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Feasibility of detecting snake envenomation biomarkers from dried blood spots

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Abstract

Biofluid proteomics is a sensitive and high throughput technique that provides vast amounts of molecular data for biomarker discovery. More recently, dried blood spots (DBS) have gained traction as a stable, noninvasive, and relatively cheap source of proteomic data for biomarker identification in disease and injury. Snake envenomation is responsible for significant morbidity and mortality worldwide; however, much remains unknown about the systemic molecular response to envenomation and acquiring biological samples for analysis is a major hurdle. In this study, we utilized DBS acquired from a case of lethal rattlesnake envenomation to determine the feasibility of discovering biomarkers associated with human envenomation. We identified proteins that were either unique or upregulated in envenomated blood compared to non-envenomated blood and evaluated if physiological response pathways and protein markers that correspond to the observed syndromes triggered by envenomation could be detected. We demonstrate that DBS provide useful proteomic information on the systemic processes that resulted from envenomation in this case and find evidence for a massive and systemic inflammatory cascade, combined with coagulation dysregulation, complement system activation, hypoxia response activation, and apoptosis. We also detected potential markers indicative of lethal anaphylaxis, cardiac arrest, and brain death. Ultimately, DBS proteomics has the potential to provide stable and sensitive molecular data on envenomation syndromes and response pathways, which is particularly relevant in low-resource areas which may lack the materials for biofluid processing and storage.

Abbreviations: DBS, Dried blood spot; DOC, Sodium deoxycholate; TCEP, Tris (2-carboxyethyl) phosphine; LC-MS/MS, Liquid chromatography-tandem mass spectrometry; NDBS, Nonenvenomated dried blood spot; EDBS, Envenomated dried blood spot; NETs, Neutrophil extracellular traps; CSR, Cytokine storm reaction; DIC, Disseminated intravascular coagulation; HSPs, Heat shock proteins; SVMPs, Snake venom metalloproteases; CRP, C-reactive protein; VWF, Von Willebrand factor; CSF, Cerebrospinal fluid; CAND1, Cullin-associated and neddylation-dissociated 1 protein; CNS, Central nervous system; KLKB1, Plasma kallikrein; TGF-β, Transforming growth factor β.

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1 | INTRODUCTION

Blood is a rich source of valuable information regarding health status, pathological syndrome, and disease progression. Blood and plasma proteomics methods represent sensitive and high throughput techniques that can provide vast amounts of molecular data which has been used for the discovery and quantification of a myriad of human disease biomarkers.[1–5] Utilizing dried blood spots (DBS) for proteomic identification of biomarkers circumvents many of the drawbacks of biofluid collection and handling while providing a relatively cheap, stable, and minimally invasive method for collecting blood, even in a non-clinical setting.[6,7]

Snake envenomation remains a worldwide scourge that primarily affects tropical and subtropical developing countries and was re-classified as a category A Neglected Tropical Disease by the World Health Organization in 2017.[8,9] Although antivenoms exist for many medically significant species, snakebite continues to be a significant cause of morbidity and mortality in these areas.[10,11] One major hurdle to improving outcomes is the production of effective antivenoms, the development of which should incorporate aspects of regionally relevant venom variation and an understanding of its relationship to the pathophysiology of envenomation.[10]

Snake venoms are complex toxic mixtures whose summative and synergistic effects trigger a cascade of physiological disruptions. Recent proteomic studies have investigated the effects of venoms or isolated toxins on mouse plasma,[12] plasma extracellular vesicles,[13] wound exudate,[14–17] and cardiac tissue,[18] each providing insight into the specific biological processes activated during envenomation. Bridging the gap between the data gathered in model organisms under experimental conditions and the information acquired in a clinical setting has the potential to provide a comprehensive picture of the mechanisms of pathophysiology triggered by envenomation as well as a suitable panel of potential biomarkers indicative of envenomation syndromes and outcomes.

In this study, we utilized DBS acquired from an individual case of lethal rattlesnake envenomation to determine the feasibility of discovering biomarkers associated with human envenomation. We identified proteins that were either unique or upregulated in envenomated blood compared to non-envenomated blood and evaluated if biological pathways and protein markers could be detected that correspond to the observed syndromes triggered by envenomation.

2 | METHODS AND MATERIALS

2.1 | Blood collection

Whole blood was collected from a 6-year-old patient envenomated by a Prairie Rattlesnake (*Crotalus viridis viridis*) who rapidly declined within minutes of being envenomated and ultimately died several days later from the sequelae of the envenomation. Written consent for the use of blood for this analysis was obtained from the patient's parents and was verbally reconfirmed with them just prior to manuscript submission. The family requested no further details of events or medical treatment be discussed. Only information regarding the envenomation syndrome that could be gleaned from public sources was discussed in this paper.

DBS from this patient were generated using whole blood drawn from the patient into EDTA-coated tubes during treatment. A small volume of blood was spotted on a Whatman 903 Proteinsaver Card (Tisch Scientific). Blood spots were dried at ambient temperature for 24 h before storage in a re-sealable zipper bag containing a humidity sponge with an indicating desiccant. Blood from a single healthy donor adult male was collected and processed in the same manner. Blood from this individual was used as a control in order to compare the blood proteomic profile of an envenomated individual to a non-envenomated individual. Samples were processed for mass spectrometry 10 days following collection and spotting onto Whatman 903 Proteinsaver Cards.

Two DBS discs (3 mm each) were punched using an Electron Microscopy Sciences (Hatfield, PA) Aluminum Punch Kit. Discs were added to 500 µl of 50 mM ammonium bicarbonate with 2% sodium deoxycholate (DOC) as in Eshghi et al.[6] Discs were incubated for 45 min at 37°C at 1400 RPM on an Eppendorf Thermomixer C (Hampton, NH). To pellet extracted discs and particulates, samples were centrifuged for 15 s and 200 µl of supernatant was transferred to a new Eppendorf tube.

The volume of supernatant corresponding to 30 µg of protein was diluted in 8 M urea/0.1 M Tris (pH 8.5) and reduced with 5 mM TCEP (tris (2-carboxyethyl) phosphine) for 20 min at room temperature. Samples were alkylated with 50 mM 2-chloroacetamide for 15 min in the dark at room temperature and then diluted four times with 100 mM Tris-HCl (pH 8.5) and trypsin digested overnight at 37°C using an enzyme/substrate ratio of 1:20. To stop the reaction, samples were acidified with 10 µl of 10% formic acid (FA), and digested peptides were purified with Pierce C18 Spin Tips (Thermo Scientific) according to the manufacturer's protocol. Samples were dried in a SpeedVac and redissolved in 0.1% FA.

2.2 | Liquid chromatography-tandem mass spectrometry analysis

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was performed using an Easy nLC 1000 instrument coupled with a Q Exactive HF Mass Spectrometer (both from ThermoFisher Scientific). Peptides were loaded on a C18 column (100 µM inner diameter x 20 cm) packed in-house with 2.7 µm Cortecs C18 resin, and separated at a flow rate of 0.4 µl/min with solution A (0.1% FA) and solution B (0.1% FA in ACN) under the following conditions: isocratic at 4% B for 3 min, followed by 4%–32% B for 102 min, 32%–55% B for 5 min, 55%–95% B for 1 min and isocratic at 95% B for 9 min. The data-dependent acquisition was performed, and the top 15 most abundant precursors were considered for MS/MS analysis. Samples from the patient and the healthy control were each analyzed by LC-MS/MS in technical triplicate.

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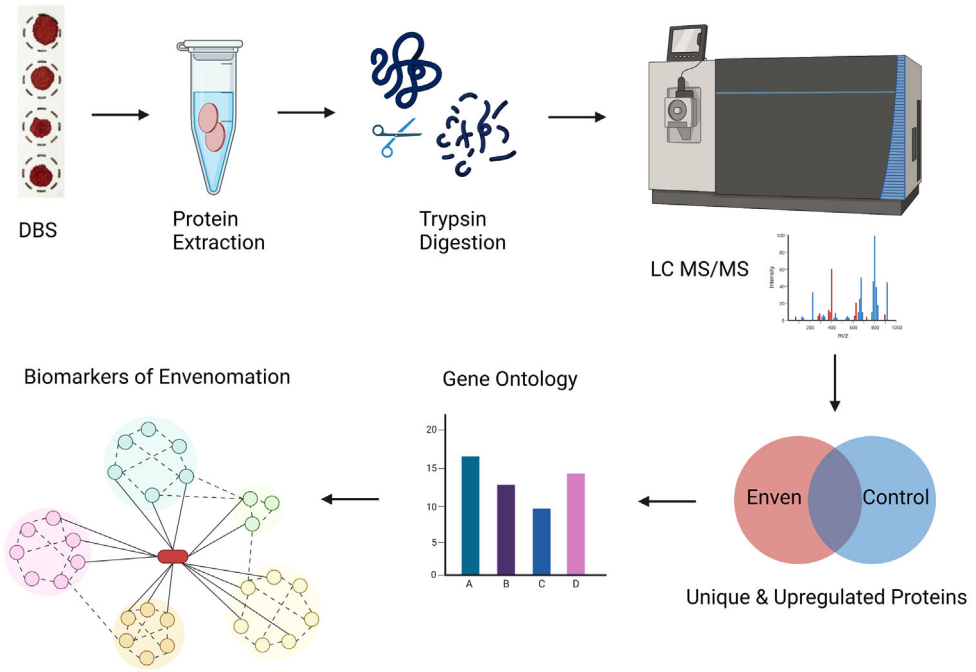

FIGURE 1 Workflow for the processing of dried blood spots from either envenomated or non-envenomated blood for bottom-up proteomics and bioinformatic analysis for envenomation biomarker identification and associated pathway analysis.

2.3 | Peptide and protein identification

Fragmentation spectra were interpreted using the MSFragger-based FragPipe computational platform[19,20] against the UniProt human proteome (ID: UP000005640) database. Reverse decoys and contaminants were included in the search database. Cysteine carbamidomethylation was selected as a fixed modification, oxidation of methionine was selected as a variable modification, and precursor-ion mass tolerance and fragment-ion mass tolerance were set at 20 ppm and 0.4 Da, respectively. Up to 2 missed tryptic cleavages were allowed and the protein-level false discovery rate (FDR) was set to <1%. The relative abundance of proteins was compared across samples using log-transformed spectral intensity in Metaboanalyst 5.0.[21]

2.4 | Gene ontology and network analysis

Overlap between proteins identified in whole blood and DBS from the non-envenomated individual (NDBS) or NDBS and envenomated DBS (EDBS) were determined using the Bioinformatics and Evolutionary Genomics Venn Diagram Tool (Figure 1; http://bioinformatics.psb.ugent.be/webtools/Venn/). Proteins found to be unique between preparation methods were input for gene enrichment analysis into ShinyGO 0.76 with an FDR cutoff of 0.05, pathway size minimum of 2 and maximum of 2000 with redundancies removed using the gene ontology (GO) Biological Process Pathway and a KEGG database.[22] Unique proteins were also input into ELIXIR gProfiler (https://biit.cs.ut.ee/gprofiler/gost) with *Homo sapiens* as the reference organism and statistical domain scope set to only annotated genes with a g:SCS significance threshold and user threshold of 0.05. Network analysis of upregulated proteins in EDBS was performed in Cytoscape 3.9.1[23,24] using the plugin BiNGO 3.0.3[25] with a hypergeometric statistical test, the Benjamini & Hochberg FDR correction at a significance level of 0.05 and using ontology terms for biological process. The output network was annotated to show broad groupings of similar processes.

3 | RESULTS

3.1 | Fresh blood versus DBS

We identified a total of 683 proteins in healthy human whole blood and DBS (Figure 2A). Of these proteins, 550 (80.5%) were able to be detected in DBS. One hundred thirty-three proteins (19%) were identified in whole blood samples but not in DBS. The majority of the proteins unique to whole blood were assigned in Panther to the gene ontology molecular function terms associated with binding (53%; GO:0005488) and catalytic activity (35.7%; GO:0003824; Figure 2A). ShinyGO 0.76.2 identified GO terms associated with the biological process as proteasome protein deneddylation (n = 4, FC = 84.4), nucleotide-excision repair/DNA damage recognition (n = 4; FC = 36.7), and protein activation cascade (n = 4, FC = 30.2) as the top 3 most enriched pathways.

3.2 | EDBS unique proteins

We identified 480 proteins total from envenomated DBSs. In DBSs from both an EDBS and an NDBS, 436 proteins were common in both samples (73%; Figure 2B). Only 44 proteins (7.4%) were unique to

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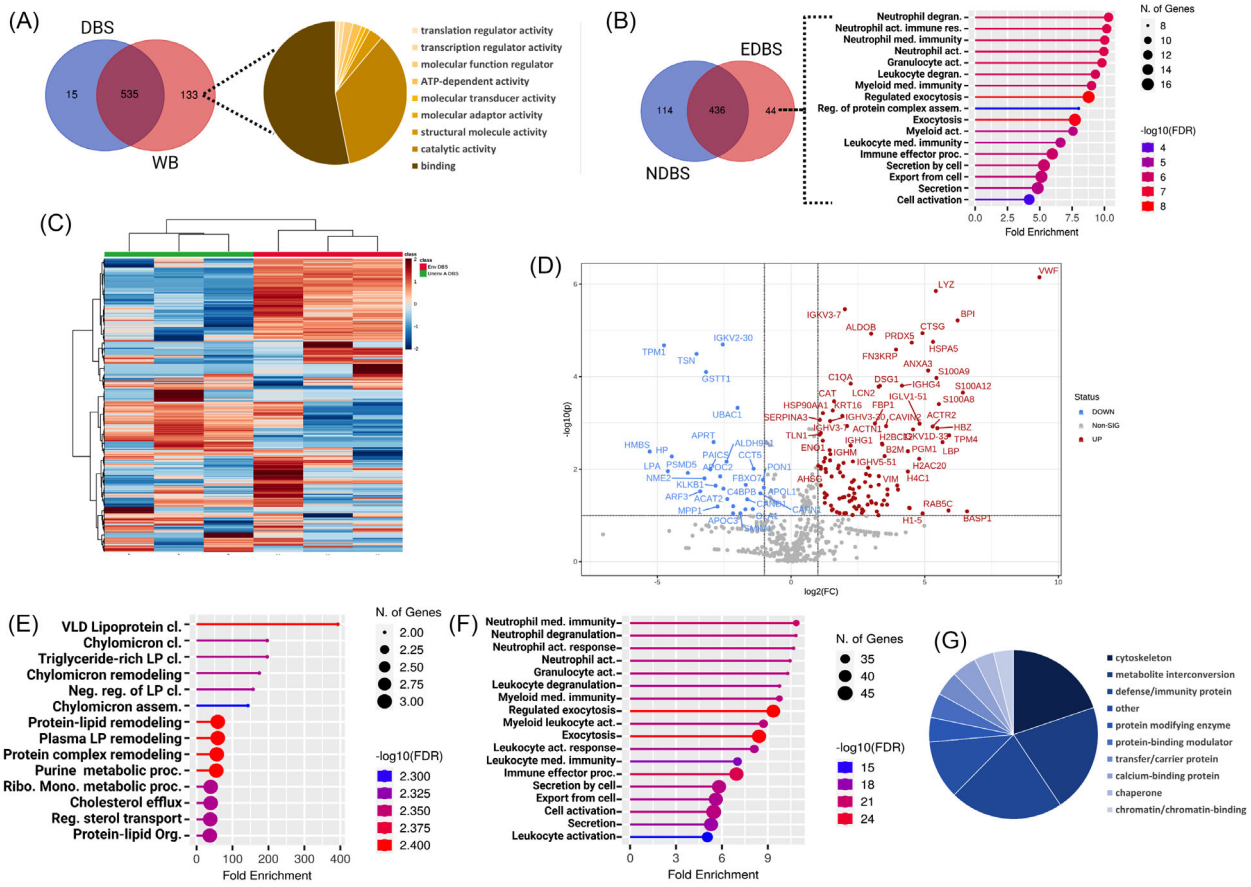

FIGURE 2 Shotgun proteomics from dried blood spots (DBS). (A) Comparison of proteomic coverage between DBS and whole blood from the non-envenomated individual blood sample and identification and distribution of gene ontology (GO) molecular function terms in Panther of proteins unique to whole blood. (B) Comparison of proteomic profile between envenomated DBS (EDBS) and non-envenomated individual (NDBS) and identification of top 20 pathways associated with proteins unique to EDBS using GO biological process terms in ShinyGO 0.76.2. (C) Heatmap cluster analysis of EDBS and NDBS run in triplicate showing clusters of overexpressed proteins in EDBS. (D) Volcano plot for identification of over- and underexpressed proteins in EDBS with a fold change > 2 and a significance threshold of 0.05. Significantly overexpressed proteins are indicated in red and significantly underexpressed proteins are indicated in blue. (E) Gene ontology and pathway enrichment analysis in ShinyGO 0.76.2 of the top 14 underexpressed proteins in DBS. (F) Gene ontology and pathway enrichment analysis of the top 20 overexpressed proteins in DBS. (G) Gene ontology analysis and abundance in Panther of all overexpressed proteins in DBS.

envenomated DBS (Table S1). Gene ontology analysis of unique proteins in EDBS showed significant enrichment for 92 pathways (Table S2). The top 20 enriched pathways associated with unique proteins are primarily involved in an acute inflammatory response and immune system activation (Figure 2B). These include neutrophil activation and degranulation ($n = 12$, FC = 10), cell activation ($n = 14$, FC = 4.2), and cell exocytosis ($n = 16$, FC = 7.7).

3.3 | Significantly upregulated and downregulated pathways in EDBS

Hierarchical clustering shows clear segregation of technical replicates of both NDBS and EDBS despite some variation between replicates (Figure 2C). There appear to be clear comprehensive differences between EDBS and NDBS including a cluster of highly upregulated proteins unique to the envenomated sample. Fold change analysis revealed the presence of 30 downregulated proteins and 118 upregulated proteins in EDBS compared to NDBS (Figure 2D and Table S3).

The 30 downregulated proteins in EDBS were associated with 158 pathways with the top pathways involving very-low-density lipoprotein particle clearance (Figure 2E and Table S4; $n = 2$, FC = 393), chylomicron remnant clearance ($n = 2$, FC = 197) remodelling ($n = 2$, FC = 174) and assembly ($n = 2$, FC = 143) and protein-lipid complex remodelling ($n = 3$, FC = 59). The identified downregulated marker involved in the kinin cascade is plasma kallikrein (KLKB1; FC = 0.14, $p = 0.02$). The markers involved in lipoprotein particle clearance and protein-lipid complex remodelling were apolipoprotein C-II (APOC2; FC = 0.16, $p = 0.01$) and C-III APOC3 (FC = 0.22, $p = 0.09$), and lipoprotein a (LPA; FC = 0.04, $p = 0.01$).

Gene ontology analysis of upregulated proteins in EDBS showed significant enrichment for a wide variety of pathways (Tables 1 and 2 and Table S5). We identified 512 pathways with an FDR under 0.05

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TABLE 1 Upregulated markers associated with immune system processes organized by gene ontology (GO) biological process or KEGG pathway terms identified in ShinyGO 0.76.2

Immune-related pathway	GO term	Gene IDs
Activation of immune response	GO:0002253	C6 CD5L CTB HSP90AA1 PSMA6 ACTR3 ARPC1B ACTR2 FCN3 PSMB4 ARPC5 MNDA C1QA ACTG1
Inflammatory response	GO:0006954	AHSG ANXA1 CD5L ELANE FN1 GSTP1 KRT16 LBP LYZ PPBP PRDX5 PSMA6 PSMB4 S100A12 S100A8 S100A9 SERPINA3 SOD1 THBS1 MSB4X
Complement activation	GO:0006956	C1QA C6 CD5L FCN3
Neutrophil-mediated immunity	GO:0002446	B2M BPI CAMP CAT CTSG DSG1 EEF1A1 ELANE GCA GSN GSTP1 SP90AA1 LBP LCN2 LTF LYZ MNDA PGM1 PKM PPBP PRTN3 RAB5C S100A12 100A8 S100A9 SERPINA3 TUBB4B VCL WDR1
NET formation	KEGG hsa04613	CTSG CAMP ELANE H4C9 H2AC20 H2BC12 VWF
Platelet activation	GO:0030168	FLNA HSPB1 TLN1 VCL VWF YWHAZ
Platelet degranulation	GO:0002576	ACTN1 AHSG F13A1 FLNA FN1 PPBP SERPINA3 SOD1 TF THBS1 TLN1 TMSB4X VCL VWF WDR1
Cytokine-mediated signalling	GO:0019221	ANXA1 B2M CAPZA1 CTSG F13A1 FN1 GSTP1 HSPA5 HSPB1 HSP90AA1 LBP LCN2 LCP1 PPBP PRTN3 PSMA6 PSMB4 SOD1 TALDO1 TMSB4X VIM YWHAZ
Cytokine production	GO:0001816	LTF HSP90AA1 GSTP1 BPI HSPB1 FN1 LBP ANXA1 THBS1 LUM SOD1 MNDA TMSB4X B2M ELANE
Cellular response to cytokine stimulus	GO:0071345	VIM HSPA5 HSP90AA1 GSTP1 CTSG PSMA6 ACTR3 FN1 CAPZA1 F13A1 LBP ANXA1 LCP1 THBS1 ACTR2 ATIC SOD1 GSN LCN2 PSMB4 MNDA PPBP CAMP YWHAZ TALDO1 ACTG1 TMSB4X B2M PRTN3
Interleukin-8 production	GO:0032637	ANXA1 BPI ELANE LBP TMSB4X
Response to interferon-γ	GO:0071346	ACTR3 ACTR2 ACTG1 B2M GSN VIM
Interleukin-1 β production		ANXA1 GSTP1 HSPB1 MNDA
Response to interleukin-1 β	GO:0070555	ANXA1 CAMP LCN2 PSMA6 PSMB4
Response to interleukin-12	GO:0071349	CAPZA1 LCP1 SOD1 TALDO1
Tumour necrosis factor production		BPI GSTP1 HSPB1 LBP LTF THBS1
Response to tumour necrosis factor	GO:0034612	CAMP GSTP1 LCN2 PSMA6 PSMB4 THBS1 MSB4X
IL-17 signalling pathway	KEGG hsa04657	HSP90AA1 LCN2 S100A8 S100A9

TABLE 2 Upregulated markers associated with various processes or pathologies organized by GO biological process or KEGG pathway terms identified in ShinyGO 0.76.2

Pathway	GO term	Gene IDs
Apoptosis	GO:0042981	ACTN1 ANXA1 C1QA CAT CTSG ENO1 FCN3 FLNA GSN GSTP1 HINT1 HSP90AA1 HSPA5 HSPB1 LTF MNDA PRDX3 PRDX5 S100A8 S100A9 SOD1 THBS1
YWHAZ		
Response to hypoxia/hypoxia-inducible factor 1 signalling	GO:0001666	
KEGG hsa04066	ALDOB CAT ENO1 PSMA6 PSMB4 THBS1 TF	
Response to oxidative stress	GO:0006979	GSTP1 HSPB1 CAT PRDX5 ANXA1 SOD1 LCN2 PRDX3 GPX3
Blood coagulation	GO:0007596	F13A1 FLNA FN1 HBG2 HSPB1 PRTN3 THBS1 TLN1 VCL VWF YWHAZ
Blood vessel development	GO:0001568	ACTG1 ANXA1 ANXA3 CAMP FLNA FN1 HSPB1 PKM THBS1 YWHAZ
Brain development	GO:0007420	ACTB ANXA3 ARPC5 ATIC B2M BASP1 FLNA
HSPA5 UGP2		
Maintenance of the blood-brain barrier	GO:0035633	VCL ACTB ACTG1
Pathways of neurodegeneration	KEGG hsa05022	HSPA5 PSMA6 CAT SOD1 PSMB4 TUBB4B GPX3
Hypertrophic cardiomyopathy	KEGG hsa05410	ACTB ACTC1 TPM4 ACTG1

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associated with the 118 identified upregulated proteins in EDBS. The top 20 enriched pathways for upregulated proteins (Figure 2F) were significantly enriched for markers associated with an acute inflammatory response and immune system activation, including neutrophil activation and degranulation ($n = 31$ genes, FC = 10), cell activation ($n = 45$, FC = 5), and cell exocytosis ($n = 43$, FC = 8).

EDBS were also significantly enriched for proteins associated with the broad categories of an inflammatory response ($n = 20$, FC = 4.24; Table 1 and Table S5), activation of immune response ($n = 14$, FC = 4.77), and complement activation ($n = 4$, FC = 8.5). Immune system pathways included platelet activation ($n = 9$, FC = 10.2; Table 1 and Table S5) and degranulation ($n = 15$, FC = 22), cytokine production ($n = 15$, FC = 3), signaling ($n = 20$, FC = 4.17), and response ($n = 29$, FC = 4.6). More specifically GO analysis in ShinyGo identified cytokine-associated production and/or response pathways that were significantly enriched for interleukin-1 beta, interleukin-8, interleukin-12, tumour necrosis factor and interferon-gamma (Table 1 and Table S4). ShinyGO KEGG pathway analysis (Table S6) and network analysis in Cytoscape (Table S7) identified interleukin-17 and interleukin-6 production, respectively. There was also significant enrichment in apoptosis pathways (Table 2; $n = 20$, FC = 2.5), blood coagulation ($n = 14$, FC = 7), cellular detoxification ($n = 9$, FC = 15), wound healing ($n = 18$, FC = 6), blood vessel development ($n = 10$, FC = 2.8), hemopoiesis ($n = 13$, FC = 2.5) and response to hypoxia ($n = 5$, FC = 2.7). Last, numerous nervous system and brain-associated markers were significantly enriched, including astrocyte development ($n = 4$, FC = 14.6), blood-brain barrier ($n = 3$, FC = 16.8), brain-specific development ($n = 9$, FC = 2.2) and neuron development ($n = 12$, FC = 2).

The markers with the highest positive fold change in EDBS are primarily associated with wound healing and blood coagulation, brain-specific processes, apoptosis, and inflammatory processes including cytokine production and neutrophil activity (Table S3 and S5). The most upregulated markers of coagulation and brain-specific processes were von Willebrand factor (VWF; FC = 625.16, $p < 0.0001$; Table S3) and brain abundant membrane attached signal protein 1 (BASP1; FC = 95.8, $p = 0.08$), respectively. Numerous inflammatory markers associated with cytokine production and neutrophil activity had high fold enrichment. Markers of cytokine production included actin-related protein 2 (ACTR2; FC = 39, $p = 0.0012$), bactericidal permeability-increasing protein (BPI; FC = 75, $p < 0.0001$), lipopolysaccharide-binding protein (LBP; FC = 50, $p = 0.0026$). Highly enriched markers of neutrophil activity included Ras-related protein (RAB5C; FC = 59, $p = 0.078$), S100A8 Protein (S100-A8; FC = 46; $p = 0.0004$), S100A9 Protein (S100A9; FC = 43, $p = 0.0001$), S100A12 Protein (S100A12; FC = 86, $p < 0.001$), and lysozyme C (LYZ; FC = 42; $p < 0.0001$). Markers of apoptosis included endoplasmic reticulum chaperone BiP (HSPA5; FC = 39; $p < 0.0001$).

Analysis of upregulated proteins in Panther by protein class revealed that the majority of markers were associated with the cytoskeleton (Figure 2G; GO: PC00085; $n = 21$, 20%), defence and immunity (GO: PC00090; $n = 23$, 22%), and metabolite conversion (GO: PC00262; $n = 22$, 21%). The majority of upregulated cytoskeletal elements were comprised of actin and actin-binding cytoskeletal proteins (81%) including two myosin proteins (MYL12B and MYL6) and one actin specific to the cardiac muscle (ACTC1).

4 | DISCUSSION

In this study, we analyzed the proteomic profile of DBS in order to ascertain if biomarkers associated with the pathological events following a case of lethal human envenomation could be detected. Envenomation can result in the dysregulation of many systems in the body simultaneously, and human envenomations are a complex and evolving medical emergency. By compiling a panel of both upregulated and unique proteins compared to non-envenomated blood, we identified a variety of affected pathways and flagged potential biomarkers indicative of severe envenomation in this case. We find multiple lines of evidence of a systemic and severe inflammatory response mediated by mast cell, neutrophil, and platelet activation and degranulation as well as a cytokine storm reaction (Figure 3). Further, we identified protein markers suggestive of lethal anaphylaxis, cardiac arrest, and neuroinflammation.

Gene ontology and network analysis illustrated a dramatic systemic effect triggered by envenomation with a dominant signal of immune system activation (Figures 2B,F and 3; Table 1). We also identified enrichment for pathways of apoptosis, blood coagulation, and those associated with a response to hypoxia along with markers that may be associated with brain damage and cardiac failure (Table 2). Our findings shed light on the specific mechanisms responsible for the syndromes observed following a lethal envenomation and demonstrate the utility of DBS to provide critical proteomic data on the pathophysiology of snakebites. This study is, to our knowledge, the first proteomic characterization of pathophysiology from envenomated human blood and the first investigation of envenomation biomarkers using DBS.

In this specific case, envenomation by *C. v. viridis* triggered an apparent and lethal rapid anaphylactic reaction characterized by sudden collapse, cardiac arrest, and eventual brain death. It is unlikely that the initial inflammatory response was triggered by the specific action of venom toxins. Rarely, acute and severe anaphylactic reactions have occurred following venomous snake bites, including bites from rattlesnakes.[26,27] Clinical manifestations of venom-induced anaphylaxis include respiratory distress, hypotension, arrhythmia, shock, airway closure, and allergic myocardial infarction.[26,28–30] Hypersensitivity in these cases occurred primarily in individuals with previous snake bites or long-term exposure to venoms and is thought to be IgE-mediated and therefore requiring previous sensitization.[31,32] However, cross-reactivity with unrelated antigens could result in severe venom hypersensitivity in patients without prior exposure.[30,33,34]

Regardless of the mechanism of induction, immune system processes dominated the top 20 enriched pathways for both unique and upregulated proteins and we further identified multiple signatures associated with inflammatory signalling and response (Table 1 and Figure 2). Similar to previous studies on biofluids investigating the effects of envenomation, we identified differences in proteins associated with a thromboinflammatory reaction including activation of the

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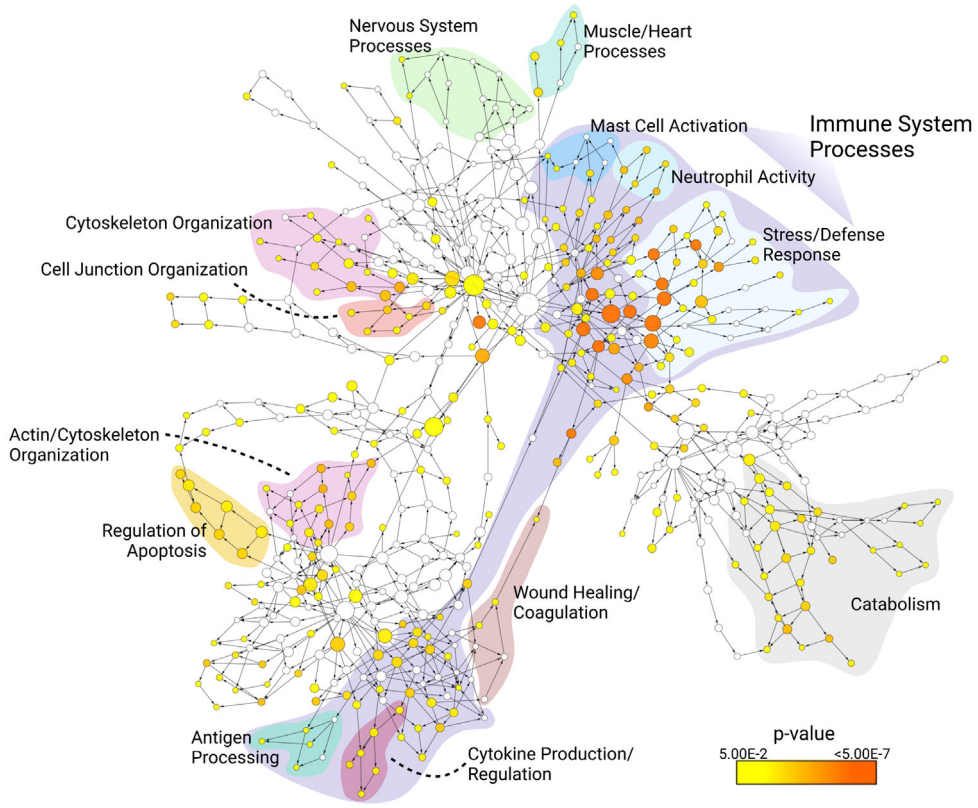

FIGURE 3 Gene ontology (GO) term network analysis from Cytoscape. Node size indicates the number of markers associated with a given term and node colour indicates the *p*-value. Nodes were annotated to show major biological systems and pathways affected.

complement system, coagulation, oxidative stress, neutrophil degranulation, and platelet degranulation.[12,13,35] Inflammation is a common response to envenomation. However, we did not detect the same proteins that were suggested to indicate the severity of inflammation, perhaps due to the development of anaphylaxis in this case as opposed to a less severe localized inflammatory reaction.[12] These authors attributed some of these inflammatory processes to the actions of venom toxins; however, it is also likely that early-stage immune processes (i.e. platelet degranulation, neutrophil chemotaxis) triggered by envenomation are the result of a general defence response. The unravelling of the direct mechanisms of venom toxin action versus a general defence response to foreign antigens requires further study.

Gene ontology analysis revealed enrichment for cellular pathways involved in early immune response events including mast cell and neutrophil activation and degranulation, as well as pathways triggering degranulation and aggregation of platelets, which have been shown to play an integral role in inflammation via crosstalk with leukocytes and the vascular endothelium.[36] Both mast cells and neutrophils are recruited during anaphylactic reactions depending on the type of stimulus and pathway, and neutrophil activation has been correlated with the severity of anaphylaxis in IgE-dependent reactions.[37] We also find evidence for the occurrence of NETosis, a pro-inflammatory neutrophil-mediated reaction that can occur soon after the onset of anaphylaxis.[37,38] Neutrophil extracellular traps (NETs) are cytotoxic to vascular endothelium and likely synergistically contribute to the vascular extravasation that occurs during anaphylaxis.[39–41]

EDBS-enriched proteins were closely associated with pathways involved in the production, signalling, and response to numerous inflammatory molecules linked with severe immune reactions, including histamine, cytokines (IL-1 β, IL-6, IL-8, IL-12, IL-17, IFN-γ and TNF; Table 1), growth factors (TGF-β), and C-reactive protein (CRP). Histamine released from mast cell degranulation is a key mediator of anaphylactic reactions and, depending on its downstream targets, is involved in the generation of nitric oxide and increased vascular permeability, cytokine synthesis, blood-brain barrier function, bronchospasm, and cardiac contraction.[42–44] Further, allergic reaction severity and delayed deterioration have been linked to elevated levels of histamine, IL-6, and TNF-1 receptor.[45] Mast cell expression of IL-8 acts as a magnifier of the inflammatory cascade via paracrine effects on other leukocytes involved in inflammation,[46] and CRP and IL-6 may act as late-phase mediators of hypersensitivity reactions.[47]

The enrichment for proteins involved in the production and signalling of numerous cytokines suggests the occurrence of a cytokine storm reaction (CSR), caused by the release of large quantities of cytokines including IL-8, IFNγ, TNFα, IL-1β and IL-6. In severe cases, this rapid release of inflammatory signals triggers increased endothelial permeability and vascular leakage, activation of the coagulation system causing a systemic syndrome with organ failure, disseminated intravascular coagulation, hypotension, hypoxemia, and cardiovascular

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collapse.[48] Specifically, IL-6 has been proposed as a biomarker of CSR because of its correlation with reaction severity.[48,49]

Another mediator of inflammation, heat shock proteins (HSPs), are a family of stress-induced molecules with cytoprotective actions that act as danger-signalling biomarkers.[50] EDBS showed upregulation of HSP90 and HSP27 (HSPB1) and uniquely expressed HSP70 (HSPA5). Increased levels of HSP27 in plasma have been found after acute ischemic stroke [51] and are released by myocardial cells after ischemia.[52] HSP70 has been shown to stimulate both arms of the immune system and trigger the release of cytokines[50] (IL-1β, IL-6, IL-12 and IFNγ). There is also evidence that these HSPs play a protective role during neuroinflammation by suppressing astrocyte inflammatory signals or interacting with ASK1 and inhibiting the ASK1-MKK4-JNK pathway.[51,53–55] Notably, serum levels of HSP70 have been directly linked to mortality in patients with CSR and septic shock.[56] This suggests that the presence of HSP70 was a strong indicator of the outcome in this case.

VWF was an upregulated protein with the highest fold increase. VWF is a mediator of coagulation and clot formation and exerts significant influence on inflammation via neutrophil recruitment, regulation of endothelial wall permeability, and altering leukocyte extravasation.[57] Another mediator of coagulation and inflammation, plasma kallikrein, KLKB1, was significantly downregulated. KLKB1 deficiency has been linked to delayed clotting,[58] while high levels of VWF have been linked to thrombotic disorders,[59] suggesting overall systemic hemostatic dysregulation in this case.

Von Willebrand factor is also a regulator of the response to endothelial cell damage, platelet activation, and platelet plug formation at the site of injury.[60–63] *C. v. viridis* venom in southern Colorado contains high levels of snake venom metalloproteases (SVMPs; Smith et al., in review), which are known to attack microvessel structure by proteolytically degrading the basement membrane and disrupting endothelial cell adhesion leading to vessel damage and fluid extravasation.[64–66] This ultimately can result in local and systemic bleeding, tissue destruction, and ischemia.[67,68] SVMPs also have been shown to play a role in blood coagulation activation,[69,70] apoptosis,[71–73] inhibition of platelet aggregation,[74,75] and inflammation.[68,74] It is possible that the high levels of VWF are indicative of a response to vascular damage caused by the abundance of SVMPs in *C. v. viridis* venom and/or that SVMPs had a synergistic effect on inflammation, blood coagulation, and apoptosis with the anaphylactic reaction observed.

There were multiple brain-derived proteins identified in EDBS that have previously been investigated in CSF as biomarkers of neuropathies associated with Alzheimer's Disease (BASP1, YWHAZ, SOD1 and PKM), and YWHAZ specifically was identified as a strong candidate for the identification of neurodegeneration.[76] The high abundance of brain-associated proteins in blood as opposed to CSF in addition to the presence of neuroprotective HSPs, may have resulted from the significant inflammation and damage to the blood-brain barrier and CNS that occurred in this case.

The downregulated protein with the highest degree of connectivity in NetworkAnalyst, CAND1 (Figure S1; Cullin-associated and neddylation-dissociated 1 protein), plays a central role in cardiac functioning by preventing the accumulation of hypertrophic proteins, thereby protecting against stress-induced cardiac hypertrophy and heart failure.[77] The direct role of this protein in cardiac function merits its investigation as a biomarker of anaphylaxis-induced heart failure.

While there is significant evidence for the pathways and markers elucidated here, there remain inherent limitations to the current study. First, this study only includes data from a single case of envenomation and a single non-envenomated control; therefore, individual or age-related variation could be responsible for some of the differences in protein presence or abundance observed when comparing blood profiles. While this is likely the case for a number of identified proteins, we detected numerous signatures in EDBS unlikely to result from individual differences in baseline healthy physiological processes (i.e. the abundance of HSP). Further, the sheer number of activated pathways and upregulated markers associated with inflammation and immune system activation is indicative of an overall trend, despite the potential for false positives. It is also unclear if the other significantly enriched pathways, including apoptosis, response to hypoxia, blood coagulation, and blood vessel development, are downstream effects of anaphylaxis or if they are the result of the action of venom toxins.[31] It is likely that the broad systemic effects of anaphylaxis masked the direct action of venom toxins, and ultimately, this limits our ability to correlate specific venom toxin action with clinical presentation in this instance.

Biofluid proteomics is a robust tool for the investigation of the pathological events of envenomation and has promise for the development of biomarkers associated with envenomation syndrome. Here we demonstrate that DBS provide useful proteomic information on the systemic processes that are triggered by envenomation. We find evidence for a massive and systemic inflammatory cascade, combined with coagulation dysregulation, complement system activation, hypoxia response activation, and apoptosis. We also identified potential markers indicative of lethal anaphylaxis, cardiac arrest, and brain death. The utilization of proteomic data from DBS has significant implications for the investigation of envenomation syndromes in limited-resource and rural settings, which bear the highest costs of snakebite envenoming worldwide. An enduring challenge will be to bring the benefits indicated by MS-based analysis of DBS to those areas that could benefit most from this technology.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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