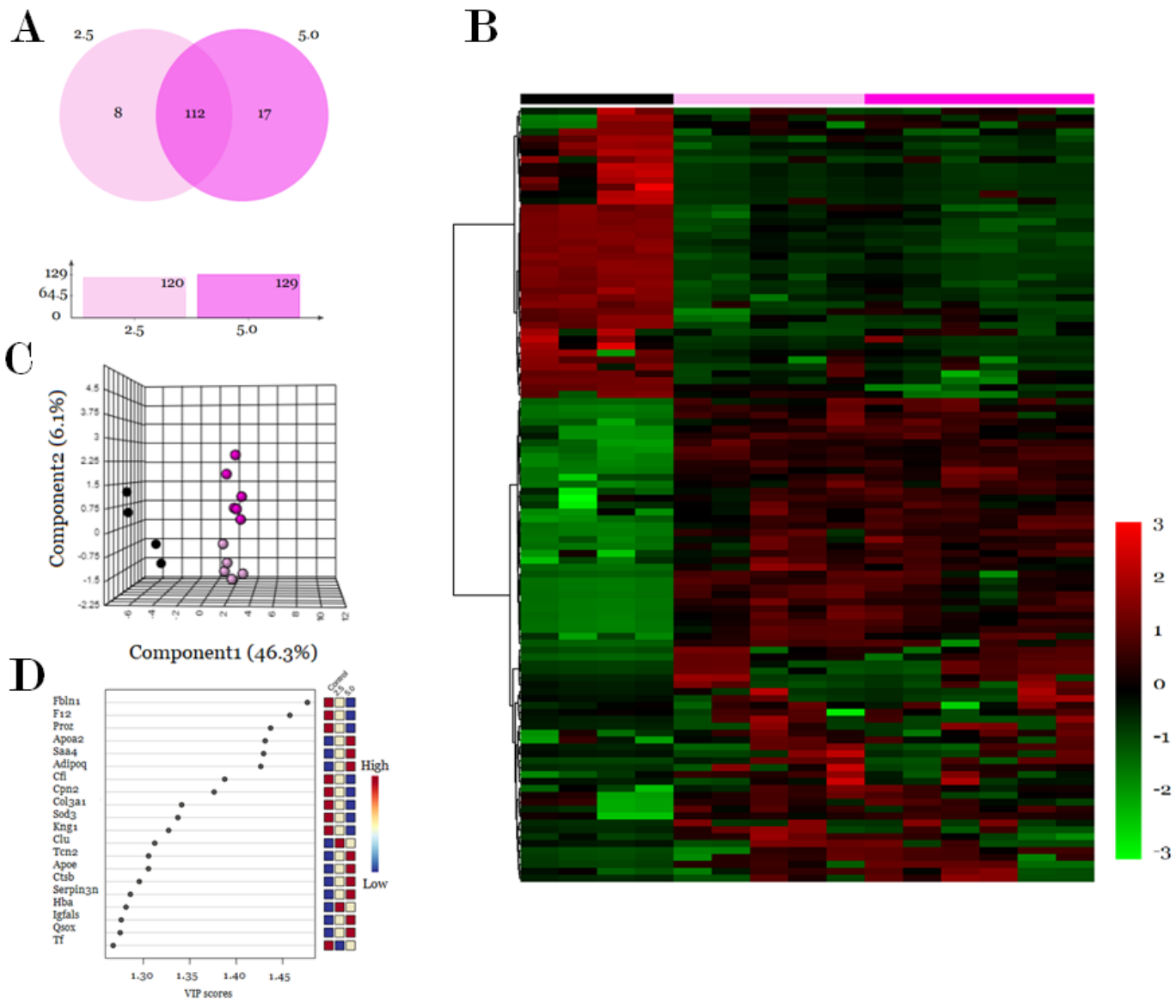
748 **Figure 2**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8
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17
129
64.5
0
120
129
2.5
5.0
C
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Component1 (46.3%)
D
Fbln1
F12
Proz
Apoa2
Saa4
Adipoq
Cfi
Cpn2
Col3a1
Sod3
Kng1
Clu
Tcn2
Apoe
Ctsb
Serpin3n
Hba
Igfals
Qsox
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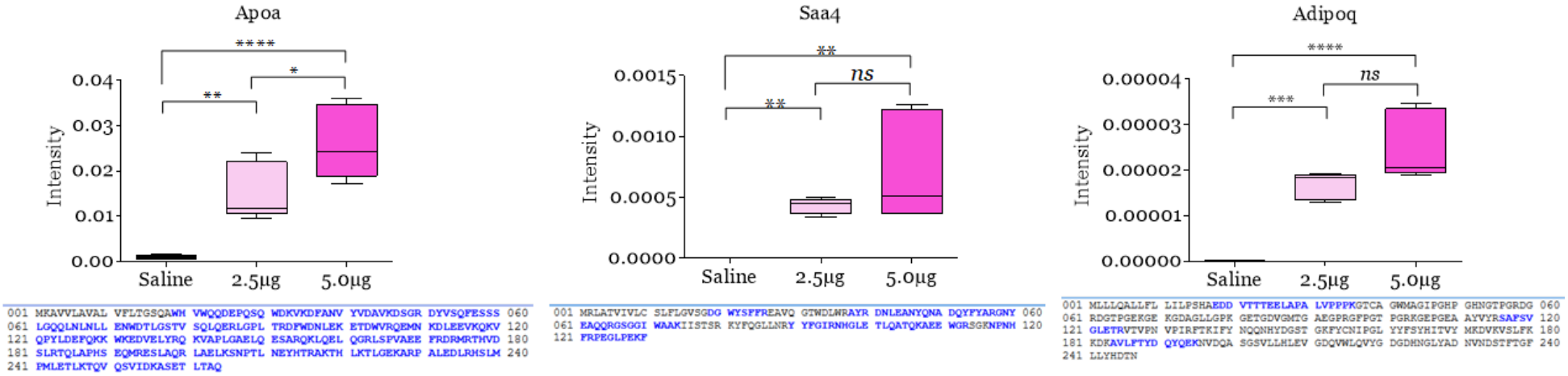
Figure 3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0.02
0.01
0.00
****
*
**
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5.0µg
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121 QPYLDEFQKK WKEDVELYRQ KVAPLGAELQ ESARQKLQEL QGRLSPVAEE FRDRMRTHVD 180
181 SLRTQLAPHS EQMRESLAQR LAELKSNPTL NEYHTRAKTH LKTLGEKARP ALEDLRHSLM 240
241 PMLETLKTQV QSVIDKASET LTAQ
Saa4
Intensity
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0.0010
0.0005
0.0000
**
ns
**
Saline
2.5µg
5.0µg
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061 EAQQRGSGGI WAAKIISTSR KYFQGLLNRY YFGIRNHGLE TLQATQKAEE WGRSGKNPNH 120
121 FRPEGLPEKF
Adipoq
Intensity
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0.00003
0.00002
0.00001
0.00000
****
ns
***
Saline
2.5µg
5.0µg
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181 KDKAVLFTYD QYQEKNVDQA SGSVLLHLEV GDQVWLQVYG DGDHNGLYAD NVNDSTFTGF 240
241 LLYHDTN
Fbln1
Intensity
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0.0002
0.0001
0.0000
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ns
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2.5µg
5.0µg
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061 CRMVQEQCCH NQLEELHCAT GINLASEPEG CASLHSYNSS LETIFIKRCC HCCMLGKASL 120
121 ARDQTCEPIV MISYQCGLVF RACCVKAREN SDFVQGNGAD LQDPAKIPDE EDQEDPYLND 180
181 RCRGGGPCKQ QCRDTGDEVI CSCFVGYQLQ SDGVSCEDIN ECITGSHNCR LGESCINTVG 240
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301 IQDALGNCID INECLSISAP CPVGQTCINT EGSYTCQKNV PNCGRGYHLN EEGTRCVDVD 360
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481 CEDIDECALP TGGHICSYRC INIPGSFQCS CPSSGYRLAP NGRNCQDIDE CVTGIHNCSI 540
541 NETCFNIQGS FRCLSFECPE NYRRSADTFR QEKTDTVRCI KSCRPNDEAC VRDPVHTVSH 600
601 TVISLPTFRE FTRPEEIIFL RAVTPLYPAN QADIIFDITE GNLRDSFDII KRYEDGMTVG 660
661 VVRQVRPIVG PFYAVLKLEM NYVLGGVVSH RNVVNVHIFV SEYWF
F12
Intensity
0.0015
0.0010
0.0005
0.0000
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ns
Saline
2.5µg
5.0µg
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121 CPEHLTGKHC QKEKCFEPQL LKFFHENELW FRTGPGGVAR CECKGSEAHC KPVASQACSI 180
181 NPCLNGGSCL LVEDHPLCRC PTGYTGYFCD LDLWATCYEG RGLSYRQQAG TTQSGAPCQR 240
241 WTVEATYRNM TEKQALSWGL GMHAPCRNPD NDTRPWCFVW SGDRLSWDYC GLEQCQTPTF 300
301 APLVVPESQE ESPSQAPSLS HAPNDSTDHQ TSLSKTNTMG CGQRFRKGLS SFMRVVGGLV 360
361 ALPGSHPYIA ALYWGNNFCA GSLIAPCWVL TAAHCLQNRP APEELTVVLG QDRHNQSCEW 420
421 CQTLAVRSYR LHEGFSSITY QHDLALLRLQ ESKTNSCAIL SPHVQPVCLP SGAAPPSETV 480
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Proz
Intensity
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0.0004
0.0003
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0.0001
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ns
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5.0µg
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752 **Figure 4**

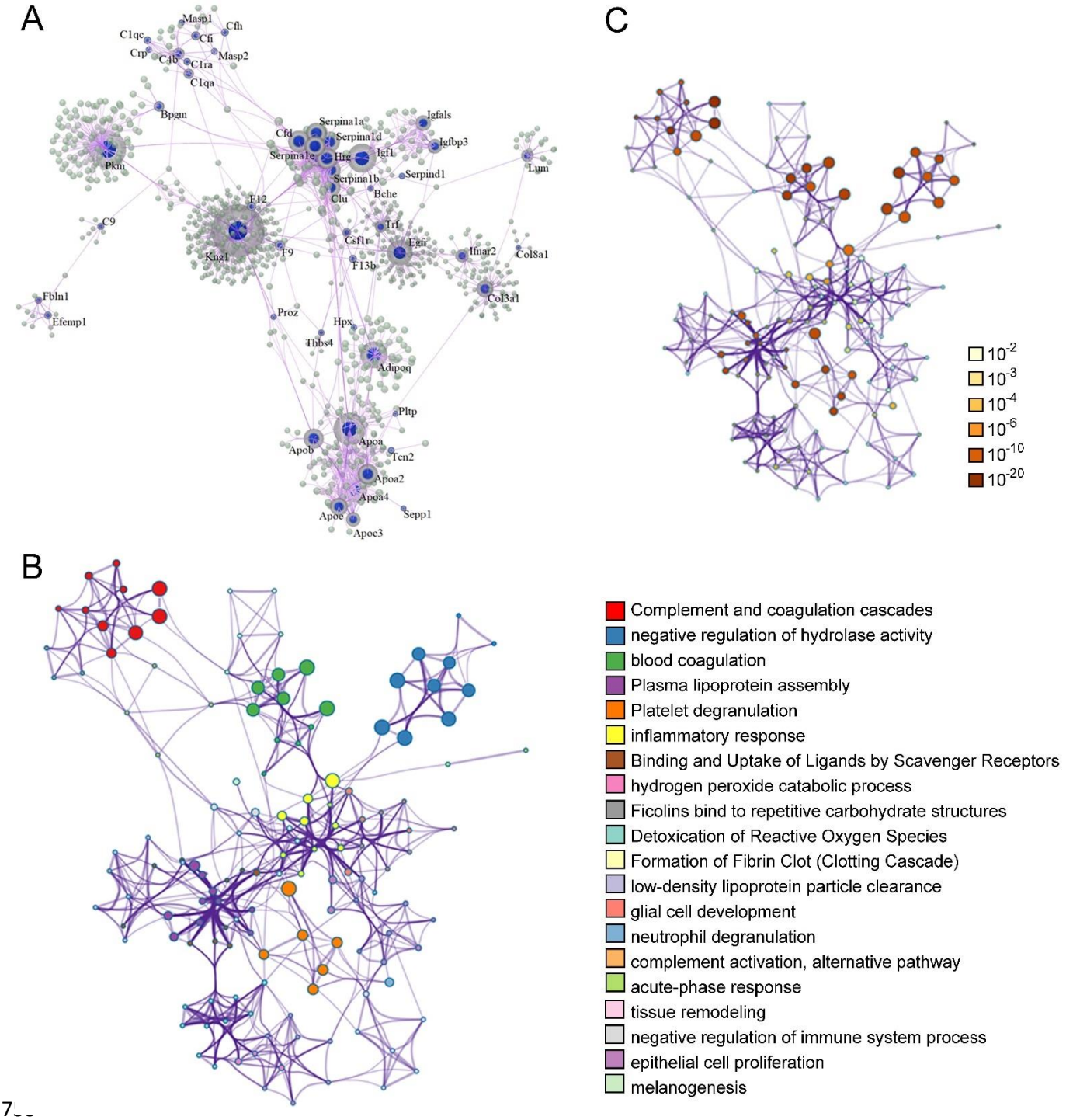

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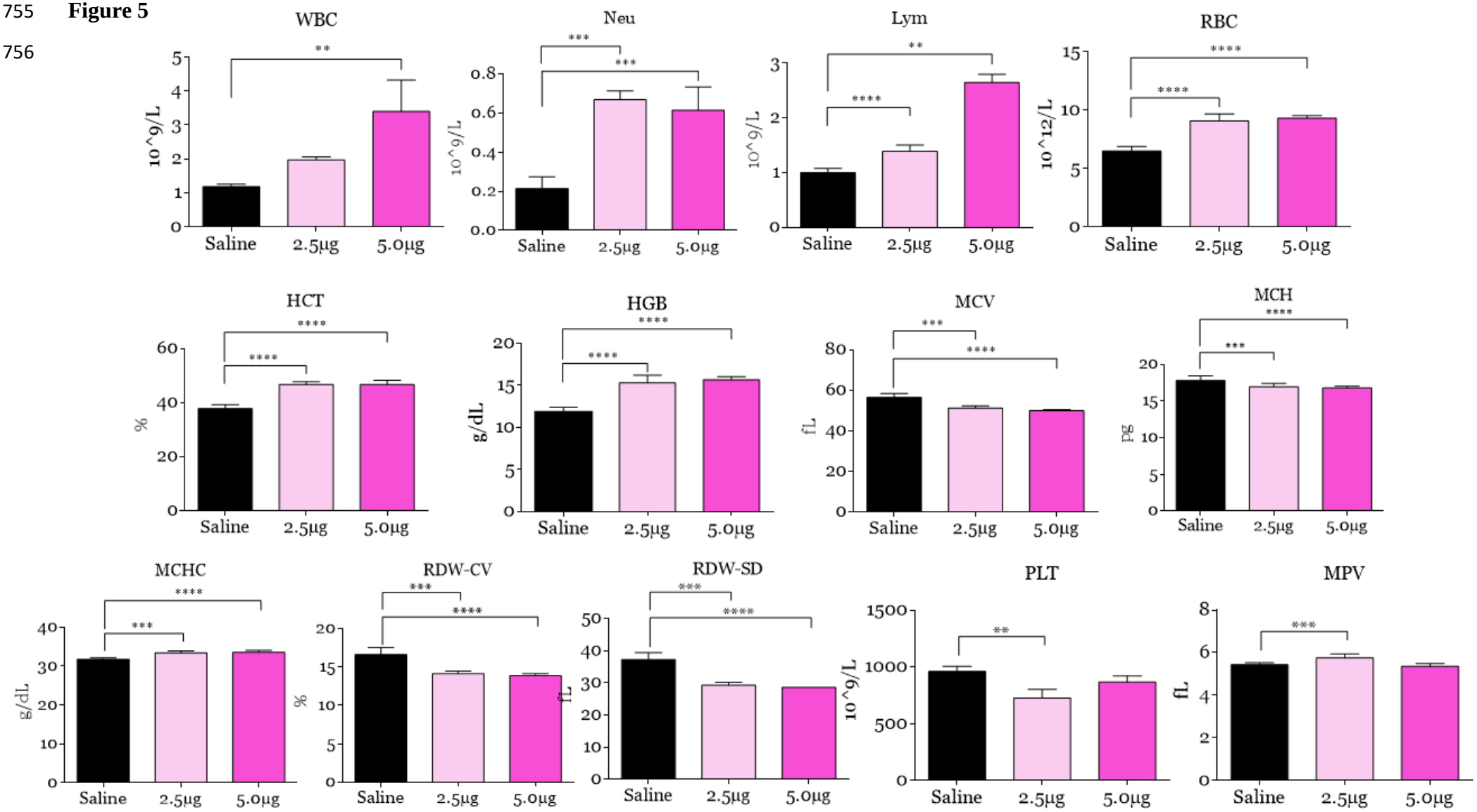
Figure 5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Neu
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%
HGB
g/dL
MCV
fL
MCH
pg
MCHC
g/dL
RDW-CV
%
RDW-SD
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PLT
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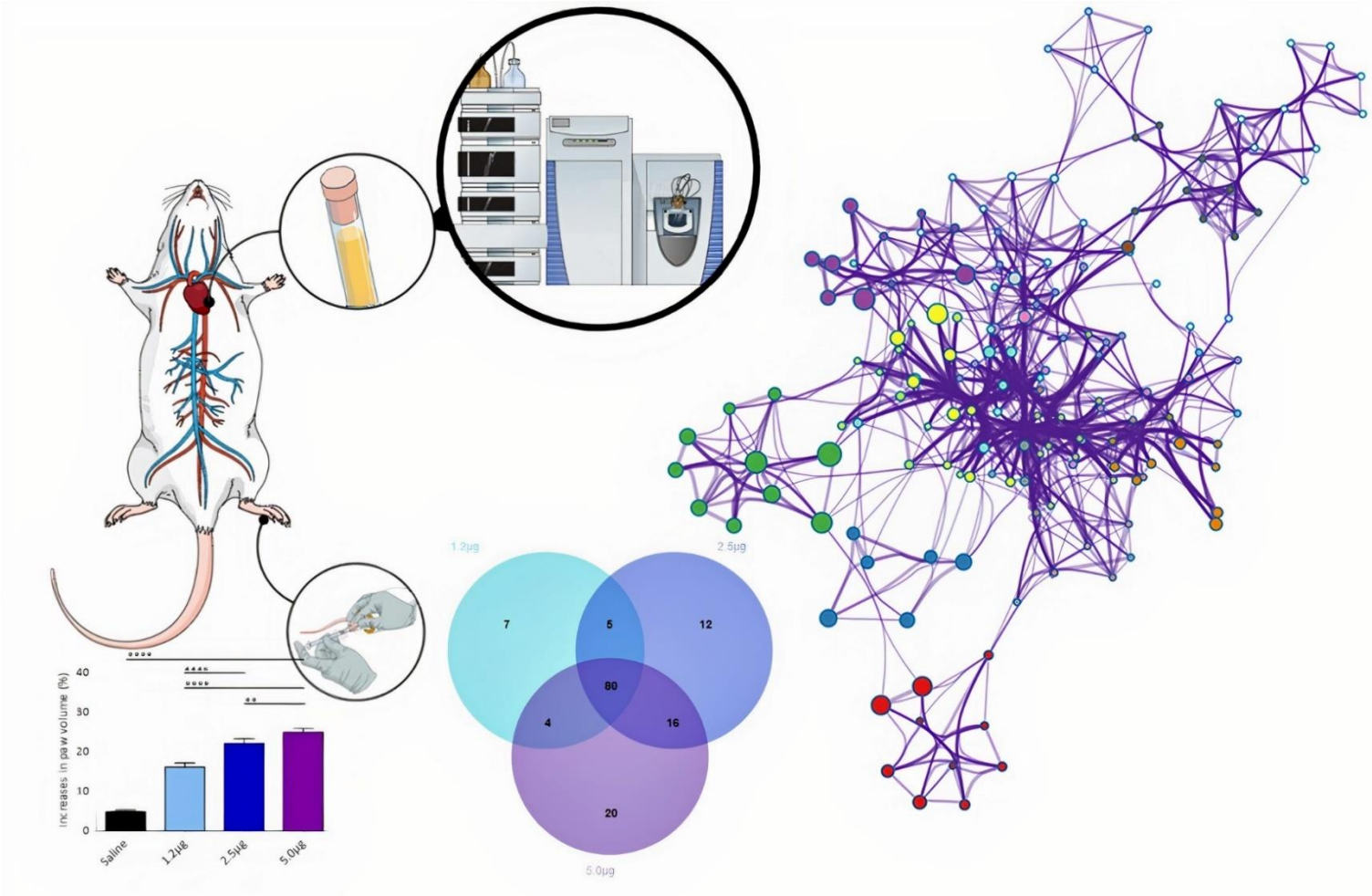
MPV
fL

04

A fingerprint of plasma proteome alteration after local tissue damage induced by *Bothrops leucurus* snake venom in mice



Graphical abstract was developed by Microsoft PowerPoint (Journal requirement). This figure represents a summary of the main findings of the study.

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A fingerprint of plasma proteome alteration after local tissue damage induced by *Bothrops leucurus* snake venom in mice

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Thromboinflammation

ABSTRACT

Bothrops spp. is responsible for about 70% of snakebites in Brazil, causing a diverse and complex pathophysiological condition. *Bothrops leucurus* is the main species of medical relevance found in the Atlantic coast in the Brazilian Northeast region. The pathophysiological effects involved *B. leucurus* snakebite as well as the organism's reaction in response to this envenoming, it has not been explored yet. Thus, edema was induced in mice paw using 1.2, 2.5, and 5.0 μg of *B. leucurus* venom, the percentage of edema was measured 30 min after injection and the blood plasma was collected and analyzed by shotgun proteomic strategy. We identified 80 common plasma proteins with differential abundance among the experimental groups and we can understand the early aspects of this snake envenomation, regardless of the suggestive severity of an ophidian accident. The results showed *B. leucurus* venom triggers a thromboinflammation scenario where family's proteins of the Serpins, Apolipoproteins, Complement factors and Component subunits, Cathepsins, Kinases, Oxidoreductases, Proteases inhibitors, Proteases, Collagens, Growth factors are related to inflammation, complement and coagulation systems, modulators platelets and neutrophils, lipid and retinoid metabolism, oxidative stress and tissue repair. Our findings set precedents for future studies in the area of early diagnosis and/or treatment of snakebites.

Significance: The physiopathological effects that the snake venoms can cause have been investigated through classical and reductionist tools, which allowed, so far, the identification of action mechanisms of individual components associated with specific tissue damage. The currently incomplete limitations of this knowledge must be expanded through new approaches, such as proteomics, which may represent a big leap in understanding the venom-modulated pathological process. The exploration of the complete protein set that suffer modifications by the simultaneous action of multiple toxins, provides a map of the establishment of physiopathological phenotypes, which favors the identification of multiple toxin targets, that may or may not act in synergy, as well as favoring the discovery of biomarkers and therapeutic targets for manifestations that are not neutralized by the antivenom.

1. Introduction

Approximately 2.7 million people suffer from snakebite envenoming annually worldwide, resulting in 81,000–138,000 fatalities and 400,000 morbidity cases. Snakebite is a disease that falls under category A of neglected diseases by the World Health Organization [1,2] that mainly

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affects tropical and subtropical developing countries [3,4] especially Asian and African [5,6], American [7], and oceanic countries [8].

In Brazil, approximately 27,000 accidents are reported each year, with the *Bothrops* genus being mainly responsible for snakebite envenoming cases due to the great diversity of species and wide distribution of the genus [1,7]. Among the high diversity of *Bothrops* sp. found in Brazil, *Bothrops leucurus* is the most common viper species in the Brazilian Northeast, considered the main species of medical relevance in the region [9,10]. Although the importance of *B. leucurus* for public health has been reported, little research has been carried out when considering the effects involved in snakebite caused by this species.

B. leucurus venom presents a proteome composed of snake venom metalloproteinases (SVMP), snake venom proteinases (SVSP), L-amino acid oxidases (LAAO), phospholipases B (PLB), type-C lectins (CTL), phospholipases A_2 (PLA_2), aminopeptidases (APases), 5′-nucleotidases (5'-Nase), and phosphodiesterases (PDE) [9–11]. Naturally, snake venom is a well-known "complex pharmacological weapon," composed of a multitude of toxic components capable of establishing multiple biochemical interactions [12,13] disturbing numerous endogenous signaling systems in the prey or victim, which results in the dysregulation of homeostasis, function and cell survival [14].

The pathophysiological effects involved in the envenoming are a condition mediated by toxins from the snake venom and the organism's reaction in response to them [14,15]. In this context, the cooptation of endogenous processes allows venoms to use the cellular machinery of organisms to interrupt neurotransmission, deregulate coagulation, and act in the production of inflammation mediators by the organism's cells [16]. Understanding the secondary causes of snake venom toxicity has been a challenge to better know the coordinated activation of inflammation and hemostatic responses, characterizing a possible thrombo-inflammatory scenario.

Thromboinflammation is a phenomenon generated by the activation of cascade systems present in the blood, complement, contact, coagulation, and fibrinolytic systems, in association with a multicellular system composed of platelets, endothelial cells, and leukocytes [17,18]. Although the activation of inflammation and hemostasis after tissue injury is a defense mechanism with a role in maintaining homeostasis, eliminating pathogens, tissue remodeling, and repair, their destabilization and/or amplification, when co-opted by venoms, can increase potential damage in the body [19].

Considering that the inflammatory response is mediated by signaling molecules carried from the sites of injury or infection through the blood, blood plasma thought proteomics analyses have attracted substantial attention for identifying candidates for biomarkers in different models of human diseases [20–25]. Plasma proteins have been used to provide new insights into disease mechanisms and for early diagnosis, differentiation between diseases with very similar etiology and clinical manifestations. Furthermore, these proteins monitor disease progression as many components of the blood plasma proteome generally exhibit changes in abundance and show an excellent correlation with the severity or disease progression [25].

In addition, the proteomic analysis associated with interaction networks analysis makes it possible to identify disturbances in biological processes and key proteins indispensable for the genesis and maintenance of diseases. That can assist in the understanding of pathological phenotypes and the design of pharmacological interventions selective [26–31].

In this scenario, we aimed to identify plasma proteins of mice that are quantitatively altered by *B. leucurus* envenomation. Besides, we evaluated the early aspects in the biological and pathophysiological processes of this envenomation regarding the thrombo-inflammation associated with the local tissue damage in vivo model.

2. Material and methods

2.1. B. leucurus snake venom

B. leucurus venom pool was obtained from 11 specimens from Scientific breeding Center for the Study of Venoms and Venomous Animals (CEVAP) at São Paulo State University (UNESP), Botucatu, SP, Brazil, under authorization from the Brazilian Institute for the Environment and Renewable Natural Resources (IBAMA 1/35/92/0044–1, Proc. No. 02001.005670/90–77). This research project is registered at the National System of Genetic Heritage and Associated Traditional Knowledge (SISGEN) under n° A54E8E8. The snakes were anesthetized with the aid of carbon dioxide (CO_2) and the venom extracted, diluted in 0.9% (*w/v*) saline, filtered with 0.45 μm pore support, and centrifuged at 8000 ×*g* for 15 min. The supernatant was collected, lyophilized, and stored at −20 °C in a pool until the moment of use [32].

2.2. Animals

All procedures involving animals were carried out according to the National Council's recommendations for the Control of Animal Experimentation (CONCEA) and the Animal Use Ethics Committee of the Biotechnology Institute of the São Paulo State University (CEUA- IBTEC) have approved the experimental protocols (Certificate number 08/2019). Male *Swiss* mice (20–22 g, 6–8 weeks of age) were used, which was provided by the Animal Breeding and Experimentation Laboratory at the Federal University of ABC. The animals were housed in polypropylene cages (micro isolators) and kept under controlled temperature (22 ± 2 °C) in a 12-h light/dark cycle and fed with standard laboratory extruded feed and water *Ad libitum*. All experiments were carried out in the morning.

2.3. Edema-inducing activity

Edema were induced in mice ($n = 6$/group) using 1.2, 2.5, and 5.0 μg of *B. leucurus* venom in 15 μL of 0.9% (*w/v*) sterile saline injected via intraplantar. The three doses were chosen to simulate the classification of experimental envenoming at different levels based on the measures of paw edema with significance without producing hemorrhage or other local effects. The animals in the control group received an equal volume of sterile saline. The individual thickness of the right hind paw was measured before the venom injection (baseline) and 30 min after the edema induction using a digital caliper (Digimess, São Paulo, SP, Brazil). The edema was expressed as the percentage difference between the paw thickness after and before the venom injection calculated with the following formula: (Te-T0) / T0 * 100. The statistical analysis was performed using the *t*-test, one Way-ANOVA, followed by the multiple comparison test ($p \leq 0.05$) and expressed as mean and standard deviation.

2.4. Collection, selection, and grouping of plasma samples

The mice were anesthetized with ketamine (80 mg/kg, i.p.) and xylazine (8 mg/kg, i.p.) and the chest cavity was opened. Approximately 700 μL of blood was collected from each animal by cardiac puncture and immediately transferred to tubes with EDTA, placed in an ice bath, and then centrifuged at 5.000 rpm for 10 min at 4 °C. The plasma samples were collected and stored until use at -80C. For protein profile analysis via shotgun (LC-MS/MS), three pools containing two mice plasma each were prepared from each dose of venom tested.

2.5. In-solution protein digestion

Thirty micrograms of proteins from each pool were solubilized in 60 μL of 50 mM buffer ammonium bicarbonate and 10 μL of 1% (*w/v*) RapiGest SF Surfactant (Waters) were added. Then, the samples were

incubated for 60 min at 37 °C. The samples were reduced with 2.5 μL of 200 mM dithiothreitol (Calbiochem) (37 °C, 40 min) and alkylated with 2.5 μL of 375 mM iodoacetamide (30 min, light-free room temperature (Sigma-Aldrich, Munich, Germany). Then, 10 μL of trypsin solution at 0.1 μg/uL (Promega, Mannheim, Germany) (1:30 enzyme: protein) was added and the samples were incubated for 12 h at 37 °C. The enzymatic reaction was stopped with the addition of 5% (*v*/v) trifluoroacetic acid (Roth, Mannheim, Germany). Tryptic digests were obtained by centrifuging the samples at 16000 ×g for 30 min and the supernatants were collected, dried, and stored at −20 °C until further analysis.

2.6. LC-MS/MS analysis

The peptides were resuspended in 50 mM ammonium bicarbonate in 50% (*v*/v) acetonitrile (ACN) at room temperature and desalinized using Peptide Cleanup C18 Spin Tubes (Agilent) according to the manufacturer's instructions. The peptides were dried and resuspended in 0.1% (*v*/v) formic acid and subjected to nano-ultra-performance liquid chromatography by an Ultimate 3000 LC (Dionex, Germering, Germany) using 0.1% (v/v) formic acid in Mili-Q water as solvent A and 0.1% (v/v) formic acid in ACN as solvent B. The peptides were loaded onto a C18 column, 30 μm × 5 mm (ThermoFisher Scientific) under an gradient of 2–40% buffer B for 120 min at a flow of 300 nL/min and eluted by a Reprosil-Pur C18-AQ analytical column, 3 μm, 120 Å, 105 mm (PICOCHIP, New Objective), followed by 55% to 90% B for 1 min, maintained at 90% B for 10 min and rebalanced to 2% B for 10 min. The peptide elution was coupled directly to a Q-Exactive mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) set for Data Dependent Acquisition, with the MS spectra acquired from 200 to 2000 *m/z*, in a resolution of 70.000 and injection time of 100 ms. The fragmentation chamber was conditioned with collision energy between 29 and 35%. MS/MS spectra were acquired with 17,500 resolution, injection time of 50 ms, 2.0 *m/z* isolation window and 45 s dynamic exclusion time. The raw MS files were acquired using the Thermo Xcalibur software (version 4.0.27.19, ThermoFisher Scientific Inc.).

2.7. Identification and quantification of proteins

The RawVegetable software [33] was used for quality control of the MS raw files, and PatternLab for Proteomics v.4 was utilized to perform the proteomic data analysis (http://patternlabforproteomics.org/) [34]. For this purpose, a FASTA file with proteins corresponding to the reviewed SwissProt entries for the *Mus musculus* proteome from Uniprot was used (UP000000589) plus 123 common contaminants added by PatternLab. Protein quantification was performed using the identified peptides' extracted ion chromatograms (XIC). Protein fold changes (log2-Fc) were calculated for each sample in relation to the control group and the results were presented with mean and standard deviation obtained by the MetaboAnalyst software (https://www.metaboanalyst.ca/). *t*-test ($p \leq 0.05$) was used to compare the mean protein abundances between each two experimental groups.

Further information regarding quality control in RawVegetable and the PatternLab search and filtering parameters can be found in Supplementary Methods.

2.8. Classification of differentially abundant proteins

After identifying the differentially abundant proteins, we classify these based on the distribution among the groups. Three groups of proteins were identified: (i) common plasma proteins (CPPs), proteins whose abundances were altered in all groups regardless of the dose of venom used; (ii) intergroup plasma proteins (IPPs) whose abundances were disturbed in only two envenoming groups (1.2/2.5, 1.2/5.0, 2.5/5.0), and (iii) exclusive plasma proteins (EPPs), whose abundances were disturbed in only one group.

2.9. Gene ontology and network protein-protein analysis

The enriched ontological gene processes were evidenced with significant fold-change using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) v6.8 tool [35]. The GOplot R package was used for data visualization [36]. Differential abundance proteins (DAPs) were mapped to the corresponding molecular interaction databases using the NetworkAnalyst v3.0 tool (https://www.networkanalyst.ca/) [35] and the analysis of generic interaction networks for predicting and identifying Reactome processes and enriched KEGG (Kyoto Encyclopedia of Genes and Genomes) was performed using the STRING database (STRING: functional protein association networks v.11.0, available on https://string-db.org/) interactome, with proteins with at least three interactions being validated. In addition, a specific analysis of interactions was also carried out only between the differentially abundant ones to identify other enriched paths of interest. Moreover, Metascape (available on https://metascape.org) was used to analyze only physical interactions with the application of Molecular Complex Detection (MCODE) algorithm to identify densely connected network components and expand the relationships between ontological gene terms [37].

3. Results

We induced an edema in mice using 1.2, 2.5, and 5.0 μg of venom in hind paw and these were measured 30 min after injection. *B. leucurus* venom showed an ascending dose-response relationship in which 1.2 μg of *B. leucurus* venom induced an increase in paw volume of 16.0% ± 0.44 ($p < 0.0001$), 2.5 μg induced 22.0% ± 0.54 ($p < 0.0001$) and 5.0 μg of venom induced 24.8% ± 0.48 ($p < 0.0001$) of edema (Fig. 1A). After evaluating the edematogenic activity, the blood was collected to continue the evaluation of the plasma proteome of these animals.

For mass spectrometry analysis, we identified up to 226 proteins hits (Supplementary Table 1) per sample analyzed. The proteins were filtered for changes in their abundance ($p < 0.05$), considering those with the value of log-FC ≥1, allowing the validation of 96 proteins for the dose of 1.2 μg, 113 proteins for 2.5 μg and 120 proteins for 5 μg (Supplementary Table 2A). Then, a qualitative cross-comparison among the proteomes for each group was performed to identify the unique proteins in each level of envenoming.

Venn Diagram analysis allowed identifying the matching relations among sets and the intersection of proteins and proteomes (Supplementary Table 2B). Our analysis resulted in 80 common plasma proteins (CPPs) among three doses tested as differentially abundant proteins (DAPs) (Fig. 1 B). Heatmap analysis revealed proteins with similar quantitative profiles among the experimental groups but dissimilar profiles when compared to control group. Among these 80 commons proteins, 36 showed a reduction (different shades of green) in their abundance after experimental envenoming, while another 44 increased (different shades of red) when compared with control group (Fig. 1 C).

The main common plasma proteins with differential abundance identified were: i) serine protease inhibitors (SerpinA1A, SerpinA1B, SerpinA1C, SerpinA1D, SerpinA6, SerpinA10, SerpinA3M, and SerpinA3N), which several ones were some up regulated; ii) other up regulated proteins after experimental envenoming were Apolipoproteins (ApoA1, ApoA2, ApoA4, ApoC1, ApoC3, ApoC4, and ApoE). Interestingly, members of the complement system (C1qc, Cfd, Cfh, Cfi) showed down regulated in mice plasma, while C4b and C1qa showed up regulated (Fig. 1 C).

To assign a biological context to the proteomic results, an association with several physiological pathways and connection of CPPs with the pathophysiology of envenoming were carried out through the results obtained in DAVID and STRING software. DAVID protein classes analysis for the molecular function classification of DAPs have indicated that this panel of proteins is composed mainly of hydrolases, serine endopeptidase inhibitors, calcium ion ligands, lipid ligands and binding

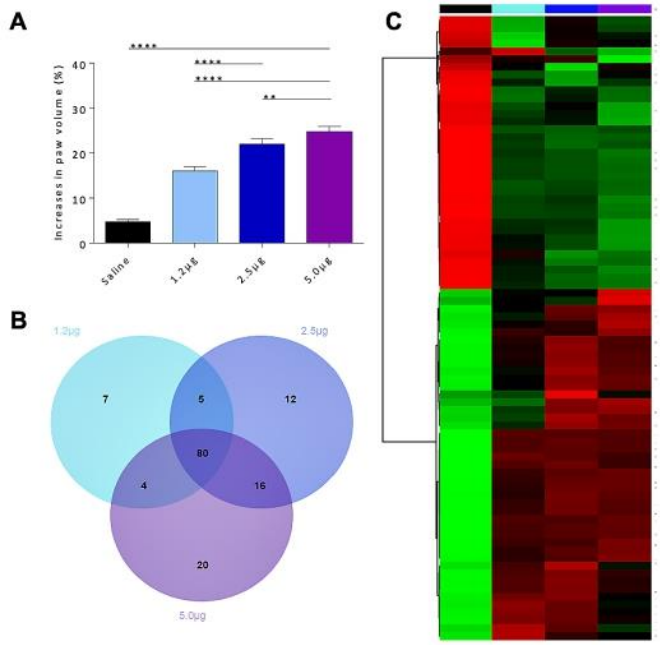


Fig. 1. *A)* Edematogenic profile of *B. leucurus* venom by dose-response: *B. leucurus* venom or sterile saline were injected into the right rear paw of male *Swiss* mice ($n = 6$). Paw edema was measured 30 min after injection with a digital caliper. The increase in paw volume was expressed as a percentage (%) and calculated using the following formula: (Te-T0) / T0 * 100. Statistical analysis was performed using one Way-ANOVA, followed by Tukey's multiple comparison test ($p \leq 0.05$) ± SD. *B)* Venn diagrams that describe the distribution of proteins with differential abundance (DAPs) among the groups. *C)* Heatmap presents the turnover of common plasma proteins (CPP) obtained from mice after injection with 1.2, 2.5, and 5.0 µg of *B. leucurus* venom. High concentrations of proteins are shown in shades of red and low concentrations are shown in shades of green. The abundance of proteins is demonstrated according to the right sidebar, in which the abundance increases in an upward direction (red tones) and tends to decrease in a downward direction (green tones). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

proteins antigens (Supplementary Table 3).

The analysis of the regulatory tendencies of the main biological processes altered after the injection of venom, in which CPPs are involved, it was explored using the GOplot package. First, the number of up-regulated and down-regulated abundance of CPPs in each category was shown individually as red and blue dots, respectively (Fig. 2). The internal rectangles are sized to positively correlate with the meaning of each GO term and colored to represent the general direction of change for each term.

Thus, it was found that the terms phospholipid efflux, cholesterol efflux, high-density lipoprotein particle remodeling, lipoprotein metabolic process, acute-phase response, lipid transport, negative regulation of peptidase activity, and response to peptide hormone have a generally positive regulation. In contrast, those related to hemostasis have a general negative regulation. In contrast, the term GO complement activation, the classical pathway has not been up regulation or negatively regulated (Fig. 2). In line with these findings, we not observed a general regulation of biological processes over systemic inflammation caused by the venom but we were able to visualize associated and modulated proteins within those processes.

Analysis of the functional pathway via DAVID revealed associations of CPPs in complement and coagulation cascades in the KEGG category; while the complement pathway and the intrinsic prothrombin activation pathway and focal adhesion were the main candidates for BIOCARTA. In addition, the main pathways in the Reactome category when analyzed via DAVID and STRING were platelet degranulation, neutrophil degranulation, retinoid metabolism, and transport, classical antibody-mediated complement activation, complement trigger, intrinsic fibrin clot formation pathway, and detoxification of reactive oxygen species (Table 1; Supplementary Fig. 1A-E).

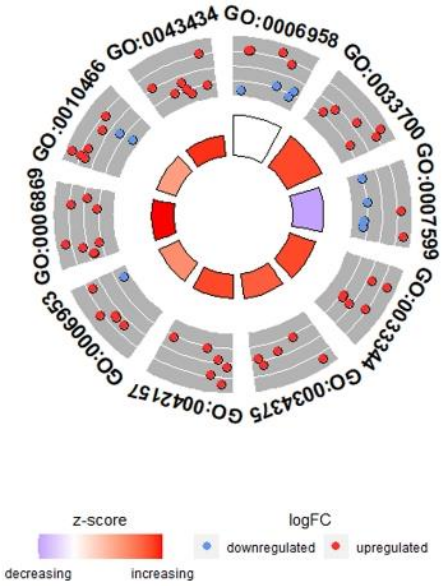
The proteins that stood out among the network of interactions with the CPPs analyzed by the NetworkAnalyst software were members of the ApoA, Serpinas, and Igf families, in addition to Adipoq, Cfd, Clu, Col3a1, Egfr, Hrg, Kng1, Pkm, Trf, Lum and Ttr showing ≥3 interactions (Fig. 3A). As for the REACTOME category analyzed after setting up the network, new enrichment paths could be evidenced, among them: extracellular matrix organization, degradation of the extracellular matrix, degradation of collagen, collagen formation and activation of matrix metalloproteinases. In addition, the biological processes related to wound healing, cell migration, blood pressure regulation, body fluid levels regulation, leukocyte chemotaxis, and other processes were also highlighted (Supplementary Table 4).

Then, we expanded the pathway and process enrichment analysis utilizing Metascapewith the following ontological gene annotations data banks: GO Biological Processes, KEGG Pathway, Reactome Gene Sets, CORUM, TRRUST, PaGenBase, WikiPathways e PANTHER Pathway. All proteome entries were used as an enrichment background and terms with a p-value <0.01, a minimum count of 3, and an enrichment factor > 1.5 were selected and grouped into clusters based on their association similarities. We identified the top 20 clusters with their representative enriched terms (one per cluster) confirming our previous findings (Data not shown). A group of enrichment terms was selected and rendered as a protein-protein network graph to expand relations between the terms. The term with >0.3 similarity is connected by edges. We selected the term according to the p-value to demonstrate the principal clusters (Fig. 3B).

For each list of genes provided, the protein-protein interaction enrichment analysis was performed. Only physical interactions in STRING (physical score > 0.132) and BioGrid were used. The resulting network contains the subset of proteins, mainly Serpins and Apolipoproteins, which form physical interactions with at least one other protein (Fig. 4). The molecular complex detection algorithm (MCODE) was applied to identify network components. The pathway and process enrichment analysis were applied to each MCODE component independently, and the terms with the best score by p-value were maintained as the functional description of the corresponding components (Fig. 4).

We also observed distinct and specific changes in Inter Plasma Proteins (IPPs) for each two doses of venom: 5 proteins between groups treated with 1.2 µg and 2.5 µg, 4 proteins between 1.2 µg/5.0 µg and 16 proteins between 2.5 µg/5.0 µg, suggesting that these may represent specific proteins to the degree of snakebite envenoming. In general, we found that only two IPPs from 1.2 µg/2.5 µg, it was possible to infer a biological process (complement and coagulation cascades). While IPPs from 1.25 µg/5.0 µg allowed the inference of complement activation, classical pathway. Finally, IPPs from 2.5 µg/5.0 µg indicated that they were involved in the inflammatory response and wound healing (Supplementary Table 2B; Supplementary Fig. 2A-C).

Seven, twelve and twenty Exclusive Plasma Proteins (EPPs) were evidenced in the groups treated with 1.2 µg, 2.5 µg and 5.0 µg of *B. leucurus* venom, respectively (Supplementary Table 2B). Most EPPs of the group with 1.2 µg have showed a negative abundance in relation to control group and no interaction among these proteins was observed (Supplementary Fig. 3A). On the other hand, the analysis of the EPPs of animals injected with 2.5 µg of venom showed a positive regulation of proteins. However, the pathophysiological functions are not clear, since the modulation of interactions among the proteins can be done, but most of them are not being attributed to any significant processes. However, C1s2 and Ficolin-1 (Fcna) interaction stood out, related mainly to complement activation, lectin pathway (Supplementary Fig. 3B). Finally, EPPs of animals injected with 5 µg of venom have showed a profile of up-regulated for interactions related to a negative regulation of protein metabolic process and negative regulation of catalytic activity



ID	Description
GO:0006958	complement activation, classical pathway
GO:0033700	phospholipid efflux
GO:0007599	hemostasis
GO:0033344	cholesterol efflux
GO:0034375	high-density lipoprotein particle remodeling
GO:0042157	lipoprotein metabolic process
GO:0006953	acute-phase response
GO:0006869	lipid transport
GO:0010466	negative regulation of peptidase activity
GO:0043434	response to peptide hormone

Fig. 2. Essential biological processes related to CPPs enriched in the plasma of mice after experimental injection with the venom of *B. leucurus*. Identification of the main ontological gene (GO) terms differentially regulated, displayed graphically according to significance, with a general regulation measure up or down for the category. The outer circle shows the number of proteins, the colour corresponding to the logarithmic change, with red representing the increase in abundance and blue representing the decrease. The internal rectangles represent the *p*-value of the GO term and are colored according to the z score to represent the general direction of change for each term. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

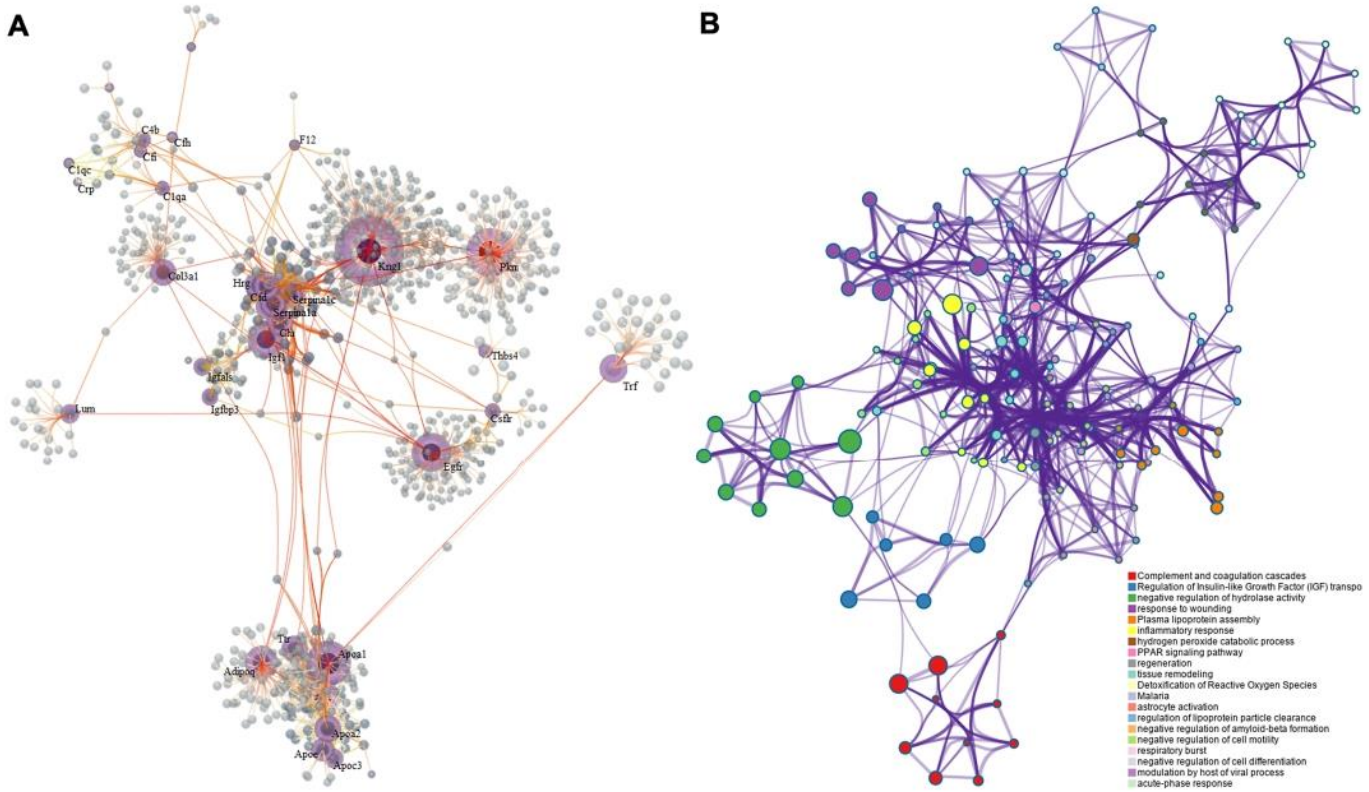
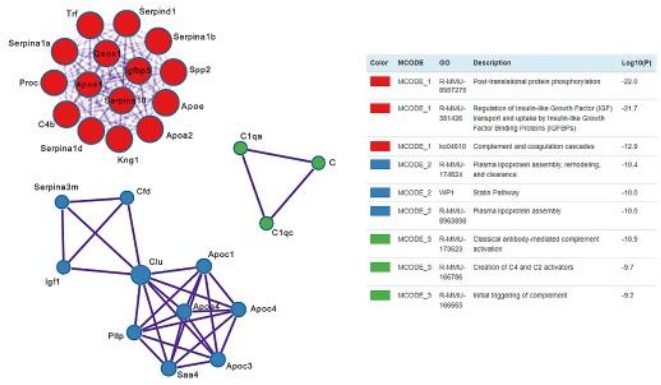


Fig. 3. Network of protein-protein interactions with the main common plasma proteins (CPPs) differentially abundant in the plasma of mice 30 min after intraplantar injection with the venom of *B. leucurus*: A - The proteins were mapped to the interaction database (NetworkAnalyst) to produce a large subnet ("continent") with several smaller subnets ("islands"). Subnets of predictive interactions with at least three nodes starting from the proteins of interest are presented. Only proteins that presented at least 3 interactions were considered for analysis, this being the criterion for identifying the main CPPs. B - Network of enriched terms colored by cluster ID, where nodes that share the same cluster ID are typically close to each other.

Table 1
Different pathways associated with CPPs in mice plasma after tissue damage caused by the *B. leucurus* venom under analysis using DAVID functional annotation tools.

Pathways	Category	Protein IDs of test set in subcategory	Observed number of proteins	*p*-Value	Benjamini
Neutrophil degranulation	STRING	Cfd, Ctsb, Pkm, Qsox1, Serpina1A, Serpina1B, Serpina1D, Serpina3M, Ttr	9	–	0.00013
Complement Pathway	BIOCARTA	C1qa, Cfd, Masp1	3	1,7E-2	4,7E-1
Intrinsic Prothrombin Activation Pathway	BIOCARTA	F12, Kng1, Proc	3	2,8E-2	4,7E-1
Complement and coagulation cascades	KEGG	F12, C1qc, C1qa, Cfh, Cfi, Cfd, Kng1, Masp1, Proc, Serpina1A, Serpina1C, Serpina1D, Serpind1, Serpina1B	14	8,5E-18	7,1E-16
Focal adhesion	KEGG	Col3a1, Egfr, Igf1, Thbs4	4	7,7E-2	8,0E-1
Platelet degranulation	REACTOME	Apoa1, Clu, Cfd, Igf1, Kng1, Qsox1, Spp2, Selenop, Serpina3M, Serpina3N, Tf	11	4,7E-9	3,4E-7
Retinoid metabolism and transport	REACTOME	Apoa1, Apoa2, Apoa4, Apoc3, Apoe, Ttr	6	1,1E-5	2,8E-4
Classical antibody-mediated complement activation	REACTOME	Crp, C1qc, C1qa, Igh-3	4	4,4E-5	8,0E-4
Intrinsic Pathway of Fibrin Clot Formation	REACTOME	F12, Kng1, Proc, Serpind1	4	3,4E-4	4,1E-3
Detoxification of Reactive Oxygen Species	REACTOME	Gpx3, Gsr, Prdx2, Sod3	4	9,2E-4	9,5E-3
Initial triggering of complement	REACTOME	C1qc, C1qa, Igh-3	3	3,6E-3	3,2E-2



Color	MCODE	GO	Description	Log10(P)
	MCODE_1	R-MMU-8957275	Post-translational protein phosphorylation	-22.0
	MCODE_1	R-MMU-381426	Regulation of Insulin-like Growth Factor (IGF) transport and uptake by Insulin-like Growth Factor Binding Proteins (IGFBPs)	-21.7
	MCODE_1	ko04610	Complement and coagulation cascades	-12.9
	MCODE_2	R-MMU-174824	Plasma lipoprotein assembly, remodeling, and clearance	-10.4
	MCODE_2	WP1	Statin Pathway	-10.0
	MCODE_2	R-MMU-8963898	Plasma lipoprotein assembly	-10.0
	MCODE_3	R-MMU-173623	Classical antibody-mediated complement activation	-10.9
	MCODE_3	R-MMU-166786	Creation of C4 and C2 activators	-9.7
	MCODE_3	R-MMU-166663	Initial triggering of complement	-9.2

Fig. 4. Physical network analysis between densely connected proteins. The pathway and process enrichment analysis were applied to each MCODE component independently, and the three terms with the best *p*-value score were maintained as the functional description of the corresponding components. Only physical interactions in STRING (physical score > 0.132) and BioGrid were used.

(Supplementary Fig. 3C).

4. Discussion

Bothropic envenomation is characterized by the rapid development of edema and inflammation at the site of venom inoculation. This local effect leads to the synergistic action, among toxins contained on the venom, and release of endogenous mediators secondary causing systemic manifestations [14-16]. Our findings demonstrated that the *B. leucurus* venom-induced edema in all doses used in this study, corroborating with previous studies, which showed rapid development of edema and inflammation at the site of venom inoculation, even at low dose, with the maximum edematogenic response at 30 min [38–40]. Considering this evidence, we chose at the same time to analyze. Correlated with clinical data, the volume of venom inoculated on snakebite is an important factor to the seriousness of this problem, thus, the second step was to identify the plasma common proteins (CPPs) released by different doses. Previous studies demonstrated that *B. leucurus* promotes edema, tissue necrosis, coagulant and hemorrhagic activity [9]. Our proteomic analysis showed that the different doses caused changes in plasma proteins, once inflammatory response is mediated by signaling molecules transported from the lesion into the bloodstream, at an early interval in face of possible clinical manifestations triggered by bothropic snake venoms. As this is a preliminary study on plasma proteome alterations involving various concentrations of *B. leucurus* snake venom, the authors have chosen to elucidate the roles of plasma proteins common (CPPs) with differential abundances among the 3 experimental groups to consolidate the early aspects of snake envenomation, regardless of the suggestive severity of an ophidian accident.

Our findings suggest induction of a thromboinflammation scenario already reported to *Bothrops* venoms [19,41,42]. Upon inflammatory stimuli, the endothelial cells lead adhesion molecules to promote binding and transmigration of leukocytes, increasing its thrombogenic potential, through transition to more pro-coagulatory phenotype [43]. We observed after venom injection on paw mice, plasma alterations related to proteins of the inflammation, complement and coagulation systems, modulators platelets and neutrophils, signaling molecules that were up or down regulated conducting the inflammatory response [44,45]. It was also observed a mechanism to adapt and reduce tissue damage [46]. So, the snake venom composition could be key to drive hemostatic disorders and pathophysiological thrombosis such as SVMPs inducing hemorrhage, blood coagulation, degradation of the extracellular matrix and interaction with Aα-fibrinogen chain [47].

Our results showed a probable cross-talk between platelet activation and complement factors by plasma proteins: Apoa1, Clu, Cfd, Igf1, Kng1, Qsox1, Spp2, Selenop, Serpina3M, Serpina3N, Tf, which they are key elements involved in the activation. Once C1q mediates activation and platelet aggregation, contributing to inflammation, leukocyte recruitment and cells from innate and adaptive response, leading tissue remodeling, considering its involvement as an important mediator on early response [48]. The activation of the complement system was demonstrated by other animal toxins [49–53]. In the present study, we identified C1q in the classical pathway, and when it interacts with Factor XII, also demonstrated here, can exert an inhibitor effect clot [54]. Complement C1q subcomponent subunit A, known as C1qa, was up-regulated in the present study. Transforming growth factor-beta-induced protein (TGFBIp/beta ig-h3) by us reported, it activates platelets and promotes thrombogenesis. Complement factor H (CFH) is the major inhibitor of the alternative pathway of the complement system [55], and it was down-regulated. Several serine protease inhibitors (SERPINs) exert regulation on the complement cascade, we observed the involvement of SERPINA1A, SERPINA1A, SERPINA1B, SERPINA1C, SERPINA1D, SERPINA3M, SERPINA3N and SERPIND1. Proteins related to clinical findings, involved with several systems during envenomation, sometimes here absence, can be due technical limitations regarding number of biological samples, protein loss during the manipulation of samples and low abundance and/or hydrophobicity of some proteins

[56,57].

Several snake venoms present anti- and coagulation activity and/or interfere in the hemostatic system [58]. Consumption coagulopathy is induced by diverse compounds containing these venoms [59]. After snakebite, through damaging endothelial cells, occurs platelet aggregation, activating coagulation cascade, with cleave fibrinogen and already formed blood clots [60]. We have identified early alterations in the intrinsic prothrombin activation and intrinsic fibrin clot formation pathways and it was noted a decrease of Factor XII after the envenomation by *B. leucurus* venom. The intrinsic prothrombin activation pathway starts with the cleavage of Factor XII zymogen into Factor XIIa, which then catalyzes the activation of Factor IX into Factor IXa. In contrast, the intrinsic pathway of fibrin clot formation is the shortest pathway of the coagulation cascade, initiating after the release of tissue factor by endothelial cells after damage to a vessel, which culminates in the activation of Factor X [61]. It was possible to evidence a decrease in Coagulation Factor V (FV), which acts in the regulation of blood coagulation and platelet activation, displaying pro and anti-inflammatory roles [62,63]. Beyond its hemostatic properties, FV has been suggested as a cofactor of activated C protein's anti-inflammatory role [64]. Previous study reported that the PLA_2 from snake venoms inhibits coagulation by enzymatic and non-enzymatic mechanisms, and also inhibits the extrinsic tenase complex (TF–FVIIa) [65]. Also, it was reported that C-type lectin interacts with factors of coagulations such as FIXa, FXa and thrombin [66]. Phosphodiesterases and nucleotidases showed dual antiplatelet and anti-thrombotic activity [67].

In the present study, we identified a dysregulation on coagulation cascade (as C1q, F12, Cfh. Cfi, Cgfd, among others), platelet degranulation (as Apoa1, Kng1, SerpinA3M, among others) and already reported, complement system (C1q, Igh3, Masp 1, among others), demonstrating the crosstalk among pathways (all described in Supplementary Materials). These findings corroborate with Magalhães and collaborators [68] and Bello and collaborators [69] that suggested the anti and pro-coagulant and fibrinolytic activities of *B. leucurus* venom. There is evidence in patients bitten by *Bothrops* envenomation coagulation disorders and inflammatory reactions, associated with the consumption of fibrinogen [70]. Proteolysis of blood coagulation factor by *B. leucurus* can suggest the activation of factors or its combination to form the prothrombinase complex, due mainly by SVSPs and SVMPs. LAAOs are also related in disruption hemostasis [71]. The Serpins also exert regulation of procoagulant enzymes, capable of antagonizing the pro coagulation cascade, as the FXa activity, inhibiting generation of thrombin and the subsequently hemostasis disorder (extrinsic or intrinsic pathways) [72]. According to Huang and collaborators [73], protein *Z*-dependent protease inhibitor (ZPI), up-regulated in this study, is a FXa-specific serpin inhibitor and when complexed with its cofactor, protein Z (PZ), exert an effect inhibitor of prothrombinase-bound FXa during prothrombin activation.

It is possible to affirm the involvement of platelet hyperreactivity and thrombogenic and immune response caused by *B. leucurus* venom. Our results corroborate with reported to *B. jararaca* venom that activates platelets, causing platelet sequestration and the formation of pulmonary thrombi composed of platelet aggregates and dense fibrin sheaths, as well as fibrin in the kidneys [42]. In the context of pro-inflammatory and thrombotic scenarios, the organ failure can due to excessive platelet activation, coagulation, and fibrin deposition in the microvasculature, degranulation of neutrophils and neutrophil extracellular traps (NETs) [74]. The NETs are upregulated upon inflammation and promote coagulatory response [43]. Neutrophils are present in myonecrotic and hemorrhagic areas or even in the inflammatory infiltrate [45]. The platelets and neutrophils may represent an important role in thrombus formation, with oxidative tissue damage and disseminated intravascular coagulation [75]. Maia-Marques and collaborators [76], demonstrated the involvement of inflammatory mediators, as biogenic amines, nitric oxide and eicosanoids by *B. leucurus* venom through intraplantar injection in mice, but we provided for the first time the changes in plasma proteins at an early interval in face of possible clinical manifestations triggered by this snake venom. The PLA_2s, that are abundant components of *B. leucurus* venom, can be involved in the inflammation, with release of proinflammatory cytokines [77].

Considering the ability of Serpins, SerpinA1, for example, is a potent neutrophilic elastase inhibitor, which influence over the complement system to acute inflammatory immune responses [78], and negatively regulating the production of pro-inflammatory mediators while positively regulating the production of anti-inflammatory cytokines [79–82]. SerpinA3 is a potent immune system kinase inhibitor as cathepsin G and mastocyte kinases [83], which prevents degradation of extracellular matrix (EM) by proteases released during inflammation [84]. Our results showed both, SerpinA1 (SerpinA1C and SerpinA1D) and SerpinA3 positively regulated. These data corroborate with previous reports that reveal an extensive degradation of extracellular matrix's components and its contribution to the development of *Bothrops* toxin-induced injuries [44,85–88]. Escalante and collaborators [89] demonstrated that *B. leucurus* caused degradation of EM. We suggest that direct and indirect effects promoted by HF3 contributed to tissue injury of *B. leurucus* that promoted destabilization of the mice skin integrity, consequently leading to degradation of the EM. Asega and collaborators [90] showed that HF3-induced hemorrhagic process. To further illustrate, HF3 from *B. jararaca* and atroxlysin and batroxrhagin from *B. atrox*, all SVMPs, induce the degradation of extracellular matrix's components, releasing mediators and chemotaxis-inducing peptides [91]. It is noteworthy that the degradation of extracellular matrix components affects not only vessel stability, but also tissue architecture in general, facilitating the process of diffusion and translocation of venom toxins from interstitial compartments into circulation.

We have identified through network analysis, an enrichment of extracellular matrix-related reactome categories, particularly: degradation of the extracellular matrix, degradation of collagen and activation of matrix metalloproteinases, which are supported by growing evidence regarding the action of *Bothrops* venoms and toxins over the extracellular matrix's components. The enrichment of the degradation of the extracellular matrix category suggests a critical role in the destabilization of murine skin integrity promoted by *B. leucurus* venom, which can probably be followed by the generation of mediators that enhance inflammation. Vascular permeability is altered in response to chemotactic factors and extracellular matrix's degradation products released at the bite site, contributing to edematogenesis [85,86,91]. These events may progress to resolution, but in many cases, the inflammatory response to snakebite tends to be excessive and out of control, involving the overactivation of endogenous signaling pathways [14], resulting in hypovolemia, ischemia, and neural compression, contributing to cell death, tissue loss and amputations [15].

Another important finding in the present work was the identification of Apolipoproteins (Apo) (as Apoa1, Apoa2, Apoa4, Apoc3, Apoe) involved in several pathways. For example, ApoA1 is negatively regulated at the start of and throughout inflammation, favoring an out-of-control inflammatory scenario [92]. Previous study also reported crosstalk between inflammation and hemostasis alterations to effect of snake venom, as *B. jararacussu*, despite to suggest that this mechanism is mediated by the toll-like receptor 4 (TLR4) and NFκB signaling leading to tissue factor expression and procoagulant activity [93]. On the other hand, a pro-inflammatory role by the TLR has been attributed to ApoA1, suggesting its reconversion into an inflammatory Apo. ApoA1 affects NF-κB and MAPK downstream pathways and induces IκBα and JNK phosphorylation [94]. It has been recently reported that ApoC3 can induce the gradual release of human monocyte IL-1β and its role as an endogenous mediator in activating the alternative NLRP3 inflammasome, underscoring its interaction with monocytes a physiopathological process that mediates damage in inflamed organs [95]. Although present in large amounts in the blood, the function of ApoC4 is still speculative. Some findings have suggested that ApoA4 acts in lipid metabolism, glucose metabolism, antioxidative and anti-inflammatory processes,

platelet aggregation and thrombosis [96,97]. ApoE modulates macrophages, suppresses T cell proliferation, inhibits smooth muscle cell proliferation, positively regulates nitric oxide production (NO) in platelets, facilitates lipid antigen presentation by CD1 molecules to natural killer T cells (NKT), as well as affects inflammatory disease modulation due to its capacity for cytokine interaction capacity [98].

The platelet hyperreactivity and thrombogenic response caused by *B. leucurus* venom may be involved with dyslipidemia, beside oxidative stress [99]. We observed the involvement of lipid transport, lipoprotein metabolism, cholesterol efflux, among others by *B. leucurus* venom. Fatty acid binding proteins (FABPs) are key regulators of lipid metabolism, energy homeostasis, inflammation and we demonstrated that Fatty acid binding protein 7 (FABP7), a brain-specific intracellular lipid binding protein, is up-regulated. This protein belongs to a large family of hydrophobic ligand-binding proteins that promote cellular uptake and transport of fatty acids, regulation of metabolic pathways. So, our data corroborate with Willard and collaborators [71] that showed similar lipid metabolism perfil to *Crotalus atrox* and *C. oreganus helleri* by proteomic analysis. It is known that PLA_2s causes membrane phospholipids hydrolysis, with release of precursors of eicosanoids and consequently lipid mediators [100]. Thus, PLA_2s from *B. leucurus* can be involved in this effect, as already reported to others snake venoms [101]. Alterations related to processes involving lipids appear mainly due to the activation of immune cells and the action of PLA_2s from snake venoms when hydrolyzing the membrane glycerophospholipid ester bonds in the sn-2 position, which produces high levels of arachidonic acid and lipid mediators (eicosanoids, prostaglandins, leukotrienes, and thromboxanes), that, in association with cytokines and chemokines trigger pain, edema and systemic inflammation [17,102–104].

Among the proteins identified in this study related to the signaling pathway of Detoxification Reactive Oxygen Species, we emphasized the important roles of some plasma proteins in the *B. leucurus* envenomation. Up regulation of Gpx3 protein acts in the detoxification of hydrogen peroxide, lipidic peroxides and organic hydroperoxide; Gsr prevents the buildup of excessive amounts of ROS, the oxidative damage, the regulation of development of the cytokine storm, along with phagocytosis and chemotaxis defects [105–107]; Prdx2 reduces hydrogen peroxide, alkyl hydroperoxides and peroxynitrite generated during inflammation and induces TNF-α production [108,109]; and Sod3 shows anti-inflammatory properties, especially in ischemic damage acting by negative regulation of migrating cells that produce reactive oxygen [110,111]. Besides this, it is known neutrophil extracellular traps (NETs) also act in tissue repair [112–118]. We have identified an up regulated of quiescin sulfhydryl oxidase 1 (Qsox1), a protein located in the neutrophil degradation pathway, which suggests the induction of oxidative stress by neutrophils in the snakebite envenoming by *B. leucurus*. We suggest an induction of redox status imbalance. This outlines the importance of knowing the mechanism involved in oxidative stress, due to its capacity to positively regulate the inflammatory processes and aggravate tissue damage.

In general, we have concluded that protein families of the Serpins, Apolipoproteins, Complement factors and Component subunits, Cathepsins, Kinases, Oxidoreductases, Proteases inhibitors, Proteases, Collagens, Growth factors have explained this thromboinflammatory scenario caused by *B. leucurus*. So, we have proposed the Fig. 5 as a response mechanism of mices injured by *B. leucurus* snake venom.

For registration purposes, we have verified the presence of Inter Plasma proteins (IPPs – proteins identified between each 2 experimental groups), indicating that the plasma protein profile can correlate with the transition of envenomation severity as well as the involvement of certain biological processes that may contribute to the progress or mitigation of the envenomation's severity. Our data suggest the necessity to explore these proteins' turnover in different envenomation conditions. Once associated with clinical-pathological parameters, these proteins may assist in the discovery of new mechanisms and pharmacological targets for envenomation treatments. Additionally, other proteins can appear and indicate the severity of the envenomation, which was elucidated in this study by Exclusive Plasma Proteins (EPPs) in each experimental envenomation group.

The physiopathological effects involved in the envenomation by

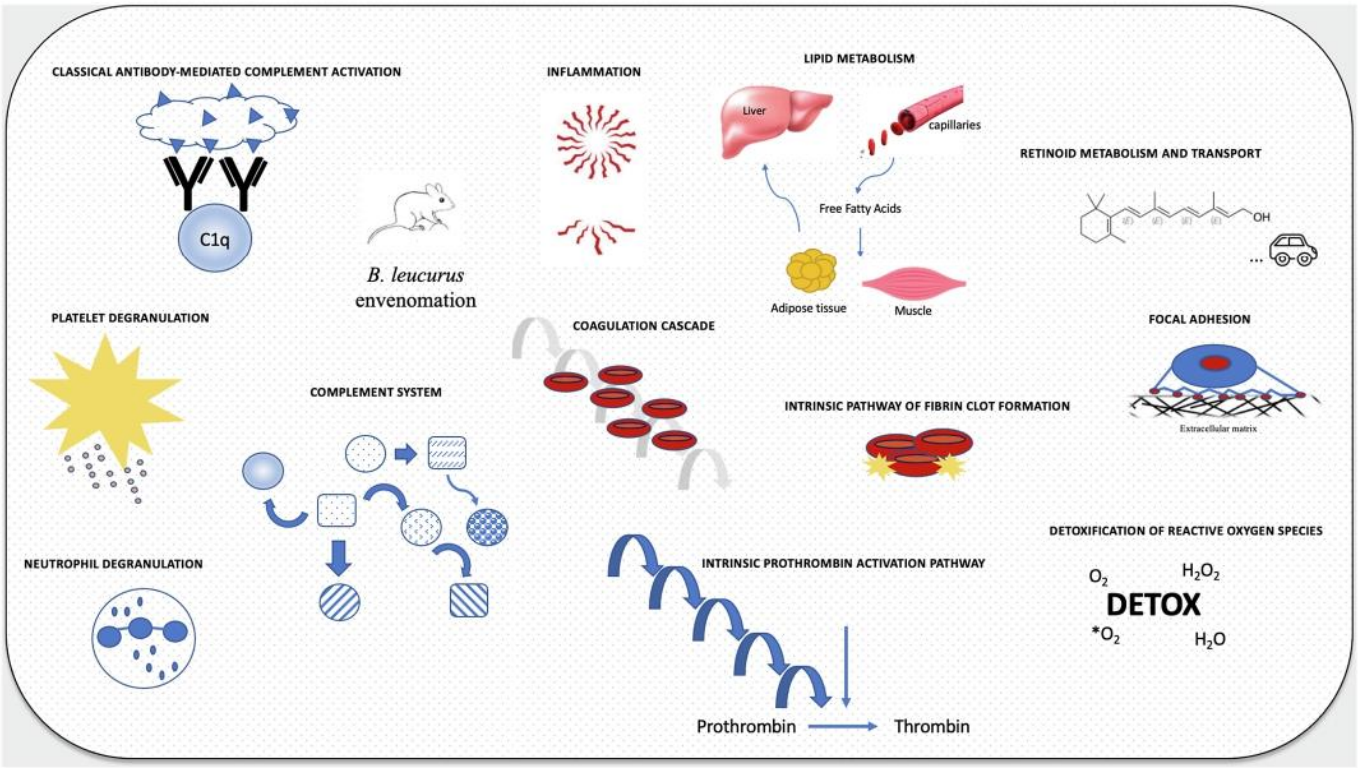


Fig. 5. Response mechanism of mices injured by *B. leucurus* snake venom: a thrombo-inflammatory scenario.

snakes is not only a condition mediated by toxins, but also by the envenomed organism's response. We used mass spectrometry and bioinformatics analysis to understand the biological processes altered in the plasma of mice during the envenomation by *B. leucurus*. The biological processes are responsible for the signal transduction in response to the condition to which the cell/tissue/organism is being exposed to [119–124]. So, the capacity of organism respond to these conditions is due to the interaction among multiple proteins, which can promote the healthy and/or survival.

5. Conclusions

For the first time, we present alterations in the blood plasma proteome of mice injured by *B. leucurus*. These changes point towards the regulation of multiple biological processes after tissue damage caused by this snake venom. We have mapped pathways related to thrombo-inflammation in the snakebite by *B. leucurus* and were able to correlate the changes of plasma proteins at an early interval in face of possible clinical manifestations triggered by this bothropic snake venom. So, our findings set precedents for future studies in the area of early diagnosis and/or treatment of snakebites.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2021.104464.

Data availability statement

The mass spectrometry data this manuscript have been uploaded to the MassIVE Repository from Computer Science and Engineering University of California, San Diego (ftp://massive.ucsd.edu/MSV000087544/, accessed on 30 may 2021) with the dataset identifier MSV000087544.

Author's contributions

Joeliton S. Cavalcante: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Visualization and Writing - original draft. *Cayo A. S. Almeida:* Methodology, Research and Writing - original draft. *Milan A. Clasen:* Methodology, Software, Validation, Formal analysis and Writing - original draft. *Emerson Lucena da Silva:* Formal analysis, Visualization and Writing - original draft. *Luciana B. Curtolo:* Methodology, Research and Writing - original draft. *Aline D. Marinho:* Writing - original draft and Writing – Review & Editing. *Bruno Cesar Rossini:* Methodology, and Writing – Review & Editing. *Celso Luís Marino:* Methodology and Writing – Review & Editing. *Paulo Costa Carvalho:* Data analysis and Writing – Review & Editing. *Roberta J. B. Jorge:* Conceptualization, Supervision, Writing - original draft and Writing – Review & Editing, Project management. *Lucilene D. Santos*: Project management, Resources, Methodology, Funding Acquisition and Writing – Review & Editing.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest, financial or otherwise.

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