**Venomics of the asp viper *Vipera aspis aspis* from France**

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**Abstract**

The asp viper *Vipera aspis a*spis is a venomous snake found in France, and despite its medical importance, the complete toxin repertoire produced is unknown. Here, we used a venomics approach to decipher the composition of its venom. Transcriptomic analysis revealed 80 venom-annotated sequences grouped into 16 gene families. Among the most represented toxins were snake venom metalloproteases (23%), phospholipases A2 (15%), serine proteases (13%), snake venom metalloprotease inhibitors (13%) and C-type lectins (12%). LC-MS of venoms revealed similar profiles regardless of the method of extraction (milking vs defensive bite). Proteomic analysis validated 57 venom-annotated transcriptomic sequences (>70%), including one for each of the 16 families, but also identified 7 sequences not initially annotated as venom proteins, including a serine protease, a disintegrin, a glutaminyl-peptide cyclotransferase, a proactivator polypeptide-like and 3 aminopeptidases. Interestingly, phospholipases A2 were the dominant proteins in the venom, among which included an ammodytoxin B-like sequence, which may explain the reported neurotoxicity following some asp viper envenomations. In total, 87 sequences were retrieved from the *Vipera aspis aspis* transcriptome and proteome, constituting a valuable resource that will help in understanding the toxinological basis of clinical signs of envenoming and for the mining of useful pharmacological compounds.

*Biological significance*: The asp viper (*Vipera aspis aspis*) causes several hundred envenomations annually in France, including unusual cases with neurological signs, resulting in one death per year on average. Here, we performed the first proteotranscriptomic analysis of *V. a. aspis* venom in order to provide a better understanding of its venom composition. We found that, as in other *Vipera* species, phospholipase A2 dominates in the venom, and the presence of a sequence related to ammodytoxin B may explain the reported neurotoxicity following some asp viper envenomations. Thus, this study will help in informing the toxinological basis of clinical signs of envenoming.

**Keywords:** Proteomics; transcriptomics; venom; toxin; snake; Vipera

**1. Introduction**

Several venomous snakes occur in France, including a rear-fanged snake (Lamprophiidae, *Malpolon monspessulanus*) and four species of vipers (Viperidae). Among the latter, *Vipera* *berus berus* (*Vbb*) and *Vipera* *aspis aspis* (*Vasp*) are the most common and are responsible for the majority of reported envenomations. In particular, the asp viper *Vipera aspis aspis* is of medical importance in France, with up to 1000 recorded bites and one death per year on average [1]. Symptoms following envenomation include local signs such as pain and oedema, combined in severe cases with extensive swelling and/or systemic signs such as gastrointestinal disorders and hypotension [2]. A French grading system for viper envenomation has been established according to several clinical report descriptions [3]. Most cases stabilize at grade 1 (minor), with only 15 to 20% progressing to grade 2 (moderate), whereas grade 3 (severe) is defined as grade 2 symptoms that continue to progress further [3]. Consistent with the potential severity of symptoms observed in some snakebite victims, the venom of these vipers is known to contain some procoagulant compounds, kinin-releasing factors, other hypotensive factors, proteases and hyaluronidases [3–7].

From the 1990s, the Marseille Poison Center repeatedly witnessed unusual cases of envenomation after *Vasp* bites that included neurological signs such as ptosis, drowsiness, diplopia and ophtalmoplegia [8]. Given these neurotoxic symptoms, De Haro *et al.* made the hypothesis that the toxins causing neurological damages in *Vasp* bites might be similar to the PLA2-I found in the venom of *Vipera* *aspis zinnikeri* and *Vipera ammodytes ammodytes* (*Vaa*) [8]. The phospholipase PLA2-I is also highly homologous to the vipoxin of the *Vipera* *ammodytes meridionalis*, and these toxins are indeed known to block the neuromuscular junction at the presynaptic or postsynaptic level[9]. Accordingly, the same group later identified two neurotoxins in the venom of *Vasp,* namely ammodytoxin B (monomeric PLA2 originally found in the venom of *Vipera* *ammodytes ammodytes, Vaa*)and a novel non-covalent PLA2 heterodimer named vaspin, which is analogous to vipoxin and PLA2-I [10]. Ferquel *et al*. screened for venom PLA2 at the genome and transcriptome levels and identified three different subpopulations of *Vasp*, based on their neurotoxic venom PLA2 content[11]. The first group is defined by the presence of genes encoding AmI1 and AmI2, the second group by genes encoding AmI1, AmI2, vasA, vasB and the third group by genes encoding AmI1 and vaspin. Overall, it was concluded that the genome of *Vipera* *aspis aspis* comprises a repertoire of PLA2 neurotoxins ready to be expressed under stimuli that remain to be identified. In addition, the apparent intra-species variation might also be due to hybridization phenomenon, which could be an important mechanism of the evolution of the composition of snake venoms [11].

Importantly, the venom variability within the different *Vasp* populations has significant implications for the design of antivenom. Indeed, intraspecific variations in venom composition is well known to cause major issues in the preparation of effective antivenoms [12]. Viperfav has been approved for marketing and is the only licensed antivenom currently readily available in France. It contains F(ab’)2 immunoglobulin fragments produced by horses against *Vipera berus*, *Vipera aspis* and *Vipera ammodytes* venoms [3]. Fortunately, since Viperfav was also designed against *Vipera ammodytes* venom, this makes it potentially effective against neurotoxic *Vipera aspis* populations because the two main neurotoxin types of *Vipera aspis* are also found in *Vipera* *ammodytes* [3,13]. However, the efficacy of Viperfav against the neurotoxic effects of *Vipera* venoms was found to be variable [14].

Despite these toxin composition and antivenom efficacy observations, to date no comprehensive global analysis of the venom protein composition of *Vasp* venom has been performed. To redress this limitation, in this study, we characterized *Vasp* venom composition through a venomics approach that combined transcriptomic and proteomic analyses [15]. The powerful technology of next generation sequencing allows for the rapid identification of transcripts produced in the venom gland, facilitating the matching of MS/MS spectra to protein/toxin sequences [16]. This work will help provide the medical and research communities with a better understanding of the composition, toxicity and evolution of the venom of this species, which in turn could lead to the future design of improved antivenom therapy for treating snakebite [17,18].

**2. Materials and methods**

*2.1. Vipera aspis aspis venom and venom glands*

Venoms glands were dissected from a single, adult, captive-bred specimen of *Vipera aspis aspis* housed and maintained in the Herpetarium of the Liverpool School of Tropical Medicine. The facility and protocol are inspected and approved by the UK Home Office and the LSTM Animal Welfare and Ethical Review Board. The week prior to dissection the specimen was not fed, and three days prior to dissection venom was extracted. Subsequently, the specimen was euthanised via an overdose of injectable pentobarbitone (Pentoject, Animalcare Ltd), and following confirmation of death, the venom glands were dissected, immediately flash frozen in liquid nitrogen, and stored cryogenically until RNA extraction. Venom was also collected from this same animal on a number of occasions prior to euthanasia. The venom was lyophilised, pooled and stored at 4°C until use. In addition, several samples of venom were collected from a wild-caught specimen of *Vipera aspis aspis* from South of France in order to test for the possible variations due to the sampling procedure, and to support the pertinence of our results to clinical symptoms of envenomation. The successive venom collections were carried out in one session of less than 1 h. For the first venom sample, the snake was simply aggravated and induced to strike a glass covered with parafilm mounted on a stick, to mimic a “defensive bite”. Next, the snake was held behind the neck and let to freely bite a container covered with parafilm (“milking”). Finally, the snake was held as previously but forcefully induced to bite the container while massaging the venom glands (“forced milking”). All venom samples were immediately freeze-dried and stored at –20°C until use.

*2.2. RNA isolation, library preparation and sequencing*

The *Vasp* venom gland transcriptome was constructed using methods previously applied to other venomous snake species [19–21]. Venom glands were homogenised under liquid nitrogen using a pestle and mortar before the TRIzol Plus RNA Purification System (Thermofisher) was used according to the manufacturer’s instructions. From this purification step, the resulting RNA samples were DNAse-treated using On-Column PureLink DNAase (Life Technologies) and total RNA was eluted in 30 µl of nuclease free water, before undergoing two rounds of poly(A) selection for mRNA using the Dynabeads mRNA DIRECT purification kit protocol (Life Technologies). Fifty nanograms of the mRNA-enriched sample was next used to construct an RNA-seq library using the Script-Seq v2 RNA-Seq Library preparation kit (epicentre), following 12 cycles of amplification. This library was purified using AMPure XP beads (Agencourt) and quantified using the Qubit dsDNA HS Assay kit (Life Technologies). The size distribution was then assessed using a Bioanalyser (Agilent). The sequencing library was then multiplexed alongside others not reported in this study, and sequenced on an Illumina MiSeq instrument (six libraries per lane) with 2 × 250 bp paired-end sequencing technology (Centre for Genomics Research, University of Liverpool).

The resulting reads were assembled using the transcriptome assembly program VTBuilder [22], which has previously been successfully applied to venom gland transcriptomic studies [19–21]. For assembly, we used the following parameters: minimum transcript length 150 bp, minimum read length 150 bp, isoform similarity 96%. As part of this process, the VTBuilder algorithm performs a normalised read mapping approach to generate relative transcript expression data, which is expressed for each assembled contig as a percentage of total transcriptome expression. Assembled contigs were batch annotated with BLAST2GO Pro v3 [23] using the BLASTx-fast algorithm with a significance threshold of 1e-5 against the NCBI non-redundant (NR) protein database (41 volumes, Nov. 2015). Annotated sequences were then exported for further examination. The raw sequencing reads and transcriptome assembly have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) and Transcriptome Shotgun Assembly (TSA) databases respectively, and are associated with the BioProject identifier PRJNA594707.

*2.3 Transcriptome assembly, annotation and toxin transcript quantification*

The resulting BLAST annotations of assembled contigs were examined manually and sorted into three categories: “toxins”, “non-toxins” and “unknowns” based on annotation similarity to previously described snake venom toxins. Contigs identified as toxins were assigned to specific toxin families based on their annotations and each gene family exhibiting total summed expression of greater than or equal to 1% were used to generate sequence alignments in MEGA v7 [24]. Sequence alignments were produced for: C-type lectins (CTL), cysteine-rich secretory proteins (CRISP), disintegrins (Dis), kunitz-type serine protease inhibitors (kunitz), nerve growth factors (NGF), phospholipases A2 (PLA2), snake venom serine proteases (SVSP), snake venom metalloproteinases (SVMP), snake venom metalloproteinase inhibitors (SVMPi) and vascular endothelial growth factor (VEGF). For each of these toxin families, the correct reading frames were identified for individual contigs using MEGA v7, facilitated by manual inspection of blastn matches and the online ExPASy Translate portal [25]. To produce high-quality alignments, quality control measures were implemented wherein contigs were discarded if: i) sequence length matching known toxins was less than 100 bp long, ii) 50% or more of the contig did not match a known toxin in a blastn search, iii) the first 50% of the contig was interrupted by a stop codon, iv) the sequence was made up either of two exonic regions interspersed by an intron (indicative of genomic DNA contamination) or two distinct sequences, based on annotation, that were assembled continuously (indicative of chimeric misassembly). To resolve the issue of “underclustering”, a common by-product of assembling isoform-rich gene data [22], contigs were merged (and expression summed) if they exhibited >98% sequence similarity over an overlapping region of >50 bp. All curated datasets were trimmed to the open reading frame (i.e. start methionine and stop codon) in MEGA v7 and then aligned using the MUSCLE algorithm [26] under default standard parameters.

*2.4 Liquid chromatography coupled mass spectrometry*

Reverse-Phase Ultra Performance Liquid Chromatography (RP-UPLC) was operated on an Acquity H-Class ultrahigh performance liquid chromatography (UPLC) system (Waters, Corp., Milford, MA, United States) fitted with a UV detector (PDA detector) under the control of Waters MassLynx software (version 4.1). Separation of 300 µg crude *Vasp* venom was achieved using a Kinetex C18 100 Å column (2.1 x 150 mm, 3 µm) fitted with a pre-column. Elution was carried out using a gradient of 0-80% B (0.1% formic acid in ACN) in 80 min. Samples eluting from the UPLC were introduced into the mass spectrometer at a flow rate of 500 µl/min. Acquisitions were carried out over the range 50 Da to 1800 Da *m/z* every 0.1 second on a Synapt-G2-S high-definition MS system (Waters, Corp., Milford, MA, United States). To obtain the molecular masses of the venom components eluting between 0 and 70 min, each peak from the total ion current (TIC) chromatogram was analysed with Waters Mass Lynx software (version 4.1).

*2.5 Shotgun proteomics*

2.5 µL of crude *Vipera aspis aspis* venom at 20.5 mg/mL in milli-Q water (or about 50 µg) were diluted 20 times in 100 mM NH4HCO3 pH 7.8 and reduced with 5 µL of 500 mM dithiothreitol in milli-Q water for 1 hour, at 56◦C, under shaking at 300 rpm. The sample was then alkylated with 10 µL of 500 mM iodoacetamide for 1 hour, at room temperature, in the dark. The venom sample was submitted to trypsin digestion by adding 5 µL of Pierce™ mass spectrometry grade trypsin (Thermo Scientific, Waltham, MA, USA) at 0.1 mg/mL in 100 mM ammonium bicarbonate buffer (4 h at 37°C). The reaction was stopped by acidifying the media with the addition of 6 µL of TFA 10% (v/v) in milli-Q water to the reaction mixture. The sample was desalted on a ZipTip™ pipette tip filled with C18 resin, using an acetonitrile/water/TFA (49.8/50/0.2 v/v) solution as eluent. A total of 0.5 µg from the digested material was analysed by the Acquity UPLC® M-Class (Waters, Milford, MA, USA) coupled to the Q-Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (Thermo Scientific, Bremen, Germany). The chromatographic system was equipped with a monolithic PepSwift Capillary column 100 µm x 25 cm (Thermo Scientific, Waltham, MA, USA). Peptides were eluted using a gradient of 3–50% of solution B in 100 min and 50-80% of solution B in 30 min (A: water/0.1% formic acid; B: acetonitrile), at a flow rate of 0.6 mL/min, and data were acquired in the positive-ion mode. Concerning mass spectrometry, all the analyses were performed in data dependent analysis (DDA) mode that automatically triggers the MS/MS experiments. The automatic gain control (AGC) target values were 3.106 for MS spectra and 2.105 for MS/MS spectra. The maximum injection times were set at 200 ms for the MS step and 1000 ms for MS/MS events. For MS/MS, a “Top 12” experiment was applied, meaning that the twelve most intense ions of each MS scan have been selected for fragmentation. Singly charged ions, ions with undetermined charge (for example, electronic noise) and ions with signal intensities below the AGC threshold set at 1e3 were excluded from the selection. For precursors ions, the selection windows were 2.0 m/z, the AGC target was 1e5 (or 50ms as a maximum of injection time) and the resolving power of 17,500 @m/z 200. Normalized collision energy was 25. A dynamic exclusion of 10s was also applied to avoid the redundancy of MS/MS spectra of the same ions.

*2.6 Bioinformatic analysis of proteomic data*

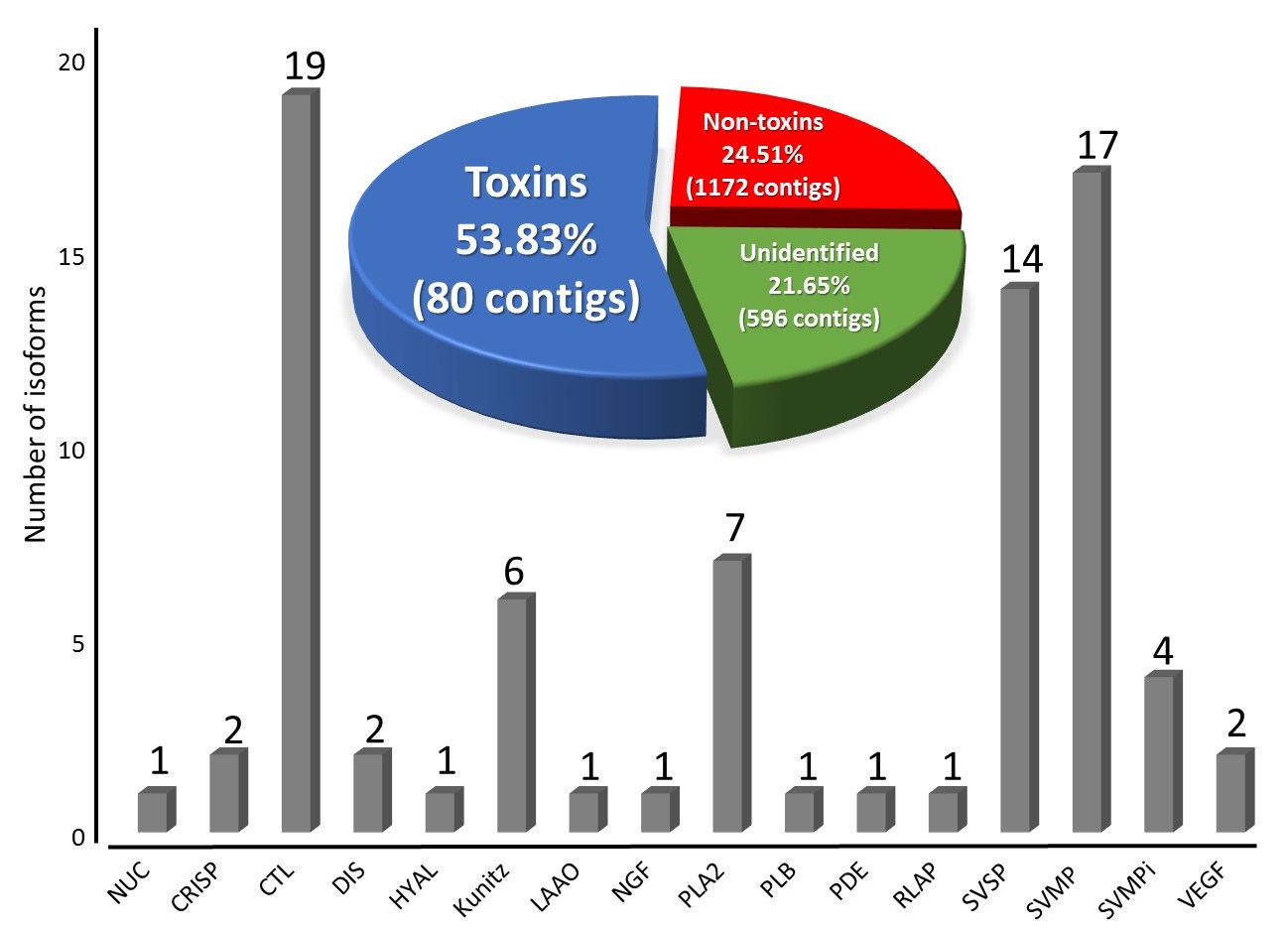
PEAKS Studio 8.5 (Bioinformatics solutions, Waterloo, ON, Canada) a *de novo* assisted database software [27,28] was chosen to analyze MS/MS data from *Vasp* venom. Indeed, by using the PEAKS studio software we were able to match MS/MS spectra obtained from proteomic analysis of *Vasp* venom to our own database resulting from the assembled venom gland transcriptome of the same specimen translated into the six reading frames. Briefly, PEAKS studio initially produces *de novo* sequences from MS/MS spectra without relying on database. The confidence of each peptide sequence obtained by this process is given by an ALC (Average Local Confidence) score. These *de novo* sequences are then corrected by comparing to the database to provide additional information about post-translational modifications (PTM’s), mutations, homologous peptide and novel peptides. Carbamidomethylation was set as fixed modification, while oxidation (M) was set as variable modifications, with maximum missed cleavages at 3 for trypsin digestion. Parent mass and fragment mass error tolerance were set at 5 ppm and 0.015 Da, respectively. False discovery rate (FDR) of 1% [29,30] and unique peptide ≥2 were used for ﬁltering out inaccurate proteins. A −10lgP > 120 indicates that the detected proteins by enough reliable peptides MS/MS spectra. In order to identify more relevant sequences, the Spider algorithm [31] from PEAKS Studio software was used to find additional mutations or to correct the sequences. This algorithm corrects the sequences stored in transcriptomic database with *de novo* sequences based on MS/MS spectra, which allowed us to identify PTM’s and mutations. Minimum ion intensity for mutation and PTM’s was set to 5%, and ALC score ≥ 90 for *de novo* sequences leading to low precursor mass error in order to identify reliable PTM’s and potential mutations. Relative abundance of proteins families were calculated according to method described by Zybailov *et al.* [32] briefly, this method is based on the principle that most abundant proteins will give more tryptic peptides and consequently more MS/MS spectras. Therefore, spectral count of each protein families can be related to its relative abundance. To take in account that big proteins will give more proteotryptic peptides the spectral count is normalized by the protein masses. This method has been used to estimate protein abundance in many label-free shotgun proteomic [33–35]. Therefore in our proteomic study the percentage of abundance of a protein family is the sum of the SpC/L of the proteins of the family over the sum of the SpC/L of all the proteins identified with SpC being the spectral count of the identified peptides and L the average mass of the protein.

**3. Results and discussion**

*3.1 Toxins identification from transcriptomic analysis*

The assembly of the >2.2 million paired-end high quality reads obtained for the *de novo* sequencing of *Vipera aspis aspis* venom gland resulted in 1848 contigs with an average length of 739 bp. Analysis of the assembled transcriptome revealed that 1172 contigs were annotated as non-toxin elements (63.42% of total contig numbers), 596 contigs were unidentified (32.25%) and 80 contigs were toxins (4.33%) (Fig. 1). However, in terms of expression levels, the toxin-annotated contigs represented 53.83% of the transcriptome, non-toxin contigs 24.51% and unidentified contigs 21.65% (Fig. 1). Thus, the mean contig expression of toxins considerably outweighs those of non-toxin and unidentified sequences (0.69 vs 0.02 and 0.04 % per contig, respectively). These findings are highly consistent with prior venom gland transcriptomic studies [19–22,36].

The 80 toxin-annotated contigs identified were classified into 16 different proteins families (Fig. 1, Fig. S1 and Table S1). Among them are many of the commonly expressed toxins found in viperid snake venoms, such as CTLs, SVMPs, SVSPs, PLA2, kunitz-type serine protease inhibitors, disintegrins, CRISPs, but also proteins such as nucleotidase, aspartic protease and phosphodiesterase. CTL, SVSP and SVMP show the highest level of diversification, with 19, 14 and 17 isoforms, respectively. In the next section, these toxins have been grouped according to their biological role in order to highlight a plausible correlation between venom composition and *Vasp* envenomation symptoms.



**Figure 1: Transcriptome expression levels and number of contigs (isoforms) for the major toxin families identified in the *Vasp* venom gland transcriptome**. Despite a relatively low number of contigs, those annotated as toxins represent more than 50% of the total gene expression captured in the venom gland. The 80 toxin-related sequences were grouped into 16 different families, where CTL, SVMP and SVSP, PLA2 and Kunitz-type toxins are the most diversified. NUC, 5'-nucleotidase; CRISP, cysteine rich secretary protein; CTL, C-type lectin; DIS, disintegrin; HYAL, hyaluronidase; kunitz, kunitz-type serine protease inhibitors; LAAO, L-amino acid oxidase; NGF, nerve growth factor; PDE, phosphodiesterase; PLA2, phospholipase A2; PL-B, phospholipase B; RLAP, renin-like aspartic protease; SVMP, snake venom metalloprotease; SVMPi, snake venom metalloprotease inhibitor; SVSP, snake venom serine protease; VEGF, vascular endothelial growth factor.

*3.1.1 Toxins perturbing hemostasis*

Snake venom metalloproteases (SVMPs) are largely responsible for the impaired hemostasis in envenomed victims [37]. SVMPs are classified into 3 different subclasses; P-III which is constituted of a protease domain, a disintegrin and a cysteine rich domain, whereas P-II lost the cysteine rich domain and P-I lost the disintegrin and the cysteine rich domains [38]. The loss of non-protease domains led to a decrease of the hemorrhagic potency of these toxins, and consequently, PIII SVMPs are typically more hemorrhagic than P-I and P-II forms [39,40]. These metalloproteases degrade the basal membrane components and the extracellular matrix such as type collagen IV, fibronectin, nidogen as well as plasma proteins involved in blood coagulation, including fibrinogen, prothrombin and factor X, thus leading to hemorrhage and coagulopathy [37]. The 17 different SVMPs expressed in the venom gland transcriptome of *Vasp* represent 23.34% of total toxin expression (Fig. 2); 11 of these belong to the P-III class, 2 to the P-II class and 4 could not be assigned to a specific class due to incomplete sequence data. The high abundance of SVMPs in the venom gland transcriptome is consistent with the hemorrhagic effect sometimes observed following bites by *Vipera aspis* [3]. Indeed Sajevic *et al.* showed that most of the hemorrhagic activity of the related *Vipera ammodytes ammodytes* (*Vaa*) venom is due to a P-IIIc metalloprotease [41].

Snake venom serine proteases (SVSPs) are also known to perturb mammalian haemostasis, by influencing blood coagulation, fibrinolysis, platelet aggregation and vasoconstriction [42,43]. SVSPs are expressed at lower levels than SVMPs in *Vasp* transcriptome, which is consistent with prior demonstration that the fibrinogenolytic activity of *Vaa* and *Vbb* venoms is mainly caused by SVMPs [42]. Nonetheless, the 14 different serine proteases detected in the transcriptome of *Vasp* represent 13.31% of total toxin expression levels (Fig. 2), and thus future work is required to assess to what extent these toxins contribute to haemotoxicity following snakebites.

Snake venom C-type lectin like proteins (CTLs), also known as snaclec, are built around a lectin scaffold, although venom CTLs diverged significantly through evolution and lost their ability to bind carbohydrates [40,44]. CTLs are non-enzymatic proteins that can affect cell adhesion, thrombosis, hemostasis, endocytosis, or pathogen neutralization [45–47]. The 19 different CTLs expressed in the transcriptome of *Vasp* represent 12.28% of total toxin expression (Fig. 2), and those that are most highly expressed show similarity with toxins that have been shown to activate platelet aggregation. Although no such agonist of platelet aggregation has been reported in *Vaa* and *Vbb* venoms, the CTLs of *Vasp* seem likely to be contributing to thrombocytopenia, which has been reported in bite victims [42].

Disintegrins are monomeric or dimeric proteins present in the venoms of various vipers that selectively block the function of integrin receptors [42,48]. They can be divided into five different groups according to their length and number of disulfide bonds [48,49]. Integrins are involved in cell-cell and cell-extracellular matrix interactions, serving as the final common pathway leading to platelet aggregation. In snake venoms, these small cysteine-rich disintegrins strongly inhibit platelet aggregation and thereby prevent blood coagulation in bite victims [50,51]. Two different disintegrins were found expressed in the venom gland transcriptome of *Vasp*,representing 4.97% of total toxin expression (Fig. 2). Both of these disintegrins are closely related to ‘lebein’, a class II disintegrin comprising ~70 amino acids with 6 disulfide bridges, isolated from the venom *Macrovipera lebetina* [52]. It seems likely that the *Vasp* disintegrins may also contribute to thrombocytopenia observed after envenoming.

Several other potential haemotoxins were found at lower levels of expression in the *Vasp* venom gland transcriptome. These included: (i) LAAOs (1.07% expression), which can inhibit ADP- and collagen-induced platelet aggregation [53], (ii) CRISPs (0.79%), which are 20 to 30 kDa proteins containing sixteen highly conserved cysteine residues [54,55] and (iii) phosphodiesterase (0.03%), which showed similarity to the platelet aggregation inhibiting phosphodiesterase 1 from *Crotalus adamanteus* venom [56].



**Figure 2: Representation of toxins families detected in the *Vasp* venom gland transcriptome according to their relative expression levels**. 5’-NUC, 5'-nucleotidase; CRISP, cysteine rich secretary protein; CTL, C-type lectin; Dis, disintegrin; HYAL, hyaluronidase; kunitz, kunitz-type serine protease inhibitors; LAAO, L-amino acid oxidase; NGF, nerve growth factor; PDE, phosphodiesterase; PLA2, phospholipase A2; PL-B, phospholipase B; RLAP, renin-like aspartic protease; SVMP, snake venom metalloprotease; SVMPi, snake venom metalloprotease inhibitor; SVSP, snake venom serine protease; VEGF, vascular endothelial growth factor.

*3.1.2 Enzyme inhibitor toxins*

Kunitz-type serine protease inhibitors have been found in diverse animal venoms such as snake, cone snails, sea anemone and spiders [57–61]. As the name suggests, the original function described for these peptides was the inhibition of diverse serine proteases, however some kunitz, particularly those found in elapid snake venoms, have evolved functional activities consistent with a toxic role, including perturbing ion channels [61]. Nonetheless, those detected here (6 different kunitz-type peptides representing 9.87% of the total toxin expression), are structurally similar to those kunitz peptides functionally annotated as serine protease inhibitors, which suggests that *Vasp* kunitz may not be employed as venom toxins. In addition to serine protease inhibitors, we also detected snake venom metalloproteinase inhibitors in the *Vasp*  venom gland transcriptome; these four contigs represented 13.20% of total toxin expression levels (Fig. 2), and one of these is highly related to an endogeneous tripeptide snake venom metalloproteinase inhibitor described from a number of other Viperinae snake species [62,63].

*3.1.3 Neurotoxic toxins*

Phospholipases A2 are multifunctional toxins that are common constituents of many different snake venoms. Their toxicity can manifest in many different ways, including neurotoxicity, myotoxicity, cardiotoxicity and anticoagulant activity [42,64]. In particular, the neurotoxicity of *Vaa* venom is attributed to the presence of three basic monomeric PLA2s, namely ammodytoxins A, B and C, which are able to block the neuromuscular junction at the presynaptic level [65,66]. The 7 different PLA2 contigs (including 3 complete sequences, see Fig. 3) retrieved from the *Vasp* venom gland transcriptome represented 15.45% of total toxin expression (Fig. 2), and the most expressed contig is closely related to ammodytoxin B, which may explain the neurotoxicity of some *Vasp* bites (Fig. 3). From the two other complete sequences, one is 100% identical to the non-neurotoxic ammodytin I2 from *Vaa*, whereas the other shows 99.28% sequence identity with ammodytin I1 from *Vasp* (only one conservative substitution of I>L). The other incomplete PLA2 sequences are similar to ammodytin I1, ammodytin L(2) and ammodytin L1 (main cardiotoxic component of *Vaa*) [67].



**Figure 3: An amino acid sequence alignment of *Vipera* PLA2 toxins**. The seven *Vasp* PLA2 sequences detected in the venom gland transcriptome are displayed (T contig numbers; three full length, four partial length) alongside previously known representatives, such as the ammodytoxins and ammodytins. The percentage identity of each sequence with Ammodytoxin B Vaa is indicated in the header, as well as the query cover percentage value being displayed in brackets.

*3.1.4 Minor toxins*

In addition to the toxins described above, a number of other minor putative venom toxin components were identified in the *Vasp* venom gland transcriptome. These included: (i) vascular endothelial growth factors (VEGFs), which are known to promote proliferation of vascular endothelial cells, and also contribute to hypotension by increasing vascular permeability [68,69], and (ii) a renin-like aspartic protease (RLAP), similar to that previously described in *Echis ocellatus*, which in turn is highly similar to human and mouse renin, and possibly facilitates the spread of the venom by inducing local hypertension [42]. One hyaluronidase sequence was also identified - this enzyme family is found in a wide range of animal venoms where it facilitates the diffusion of other toxins present in the venom through the hydrolysis of hyaluronic acid [29,70]. Finally, one nerve growth factor (NGF) and one nucleotidase were also found but the importance of their role in snake venoms remains unclear.

*3.2. Comparative LC-MS of Vasp venom*

The composition of secreted venom has been shown to vary in certain venomous lineages according to the mode of collection, such as manual versus electric stimulation or predatory versus defensive stimulus [71,72]. Therefore, in order to investigate the possible venom composition variations between the different milking procedures applied to snakes, the venom of one specimen of *Vasp* was collected sequentially using different methods and analysed by LC-MS. Overall, the venoms collected after a defensive bite, classic milking and forced milking show very similar total ion chromatogram traces (Fig. 4). Thus, the mode of venom collection does not seem to influence venom composition, confirming the unbiased nature of our venomics approach and that the results can be directly applicable to clinical symptomology observed following a snakebite. The profile of each venom sample can be separated into 3 major groups, based on the resulting retention time and molecular mass range. The first group (< 20 minutes elution) contains mostly small molecular masses (< 2 kDa), whereas the second group (~20-33 min) contains a majority of masses in the 2-4 kDa range. Yet, about half of the TIC is constituted of masses > 10 kDa, including 3 distinct subgroups. Eluting between 33 and 40 minutes, molecular masses around 13-14 kDa likely correspond to the phospholipase A2 (PLA2) enzymes and C-type lectins (CTL). The remaining subgroups (30-60 kDa and 13-30 kDa) may include snake venom metalloprotease (50-60 kDa), phospholipase B (64 kDa), L-amino acid oxidase (57 kDa), renin-like aspartic protease (43 kDa), glutaminyl-peptide cyclotransferase (42 kDa), snake venom serine protease (SVSP) (28-30 kDa), nerve growth factor (NGF) (27 kDa) and cysteine rich secretary protein (CRISP) (25 kDa).



**Figure 4: Total Ion Chromatogram profiles and determined molecular masses of *Vasp* venom**. Top panel shows the TIC of venoms collected after a defensive bite, unforced, and forced milking, which display very similar profile indicating highly similar venom compositions. On the bottom panel is reported the major molecular masses for each peak (defensive bite trace).

*3.3. Shotgun proteomics of Vasp venom*

To reduce potential bias due to intraspecific venom variations, and to allow for a direct correlation between transcriptomic and proteomic data, crude venom was also collected via classic manual milking from the animal used for venom gland transcriptomics prior to RNA extraction. The MS/MS spectra obtained after reduction, alkylation and digestion of the crude venom were analyzed by PEAKS studio software using the full assembled transcriptome translated into six reading frames (Table S2). Overall, each toxin family identified in our transcriptomic analysis was validated by proteomics (with a minimum of one sequence per family).

The relative abundance of each protein family was calculated according to the method described by Zybailov *et al.* [32], which is based on the principle that most abundant proteins will give more tryptic peptides and consequently more MS/MS spectras. Therefore, the spectral count of each protein family can be related to their relative abundance. To also take into account that larger proteins will give more proteotryptic peptides, the spectral count has been normalized by the protein masses. This method has been used to estimate protein abundance in many label-free shotgun proteomic studies [33–35], however, the ensuing results have to interpreted carefully, as this approach assumes that linearity of response is the same for every protein and it does not take into account the theoretical number of observable peptides per proteins.

A total of 64 proteins were identified in the venom using our assembled transcriptome as a reference database (Table S3). Although variable half-lives of distinct mRNA and differential regulation of the post transcription machinery can affect the relationship between transcriptome and proteome [73,74], in this instance a reasonable correlation was observed between toxin gene expression and protein abundances. For example, the transcriptomic expression levels and proteomic abundances of SVSPs and disintegrins were relatively conserved, representing around ~12% and ~5% of total abundances, respectively (Fig. 2 and 5). However, for other toxin families, differences were observed. For instance, PLA2 levels were substantially different, representing ~15% of gene expression in the venom gland transcriptome (Fig. 2) compared to a proteome abundance twice as much at ~30% (Fig. 5). Surprisingly, the most expressed PLA2 are similar to ammodytin I1, which is likely not neurotoxic considering its similarity with ammodytin I2 [67]. Therefore, the occasional reported neurotoxicity of *Vasp* bites could rather be explained by the relatively low levels of ammodytoxin B-related toxin, despite this being the most highly expressed PLA2 sequence in the transcriptome [65,66]. We also detected differences in: (i) the functionally diverse C-type lectins, hence the many isoforms detected in the transcriptome (~24% of the toxin transcriptome according to contig numbers, Fig. 2), but expression level in the proteome is much lower ~12% (Fig. 5) and (ii) the SVMPs, which are only moderately abundant in venom (representing around 11% of all venom proteins; Fig. 5), despite much higher transcriptome expression levels (~22%; Fig. 2).

Interestingly, the toxin families represented by only one transcriptomic contig were all validated by our proteomics approach, which detected the presence of 5’-nucleotidase, hyaluronidase, L-amino-acid oxidase, NGF, phosphodiesterase, PLB and renin-like aspartic proteases in *Vasp* venom, despite typically low transcriptomic expression levels. Finally, out of the 64 identified proteins, 7 were not initially annotated as venom proteins in our curated venom gland transcriptome. These included a serine protease, a disintegrin, a glutaminyl-peptide cyclotransferase, a proactivator polypeptide-like and 3 aminopeptidases (table S3).

*3.4. Comparison with Vipera berus berus venom*

*Vasp* and *Vbb* are the two vipers most commonly encountered in France, and in this section we will discuss the difference between the venom of these two species potentially explaining the differences in symptoms following envenomation.



**Figure 5: Comparison of toxins families between *Vasp* and *Vbb* venom proteomes** [75]. NUC, 5'-nucleotidase; AP, amino-peptidase; BPP, Bradykinin potentiating peptides; C-NAP, C-type natriuretic peptide; CRISP, cysteine rich secretary protein; CTL, C-type lectin/snaclec; Dis, disintegrin; HYAL, hyaluronidase; LAOO, L-amino acid oxidase; NGF, nerve growth factor; PDE, phosphodiesterase; PLA2, phospholipase A2; PL-B, phospholipase B; PP, proactivator polypeptide; QC, glutaminyl-peptide cyclotransferase; RLAP, renin-like aspartic protease; SVMP, snake venom metalloprotease; SVMPi, snake venom metalloprotease inhibitor; SVSP, snake venom serine protease; VEGF, vascular endothelial growth factor.

*Vipera berus berus* (*Vbb*)is the most widely distributed viper in Europe and causes more cases of human snakebite than any of its congeners [76,77]. The principal local effect of *Vbb* bites are hemorrhage, edema, myonecrosis, bruising and pain [76,78,79], while hypotension is the most clinically important sign of systemic envenomation [4,80–82]. However, *Vbb* envenomings rarely cause systemic hemorrhage, coagulopathy or neurotoxicity [76,82]. While there are some similarities between these two venoms, the most striking differences between their toxin compositions are the lower abundances of CTLs, PLA2s and disintegrins in *Vbb*. Given the different methodologies employed (shotgun MS and a label-free approach in *Vasp* versus normalized volumes of corresponding SDS-PAGE protein bands and normalized areas of corresponding peaks eluting from the HPLC analysis of the venom in *Vbb*) some of these differences may simply arise from the intrinsic performances of each method, highlighting the limitations of such comparison. Yet, importantly, Latinović *et al.* previously showed that there is no ammodytoxin-like PLA2 in *Vbb* venom [42]. This differs from *Vasp* venom, which exhibits detectable levels of ammodytoxin B in both the venom proteome and venom gland transcriptome – these observations may well explain the distinctions between envenoming symptoms relating to neurotoxicity following bites by these two species [8,10]. In addition, the observed lower levels of CTL, PLA2 and disintegrins in *Vbb* compared to *Vasp*, may explain the low levels of systemic haemotoxicity observed after envenoming by *Vbb*. These toxin families are all known to interfere with platelet function, and thus often contribute to thrombocytopenia clinically [83].

CTLs are the major inducers of thrombocytopenia, which is clinically manifested as excessive platelet aggregation/agglutination [42], despite low level of C-type lectin in *Vbb* venom compared to *Vasp* venom, thrombocytopenia is relatively frequently in patients envenomed by *Vbb*, which may arise from the contributions of other toxins families (SVMPs, SVSPs and PLA2)[81]. Thus, CTL from *Vasp* venom along with others perturbing hemostasis toxins actively contribute to hemorrhage and coagulopathy observed inasp viper envenomations [3,8,10]. Other differences include the presence of BPPs in *Vbb* (absent in *Vasp*) and SVMPi in *Vasp* (absent in *Vbb*). However, we suggest that BPPs detected in Vbb were most likely actually SVMPis – explaining the apparent discrepancy. Indeed, SVMPis and BPPs originate from the same gene precursor, and true vipers like Vipera only have SVMPis and not BPPs, whereas Pit vipers can have both but mostly BPPs. In support of this hypothesis, we checked the original *Vbb* data and found that the BPP sequence identified (ZRRQPPIPP) closely resembles a motif found in a sequence of our Vasp transcriptome classified as SVMPi (>T1491 containing the QRRPPEIPP motif). Overall, these differences between *Vasp* and *Vbb* venoms may not have an impact on snakebite treatments as ViperFav is made simultaneously from both venoms (and *Vaa*). Moreover, the monospecific anti-*Vbb* antivenom ViperaTAb has been shown to cross-react extensively with *Vasp* venom and was also capable of preclinically protecting mice from venom-induced lethality [84]. Thus, it seems that despite the differences observed, there appears to be sufficient similarities in venom composition between these species for an antivenom to cross-neutralise the various toxic components. Finally, it should be noted that this comparison cannot be generalized as representative of inter-species differences since only one specimen was used.

**Conclusions**

Highly similar LC-MS profiles of *Vasp* venoms collected via three different venom extraction techniques reveal that the mode of venom extraction has no influence on venom composition. Transcriptomic analysis of the *Vasp* venom gland identified 80 toxin-annotated contigs that grouped in to 16 different gene families. Of these, a total of 64 proteins were confirmed by proteomic analysis as being present in *Vasp* venom. The elucidated venom composition is largely consistent with the symptoms of envenoming observed after snakebite by *Vasp*, with various haemotoxins, such as PIII SVMPs, CTLs and disintegrins, detected in considerable abundances, and the presence of an ammodytoxin B-like PLA2 may potentially be responsible for reports of neurotoxicity. Comparisons with *Vbb* revealed a reasonable degree of similarity in venom composition between the two congeners, although notable differences in abundance were observed for both CTL and disintegrin toxins. Overall, this proteotranscriptomic analysis of *Vasp* venom provides new insight into the venom composition of this species, which may ultimately help us to better understand the pathology of envenoming and the neutralizing capability of antivenom therapies used to treat snakebites by European vipers.

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**References**

[1] V. Jollivet, J.F. Hamel, L. de Haro, M. Labadie, J.M. Sapori, L. Cordier, A. Villa, P. Nisse, E. Puskarczyk, L. Berthelon, P. Harry, D. Boels, European viper envenomation recorded by French poison control centers: A clinical assessment and management study, Toxicon. 108 (2015) 97–103. https://doi.org/10.1016/j.toxicon.2015.09.039.

[2] F. Audebert, M. Sorkine, C. Bon, Envenoming by viper bites in France: clinical gradation and biological quantification by ELISA, Toxicon. 30 (1992) 599–609. https://doi.org/10.1016/0041-0101(92)90854-x.

[3] L. De Haro, M. Glaizal, L. Tichadou, I. Blanc-Brisset, M. Hayek-Lanthois, Asp Viper (Vipera aspis) Envenomation: Experience of the Marseille Poison Centre from 1996 to 2008, Toxins. 1 (2009) 100–112. https://doi.org/10.3390/toxins1020100.

[4] L. Calderón, B. Lomonte, J.M. Gutiérrez, A. Tarkowski, L.A. Hanson, Biological and biochemical activities of Vipera berus (European viper) venom, Toxicon. 31 (1993) 743–753. https://doi.org/10.1016/0041-0101(93)90380-2.

[5] M. Samel, J. Siigur, Isolation and characterization of hemorrhagic metalloproteinase from Vipera berus berus (common viper) venom, Comp. Biochem. Physiol. C. 97 (1990) 209–214. https://doi.org/10.1016/0742-8413(90)90129-w.

[6] Y. Komori, H. Sugihara, Purification and physiological study of a hypotensive factor from the venom of Vipera aspis aspis (aspic viper), Toxicon. 28 (1990) 359–369. https://doi.org/10.1016/0041-0101(90)90073-g.

[7] Y. Komori, T. Nikai, H. Sugihara, Isolation and characterization of procoagulant from the venom of Vipera aspis aspis, Int. J. Biochem. 25 (1993) 761–767. https://doi.org/10.1016/0020-711x(93)90363-j.

[8] L. de Haro, A. Robbe-Vincent, B. Saliou, M. Valli, C. Bon, V. Choumet, Unusual neurotoxic envenomations byVipera aspis aspis snakes in France, Hum. Exp. Toxicol. 21 (2002) 137–145. https://doi.org/10.1191/0960327102ht226oa.

[9] I. Mancheva, T. Kleinschmidt, B. Aleksiev, G. Braunitzer, Sequence homology between phospholipase and its inhibitor in snake venom. The primary structure of phospholipase A2 of vipoxin from the venom of the Bulgarian viper (Vipera ammodytes ammodytes, Serpentes), Biol. Chem. Hoppe. Seyler. 368 (1987) 343–352. https://doi.org/10.1515/bchm2.1984.365.2.885.

[10] V. Jan, R. c Maroun, A. Robbe-Vincent, L. De Haro, V. Choumet, Toxicity evolution of Vipera aspis aspis venom: identification and molecular modeling of a novel phospholipase A2 heterodimer neurotoxin 1, FEBS Lett. 527 (2002) 263–268. https://doi.org/10.1016/S0014-5793(02)03205-2.

[11] E. Ferquel, L. de Haro, V. Jan, I. Guillemin, S. Jourdain, A. Teynié, J. d’Alayer, V. Choumet, Reappraisal of Vipera aspis Venom Neurotoxicity, PLoS ONE. 2 (2007). https://doi.org/10.1371/journal.pone.0001194.

[12] G. Zanetti, E. Duregotti, C.A. Locatelli, A. Giampreti, D. Lonati, O. Rossetto, M. Pirazzini, Variability in venom composition of European viper subspecies limits the cross-effectiveness of antivenoms, Sci. Rep. 8 (2018) 9818. https://doi.org/10.1038/s41598-018-28135-0.

[13] T. Lamb, L. de Haro, D. Lonati, M. Brvar, M. Eddleston, Antivenom for European Vipera species envenoming, Clin. Toxicol. 55 (2017) 557–568. https://doi.org/10.1080/15563650.2017.1300261.

[14] M. Guiavarch, M. Médus, L. Tichadou, M. Glaizal, L. de Haro, Efficacité variable de l’antivenin Viperfav® pour traiter les envenimations vipérines avec neurotoxicité, Presse Médicale. 40 (2011) 654–656. https://doi.org/10.1016/j.lpm.2011.01.023.

[15] J.J. Calvete, B. Lomonte, A bright future for integrative venomics, Toxicon. 107 (2015) 159–162. https://doi.org/10.1016/j.toxicon.2015.10.024.

[16] D. Pla, D. Petras, A.J. Saviola, C.M. Modahl, L. Sanz, A. Pérez, E. Juárez, S. Frietze, P.C. Dorrestein, S.P. Mackessy, J.J. Calvete, Transcriptomics-guided bottom-up and top-down venomics of neonate and adult specimens of the arboreal rear-fanged Brown Treesnake, Boiga irregularis, from Guam, J. Proteomics. 174 (2018) 71–84. https://doi.org/10.1016/j.jprot.2017.12.020.

[17] J.J. Calvete, Venomics: integrative venom proteomics and beyond, Biochem. J. 474 (2017) 611–634. https://doi.org/10.1042/BCJ20160577.

[18] J.J. Calvete, L. Sanz, Y. Angulo, B. Lomonte, J.M. Gutiérrez, Venoms, venomics, antivenomics, FEBS Lett. 583 (2009) 1736–1743. https://doi.org/10.1016/j.febslet.2009.03.029.

[19] D. Pla, L. Sanz, G. Whiteley, S.C. Wagstaff, R.A. Harrison, N.R. Casewell, J.J. Calvete, What killed Karl Patterson Schmidt? Combined venom gland transcriptomic, venomic and antivenomic analysis of the South African green tree snake (the boomslang), Dispholidus typus, Biochim. Biophys. Acta BBA - Gen. Subj. 1861 (2017) 814–823. https://doi.org/10.1016/j.bbagen.2017.01.020.

[20] S. Ainsworth, D. Petras, M. Engmark, R.D. Süssmuth, G. Whiteley, L.-O. Albulescu, T.D. Kazandjian, S.C. Wagstaff, P. Rowley, W. Wüster, P.C. Dorrestein, A.S. Arias, J.M. Gutiérrez, R.A. Harrison, N.R. Casewell, J.J. Calvete, The medical threat of mamba envenoming in sub-Saharan Africa revealed by genus-wide analysis of venom composition, toxicity and antivenomics profiling of available antivenoms, J. Proteomics. 172 (2018) 173–189. https://doi.org/10.1016/j.jprot.2017.08.016.

[21] G. Whiteley, N.R. Casewell, D. Pla, S. Quesada-Bernat, R.A.E. Logan, F.M.S. Bolton, S.C. Wagstaff, J.M. Gutiérrez, J.J. Calvete, R.A. Harrison, Defining the pathogenic threat of envenoming by South African shield-nosed and coral snakes (genus Aspidelaps), and revealing the likely efficacy of available antivenom, J. Proteomics. 198 (2019) 186–198. https://doi.org/10.1016/j.jprot.2018.09.019.

[22] J. Archer, G. Whiteley, N.R. Casewell, R.A. Harrison, S.C. Wagstaff, VTBuilder: a tool for the assembly of multi isoform transcriptomes, BMC Bioinformatics. 15 (2014) 389. https://doi.org/10.1186/s12859-014-0389-8.

[23] A. Conesa, S. Gotz, J.M. Garcia-Gomez, J. Terol, M. Talon, M. Robles, Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research, Bioinformatics. 21 (2005) 3674–3676. https://doi.org/10.1093/bioinformatics/bti610.

[24] S. Kumar, G. Stecher, K. Tamura, MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets, Mol. Biol. Evol. 33 (2016) 1870–1874. https://doi.org/10.1093/molbev/msw054.

[25] E. Gasteiger, ExPASy: the proteomics server for in-depth protein knowledge and analysis, Nucleic Acids Res. 31 (2003) 3784–3788. https://doi.org/10.1093/nar/gkg563.

[26] R.C. Edgar, MUSCLE: a multiple sequence alignment method with reduced time and space complexity, BMC Bioinformatics. 5 (2004) 113. https://doi.org/10.1186/1471-2105-5-113.

[27] J. Zhang, L. Xin, B. Shan, W. Chen, M. Xie, D. Yuen, W. Zhang, Z. Zhang, G.A. Lajoie, B. Ma, PEAKS DB: De Novo sequencing assisted database search for sensitive and accurate peptide identification, Mol. Cell. Proteomics. (2011) mcp.M111.010587. https://doi.org/10.1074/mcp.M111.010587.

[28] B. Ma, K. Zhang, C. Hendrie, C. Liang, M. Li, A. Doherty‐Kirby, G. Lajoie, PEAKS: powerful software for peptide de novo sequencing by tandem mass spectrometry, Rapid Commun. Mass Spectrom. 17 (2003) 2337–2342. https://doi.org/10.1002/rcm.1196.

[29] F.G. Amorim, T.R. Costa, D. Baiwir, E. De Pauw, L. Quinton, S.V. Sampaio, Proteopeptidomic, Functional and Immunoreactivity Characterization of Bothrops moojeni Snake Venom: Influence of Snake Gender on Venom Composition, Toxins. 10 (2018) 177. https://doi.org/10.3390/toxins10050177.

[30] M. Degueldre, M. Verdenaud, G. Legarda, R. Minambres, S. Zuniga, M. Leblanc, N. Gilles, F. Ducancel, E. De Pauw, L. Quinton, Diversity in sequences, post-translational modifications and expected pharmacological activities of toxins from four Conus species revealed by the combination of cutting-edge proteomics, transcriptomics and bioinformatics, Toxicon. 130 (2017) 116–125. https://doi.org/10.1016/j.toxicon.2017.02.014.

[31] Y. Han, B. Ma, K. Zhang, SPIDER: software for protein identification from sequence tags with de novo sequencing error, J. Bioinform. Comput. Biol. 3 (2005) 697–716. https://doi.org/10.1142/s0219720005001247.

[32] B. Zybailov, A.L. Mosley, M.E. Sardiu, M.K. Coleman, L. Florens, M.P. Washburn, Statistical Analysis of Membrane Proteome Expression Changes in *Saccharomyces* *c* *erevisiae*, J. Proteome Res. 5 (2006) 2339–2347. https://doi.org/10.1021/pr060161n.

[33] A. Lochner, R.J. Giannone, M. Keller, G. Antranikian, D.E. Graham, R.L. Hettich, Label-free Quantitative Proteomics for the Extremely Thermophilic Bacterium *Caldicellulosiruptor obsidiansis* Reveal Distinct Abundance Patterns upon Growth on Cellobiose, Crystalline Cellulose, and Switchgrass, J. Proteome Res. 10 (2011) 5302–5314. https://doi.org/10.1021/pr200536j.

[34] M.R. Al Shweiki, S. Mönchgesang, P. Majovsky, D. Thieme, D. Trutschel, W. Hoehenwarter, Assessment of Label-Free Quantification in Discovery Proteomics and Impact of Technological Factors and Natural Variability of Protein Abundance, J. Proteome Res. 16 (2017) 1410–1424. https://doi.org/10.1021/acs.jproteome.6b00645.

[35] M.R. Hoopmann, J.M. Winget, L. Mendoza, R.L. Moritz, StPeter: Seamless Label-Free Quantification with the Trans-Proteomic Pipeline, J. Proteome Res. 17 (2018) 1314–1320. https://doi.org/10.1021/acs.jproteome.7b00786.

[36] D.A.P. Cidade, T.A. Simão, A.M.R. Dávila, G. Wagner, I. de L.M. Junqueira-de-Azevedo, P. Lee Ho, C. Bon, R.B. Zingali, R.M. Albano, Bothrops jararaca venom gland transcriptome: Analysis of the gene expression pattern, Toxicon. 48 (2006) 437–461. https://doi.org/10.1016/j.toxicon.2006.07.008.

[37] T. Sajevic, A. Leonardi, I. Križaj, An overview of hemostatically active components of *Vipera ammodytes ammodytes* venom, Toxin Rev. 33 (2014) 33–36. https://doi.org/10.3109/15569543.2013.835827.

[38] E.F. Sanchez, R.J. Flores-Ortiz, V.G. Alvarenga, J.A. Eble, Direct Fibrinolytic Snake Venom Metalloproteinases Affecting Hemostasis: Structural, Biochemical Features and Therapeutic Potential, Toxins. 9 (2017). https://doi.org/10.3390/toxins9120392.

[39] J.M. Gutiérrez, T. Escalante, A. Rucavado, C. Herrera, Hemorrhage Caused by Snake Venom Metalloproteinases: A Journey of Discovery and Understanding, Toxins. 8 (2016). https://doi.org/10.3390/toxins8040093.

[40] B. Fry, ed., Venomous reptiles and their toxins: evolution, pathophysiology, and biodiscovery, Oxford University Press, New York, NY, 2015.

[41] T. Sajevic, A. Leonardi, L. Kovačič, M. Lang-Balija, T. Kurtović, J. Pungerčar, B. Halassy, A. Trampuš-Bakija, I. Križaj, VaH3, one of the principal hemorrhagins in Vipera ammodytes ammodytes venom, is a homodimeric P-IIIc metalloproteinase, Biochimie. 95 (2013) 1158–1170. https://doi.org/10.1016/j.biochi.2013.01.003.

[42] Z. Latinović, A. Leonardi, J. Šribar, T. Sajevic, M.C. Žužek, R. Frangež, B. Halassy, A. Trampuš-Bakija, J. Pungerčar, I. Križaj, Venomics of Vipera berus berus to explain differences in pathology elicited by Vipera ammodytes ammodytes envenomation: Therapeutic implications, J. Proteomics. 146 (2016) 34–47. https://doi.org/10.1016/j.jprot.2016.06.020.

[43] S.M.T. Serrano, The long road of research on snake venom serine proteinases, Toxicon. 62 (2013) 19–26. https://doi.org/10.1016/j.toxicon.2012.09.003.

[44] K.J. Clemetson, Snaclecs (snake C-type lectins) that inhibit or activate platelets by binding to receptors, Toxicon. 56 (2010) 1236–1246. https://doi.org/10.1016/j.toxicon.2010.03.011.

[45] T. Ogawa, T. Chijiwa, N. Oda-Ueda, M. Ohno, Molecular diversity and accelerated evolution of C-type lectin-like proteins from snake venom, Toxicon. 45 (2005) 1–14. https://doi.org/10.1016/j.toxicon.2004.07.028.

[46] P.R. Crocker, J.C. Paulson, A. Varki, Siglecs and their roles in the immune system, Nat. Rev. Immunol. 7 (2007) 255–266. https://doi.org/10.1038/nri2056.

[47] L.M. van den Berg, S.I. Gringhuis, T.B.H. Geijtenbeek, An evolutionary perspective on C-type lectins in infection and immunity: C-type lectins in infection and immunity, Ann. N. Y. Acad. Sci. 1253 (2012) 149–158. https://doi.org/10.1111/j.1749-6632.2011.06392.x.

[48] J.J. Calvete, C. Marcinkiewicz, D. Monleón, V. Esteve, B. Celda, P. Juárez, L. Sanz, Snake venom disintegrins: evolution of structure and function, Toxicon. 45 (2005) 1063–1074. https://doi.org/10.1016/j.toxicon.2005.02.024.

[49] J.J. Calvete, M.P. Moreno-Murciano, R.D.G. Theakston, D.G. Kisiel, C. Marcinkiewicz, Snake venom disintegrins: novel dimeric disintegrins and structural diversification by disulphide bond engineering., Biochem. J. 372 (2003) 725–734. https://doi.org/10.1042/BJ20021739.

[50] J.J. Calvete, The continuing saga of snake venom disintegrins, Toxicon. 62 (2013) 40–49. https://doi.org/10.1016/j.toxicon.2012.09.005.

[51] D. Chakrabarty, C. Chanda, Snake Venom Disintegrins, in: P. Gopalakrishnakone, H. Inagaki, A.K. Mukherjee, T.R. Rahmy, C.-W. Vogel (Eds.), Snake Venoms, Springer Netherlands, Dordrecht, 2015: pp. 1–11. https://doi.org/10.1007/978-94-007-6648-8\_14-1.

[52] A. Gasmi, N. Srairi, S. Guermazi, H. Dkhil, H. Karoui, M. El Ayeb, Amino acid structure and characterization of a heterodimeric disintegrin from Vipera lebetina venom, Biochim. Biophys. Acta BBA - Protein Struct. Mol. Enzymol. 1547 (2001) 51–56. https://doi.org/10.1016/S0167-4838(01)00168-6.

[53] H.-S. Chen, Y.-M. Wang, W.-T. Huang, K.-F. Huang, I.-H. Tsai, Cloning, characterization and mutagenesis of Russell’s viper venom l-amino acid oxidase: Insights into its catalytic mechanism, Biochimie. 94 (2012) 335–344. https://doi.org/10.1016/j.biochi.2011.07.022.

[54] Y. Yamazaki, T. Morita, Structure and function of snake venom cysteine-rich secretory proteins, Toxicon. 44 (2004) 227–231. https://doi.org/10.1016/j.toxicon.2004.05.023.

[55] K. Sunagar, W.E. Johnson, S.J. O’Brien, V. Vasconcelos, A. Antunes, Evolution of CRISPs Associated with Toxicoferan-Reptilian Venom and Mammalian Reproduction, Mol. Biol. Evol. 29 (2012) 1807–1822. https://doi.org/10.1093/molbev/mss058.

[56] Ullah, Ullah, Ali, Betzel, ur Rehman, The Sequence and a Three-Dimensional Structural Analysis Reveal Substrate Specificity Among Snake Venom Phosphodiesterases, Toxins. 11 (2019) 625. https://doi.org/10.3390/toxins11110625.

[57] H. Schweitz, T. Bruhn, E. Guillemare, D. Moinier, J.M. Lancelin, L. Béress, M. Lazdunski, Kalicludines and kaliseptine. Two different classes of sea anemone toxins for voltage sensitive K+ channels, J. Biol. Chem. 270 (1995) 25121–25126. https://doi.org/10.1074/jbc.270.42.25121.

[58] C.Y. Dy, P. Buczek, J.S. Imperial, G. Bulaj, M.P. Horvath, Structure of conkunitzin-S1, a neurotoxin and Kunitz-fold disulfide variant from cone snail, Acta Crystallogr. D Biol. Crystallogr. 62 (2006) 980–990. https://doi.org/10.1107/S0907444906021123.

[59] S.D. Sasaki, S.S. Azzolini, I.Y. Hirata, R. Andreotti, A.S. Tanaka, Boophilus microplus tick larvae, a rich source of Kunitz type serine proteinase inhibitors, Biochimie. 86 (2004) 643–649. https://doi.org/10.1016/j.biochi.2004.09.010.

[60] D.J. Strydom, Protease inhibitors as snake venom toxins, Nature. New Biol. 243 (1973) 88–89.

[61] C.-H. Yuan, Q.-Y. He, K. Peng, J.-B. Diao, L.-P. Jiang, X. Tang, S.-P. Liang, Discovery of a Distinct Superfamily of Kunitz-Type Toxin (KTT) from Tarantulas, PLoS ONE. 3 (2008). https://doi.org/10.1371/journal.pone.0003414.

[62] K.T. Yee, M. Pitts, P. Tongyoo, P. Rojnuckarin, M.C. Wilkinson, Snake Venom Metalloproteinases and Their Peptide Inhibitors from Myanmar Russell’s Viper Venom, Toxins. 9 (2016). https://doi.org/10.3390/toxins9010015.

[63] S.C. Wagstaff, P. Favreau, O. Cheneval, G.D. Laing, M.C. Wilkinson, R.L. Miller, R. Stöcklin, R.A. Harrison, Molecular characterisation of endogenous snake venom metalloproteinase inhibitors, Biochem. Biophys. Res. Commun. 365 (2008) 650–656. https://doi.org/10.1016/j.bbrc.2007.11.027.

[64] R. Manjunatha Kini, Excitement ahead: structure, function and mechanism of snake venom phospholipase A2 enzymes, Toxicon. 42 (2003) 827–840. https://doi.org/10.1016/j.toxicon.2003.11.002.

[65] D. Georgieva, M. Risch, A. Kardas, F. Buck, M. von Bergen, C. Betzel, Comparative Analysis of the Venom Proteomes of *Vipera ammodytes ammodytes* and *Vipera ammodytes meridionalis*, J. Proteome Res. 7 (2008) 866–886. https://doi.org/10.1021/pr070376c.

[66] J. Pungercar, I. Krizaj, N.S. Liang, F. Gubensek, An aromatic, but not a basic, residue is involved in the toxicity of group-II phospholipase A2 neurotoxins., Biochem. J. 341 (1999) 139–145.

[67] I. Krizaj, N.-S. Liang, J. Pungercar, B. Strukelj, A. Ritonja, F. Gubensek, Amino acid and cDNA sequences of a neutral phospholipase A2 from the long-nosed viper (Vipera ammodytes ammodytes) venom, Eur. J. Biochem. 204 (1992) 1057–1062. https://doi.org/10.1111/j.1432-1033.1992.tb16728.x.

[68] Y. Yamazaki, K. Takani, H. Atoda, T. Morita, Snake Venom Vascular Endothelial Growth Factors (VEGFs) Exhibit Potent Activity through Their Specific Recognition of KDR (VEGF Receptor 2), J. Biol. Chem. 278 (2003) 51985–51988. https://doi.org/10.1074/jbc.C300454200.

[69] Y. Komori, T. Nikai, K. Taniguchi, K. Masuda, H. Sugihara, Vascular Endothelial Growth Factor VEGF-like Heparin-Binding Protein from the Venom of *Vipera aspis aspis* (Aspic Viper) †, Biochemistry. 38 (1999) 11796–11803. https://doi.org/10.1021/bi990562z.

[70] D. Sannaningaiah, G.K. Subbaiah, K. Kempaiah, Pharmacology of spider venom toxins, Toxin Rev. 33 (2014) 206–220. https://doi.org/10.3109/15569543.2014.954134.

[71] S. Tobassum, H.M. Tahir, M.T. Zahid, Q.A. Gardner, M.M. Ahsan, Effect of Milking Method, Diet, and Temperature on Venom Production in Scorpions, J. Insect Sci. 18 (2018). https://doi.org/10.1093/jisesa/iey081.

[72] S. Dutertre, A.-H. Jin, I. Vetter, B. Hamilton, K. Sunagar, V. Lavergne, V. Dutertre, B.G. Fry, A. Antunes, D.J. Venter, P.F. Alewood, R.J. Lewis, Evolution of separate predation- and defence-evoked venoms in carnivorous cone snails, Nat. Commun. 5 (2014). https://doi.org/10.1038/ncomms4521.

[73] S. Haider, R. Pal, Integrated Analysis of Transcriptomic and Proteomic Data, Curr. Genomics. 14 (2013) 91–110. https://doi.org/10.2174/1389202911314020003.

[74] N.R. Casewell, S.C. Wagstaff, W. Wuster, D.A.N. Cook, F.M.S. Bolton, S.I. King, D. Pla, L. Sanz, J.J. Calvete, R.A. Harrison, Medically important differences in snake venom composition are dictated by distinct postgenomic mechanisms, Proc. Natl. Acad. Sci. 111 (2014) 9205–9210. https://doi.org/10.1073/pnas.1405484111.

[75] R.I. Al-Shekhadat, K.S. Lopushanskaya, Á. Segura, J.M. Gutiérrez, J.J. Calvete, D. Pla, Vipera berus berus Venom from Russia: Venomics, Bioactivities and Preclinical Assessment of Microgen Antivenom, Toxins. 11 (2019) 90. https://doi.org/10.3390/toxins11020090.

[76] C.J. Reading, Incidence, pathology, and treatment of adder (Vipera berus L.) bites in man., Emerg. Med. J. 13 (1996) 346–351. https://doi.org/10.1136/emj.13.5.346.

[77] J.-P. Chippaux, Epidemiology of snakebites in Europe: A systematic review of the literature, Toxicon. 59 (2012) 86–99. https://doi.org/10.1016/j.toxicon.2011.10.008.

[78] J. Valenta, Z. Stach, M. Stříteský, P. Michálek, Common Viper Bites in the Czech Republic - Epidemiological and Clinical Aspects during 15 Year Period (1999–2013), Prague Med. Rep. 115 (2014) 120–127. https://doi.org/10.14712/23362936.2014.42.

[79] A. Garkowski, P. Czupryna, A. Zajkowska, S. Pancewicz, A. Moniuszko, M. Kondrusik, S. Grygorczuk, P. Gołębicki, M. Letmanowski, J. Zajkowska, Vipera berus bites in Eastern Poland – a retrospective analysis of 15 case studies, Ann. Agric. Environ. Med. 19 (2012) 6.

[80] T. Malina, L. Krecsak, D.A. Warrell, Neurotoxicity and hypertension following European adder (Vipera berus berus) bites in Hungary: case report and review, QJM. 101 (2008) 801–806. https://doi.org/10.1093/qjmed/hcn079.

[81] J. Magdalan, M. Trocha, A. Merwid-Ląd, T. Sozański, M. Zawadzki, Vipera berus Bites in the Region of Southwest Poland—A Clinical Analysis of 26 Cases, Wilderness Environ. Med. 21 (2010) 114–119. https://doi.org/10.1016/j.wem.2010.01.005.

[82] D.A. Warrell, Treatment of bites by adders and exotic venomous snakes, BMJ. 331 (2005) 1244–1247. https://doi.org/10.1136/bmj.331.7527.1244.

[83] J. Slagboom, J. Kool, R.A. Harrison, N.R. Casewell, Haemotoxic snake venoms: their functional activity, impact on snakebite victims and pharmaceutical promise, Br. J. Haematol. 177 (2017) 947–959. https://doi.org/10.1111/bjh.14591.

[84] N. Casewell, I. Al-Abdulla, D. Smith, R. Coxon, J. Landon, Immunological Cross-Reactivity and Neutralisation of European Viper Venoms with the Monospecific Vipera berus Antivenom ViperaTAb, Toxins. 6 (2014) 2471–2482. https://doi.org/10.3390/toxins6082471.