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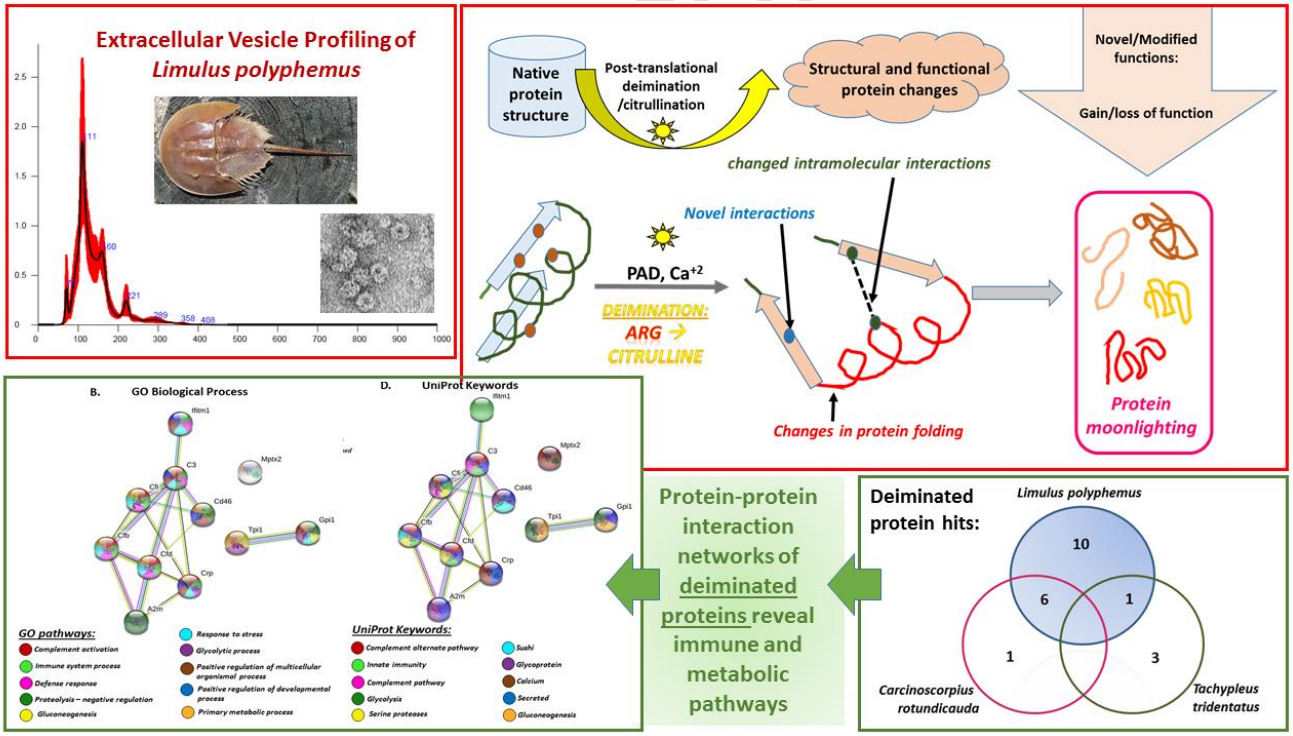
Graphic Abstract



Abstract

The horseshoe crab is a living fossil and a species of marine arthropod with unusual immune system properties which are also exploited commercially. Given its ancient status dating to the Ordovician period (450 million years ago), its standing in phylogeny and unusual immunological characteristics, the horseshoe crab may hold valuable information for comparative immunology studies.

Peptidylarginine deiminases (PADs) are calcium dependent enzymes that are phylogenetically conserved and cause protein deimination via conversion of arginine to citrulline. This post-translational modification can lead to structural and functional protein changes contributing to protein moonlighting in health and disease. PAD-mediated regulation of extracellular vesicle (EV) release, a critical component of cellular communication, has furthermore been identified to be a phylogenetically conserved mechanism. PADs, protein deimination and EVs have hitherto not been studied in the horseshoe crab and were assessed in the current study. Horseshoe crab haemolymph serum-EVs were found to be a poly-dispersed population in the 20-400 nm size range, with the majority of EVs falling within 40-123 nm. Key immune proteins were identified to be post-translationally deiminated in horseshoe crab haemolymph serum, providing insights into protein moonlighting function of *Limulus* and phylogenetically conserved immune proteins. KEGG (Kyoto encyclopaedia of genes and genomes) and GO (gene ontology) enrichment analysis of deiminated proteins identified in *Limulus* revealed KEGG pathways relating to complement and coagulation pathways, *Staphylococcus aureus* infection, glycolysis/gluconeogenesis and carbon metabolism, while GO pathways of biological and molecular pathways related to a range of immune and metabolic functions, as well as developmental processes. The characterisation of EVs, and post-translational deimination signatures, revealed here in horseshoe crab, contributes to current understanding of protein moonlighting functions and EV-mediated communication in this ancient arthropod and throughout phylogeny.

Key words: Peptidylarginine deiminases (PADs); protein deimination; Horseshoe crab (*Limulus polyphemus*); extracellular vesicles (EVs); innate immunity; CRP; complement.

1. Introduction

The Atlantic horseshoe crab (*Limulus polyphemus*) belongs to the group Merostomata under the phylum Arthropoda. Merostomata consist of the now extinct sea scorpions (Eurypterida) and the horseshoe crabs (Xiphosura). Horseshoe crabs are considered living fossils and have four extant (living) species: *Limulus polyphemus*, the Atlantic horseshoe crab, which resides along the eastern coast of North America and the Gulf of Mexico, and three species in the Indo-Pacific region: *Tachypleus gigas* (southern horseshoe crab), *Tachypleus tridentatus* (tri-spine horseshoe crab), and *Carcinoscorpius rotundicauda* (mangrove horseshoe crab). These all display similar morphology, also with relation to a Jurassic fossil specimen (Xia, 2000).

Along the US Atlantic coast, the Atlantic horseshoe crab is of importance for the ecosystem due to its foraging habits and its eggs provide amongst other valuable nutrition for migrating birds (Berkson and Shuster, 1999). Horseshoe crab blood is highly valuable for its specific ability to identify Gram-negative bacteria via their endotoxin, a process which rapidly induces coagulation of *Limulus* amebocyte lysate (Levin and Bang 1964 and 1968; Kawabata, 2010). This ability has been utilised widely in the medical field (Pierrakakis et al., 1990; Novitsky, 1994) for the detection of bacterial endotoxin in quality control of pharmaceuticals and drugs (Liu et al., 1994). *Limulus* is also a valuable model for research in neurobiology, including visual physiology (Hartline et al., 1956; Barlow, 1983; Watson et al., 2008; Battelle, 2016; Battelle et al., 2016) and circadian rhythm (Chabot et al., 2004; Chabot and Watson, 2010; Chabot et al., 2016; Chesmore et al., 2016). The genome and transcriptome of the Atlantic horseshoe crab has recently been drafted with particular relation to the circadian clock (Simpson et al., 2017). Due to the unique position of the horseshoe crab in phylogeny, and its unusual immunological and physiological characteristics, it is considered a valuable model organism holding information for molecular pathways underlying such traits.

Peptidylarginine deiminases (PADs) are a group of calcium-dependent enzymes, which are phylogenetically conserved and have been described in a range of taxa. While a group of 5 isozymes are described in mammals, 3 are known in chicken and alligator, one in fish (bony and cartilaginous fish) (Vossenaar et al., 2003; Rebl et al., 2010; Magnadottir 2018a, Magnadottir et al., 2019a; Criscitiello et al., 2019; Criscitiello et al., 2020a,b), and PAD homologues (arginine deiminases, ADI; Novák et al., 2016) have been identified in parasites (Gavinho et al., 2019), fungi (El-Sayed et al., 2019) and bacteria (Bielecka et al., 2014; Kosgodage et al., 2019). PADs or PAD homologues arginine deiminases have hitherto not been studied in Merostomata.

As PADs post-translationally and irreversibly convert arginine into citrulline in a number of cytoplasmic, mitochondrial and nuclear target proteins, this can cause structural changes in these

proteins and affect their function (Vossenaar, 2003; György et al., 2006; Alghamdi et al., 2019). This leads to changes in protein function, downstream protein-protein interactions as well as effects on gene regulation and contribution to neo-epitope generation, resulting inflammatory responses (Bicker and Thompson, 2013; Wang and Wang, 2013; Witalison et al., 2015; Yang et al., 2016; Lange et al., 2017; Mondal and Thompson, 2019). Such post-translational changes in proteins may furthermore facilitate moonlighting abilities of proteins, an evolutionary acquired phenomenon where one protein can display several physiologically relevant functions within one polypeptide chain (Henderson and Martin, 2014; Jeffrey, 2018). Interestingly, proteins of higher disorder have been found to be more prone to deimination and the position of the arginine has also been found to be of importance (György et al., 2006; Tarsca et al., 1996). While the bulk of research on PADs and associated post-translational deimination and downstream effects has hitherto focussed on human pathologies, roles in normal physiology are receiving increasing attention – including in a range of taxa throughout the phylogenetic tree (Magnadottir et al., 2018a, 2019a,b, 2020a,b,c; Phillips et al., 2020; Pamenter et al., 2019; Criscitiello et al., 2019,2020a,b). Hitherto, no studies have been carried out on PAD/ADI protein function or physiological relevance for PAD/ADI-mediated post-translational deimination in Merostomata.

In relation to pathological responses, PADs and associated protein deimination are recognized as crucial players in cancer, inflammatory, autoimmune, and neurodegenerative diseases (Mohanan et al., 2012; Wang and Wang, 2013; Witalison et al., 2015; Lange et al., 2017; Uysal-Onganer et al, 2020; Darrah and Andrade, 2018; Tilvawala et al., 2018; Ruiz-Romero et al., 2019; Fert-Bober et al., 2020; Martinez-Prat et al., 2019; Svärd et al., 2019; Mastronardi et al., 2006; Moscarello et al., 2013; Wei et al., 2013; Yang et al., 2016; Faigle et al., 2019; Méchin et al., 2020;) as well as in relation to ageing (Ding et al., 2017; Wong and Wagner, 2018). There is also a considerable interest in roles for PADs in tissue regeneration, including in the CNS and in response to hypoxia (Lange et al., 2011; Lange et al., 2014; Lange, 2016; Sase et al., 2017; Yu et al., 2018), as well as in wound healing (Wong et al., 2015; Fadini et al., 2016). In addition, PADs play important roles in infection, including sepsis and endotoxemia (Pan et al., 2017; Biron et al., 2018; Claushuis et al., 2018; Costa et al., 2018; Liang et al., 2018; Muraro et al., 2018; Stobernack et al., 2018; Saha et al., 2019), as well as in other anti-pathogenic, including anti-viral, responses (Muraro et al., 2018; Casanova et al., 2020). Roles for PADs in mucosal, innate and adaptive immunity have also recently been studied in a range of taxa from bacteria to mammals (Kosgodage et al., 2019; Lange et al., 2019; Magnadottir et al., 2018a and 2018b, 2019a; 2020a,b,c; Criscitiello et al., 2019, 2020a,b,c; Pamenter et al., 2019; Phillips et al., 2020).

One phylogenetically conserved function identified for PADs is the regulation of extracellular vesicle (EV) release (Kholia et al., 2015; Kosgodage et al., 2017, 2018, 2019). EVs are present in, and can be isolated from, most body fluids where they participate in cellular communication in health and disease via transfer of cargo proteins and genetic material (Inal et al., 2013; Colombo et al., 2014; Lange et al., 2017; Turchinovich et al., 2019; Vagner et al., 2019). EV cargo signatures, holding information from their cells of origin, can be utilised as biomarkers and are readily isolated from both serum and plasma (Hessvik and Llorente, 2018; Ramirez et al., 2018). Little is known about EVs in Merostomata and EVs have not been characterised in horseshoe crab before. Overall, work on EVs has largely focussed on human pathologies, while recently an increasing body of comparative studies in a range of taxa has emerged with respect to EVs and EV cargo, including from our group (Iliev et al., 2018; Gatien et al., 2019; Montaner-Tarbeset al., 2019; Šimundić et al., 2019; Magnadottir et al., 2019b, 2020a, 2020b,c; Criscitello et al., 2019 and 2020a and 2020b; Lange et al., 2019; Pamenter et al., 2019).

Horseshoe crab haemolymph is well known for its exceptional anti-microbial activity, while roles for post-translational modifications in relation to their immunity and physiology have received less attention. In the light of our ongoing studies on deimination signatures and EV characterisation throughout phylogeny, including in animals with unusual immune and metabolic functions, and due to the horseshoe crab's unusual position in the phylogenetic tree, a study on these parameters in *Limulus* was warranted.

In the current study post-translational deiminated protein signatures were assessed in haemolymph serum of the Atlantic horseshoe crab (*L. polyphemus*), and furthermore, EVs were isolated and characterised by nanoparticle tracking analysis, western blotting and transmission electron microscopy. This is the first report of post-translational deimination in *Limulus*, indicating some key immune proteins and reporting haemolymph serum EV signatures. Our findings provide novel insights into the unusual immunological traits of the horseshoe crab and the adaption of immune functions throughout phylogeny, through post-translational modifications of phylogenetically conserved proteins and of pathways underlying anti-pathogenic responses.

2. Materials and Methods

2.1 Serum Sampling from Horseshoe crab

The horseshoe crabs were housed in a 4x15' tank in ~2ft of water with sand bottom, with flow through seawater. *Limulus* were held on temperature treated loops at 10 °C, blood (haemolymph) was collected from three healthy horseshoe crabs (*L. polyphemus*), ~20cm carapace width and ~35cm length (generously donated by Marine Biological Laboratory, Woods Hole, MA, USA). Procedures for blood-collection and processing were according to previously described protocols (Armstrong and Conrad, 2008). Following collection, the haemolymph was kept on ice for 24 h and then frozen at -80 °C until used for further individual experiments.

2.2 Isolation of Extracellular Vesicles and Nanoparticle Tracking Analysis (NTA)

Limulus haemolymph serum-EVs were prepared from haemolymph serum of individual horseshoe crabs (n=3), using sequential centrifugation and ultracentrifugation according to previously described protocols (Kosgodage et al., 2018; Criscitiello et al., 2019; Pamenter et al., 2019; Phillips et al., 2020; Criscitiello et al., 2020b) and according to the recommendations of MISEV2018 (the minimal information for studies of extracellular vesicles 2018; Théry et al., 2018). For each individual serum-EV preparation, 100 µl of horseshoe crab serum were diluted 1:5 in Dulbecco's PBS (DPBS, ultrafiltered using a 0.22 µm filter, before use) before centrifugation at 4,000 *g* for 30 min at 4 °C, for removal of aggregates and apoptotic bodies. Thereafter the supernatants were collected and centrifuged again using ultracentrifugation at 100,000 *g* for 1 h at 4 °C. The resulting EV-enriched pellets were resuspended in 500 µl DPBS and ultracentrifuged again at 100,000 *g* for 1 h at 4 °C. The final washed EV pellets were then resuspended in 100 µl DPBS and frozen at -80 °C until further use. For the generation of serum-EV size distribution profiles and for quantification of serum-EVs, NTA analysis based on particle size assessment by Brownian motion was carried out using the NanoSight NS300 system (Malvern, U.K.). The EV samples were diluted 1/100 in DPBS (10 µl of EV preparation diluted in 990 µl of DPBS) and applied to the NanoSight NS300 using a syringe pump to ensure continuous flow of the sample. Five repetitive reads, each lasting 60 sec, were recorded for each sample. The number of particles per frame was kept in-between 40 to 60, samples were recorded at camera level 12 and post-analysis was carried out at threshold 3. Replicate histograms were generated from these videos using the NanoSight software 3.0 (Malvern), representing mean and confidence intervals of the 5 recordings for each sample.

2.3 Transmission Electron Microscopy (TEM)

A pool of EVs, isolated from serum of the three individual animals as described above, was used for morphological analysis using TEM according to previously described protocols (Criscitiello et al., 2020b; Phillips et al., 2020). Following isolation, the EVs were resuspended in 100 mM sodium cacodylate buffer (pH 7.4) and a drop (~3-5 µl) of the suspension was placed onto a grid with previously glow discharged carbon support film. After the suspension had partly dried, the EVs were fixed by placing the grid onto a drop of a fixative solution (2.5 % glutaraldehyde in 100 mM sodium cacodylate buffer (pH 7.0)) for 1 min at room temperature and washed afterwards by touching the grid to the surface of three drops of distilled water. Excess water was removed by touching the grid to a filter paper. Next, the EVs were stained with 2 % aqueous Uranyl Acetate (Sigma-Aldrich) for 1 min, the excess stain was removed by touching the grid edge to a filter paper and the grid was allowed to air dry. Imaging of EVs was performed using a JEOL JEM 1400 transmission electron microscope (JEOL, Tokyo, Japan) operated at 80 kV at a magnification of 30,000x to 60,000x. Digital images were recorded using an AMT XR60 CCD camera (Deben, UK).

2.4 Isolation of Deiminated Proteins using F95-enrichment

Immunoprecipitation and isolation of deiminated proteins in serum was carried out using the Catch and Release®v2.0 immunoprecipitation kit (Merck, U.K.) in conjunction with the F95 pan-deimination antibody (MABN328, Merck), which has been developed against a deca-citrullinated peptide and specifically detects proteins modified by citrullination (Nicholas and Whitaker, 2002). Horseshoe crab serum pools of three individual animals (3 x 50 µl) were used for F95-enrichment. Immunoprecipitation (F95-enrichment) was carried out overnight at 4 °C on a rotating platform. The F95 bound proteins were thereafter eluted using denaturing elution buffer (Merck), according to the manufacturer's instructions (Merck), diluted 1:1 in 2xLaemmli sample buffer (BioRad, UK) and kept frozen at -20 °C until further analysis by SDS-PAGE, followed by silver staining or western blotting, or by in-gel digestion followed by LC-MS/MS analysis.

2.5 Silver Staining

F95-enriched protein eluates from horseshoe crab serum were silver stained following SDS-PAGE (using 4–20 % gradient TGX gels, BioRad, U.K.) under reducing conditions, using the BioRad Silver Stain Plus Kit (1610449, BioRad, U.K.), according to the manufacturer's instructions (BioRad).

2.6 Western Blotting Analysis

Horseshoe crab sera and serum-EVs were diluted 1:1 in denaturing 2x Laemmli sample buffer (containing 5 % beta-mercaptoethanol, BioRad, U.K.) and heated for 5 min at 100 °C. The proteins

were separated by SDS-PAGE using 4-20 % gradient TGX gels (BioRad U.K.). Western blotting was carried out using the Trans-Blot® SD semi-dry transfer cell (BioRad, U.K.); even transfer was assessed by staining the membranes with PonceauS (Sigma, U.K.). Blocking was performed for 1 h at room temperature using 5 % bovine serum albumin (BSA, Sigma, U.K.), in Tris buffered saline (TBS) containing 0.1 % Tween20 (BioRad, U.K.; TBS-T). Following blocking, the membranes were incubated overnight at 4 °C on a shaking platform with the primary antibodies, which were diluted in TBS-T. For detection of deiminated/citrullinated proteins, the F95 pan-deimination antibody was used (MABN328, Merck; diluted 1/1000). For detection of *Limulus* PAD protein homologue, the anti-human PAD2 antibody was used (anti-PAD2, ab50257, Abcam; diluted 1/1000); as this is the phylogenetically most conserved PAD isozyme and has previously been shown to cross-react with PAD homologues in a range of taxa (Lange et al., 2011; Lange et al., 2014; Magnadottir et al., 2018a and 2019a; Criscitiello et al., 2019 and 2020a,b; Pameneter et al., 2019; Phillips et al., 2020; Magnadottir et al., 2020). *Limulus* serum-EV isolates were blotted against two phylogenetically conserved EV-specific markers: CD63 (ab216130, Abcam, U.K.; diluted 1/1000) and Flotillin-1 (Flot-1, ab41927; diluted 1/2000), for further characterisation of EVs. After primary antibody incubation the membranes were washed for 3 x 10 min in TBS-T at RT and incubated for 1 h, at RT with HRP-conjugated secondary antibodies (anti-rabbit IgG (BioRad) or anti-mouse IgM (BioRad) respectively, diluted 1/3000 in TBS-T). The membranes were then washed in TBS-T for five times 10 min and positive proteins bands were visualised digitally, using enhanced chemiluminescence (ECL, Amersham, U.K.) and the UVP BioDoc-ITTM System (Thermo Fisher Scientific, U.K.).

2.7 Liquid Chromatography with Tandem Mass Spectrometry (LC-MS/MS) Analysis of Deiminated Protein Candidates

The F95-enriched eluate from a pool (n=3) of *Limulus* sera was analysed by liquid chromatography with tandem mass spectrometry (LC-MS/MS) according to previously described methods (Phillips et al., 2020; Criscitiello et al., 2020b). In preparation for LC-MS/MS analysis, the F95-enriched eluates were first run 0.5 cm into a 12 % TGX gel (BioRad, U.K.), the band cut out, and then trypsin digested and subjected to proteomic analysis using a Dionex Ultimate 3000 RSLC nanoUPLC (Thermo Fisher Scientific Inc, Waltham, MA, U.S.A.) system and a QExactive Orbitrap mass spectrometer (Thermo Fisher Scientific Inc, Waltham, MA, U.S.A.). Separation of peptides was performed by reverse-phase chromatography at a flow rate of 300 nL/min and a Thermo Scientific reverse-phase nano Easy-spray column (Thermo Scientific PepMap C18, 2 µm particle size, 100A pore size, 75 µm i.d. x 50 cm length). Peptides were loaded onto a pre-column (Thermo Scientific PepMap 100 C18, 5 µm particle size, 100A pore size, 300 µm i.d. x 5 mm length) from the Ultimate 3000 autosampler with 0.1 %

formic acid for 3 minutes at a flow rate of 10 µL/min. After this period, the column valve was switched to allow elution of peptides from the pre-column onto the analytical column. Solvent A was water + 0.1 % formic acid and solvent B was 80 % acetonitrile, 20 % water + 0.1 % formic acid. The linear gradient employed was 2-40 % B in 30 minutes. The LC eluant was sprayed into the mass spectrometer by means of an Easy-Spray source (Thermo Fisher Scientific Inc.). All m/z values of eluting ions were measured in an Orbitrap mass analyzer, set at a resolution of 70000 and was scanned between m/z 380-1500. Data dependent scans (Top 20) were employed to automatically isolate and generate fragment ions by higher energy collisional dissociation (HCD, NCE:25 %) in the HCD collision cell and measurement of the resulting fragment ions was performed in the Orbitrap analyser, set at a resolution of 17500. Singly charged ions and ions with unassigned charge states were excluded from being selected for MS/MS and a dynamic exclusion window of 20 seconds was employed. Post-run, the data was processed using Protein Discoverer (version 2.1., Thermo Scientific) and all MS/MS data were converted to mgf files. For identification of deiminated protein hits, the files were then submitted to the Mascot search algorithm (Matrix Science, London, U.K.) and searched against the UniProt database common for Merostomata (Merostomata_ 20170607 database; 24148 sequences; 40594 residues), identifying hits with Atlantic horseshoe crab (*L. polyphemus*), Mangrove horseshoe crab (*Carcinoscorpius rotundicauda*) and tri-spine horseshoe crab (*Tachypleus tridentatus*). A search was also conducted against a common contaminant database, containing sequences 123 sequences and 40594 residues (cRAP 20190401). The peptide and fragment mass tolerances were set to 20 ppm and 0.1 Da, respectively. A significance threshold value of $p < 0.05$ and a peptide cut-off score of 16 were also applied (carried out by Cambridge Proteomics, Cambridge, U.K.).

2.8 FoldIndex© Analysis of Deiminated Proteins in Horseshoe crab serum

Deiminated protein hits were assessed for disordered regions (as disordered proteins are more susceptible to deimination) using FoldIndex© analysis (Uversky et al., 2000; Prilusky et al., 2005; https://fold.weizmann.ac.il/fldbin/findex). This was used to identify numbers and lengths of disordered regions in the deimination protein hits, as well as the number of arginines present in the uniprot sequences identified in *Limulus* and with other Merostomata.

2.9 Protein-protein interaction Network Analysis

For the identification and prediction of putative protein-protein interaction networks for deiminated proteins identified in *Limulus* serum, STRING analysis (Search Tool for the Retrieval of Interacting Genes/Proteins; https://string-db.org/) was carried out. The protein networks were built based on

the protein IDs and using the function of "search multiple proteins" in STRING (https://string-db.org/), choosing "*Mus musculus*" as the species database, as no protein database is available for *Limulus* or other Merostomata in STRING. For protein interactions, "basic settings" and "medium confidence" were applied in STRING, with colour lines between nodes indicating evidence-based interactions for network edges as follows: "known interactions" (based on curated databases, experimentally determined), "predicted interactions" (based on gene neighbourhood, gene fusion, gene co-occurrence or via text mining, co-expression or protein homology). Identified KEGG (Kyoto Encyclopaedia of Genes and Genomes) and gene ontology (GO) pathways were highlighted in the identified protein networks for deiminated proteins (see colour code for nodes and connective lines included in the figures).

2.10 Statistical Analysis

NTA curves were generated using the Nanosight 3.0 software (Malvern, U.K.) NTA curves represent mean and standard error of mean (SEM), indicated by confidence intervals. Histograms were generated using GraphPad Prism version 7, error bars show standard deviation (SD). STRING analysis (https://string-db.org/) was used for prediction of protein-protein interaction networks using basic settings and medium confidence. Significance was considered as $p \leq 0.05$.

3. Results

3.1 Characterisation of Horseshoe Crab Serum-EVs

Haemolymph serum-EVs were assessed by NTA for particle numbers and size distribution using the NanoSight NS300, revealing a poly-dispersed population of EVs in the size range of 20-400 nm, with main peaks at approximately 40, 70, 110, 160 and 220 nm and the majority of EVs in the range of 40-123 nm (Figure 1A). EVs were also assessed for two phylogenetically conserved EV-specific markers by western blotting using anti-CD63 and anti-Flot-1 antibodies, with CD63 showing a strong positive reaction, while Flot-1 showed very low positive detectable response (Figure 1B). Morphological characterisation was carried out by transmission electron microscopy (TEM), confirming typical EV morphology, including "cup-shaped" EVs (see composite EV figures in Figure 1C). Some variation was observed between the three individuals with respect to EV yield from haemolymph serum (6.34 x 10^9, 6.75 x 10^9 and 9.21 x 10^9, respectively) and modal EV size, which fell in the range of 110.4-122.6 nm.

3.2 PAD Protein Homologue and Deiminated Proteins in Horseshoe Crab Serum

For assessment of a horseshoe crab PAD protein homologue, anti-human PAD2 specific antibodies were used in western blotting, identifying a positive protein band at the expected approximate 70 kDa size, similar to mammalian PAD, in horseshoe crab haemolymph serum (Figure 2A). For assessment of total deiminated proteins present in haemolymph serum, SDS-PAGE followed by western blotting showed a prominent band between 50-75 kDa (Figure 2B), while silverstaining of fractions following immunoprecipitation with the F95 antibody revealed F95-enriched protein bands between 15-150 kDa also with the most prominent band at a similar size as seen in western blotting (Figure 2C).

3.3 LC-MS/MS Analysis of Deiminated Proteins in Horseshoe crab Serum

Protein identification of deiminated proteins in horseshoe crab serum was carried out following F95-enrichment using LC-MS/MS analysis. Species-specific protein hits with *L. polyphemus*, as well as hits with other Merostomata were identified using the UniProt Merostomata database (Table 1; see Supplementary Table S1 for full details on all peptide hits). Overall, 17 species-specific *L. polyphemus* deiminated protein hits were identified. Further 7 hits were identified for *C. rotundicauda*, whereof 6 were in common with *L. polyphemus*, but one specific to *C. rotundicauda* (galactose-binding protein). Hits identified for *T. tridentatus* indicated pentaxin in common with *L. polyphemus*, while 3 were specific *to T. tridentatus* (complement component 3, plasma carcinolectin CL5B1 and tachylectin-P) (Figure 3 and Table 1; see Supplementary Table S1 for full details on peptide hits).

Table 1. Deiminated proteins in serum of horseshoe crab (*Limulus polyphemus*), as identified by F95-enrichment and LC-MS/MS analysis. Deiminated proteins were isolated from horseshoe crab sera (n=3) by immunoprecipitation using the pan-deimination F95 antibody. The resulting F95-enriched eluate was then analysed by LC-MS/MS and peak list files submitted to mascot, using a common Merostomata database. Both *Limulus polyphemus* species-specific peptide sequence hits, as well as hits with other Merostomata are listed, showing number of sequences for protein hits and total score. A full list of protein sequence hits and peptides is further provided in Supplementary Table S1.

Protein name	Symbol	Sequences (Matches)	Total score (p<0.05)[‡]
	Atlantic horseshoe crab (*Limulus polyphemus)*		
Hemocyanin subunit IV	A2AX58_LIMPO	65 (1011)	4816
Hemocyanin subunit II	A2AX56_LIMPO	62 (636)	4492
Hemocyanin subunit IIIa	A2AX57_LIMPO	64 (827)	4250
Hemocyanin subunit IIIb	G8YZR0_LIMPO	55 (886)	4151
Hemocyanin subunit VI	A2AX59_LIMPO	57 (672)	4066
C-reactive protein 1.1	CRP1_LIMPO	11 (135)	767
C-reactive protein 1.4	CRP4_LIMPO	9 (80)	641
Limulin	LIMU_LIMPO	5 (64)	335
Hemocyanin subunit I	Q7M4H2_LIMPO	3 (66)	277
Endotoxin-binding protein-protease inhibitor	Q25387_LIMPO	2(4)	123
Coagulogen	COAG_LIMPO	2(2)	106
Pentaxin	Q8WQK3_LIMPO	2(2)	85
Alpha-2-macroglobulin	Q7M430_LIMPO	1(1)	78
Hemocyanin subunit V	Q7M490_LIMPO	1(10)	38
Hemagglutinin/amebocyte aggregation factor	HAAF_LIMPO	1(2)	35
Putative integrin-linked protein kinase	A9XXT7_LIMPO	1(1)	21
Glucose-6-phosphate isomerase	A9Y038_LIMPO	1(1)	20
	Mangrove horseshoe crab (*Carcinoscorpius rotundicauda*):		
Hemocyanin subunit IV	A1X1V5_CARRO	32 (424)	1918
Hemocyanin subunit IIIb	A1X1V4_CARRO	25 (313)	1436
Hemocyanin subunit IIIa	A1X1V3_CARRO	24 (272)	1393
Hemocyanin subunit II	A1X1V2_CARRO	19 (204)	1292
Hemocyanin subunit V	A1X1V6_CARRO	18 (221)	1125
Hemocyanin subunit I	A1X1V1_CARRO	14 (286)	864
Galactose-binding protein	Q2TS30_CARRO	3 (4)	232
	Tri-spine horseshoe crab (*Tachypleus tridentatus*):		
Complement component 3	B6ZH52_TACTR	6 (6)	267
Pentaxin	Q9U8Z6_TACTR	3 (49)	191
Plasma carcinolectin CL5B1	A1KYQ1_CARRO	3 (5)	134
Tachylectin-P	Q9U5E9_TACTR	1 (1)	36

[‡]Ions score is -10*Log(P), where P is the probability that the observed match is a random event. Individual ions scores > 16 indicated identity or extensive homology (p < 0.05). Protein scores were derived from ions scores as a non-probabilistic basis for ranking protein hits.

3.4 Protein-protein Interaction Network Identification of Deiminated Proteins in Horseshoe Crab Serum

For the prediction of protein-protein interaction networks of the deimination candidate proteins identified in *Limulus*, the protein IDs were submitted to STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) analysis (https://string-db.org/) (Figure 4). Protein interaction networks were based on known and predicted interactions and represent all deiminated proteins identified in *Limulus* haemolymph serum and their interaction partners present in the STRING database, based on networks for mouse (*Mus musculus*), as protein identifiers for *Limulus* were not available in STRING (Figure 4). The PPI enrichment *p*-value (based on protein name as networks had to be built on mouse

homologous proteins) was found to be $p = 0.00333$, indicating more interactions than expected for a random set of proteins of similar size, drawn from the genome.

KEGG pathways for the deiminated serum proteins identified related to "complement and coagulation pathways", "*Staphylococcus aureus* infection", "glycolysis/gluconeogenesis" and "carbon metabolism" (Figure 4A), while GO biological pathways identified included "regulation of complement activation", "immune system processes", "defence response", "negative regulation of proteolysis", "gluconeogenesis", "response to stress", "glycolytic process", "positive regulation of multicellular organismal process", "positive regulation of developmental process" (Figure 4B).

GO molecular pathways identified included "oxidoreductase activity", "serine-type endopeptidase activity", "complement binding", "endopeptidase inhibitor binding", "signalling receptor binding", "ubiquitin protein ligase binding" (Figure 4C).

UniProt keywords identified in the protein networks for deiminated proteins included "complement alternate pathway", "secreted", "innate immunity", "gluconeogenesis", "serine protease", "complement pathway", "glycolysis", "sushi", "glycoprotein", "calcium" (Figure 4D).

Reactome pathways identified in the protein networks for deiminated proteins included "complement cascade", including regulation and alternative pathway activation, "Immune system", "Innate immune system", "gluconeogenesis", "glycolysis", "immunoregulatory interactions", "neutrophil degranulation", "platelet degranulation" (Figure 4E).

SMART protein domains identified in the protein networks for deiminated proteins included "alpha-macroglobulin receptor", "alpha-2-macroglobulin family", "pentraxin/CRP family", "trypsin-like serine protease", "domain abundant in complement control proteins" (Figure 4F).

PFAM protein domains identified in the protein networks for deiminated proteins included "alpha-macroglobulin tiolester bond-forming region", "alpha-macroglobulin complement component", "MG2 domain", "pentaxin family", "alpha2-macroglobulin family", "trypsin", "sushi repeat" (Figure 4G).

INTERPRO protein domains identified in the protein networks for deiminated proteins included "serine protease, trypsin family", "alpha-2-macroglobulin conserved site", "Peptidase", "pentraxin-related", "sushi/SCR/CCP superfamily", "concanavalin-A like lectin/glucanase domain superfamily" (Figure 4H).

3.5 FoldIndex© Analysis of Deiminated Proteins in Horseshoe Crab Serum

Deiminated protein hits were assessed for number and length of disordered regions using FoldIndex© analysis (https://fold.weizmann.ac.il/fldbin/findex). These are presented, alongside

number of arginines present in the uniprot sequences for the identified deimination protein candidates in *L. polyphemus* and other Merostomata.

Table 2. FoldIndex© analysis of deiminated proteins identified by F95 enrichment in serum of horseshoe crab (*Limulus polyphemus*). The number of disordered regions, residue length of the longest disordered region, total number of disordered residues, as well as number of arginines present in the total number of residues for the individual protein hits is shown.

Protein name	Number disordered regions	Longest disordered region	Number disordered residues	Number of arginines
	Atlantic horseshoe crab (*Limulus polyphemus)*			
A2AX58_LIMPO Hemocyanin subunit IV	16	83	289	**35** (out of 624 residues)
A2AX56_LIMPO Hemocyanin subunit II	11	81	230	**31** (out of 629 residues)
A2AX57_LIMPO Hemocyanin subunit IIIa	15	90	309	**33** (out of 627 residues)
G8YZR0_LIMPO Hemocyanin subunit IIIb	9	80	198	**29** (out of 628 residues)
A2AX59_LIMPO Hemocyanin subunit VI	15	67	285	**32** (out of 638 residues)
CRP1_LIMPO C-reactive protein 1.1	2	11	18	**4** (out of 242 residues)
CRP4_LIMPO C-reactive protein 1.4	1	32	32	**2** (out of 242 residues)
LIMU_LIMPO Limulin	0	0	0	**2** (out of 84 residues)
Q7M4H2_LIMPO Hemocyanin subunit I	11	81	230	**30** (out of 628 residues)
Q25387_LIMPO Endotoxin-binding protein-protease inhibitor	1	7	7	**8** (out of 136 residues)
COAG_LIMPO Coagulogen	3	30	68	**13** (out of 195 residues)
Q8WQK3_LIMPO Pentaxin	4	19	45	**8** (out of 234 residues)
Q7M430_LIMPO Alpha-2-macroglobulin	15	110	402	**53** (out of 1507 residues)
Q7M490_LIMPO Hemocyanin subunit V	0	0	0	**1** (out of 24 residues)
HAAF_LIMPO Hemagglutinin/amebocyte aggregation factor	1	139	139	**13** (out of 172 residues)
A9XXT7_LIMPO Putative integrin-linked protein kinase (partial)	2	42	55	**16** (out of 312 residues)
A9Y038_LIMPO Glucose-6-phosphate isomerase	1	31	31	**16** (out of 599 residues)
	Mangrove horseshoe crab (*Carcinoscorpius rotundicauda*):			
A1X1V5_CARRO Hemocyanin subunit IV	12	84	227	**36** (out of 624 residues)
A1X1V4_CARRO Hemocyanin subunit IIIb	11	58	215	**31** (out of 628 residues)
A1X1V3_CARRO Hemocyanin subunit IIIa	16	98	341	**35** (out of 631 residues)
A1X1V2_CARRO	8	80	207	**29**

Hemocyanin subunit II				(out of 629 residues)
A1X1V6_CARRO Hemocyanin subunit V	12	66	330	**35** (out of 638 residues)
A1X1V1_CARRO Hemocyanin subunit I	9	51	165	**31** (out of 624 residues)
Q2TS30_CARRO Galactose-binding protein	3	25	52	**9** (out of 256 residues)
	Tri-spine horseshoe crab (*Tachypleus tridentatus*):			
B6ZH52_TACTR Complement component 3	16	52	321	**82** (out of 1737 residues)
Q9U8Z6_TACTR Pentaxin	2	7	13	**7** (out of 202 residues)
A1KYQ1_CARRO Plasma carcinolectin CL5B1 (fragment)	6	71	129	**14** (out of 267 residues)
Q9U5E9_TACTR Tachylectin-P (Partial)	4	27	67	**8** (out of 203 residues)

4. Discussion

The current study is the first to profile extracellular vesicles (EVs) and deiminated protein signatures in serum of a Merostomata, using *L. polyphemus* as a model species. EV profiles of the Atlantic horseshoe crab showed similar size distribution as observed for human EVs, in the range of 20-400 nm, although a high proportion was observed for small EVs in the range of 40-123 nm, which furthermore were mainly CD63 positive, indicating a majority of exosomes compared to microvesicles in horseshoe crab haemolymph sera. CD63 is here for the first time assessed in horseshoe crab EVs, and shows protein bands in the size range observed for CD63 in a range of taxa (Iliev et al., 2018; Lange et al., 2019; Pamenter et al., 2019; Criscitiello et al., 2019, 2020a, 2020b; Kosgodage et al., 2018; Phillips et al., 2020). Furthermore, as CD63 does not show positive for either bacterial membrane vesicles (Kosgodage et al., 2019) or EVs from *Giardia intestinalis* (Gavinho et al., 2019), any unspecific reaction for CD63 in horseshoe crab EVs in the current study can be excluded. Morphological analysis by TEM also revealed a high proportion of small EVs, including "cup-shaped" EVs. Therefore it may be possible that the larger peaks observed by NTA analysis may be aggregated small EVs (exosomes), although aggregation was not prominent in TEM analysis.

F95-enrichment for deiminated proteins from horseshoe crab haemolymph serum revealed a range of immunological and metabolic proteins as candidates for this post-translational modification. Our findings indicate hitherto under-recognized modes for protein-moonlighting of these proteins in horseshoe-crab physiology and immunity, and roles for such deimination-mediated changes in proteins with phylogenetically conserved roles in immunity and metabolism. A PAD protein homologue was identified in horseshoe crab haemolymph serum via cross-reaction to the anti-

human PAD2 antibody, which has previously been shown to cross-react with PADs and PAD homologues from diverse taxa (Magnadottir et al., 2018a; 2019a; Criscitiello et al., 2019, 2020a,b; Pamenter et al., 2019; Phillips et al., 2020). Such cross-reactivity with anti-human PAD2 is also in accordance with PAD2 being reported to be the most phylogenetically conserved PAD isozyme (Vossenaar et al., 2003; Magnadottir et al., 2018a; 2019a; Criscitiello et al., 2019,2020a,b; Pamenter et al., 2019). A PAD or PAD homologue has not been previously reported in *Limulus*, searching genetic and proteomic databases, while arginine kinase isoenzymes have been described (Blethen, 1972). The current study is the first to assess post-translationally deiminated proteins, indicative of PAD-mediated deimination protein products, in *Limulus* and any Merostomata. Indeed, many of the identified deimination candidates in the current study showed a high level of disorder, as assessed by number of disordered regions using FoldIndex analysis. Protein structures that have been identified to be most prone to undergo deimination are intrinsically disordered proteins and beta-sheets (Tarsca et al., 1996; György et al., 2006). The position of the arginine furthermore is of importance as arginines placed next to aspartic acid residues are most prone to deimination/citrullination, but arginines that are next to glutamic acid residues are rarely deiminated/citrullinated and arginines that are flanked by proline are poorly deiminated/citrullinated (Nomura, 1992; György et al., 2006).

A number of species-specific deiminated protein candidates for *Limulus polyphemus*, and with other Merostomata, were identified in the current study in horseshoe crab serum using F95-enrichment in tandem with LC-MS/MS analysis. The role of these proteins and their function, and therefore putative effects via post-translational deimination changes are discussed below:

Hemocyanin was identified as a deimination candidate in horseshoe crab, both species-specific for *Limulus* as well as in other horseshoe crabs. This included hemocyanin subunit II, IIIa, IIIb, IV, V and VI as species specific-for *L. polyphemus*, and in addition subunit I was identified as a protein hit for both the mangrove and tri-spined horseshoe crab. Hemocyanin works as an oxygen carrier in Mollusca and Arthropoda, similar to as haemoglobin in human blood, although it is directly suspended into the *Limulus* haemolymph, rather than being in blood cells, as is found for haemoglobin in human blood (Burmester, 2002). In hemocyanin, the central metal ion binding oxygen is copper, which contributes to the blue appearance of horseshoe crab blood when a colour change occurs between the colourless Cu(I) deoxygenated form and the blue Cu(II) oxygenated form (Coates and Nairn, 2014). Hemocyanin subunit IIIa has been assessed for broad antimicrobial effects in the Asian horseshoe crab (*Tachypleus gigas*) and found to be affective against several bacterial and fungal strains, posing as a putative novel antimicrobial, including against resistant strains (Jolly

et al., 2019). It must be noted that horseshoe crab hemocyanin, which has a unique oligomeric structure, is present in hemolymph plasma at very high concentration at ~100 mg/ml, and was here identified as a major candidate for deimination in both *Limulus*, as well as other Merostomata. It cannot be excluded that hemocyanin, which is a very sticky protein, may interact unspecifically. Nonetheless, in the current study all hemacyanins which were identified to be deiminated also turned out to be highly disordered, as assessed by FoldIndex analysis, and to contain a large proportional number of arginines (~5 %). These features contribute to a protein's ability to carry out moonlighting functions as disordered proteins are also most susceptible to post-translational deimination. Deimination of hemocyanin is here described for the first time and may contribute to its multifaceted functions in Mollusca and Arthropoda.

C-reactive protein (CRP 1.1. and 1.4) was identified as a deimination candidate in *L. polyphemus*. Furthermore **Pentaxin**, also pentraxin, and therefore in the CRP family, was identified as deiminated. CRP is evolutionary conserved throughout phylogeny from arthropod to humans (Armstrong, 2015; Magnadottir et al., 2018b; Pathak and Agrawal, 2019). In arthropods, it is a constitutively expressed protein and in human belongs to acute phase proteins, and therefore the acute phase immune response (Pathak and Agrawal, 2019). *Limulus* pentraxins have been shown to from membrane pores and to permeabilise mammalian erythrocytes as well as lipid bilayers (Harrington et al., 2008). Both functional and structural diversities have been described for C-reactive proteins present in horseshoe crab haemolymph (Iwaki et al., 1999). While roles for glycosylation have been implicated to contribute to structural changes in CRP and its function in human and other taxa (Paul et al., 2001; Das et al., 2003; Das et al., 2004; Ansar et al., 2009; Gisladottir et al., 2009), less is known about other post-translational modifications. Previously, we have identified CRP to be deiminated in cod (*Gadus morhua* L.), a teleost fish (Magnadottir et al., 2018b), and have in that study discussed putative roles for deimination in the conserved and diverse roles for CRP and other pentraxins throughout phylogeny, including in the horseshoe crab (Magnadottir et al., 2018b). CRP has also been described to be exported in mucosal extracellular vesicles in Atlantic cod (Magnadottir et al., 2019b). The fact that CRP and pentaxin both came up as deimination candidates in horseshoe crab in the current study therefore supports our speculations on post-translationally mediated moonlighting functions of pentraxins via deimination. The ancient status of CRP in evolution of the immune system and modulation of its function via post-translational modifications, such as deimination identified here, may be of some relevance for furthering understanding of its function in a range of human pathological conditions (Das et al., 2003; Das et al., 2004; Ansar et al., 2009).

Limulin was identified as a deimination candidate in *Limulus*. Limulin belongs to the pentraxin family (Ying et al., 1992) and is a sialic acid-binding lectin which is central to mediating the plasma-based cytolytic system (Armstrong et al., 1996; Swarnakar et al., 1996; Asokan and Armstrong, 1999). It is the mediator of the Ca^{2+}-dependent haemolytic activity present in the plasma of *Limulus*, also with ability for cytolysis of foreign cells (Armstrong et al., 1996). Limulin has furthermore been shown to have the ability to bind to, and to discriminate between, lower and higher level metastatic cancer cells *in vitro*, via selective agglutination of sialidase-treated cells (Fischer and Brossmer, 1995). Post-translational deiminatinon is for the first time identified in limulin in the current study and such post-translational modification may contribute to changes in limulin folding and protein function via deimination of the two arginines identified in the 84 aa protein sequence of limulin. It must be noted that while no disorded regions where identified in limulin using FoldIndex analysis, disorder is not a requirement for deimination.

Endotoxin-binding protein-protease inhibitor (LEBP-PI) was identified as a deimination candidate via F95 enrichment in *L. polyphemus* haemolymph serum in the current study. This protein was originally identified purified from *Limulus* amebocytes by binding to a lipopolysaccharide (LPS) affinity column and using ion exchange chromatography (Minetti et al., 1991). LEBP-PI is a major component (1%) of the cytoplasmic proteins in *Limulus* and has the ability to bind to *Escherichia coli* (Minetti et al., 1991). LEBP-PI is found in the secretory granules of the amebocytes, a site for the enzymes and substrates of the clotting cascade (Minetti et al., 1991), and contributes to haemolymph clotting in concert with CRP, limunectin and coagulin (Liu et al., 1994). Therefore LEBP-PI plays important roles in assisting recognition and removal of invading microorganisms (Liu et al., 1994). Indeed, one disordered region and 8 arginines were here identified in LEBP-PI using FoldIndex analysis. The deimination of LEBP-PI identified here may contribute to LEBP-PI function and interaction with CRP and coagulin, also identified here to be post-translationally deiminated in *Limulus*.

Coagulogen was identified as a deimination candidate in *L. polyphemus*. Coagulogen forms part of the endotoxin-mediated coagulation pathway – which is utilised for the Limulus test for detection of bacterial endotoxins (Iwanaga, 2007). In horseshoe crabs, the proteolytic coagulation cascade coagulins are cross-linked on hemocyte cell surface proteins (proxins), which is the final stage of haemolymph coagulation and forms an important part of the horseshoe crab innate immune system (Osaki and Kawabata, 2004). Coagulogen is considered a functional homologue of vertebrate fibrinogen, indicating that an ancestor of fibrinogen may have functioned as a non-self-recognizing

protein (Gokudan et al., 1999). Human fibrinogen is indeed a well-known deimination target and has been associated with inflammatory diseases (Sharma et al., 2019), while a recent study has identified that deiminated fibrinogen impairs fibrin clot structure (Damiana et al., 2020). Horeseshoe crab coagulogen was here further analysed using FoldIndex analysis, revealing 3 disordered regions, with a total of 68 disordered residues and 13 arginines (out of 195 residues) which can act as putative candidates for conversion or arginine into citrulline. The deimination of coagulogen identified here in horseshoe crab may contribute to the function of coagulogen in *Limulus* immunity and remains to be further investigated.

Alpha-2-macroglobulin is a thioester containing protein and a broad-spectrum protease-binding protein, which is a phylogenetically conserved part of the innate immune system (Armstrong and Quigley, 1999). It was characetrised at the protein level in 1990, indicating 67 % identity with human alpha-2-macroglobulin (Sottrup-Jensen et al., 1990) and cloned from *Limulus* in 1996 (Iwaki et al., 1996). Alpha-2-macroglobulin is the third-most abundant plasma protein and can, in addition to functions including protease inhibitory activity, it can participate in the haemolytic system and inhibit and modulate the cytolytic pathway of limulin (Enghild et al., 1990; Armstrong and Quigley, 1999; Swarnakar et al., 2000). Alpha-2-macroglobulin is furthermore closely related to complement proteins C3, C4 and C5, which are also thioester-containing proteins (Davies and Sim, 1981; Sottrup-Jensen et al., 1985; Dodds and Law, 1998). Alpha-2-macroglobulin has previously been identified as a deimination candidate in serum and plasma of a range of taxa (including camelid, birds and alligator) (Criscitiello et al., 2020a and b; Phillips et al., 2020) and such deimination may contribute to its immunological functions throughout phylogeny. Indeed, in *Limulus* 15 disordered regions are identified here, with a total of 402 disordered residues and 53 arginines (out of 1507 residues) which can pose as putative deimination sites.

Hemagglutinin/amebocyte aggregation factor was here identified as deiminated in *L. polyphemus* haemolymph serum. The horseshoe crab has only one type of circulating blood cell, the amebocyte, which is a granular cell which forms the primary defence mechanism against invading pathogens. Upon degranulation the coagulin clotting protein is released alongside proteases leading to fibrous clots that seal the site of infection off (Coursey et al., 2003). Hemagglutinin is another protein from the amebocytes which can cause aggregation of amebocytes, as well as agglutination of erythrocytes (Fuji et al., 1992). It was originally described as a non-glycosylated, single chain polypeptide protein, stored in the large granules secreted from amebocytes (Fuji et al., 1992). Furthermore, a homologuous form with alternative phospholipidase A2 activity has also been described

(MacPherson and Jacobs,2000). Post-translational deimination identified here for hemagglutinin may therefore be of considerable interest for its function. Using FoldIndex analysis, one large disordered region comprised of 139 disordered residues was identified and the protein contains 13 arginines (out of 172 residues) that can pose as deimination sites and therefore influence protein structure and function.

Putative integrin-linked protein kinase was identified as deiminated in *L. polyphemus*. Integrin-linked kinase (ILK) plays multifaceted roles in cellular functions such as cell migration, differentiation, survival, and division and importantly has been highlighted as a key regulator of longevity and of cellular senescence induced by extracellular stressors (Olmos et al., 2017). ILK is also found to be involved in choroidal neovascularization via recruitment of endothelial progenitor cells (Yang et al., 2018), which may be of interest as horseshoe crab is utilised for studies on visual physiology (Hartline et al., 1956; Barlow, 1983; Watson et al., 2008; Battelle, 2016; Battelle et al., 2016). Deimination has indeed been linked to the visual system, both during fish and mouse development (Magnadottir et al., 2018a, Hollingsworth et al., 2018; Magnadottir et al., 2019a) as well as in relation to ocular diseases (Bhattacharya et al., 2006; Bonilha et al., 2013; Ding et al., 2017; Iannaccone and Radic, 2019; Kwon et al., 2020). Roles for ILK have been linked to a range of pathologies, including cyst growth and fibrosis in polycystic kidney disease (Raman et al., 2017), as well as roles in a number of cancers and its potential as a cancer biomarker has been highlighted (Zheng et al., 2019). Post-translational deimination identified here in ILK in horseshoe crab may contribute to some of these multifaceted functions and be of translatable value throughout phylogeny; particularly in the light of horseshoe crab being relatively long-lived animals for an arthropod, with a lifespan of 20 years.

Glucose-6-phosphate isomerase (GPI) was identified as deiminated in *L. polyphemus* haemolymph serum. GPI is a dimeric enzyme and the second enzyme in the glycolytic pathway and catalyses the interconversion of fructose-6-phosphate and glucose-6-phosphate (Achari et al., 1981). GPI has indeed been identified to be a moonlighting protein due to its ability to perform mechanistically distinct functions. In amphibians, GPI is linked to embryonic development (Miranda, 1976) and due to the importance of glycolysis pathways for the survival of a number of unicellular protozoans, their GPI's have received attention as putative targets for drug design (Cordeiro et al., 2014). While GPI functions as a glycolytic enzyme in the cytoplasm in the extracellular environment, it functions as a neurotrophic factor for skeletal motor and sensory neurones. It can also act as a lymphokine and induce immunoglobulin secretion and as a tumour-secreted cytokine and angiogenic factor. GPI has

been linked to proliferation and motility of cancer cells (Lincet and Icard, 2015), found to promote angiogenesis and participate in cancer metabolism (Singh et al., 2017), therefore playing roles in the Warburg effect (Ždralević et al., 2018). GPI has also been identified as an autoantigen in rheumatoid arthritis (RA) alongside deiminated/citrullinated proteins (Matsumoto et al., 2020) as well as to regulate hypoxia-induced angiogenesis in RA (Lu et al., 2017). Indeed, GPI-induced arthritis is a valid animal model of rheumatoid arthritis (Ebbinghaus et al., 2019) and PAD4 deficiency has been shown to decrease disease severity in the GPI-induced arthritis model (Seri et al., 2015). The role for deiminated proteins in RA is well known and the citrullinome of RA has been extensively studied (Darrah and Andrade, 2018; Tilvawala et al., 2018; Ruiz-Romero et al., 2019; Martinez-Prat et al., 2019; Svärd et al., 2019; Boberet al., 2020). GPI has indeed been identified to be deiminated in RA (Wu et al., 2016; Umeda et al., 2013). GPI deficiency is furthermore an autosomal recessive disorder which has been identified as the second most frequent erythroenzymopathy in glycolysis. It is associated both with non-spherocytic haemolytic anaemia as well as neurological impairment in some cases (Kugler and Lakomek, 2000; Fermo et al., 2019). The identification of deiminated GPI in horseshoe crab haemolymph serum in the current study highlights a role for this post-translational modification in the contribution of multifaceted functions of GPI, throughout phylogeny. In *Limulus* GPI, one disordered region was identified containing 31 disordered residues, and 16 arginines, which pose as putative sites for deimination/citrulliation, were identified out of 599 residues of the protein. Deimination of GPI, in the light of its conservation throughout phylogeny, may indeed contribute to its multifaceted functions and remains to be further investigated beyond its currently identified connection to autoimmune responses in RA.

Galactose-binding protein (GBP) was identified as deiminated in the mangrove horseshoe crab (*C. rotundicauda*). GBP is classified as a beta-propeller protein that contains tectonin domains, and has functions in antibacterial defences (Low et al., 2010). Horseshoe crab GBP binds to LPS of Gram-negative bacteria and helps in eliminating these pathogens through interactions with CRP (Ng et al., 2007; Low et al., 2010) via GBP's beta-propeller domains, as identified using protein modelling (Low et al., 2010). Furthermore, tectonin beta-propeller repeat containing proteins are linked to the interplay of bacteria and host autophagy (Ogawa et al., 2011; Chen and Zhong, 2012; Sudhakar et al., 2019). Beta-propeller proteins have multiple functions in catalysis, protein-protein interaction, cell cycle regulation, and innate immunity (Low et al., 2010). A GBP has also been identified in the tri-spined horseshoe crab (*T. tridentatus*) (Chiou et al., 2010). Horseshoe crab GBP has been identified to share both structural and functional homologies to human hTectonin, which has binding affinities to bacterial LPS and interacts with ficolins (Low et al., 2009), indicating evolutionary conservation of

these proteins over 500 million years (Low et al., 2010). As beta-sheets are more prone to undergo deimination, post-translationally mediated changes in their structure and function via deimination may contribute to the multifaceted functions of GBP. In the current study, GBP from horseshoe crab was identified to have 3 disordered regions, with a total of 52 disordered residues and 9 arginines that can pose as candidates for arg/cit conversion, out of 256 residues of the protein. To what extent deimination contributes to the functional diversity of GBP remains to be further investigated.

Complement component 3 was identified here as deiminated in Merostomata scoring with C3 from tri-spine horseshoe crab (*T. tridentatus*). C3 is evolutionarily conserved, including in horseshoe crab (Zhu et al., 2005; Tagawa et al., 2012; Zimmer et al. 2015) and has recently been identified as a deimination protein candidate in a range of taxa (teleost, shark, cetacean, camelid, birds, alligator) (Magnadottir et al., 2019a, 2020b; Criscitiello et al., 2019; Criscitiello et al., 2020a,b; Phillips et al., 2020). C3 plays central roles in the complement cascade and can furthermore be directly activated by self- and non-self surfaces (Dodds and Law, 1998; Dodds, 2002). Besides key roles for C3 in the immune response, diverse roles have been linked to C3, including in regeneration (Del Rio-Tsonis et al., 1998) and during early teleost development (Lange et al., 2004a,b, 2005, 2006). In the mangrove horseshoe crab (*C. rotundicauda*) antimicrobial effects of C3a have been studied, highlighting conserved antimicrobial properties for Gram-negative and Gram-positive bacteria (Pasupuleti et al., 2007). The recent identification of C3 deimination in diverse species, including in Merostomata in the current study, may be a hitherto under-recognized factor which contributes to the multifaceted and conserved functions of the complement system throughout phylogeny (Boshra et al., 2006; Lange et al., 2005; 2006; Sunyer and Lambris, 1998; Nakao et al., 2006; Carrol and Sim, 2011; Nakao et al., 2011; Forn-Cuní et al., 2014; Magnadottir et al., 2019). In the current study 16 unfolded regions were identified in merostomata C3, based on C3 from tri-spine horseshoe crab, with a total of 321 disordered residues and 82 arginines (out of 1737 residues of the total protein). To what extent the different sites for deimination/citrullination contribute to C3 function will remain to be further investigated.

Plasma carcinolectin CL5B1 (CL5) was identified as deiminated scoring with the tri-spined horseshoe crab (*T. tridentatus*). CL5 acts as the functional protein partner of galactose binding protein (GBP) during infection (Low et al., 2009). It has been shown to be co-purified with CRP and GBP from horseshoe crab haemolymph, using LPS-affinity chromatography, and to form pathogen recognition complexes (Ng et al., 2007). Furthermore, CL5 has been shown to be homologous to human M-ficolin, which activates the lectin-arm of the complement system via binding to CRP (Ng et al., 2007;

Low et al., 2009). In deep-sea mussels (*Bathymodiolus azoricus*), which inhabit deep-sea hydrothermal vents, carcinolectin has been identified to be an immune recognition molecule in early stages of immune responses (Bettencourt et al., 2014) and to act as a biomarker in response to *Vibrio diabolicus* challenge (Martins et al., 2015). CL5 has also been identified in the innate immune response of shrimp in response to *Vibrio anguillarium* stimulation (Wang et al., 2013). In the current study, CL5 of horseshoe crab was identified to have 6 disordered regions, with a total of 129 disordered residues, whereof the longest disordered region contained 71 residues. The number of arginines, and therefore putative sites for arg/cit conversion, were found to be 14 out of 267 residues of the total protein. These characteristic indicate that CL5 is prone to deimination, as indeed identified here, and such post-translational change may contribute to its varying functions in differing scenarios throughout phylogeny, and will remain to be further investigated.

Tachylectin-P (TL-P) was identified as deiminated in the current study in Merostomata, via a protein hit with the tri-spine horseshoe crab (*T. tridentatus*). It is a 27 kDa lectin originally identified in perivitelline fluid (Nagai et al., 1999). It has agglutination preferences for human A-type erythrocytes and been suggested to play important roles in embryonic development via interaction with endogenous glycoproteins or N-acetylhexosamines (Nagai et al., 1999). Furthermore, two forms of trachylectin (TPL-1 and TPL-2) have been isolated from the tri-spine horseshoe crab and found to bind Gram-positive and Gram-negative bacteria (Chen et al., 2001). It has been proposed that the physiological function of TPL-1 and TPL-2 may be related to their ability to form a cluster of interlocking molecules to immobilize and entrap invading organisms (Chen et al., 2001). TL-P has been found to have a similar structure (based on amino acid sequence alignment) to the TH-1 hemocyte-derived lectin, tachylectin-1 (TL-1), which has no hemagglutinating activity (Nagai et al., 1999). As TL-P was here identified to have 4 disordered regions, with a total of 67 disordered residues and 8 arginines (out of 203 residues of the total protein), deimination of TL-P identified here may contribute to differences in its functions. If such deimination and downstream function and structural changes are also applicable for TL-1 and TL-2, deimination may contribute to changes in steric form and therefore function in antibacterial responses. Tachylectins have recently also been described in acute hepatopancreatic necrosis disease (AHPND) in shrimp (Angthong et al., 2017). Roles for deimination of TL-P therefore will need further investigation in a range of taxa.

Protein networks constructed in the current study using STRING based on mouse (*Mus musculus*) homologue proteins, as *Limulus* proteins are not available in the STRING database, correlated with immunological and metabolic functions of the proteins identified to be deiminated in *Limulus*. This

692 highlights a novel aspect of post-translational deimination in regulation of these pathways in
693 *Limulus*, and also putative roles of such regulation in conserved functions, as well as moonlighting
694 functions of the proteins identified, throughout phylogeny. KEGG pathways highlighted for
695 deiminated proteins included the complement and coagulation pathways, as well as response to
696 bacterial infection (Gram-negative *S. aureus* infection), which are well known in *Limulus*, although
697 not in relation to deimination until in the current study, as discussed above. Furthermore,
698 deimination of pathways for glycolysis and gluconeogenesis relate to proteins identified to be
699 deiminated in *Limulus* in the current study (including GPI) with roles in glycolysis, amongst other
700 functions. GO biological and molecular pathways identified in the deiminated *Limulus* proteins
701 related to a range of immune and metabolic functions, as well as to developmental processes, and
702 this correlates to multifaceted functions of these deiminated protein candidates identified in
703 immunity and development, highlighting their moonlighting abilities. PFAM protein domains
704 identified in the protein networks for deiminated proteins in *Limulus* also highlighted immune
705 functions in particular, as did the UniProt Keywords, which also emphasised metabolic proteins and
706 highlighted calcium, which is a key driver of deimination (Alghamdi et al., 2019) and a key modulator
707 in a range of immunological, metabolic and developmental functions (Paupe and Prudent, 2018; King
708 et al., 2020; Puri et al., 2020). A similar relation to immunity and metabolism was seen for the
709 SMART protein domains, INTERPRO protein domains, as well as for the reactome pathways, for the
710 deiminated proteins identified in *Limulus* in the current study.

711 The characterisation of EVs and of post-translational deimination signatures revealed in horseshoe
712 crab in the current study, and for the first time in Merstomata, contributes to current understanding
713 of deimination and EV-mediated communication in this ancient arthropod. Our findings may inform
714 conserved and diverse functions of moonlighting proteins via post-translational deimination
715 throughout phylogeny.

716

717 **Conclusion**

718 This is the first study to assess PADs, protein deimination and extracellular vesicle profiles in serum
719 of a Merostomata, using the Atlantic horseshoe crab (*Limulus polyphemus*) as a model species. EV
720 profiles of *Limulus* showed a high proportion for small EVs, which were mainly CD63 positive,
721 indicating a majority of exosomes (small EVs), compared to microvesicles (larger EVs). Assessment of
722 deiminated proteins revealed a range of deiminated proteins relevant for immunological and
723 metabolic function in horseshoe crab haemolymph serum. Protein network analysis revealed KEGG
724 and GO pathways for key immunological and metabolic functions. This study highlights therefore

roles for protein deimination and associated protein moonlighting functions of key immune and metabolic proteins, some of which are conserved throughout phylogeny from horseshoe crab to man. Our findings provide novel insights into the immunity and physiology of these ancient ancestors and living fossils. Comparative studies in horseshoe crab may be of translational value for furthering current understanding of mechanisms underlying conserved physiological and pathogenic pathways, including via the diversification of protein functions facilitated by post-translational deimination.

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Author Contributions Statement

TB: Resources; Validation; Writing - review & editing.

IK: Methodology; Resources; Visualization.

SL: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Validation; Visualization; Writing -original draft; Writing - review & editing.

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References

Achari, A., Marshall, S.E., Muirhead, H., Palmieri, R.H., Noltmann, E.A., 1981. Glucose-6-phosphate isomerase. Philos. Trans. R. Soc. Lond. B Biol. Sci. 293 (1063), 145-57.

Alghamdi, M., Alasmari, D., Assiri, A., Mattar, E., Aljaddawi, A.A., Alattas, S.G., Redwan, E.M., 2019. An Overview of the Intrinsic Role of Citrullination in Autoimmune Disorders. J. Immunol. Res. 2019, 7592851.

Angthong, P., Roytrakul, S., Jarayabhand, P., Jiravanichpaisal, P., 2017. Involvement of a tachylectin-like gene and its protein in pathogenesis of acute hepatopancreatic necrosis disease (AHPND) in the shrimp, *Penaeus monodon*. Dev. Comp. Immunol. 76, 229-237.

Ansar, W., Mukhopadhyay, S., Habib, S.K., Basu, S., Saha, B., Sen, A.K., Mandal, C.N., Mandal, C., 2009. Disease-associated glycosylated molecular variants of human C-reactive protein activate complement-mediated hemolysis of erythrocytes in tuberculosis and Indian visceral leishmaniasis. Glycoconj. J. 26, 1151-69.

Armstrong, P.B., 2015. Comparative biology of the pentraxin protein family: evolutionarily conserved component of innate immune system. Int. Rev. Cell. Mol. Biol. 316, 1-47.

Armstrong, P, Conrad, M., 2008. Blood collection from the American horseshoe crab, *Limulus polyphemus*. J. Vis. Exp. (20), pii 958.

Armstrong, P.B., Swarnakar, S., Srimal, S., Misquith, S., Hahn, E.A., Aimes, R.T., Quigley, J.P., 1996. A cytolytic function for a sialic acid-binding lectin that is a member of the pentraxin family of proteins. J. Biol. Chem. 271(25), 14717-21.

Armstrong, P.B., Quigley, J.P., 1999. Alpha2-macroglobulin: an evolutionarily conserved arm of the innate immune system. Dev. Comp. Immunol. 23, 375-90.

Asokan, R., Armstrong, P.B., 1999. Cellular Mechanisms of Hemolysis by the Protein Limulin, a Sialic-Acid-Specific Lectin From the Plasma of the American Horseshoe Crab, *Limulus polyphemus*. Biol. Bull. 197(2), 275-276.

Barlow, R. B., 1983. Circadian rhythms in the *Limulus* visual system. J Neurosci. 3(4), 856-70.

Battelle, B.A., 2016. Simple Eyes, Extraocular Photoreceptors and Opsins in the American Horseshoe Crab. Integr. Comp. Biol. 56(5), 809-819.

Battelle, B.A., Ryan, J.F., Kempler, K.E., Saraf, S.R., Marten, C.E., Warren, W.C., Minx, P.J., Montague, M.J., Green, P.J., Schmidt, S.A., Fulton, L., Patel, N.H., Protas, M.E., Wilson, R.K., Porter, M.L., 2016. Opsin Repertoire and Expression Patterns in Horseshoe Crabs: Evidence from the Genome of *Limulus polyphemus* (Arthropoda: Chelicerata). Genome Biol Evol. 8 (5), 1571-89.

Berkson, J., Shuster C. N. Jr., 1999. The horseshoe crab: the battle for a true multiple-use resource, Fisheries. 24 (11), 6-10.

Bettencourt, R., Rodrigues, M., Barros, I., Cerqueira, T., Freitas, C., Costa, V., Pinheiro, M., Egas, C., Santos, R.S, 2014. Site-related differences in gene expression and bacterial densities in the mussel *Bathymodiolus azoricus* from the Menez Gwen and Lucky Strike deep-sea hydrothermal vent sites. Fish Shellfish Immunol. 39 (2), 343-53

Bhattacharya, S.K., Crabb, J.S., Bonilha, V.L., Gu, X., Takahara, H., Crabb, J.W., 2006. Proteomics implicates peptidyl arginine deiminase 2 and optic nerve citrullination in glaucoma pathogenesis. Invest. Ophthalmol. Vis. Sci. 47 (6), 2508-14.

Bicker, K.L., Thompson, P.R., 2013 The protein arginine deiminases: Structure, function, inhibition, and disease. Biopolymers. 99 (2), 155-63.

Bielecka, E., Scavenius, C., Kantyka, T., Jusko, M., Mizgalska, D., Szmigielski, B., Potempa, B., Enghild, J.J., Prossnitz, E.R., Blom, A.M., Potempa, J., 2014. Peptidyl arginine deiminase from *Porphyromonas gingivalis* abolishes anaphylatoxin C5a activity. J. Biol. Chem. 289, 32481-32487.

Biron, B.M., Chung, C.S., Chen, Y., Wilson, Z., Fallon, E.A., Reichner, J.S., Ayala, A., 2018. PAD4 deficiency leads to decreased organ dysfunction and improved survival in a dual insult model of hemorrhagic shock and sepsis. J. Immunol. 200, 1817-1828.

Blethen, S. L., 1972. Kinetic Properties of Arginine Kinase Isoenzymes of *Limulus Polyphemus* Arch. Biochem. Biophys. 149 (1), 244-51.

Bonilha, V.L., Shadrach, K.G., Rayborn, M.E., Li, Y., Pauer, G.J., Hagstrom, S.A., Bhattacharya, S.K., Hollyfield, J.G., 2013. Retinal deimination and PAD2 levels in retinas from donors with age-related macular degeneration (AMD). Exp. Eye Res., 111, 71-8.

Boshra, H., Li, J., Sunyer, J.O., 2006. Recent advances on the complement system of teleost fish. Fish Shellfish Immunol. 20(2), 239-62.

Burmester, T., 2002. Origin and evolution of arthropod hemocyanins and related proteins. J. Comp. Physiol. B. 172 (2), 95-107.

Carroll, M.V., Sim, R.B., 2011. Complement in health and disease. Adv. Drug Deliv. Rev. 63(12), 965-75.

Casanova, V., Sousa, F.H., Shakamuri, P., Svoboda, P., Buch, C., D'Acremont, M., Christophorou, M.A., Pohl, J., Stevens, C., Barlow, P.G. Citrullination Alters the Antiviral and Immunomodulatory Activities of the Human Cathelicidin LL-37 During Rhinovirus Infection. Front. Immunol. 2020, 11, 85.

Chabot, C.C., Kent, J., Watson, W.H. 3rd.II., 2004. Circatidal and circadian rhythms of locomotion in *Limulus Polyphemus*. Biol Bull. 207(1), 72-5.

Chabot, C.C., Watson, W.H. 3rd.II., 2010. Circatidal rhythms of locomotion in the American horseshoe crab *Limulus Polyphemus*: underlying mechanisms and cues that influence them. Curr. Zool. 56 (5), 499-517.

Chabot, C.C., Ramberg-Pihl, N. C., Watson, W.H. 3rd., 2016. Circalunidian clocks control tidal rhythms of locomotion in the American horseshoe crab, *Limulus Polyphemus*. Mar. Freshw. Behav. Physiol. 49(2), 75-91

Chen, S.C., Yen, C.H., Yeh, M.S., Huang, C.J., Liu, T.Y., 2001. Biochemical properties and cDNa cloning of two new lectins from the plasma of *Tachypleus tridentatus*: Tachypleus plasma lectin 1 and 2+. J Biol. Chem. 276(13), 9631-9.

Chen, D., Zhong, Q., 2012. A tethering coherent protein in autophagosome maturation. Autophagy. 8(6), 985-6.

Chesmore, K.N., Watson, W.H. 3rd., Chabot, C.C., 2016. Identification of putative circadian clock genes in the American horseshoe crab, *Limulus Polyphemus*. Comp. Biochem. Physiol. Part D. Genomics Proteomics. 19, 45-61.

Chiou, S.T., Chen, Y.W., Chen, S.C., Chao, C.F., Liu, T.Y., 2000. Isolation and characterization of proteins that bind to galactose, lipopolysaccharide of *Escherichia coli*, and protein A of *Staphylococcus aureus* from the hemolymph of *Tachypleus tridentatus*. J. Biol. Chem. 275(3), 1630-4.

Claushuis, T.A.M., van der Donk, L.E.H., Luitse, A.L., van Veen, H.A., van der Wel, N.N., van Vught, L.A., Roelofs, J.J.T.H., de Boer, O.J., Lankelma, J.M., Boon, L., de Vos, A.F., van 't Veer, C., van der Poll, T., 2018. Role of peptidylarginine deiminase 4 in neutrophil extracellular trap formation and host defense during *Klebsiella pneumoniae*-induced pneumonia-derived sepsis. J. Immunol. 201(4), 1241-1252.

Coates, C.J., Nairn, J., 2014. Diverse immune functions of hemocyanins. Dev. Comp. Immunol. 45 (1), 43-55.

Colombo, M., Raposo, G., Théry, C., 2014. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. Annu. Rev. Cell. Dev. Biol. 30, 255-289.

Cordeiro, A.T., Michels, P.A., Delboni, L.F., Thiemann, O.H., 2004. The crystal structure of glucose-6-phosphate isomerase from *Leishmania mexicana* reveals novel active site features. Eur. J. Biochem. 271(13), 2765-72.

Costa, N.A., Gut, A.L., Azevedo, P.S., Polegato, B.F., Magalhães, E.S., Ishikawa, L.L.W., Bruder, R.C.S., Silva, E.A.D., Gonçalves, R.B., Tanni, S.E., Rogero, M.M., Norde, M.M., Cunha, N.B., Zornoff, L.A.M., de Paiva, S.A.R, Minicucci, M.F., 2018. Peptidylarginine deiminase 4 concentration, but not PADI4 polymorphisms, is associated with ICU mortality in septic shock patients. J. Cell. Mol. Med. 22, 4732-4737.

Coursey, Y., Ahmad, N., McGee, B.M., Steimel, N., Kimble, M., 2003. Amebocyte production begins at stage 18 during embryogenesis in *Limulus polyphemus*, the American horseshoe crab. Biol. Bull. 204(1), 21-7.

Criscitiello, M.F., Kraev, I., Lange, S., 2019. Deiminated proteins in extracellular vesicles and plasma of nurse shark (*Ginglymostoma cirratum*) - Novel insights into shark immunity. Fish Shellfish Immunol. 92, 249-255.

Criscitiello, M.F., Kraev, I., Lange, S., 2020a. Deiminated proteins in extracellular vesicles and serum of llama (*Lama glama*)-Novel insights into camelid immunity. Mol. Immunol. 117, 37-53.

Criscitiello, M.F., Kraev, I., Lange, S., 2020b. Deimination Protein Profiles in *Alligator mississippiensis* Reveal Plasma and Extracellular Vesicle- specific Signatures Relating to Immunity, Metabolic Function and Gene Regulation. Front. Immunol. doi: 10.3389/fimmu.2020.00651

Damiana, T., Damgaard, D., Sidelmann, J.J., Nielsen, C.H., de Maat, M.P.M., Münster, A.B., Palarasah, Y., 2020. Citrullination of fibrinogen by peptidylarginine deiminase 2 impairs fibrin clot structure. Clin. Chim. Acta. 501,6-11.

Darrah, E., Andrade, F., 2018. Rheumatoid arthritis and citrullination. Curr. Opin. Rheumatol. 30(1), 72-78.

Das, T., Sen, A., Kempf, T., Pramanik, S.R., Mandal, C., Mandal, C., 2003. Induction of glycosylation in human C-reactive protein under different pathological conditions. Biochem. J. 373, 345-55.

Das, T., Mandal, C., Mandal, C., 2004. Variations in binding characteristics of glycosylated human C-reactive proteins in different pathological conditions. Glycoconj. J. 20, 537-43.

Davies, S.G., Sim, R.B., 1981. Intramolecular general acid catalysis in the binding reactions of alpha 2-macroglobulin and complement components C3 and C4. Biosci. Rep. 1(6), 461-8.

Del Rio-Tsonis, K., Tsonis, P.A., Zarkadis, I.K., Tsagas, A.G., Lambris, J.D., 1998. Expression of the third component of complement, C3, in regenerating limb blastema cells of urodeles. J. Immunol. 161(12), 6819-24.

Ding, D., Enriquez-Algeciras, M., Bhattacharya, S.K., Bonilha, V.L., 2017. Protein deimination in aging and age-related diseases with ocular manifestations. In: Nicholas A., Bhattacharya S., Thompson P. (eds) Protein Deimination in Human Health and Disease. Springer, Cham.

Dodds, A.W., 2002. Which came first, the lectin/classical pathway or the alternative pathway of complement? Immunobiology. 205 (4-5), 340-54.

Dodds, A.W., Law, S.K., 1998. The phylogeny and evolution of the thioester bond-containing proteins C3, C4 and alpha 2-macroglobulin. Immunol. Rev., 166, 15-26.

Ebbinghaus, M., Müller, S., Segond von Banchet, G., Eitner, A., Wank, I., Hess, A., Hilger, I., Kamradt, T., Schaible, H.G., 2019. Contribution of Inflammation and Bone Destruction to Pain in Arthritis: A Study in Murine Glucose-6-Phosphate Isomerase-Induced Arthritis. Arthritis Rheumatol. 71 (12), 2016-2026.

El-Sayed, A.S.A., Shindia, A.A., AbouZaid, A.A., Yassin, A.M., Ali, G.S., Sitohy, M.Z., 2019. Biochemical characterization of peptidylarginine deiminase-like orthologs from thermotolerant *Emericella dentata* and *Aspergillus nidulans*. Enzyme Microb. Technol. 124, 41-53.

Enghild, J.J., Thøgersen, I.B., Salvesen, G., Fey, G.H., Figler, N.L., Gonias, S.L., Pizzo, S.V., 1990. Alpha-macroglobulin from *Limulus polyphemus* exhibits proteinase inhibitory activity and participates in a hemolytic system. Biochemistry. 29(43), 10070-80.

Fadini, G.P., Menegazzo, L., Rigato, M., Scattolini, V., Poncina, N., Bruttocao, A., Ciciliot, S., Mammano, F., Ciubotaru, C.D., Brocco, E., Marescotti, M.C., Cappellari, R., Arrigoni, G., Millioni, R., Vigili de Kreutzenberg, S., Albiero, M., Avogaro, A., 2016. NETosis Delays Diabetic Wound Healing in Mice and Humans. Diabetes. 65(4), 1061-71.

882 Faigle, W., Cruciani, C., Wolski, W., Roschitzki, B., Puthenparampil, M., Tomas-Ojer, P., Sellés-Moreno, C., Zeis,
883 T., Jelcic, I., Schaeren-Wiemers, N., Sospedra, M., Martin, R., 2019. Brain citrullination patterns and T cell
884 reactivity of cerebrospinal fluid-derived CD4+ T cells in multiple sclerosis. Front. Immunol. 10, 540.

885 Fermo, E., Vercellati, C., Marcello, A.P., Zaninoni, A., Aytac, S., Cetin, M., Capolsini, I., Casale, M., Paci, S.,
886 Zanella, A., Barcellini, W., Bianchi, P., 2019. Clinical and Molecular Spectrum of Glucose-6-Phosphate
887 Isomerase Deficiency. Report of 12 New Cases. Front. Physiol. 10, 467.

888 Fert-Bober, J., Darrah, E., Andrade, F., 2020. Insights into the study and origin of the citrullinome in
889 rheumatoid arthritis. Immunol. Rev. 294 (1), 133-147.

890 Fischer, E., Brossmer, R., 1995. Sialic acid-binding lectins: submolecular specificity and interaction with
891 sialoglycoproteins and tumour cells. Glycoconj J. 12 (5), 707-13.

892 Forn-Cuní, G., Reis, E.S., Dios, S., Posada, D., Lambris, J.D., Figueras, A., Novoa, B., 2014. The evolution and
893 appearance of C3 duplications in fish originate an exclusive teleost c3 gene form with anti-inflammatory
894 activity. PLoS One. 9(6), e99673.

895 Fujii, N., Minetti, C.A., Nakhasi, H.L., Chen, S.W., Barbehenn, E., Nunes, P.H., Nguyen, N.Y., 1992. Isolation,
896 cDNA cloning, and characterization of an 18-kDa hemagglutinin and amebocyte aggregation factor from
897 *Limulus polyphemus*. J. Biol. Chem. 267 (31), 22452-9.

898 Gavinho, B., Rossi, I.V., Evans-Osses, I., Lange, S., Ramirez, M.I., 2019. Peptidylarginine deiminase inhibition
899 abolishes the production of large extracellular vesicles from *Giardia intestinalis*, affecting host-pathogen
900 interactions by hindering adhesion to host cells. bioRxiv 586438. 10.1101/586438.

901 Gisladottir, B., Gudmundsdottir, S., Brown, L., Jonsson, Z.O., Magnadottir, B., 2009. Isolation of two C-reactive
902 protein homologues from cod (*Gadus morhua* L.) serum. Fish Shellfish Immunol. 26(2), 210-9.

903 Gatien, J., Mermillod, P., Tsikis, G., Bernardi, O., Janati Idrissi, S., Uzbekov, R., Le Bourhis, D., Salvetti, P.,
904 Almiñana, C., Saint-Dizier, M., 2019. Metabolomic profile of oviductal extracellular vesicles across the estrous
905 cycle in cattle. Int. J. Mol Sci. 20 (24), pii E6339.

906 Gokudan, S., Muta, T., Tsuda, R., Koori, K., Kawahara, T., Seki, N., Mizunoe, Y., Wai, S.N., Iwanaga, S.,
907 Kawabata, S.., 1999. Horseshoe crab acetyl group-recognizing lectins involved in innate immunity are
908 structurally related to fibrinogen. Proc. Natl. Acad. Sci. USA. 96 (18), 10086-91.

909 György, B., Toth, E., Tarcsa, E., Falus, A., Buzas, E.I., 2006. Citrullination: a posttranslational modification in
910 health and disease. Int. J. Biochem. Cell. Biol. 38, 1662–77.

911 Harrington, J.M., Chou, H.T., Gutsmann, T., Gelhaus, C., Stahlberg, H., Leippe, M., Armstrong, P.B., 2008.
912 Membrane pore formation by pentraxin proteins from *Limulus*, the American horseshoe crab. Biochem. J. 413
913 (2), 305-13.

914 Hartline, H. K., Wagner, H. G., Ratliff, F., 1956. Inhibition in the eye of *Limulus*. J. Gen. Physiol.39 (5), 651–673.

915 Henderson, B., Martin, A.C., 2014. Protein moonlighting: a new factor in biology and medicine. Biochem. Soc.
916 Trans. 42(6), 1671-8.

917 Hessvik, N.P., Llorente, A., 2018. Current knowledge on exosome biogenesis and release. Cell. Mol. Life Sci. 75
918 (2), 193-208.

919 Hollingsworth, T.J., Radic, M.Z., Beranova-Giorgianni, S., Giorgianni, F., Wang, Y., Iannaccone, A., 2018. Murine
920 Retinal Citrullination Declines With Age and is Mainly Dependent on Peptidyl Arginine Deiminase 4 (PAD4).
921 Invest. Ophthalmol. Vis. Sci. 59 (10), 3808-3815.

922 Iannaccone, A., Radic, M.Z., 2019. Increased Protein Citrullination as a Trigger for Resident Immune System
923 Activation, Intraretinal Inflammation, and Promotion of Anti-retinal Autoimmunity: Intersecting Paths in
924 Retinal Degenerations of Potential Therapeutic Relevance. Adv. Exp. Med. Biol. 1185, 175-179.

925 Iliev, D., Strandskog, G., Nepal, A., Aspar, A., Olsen, R., Jørgensen, J., Wolfson, D., Ahluwalia, B.S., Handzhiyski,
926 J., Mironova, R., 2018. Stimulation of exosome release by extracellular DNA is conserved across multiple cell
927 types. FEBS J. 285 (16), 3114-3133.

928 Inal, J.M., Ansa-Addo, E.A., Lange, S., 2013. Interplay of host-pathogen microvesicles and their role in
929 infectious disease. Biochem. Soc. Trans. 41 (1), 258-62.

930 Iwaki, D., Kawabata, S., Miura, Y., Kato, A., Armstrong, P.B., Quigley, J.P., Nielsen, K.L., Dolmer, K., Sottrup-
931 Jensen, L., Iwanaga, S., 1996. Molecular cloning of *Limulus* alpha 2-macroglobulin. Eur. J. Biochem. 242 (3),
932 822-31.

933 Iwaki, D., Osaki, T., Mizunoe, Y., Wai, S.N., Iwanaga, S., Kawabata, S., 1999. Functional and structural diversities
934 of C-reactive proteins present in horseshoe crab hemolymph plasma. Eur. J. Biochem. 264(2), 314-26.

935 Iwanaga, S., 2007. Biochemical principle of *Limulus* test for detecting bacterial endotoxins. Proc. Jpn. Acad. Ser.
936 B Phys. Biol. Sci. 83(4), 110-9.

937 Jeffrey, C.J., 2018. Protein moonlighting: what is it, and why is it important? Philos. Trans. R. Soc. Lond. B. Biol.
938 Sci. 373 (1738), pii 20160523.

939 Jolly, J.J., Dzulkiply, S.K., Yusof, M.A., Kamaruding, N.A., Ismail, N., 2019. Broad-spectrum Antimicrobial Activity
940 of Purified Hemocyanin Subunit IIIA Isolated from Asian Horseshoe Crab, *Tachypleus gigas*. Pak J Biol Sci. 22
941 (9), 427-434.

942 Kawabata, S., 2010. Immunocompetent molecules and their response network in horseshoe crabs. Adv. Exp.
943 Med. Biol. 708, 122-36.

944 Kholia, S., Jorfi, S., Thompson, P.R., Causey, C.P., Nicholas, A.P., Inal, J.M., Lange, S., 2015. A novel role for
945 peptidylarginine deiminases (PADs) in microvesicle release: A therapeutic potential for PAD inhibitors to
946 sensitize prostate cancer cells to chemotherapy. J. Extracell. Vesicles. 4, 26192.

947 King, M.M., Kayastha, B.B., Franklin, M.J., Patrauchan, M.A., 2020. Calcium Regulation of Bacterial Virulence.
948 Adv. Exp. Med. Biol. 1131, 827-855.

949 Kosgodage, U.S., Trindade, R.P., Thompson, P.T., Inal, J.M., Lange, S., 2017. Chloramidine/Bisindolylmaleimide-
950 I-mediated inhibition of exosome and microvesicle release and enhanced efficacy of cancer chemotherapy. Int.
951 J. Mol. Sci. 18(5), pii E1007.

952 Kosgodage, U.S., Uysal-Onganer, P., Maclatchy, A., Nicholas, A.P., Inal, J.M., Lange, S., 2018. Peptidylarginine
953 deiminases post-translationally deiminate prohibitin and modulate extracellular vesicle release and miRNAs 21
954 and 126 in glioblastoma multiforme. Int. J. Mol. Sci. 20(1), pii E103.

955 Kosgodage, U.S., Matewele, P., Mastroianni, G., Kraev, I., Brotherton, D., Awamaria, B., Nicholas, A.P., Lange,
956 S., Inal, J.M., 2019. Peptidylarginine Deiminase Inhibitors Reduce Bacterial Membrane Vesicle Release and
957 Sensitize Bacteria to Antibiotic Treatment. Front. Cell. Infect. Microbiol. 9, 227.

958 Kugler, W., Lakomek, M., 2000. Glucose-6-phosphate isomerase deficiency. Baillieres Best Pract. Res. Clin.
959 Haematol. 13 (1), 89-101.

960 Kwon, J., Surenkhuu, B., Raju, I., Atassi, N., Mun, J., Chen, Y.F., Sarwar, M.A., Rosenblatt, M., Pradeep, A., An,
961 S., Dhall, N., Mun, C., Jain, S., 2020. Pathological consequences of anti-citrullinated protein antibodies in tear
962 fluid and therapeutic potential of pooled human immune globulin-eye drops in dry eye disease. Ocul. Surf. 18
963 (1), 80-97.

964 Lange, S., Bambir, S., Dodds, A.W., Magnadottir, B., 2004a. The ontogeny of complement component C3 in
965 Atlantic Cod (*Gadus morhua* L.)—an immunohistochemical study. Fish Shellfish Immunol. 16, 359-367.

966 Lange, S., Bambir, S., Dodds, A.W., Magnadottir, B., 2004b. An immunohistochemical study on complement
967 component C3 in juvenile Atlantic halibut (*Hippoglossus hippoglossus* L.). Dev. Comp. Immunol. 28(6), 593-601.

968 Lange, S., Dodds, A.W., Gudmundsdóttir, S., Bambir, S.H., Magnadottir, B., 2005. The ontogenic transcription of
969 complement component C3 and Apolipoprotein A-I tRNA in Atlantic cod (*Gadus morhua* L.) - a role in
970 development and homeostasis? Dev. Comp. Immunol. 29(12), 1065-77.

971 Lange, S., Bambir, S.H., Dodds, A.W., Bowden, T., Bricknell, I., Espelid, S., Magnadottir, B., 2006. Complement
972 component C3 transcription in Atlantic halibut (*Hippoglossus hippoglossus* L.) larvae. Fish Shellfish Immunol.
973 20(3), 285-94.

974 Lange, S., 2016. Peptidylarginine deiminases as drug targets in neonatal hypoxic-ischemic encephalopathy.
975 Front. Neurol. 7, 22.

976 Lange, S., Kraev,I., Magnadóttir, B., Dodds, A.W., 2019. Complement component C4-like protein in Atlantic cod
977 (*Gadus morhua* L.) - Detection in ontogeny and identification of post-translational deimination in serum and
978 extracellular vesicles. Dev. Comp. Immunol. 101, 103437.

979 Lange, S., Gögel, S., Leung, K.Y., Vernay, B., Nicholas, A.P., Causey, C.P., Thompson, P.R., Greene, N.D., Ferretti,
980 P., 2011. Protein deiminases: new players in the developmentally regulated loss of neural regenerative ability.
981 Dev. Biol. 355(2), 205-14.

982 Lange, S., Rocha-Ferreira, E., Thei, L., Mawjee, P., Bennett, K., Thompson, P.R., Subramanian, V., Nicholas, A.P.,
983 Peebles, D., Hristova, M., Raivich, G., 2014. Peptidylarginine deiminases: novel drug targets for prevention of
984 neuronal damage following hypoxic ischemic insult (HI) in neonates. J. Neurochem. 130 (4), 555-62.

985 Lange, S., Gallagher, M., Kholia, S., Kosgodage, U.S., Hristova, M., Hardy, J., Inal, J.M., 2017. Peptidylarginine
986 deiminases-roles in cancer and neurodegeneration and possible avenues for therapeutic intervention via
987 modulation of exosome and microvesicle (EMV) release? Int. J. Mol. Sci. 18(6), pii E1196.

988 Levin, J., Bang, F.B., 1964. The role of endotoxin in the extracellular coagulation of *Limulus* blood. Bull. Johns
989 Hopkins Hosp. 115, 265-74.

990 Levin, J., Bang, F.B., 1968. Clottable protein in *Limulus*; its localization and kinetics of its coagulation by
991 endotoxin. Thromb. Diath. Haemorrh. 19 (1), 186-97.

992 Liang, Y., Pan, B., Alam, H.B., Deng, Q., Wang, Y., Chen, E., Liu, B., Tian, Y., Williams, A.M., Duan, X., Wang, Y.,
993 Zhang, J., Li, Y., 2018. Inhibition of peptidylarginine deiminase alleviates LPS-induced pulmonary dysfunction
994 and improves survival in a mouse model of lethal endotoxemia. Eur. J. Pharmacol. 833, 432-440.

995 Lincet, H., Icard, P., 2015. How do glycolytic enzymes favour cancer cell proliferation by nonmetabolic
996 functions? Oncogene. 34 (29), 3751-9.

997 Liu, T.Y., Minetti, C.A., Fortes-Dias, C.L., Liu, T., Lin, L., Lin, Y., 1994. C-reactive proteins, limunectin,
998 lipopolysaccharide-binding protein, and coagulin. Molecules with lectin and agglutinin activities from *Limulus*
999 *polyphemus*. Ann. N.Y. Acad. Sci. 712, 146-54.

1000 Low, D.H., Ang, Z., Yuan, Q., Frecer, V., Ho, B., Chen, J., Ding, J.L. , 2009. A novel human tectonin protein with
1001 multivalent beta-propeller folds interacts with ficolin and binds bacterial LPS. PLoS One. 4(7), e6260.

1002 Low, D.H., Frecer, V., Le Saux, A., Srinivasan, G.A., Ho, B., Chen, J., Ding, J.L., 2010. Molecular interfaces of the
1003 galactose-binding protein Tectonin domains in host-pathogen interaction. J. Biol. Chem. 285 (13), 9898-907.

Lu, Y., Yu, S.S., Zong, M., Fan, S.S., Lu, T.B., Gong, R.H., Sun, L.S., Fan, L.Y., 2017. Glucose-6-Phosphate Isomerase (G6PI) Mediates Hypoxia-Induced Angiogenesis in Rheumatoid Arthritis. Sci.Rep. 7, 40274.

MacPherson, J.C., Jacobs, R.S., 2000. An 18.5 kDa protein from the amebocyte of *Limulus polyphemus*, homologous to the previously described amebocyte aggregation factor, expresses alternative phospholipase A2 activity. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 127 (1), 31-44.

Magnadottir, B., Hayes, P., Hristova, M., Bragason, B.T., Nicholas, A.P., Dodds, A.W., Guðmundsdóttir, S., Lange, S., 2018a. Post-translational protein deimination in cod (*Gadus morhua* L.) ontogeny – novel roles in tissue remodelling and mucosal immune defences? Dev. Comp. Immunol. 87, 157-170.

Magnadottir, B., Hayes, P., Gísladóttir, B., Bragason, B.Þ., Hristova, M., Nicholas, A.P., Guðmundsdóttir, S., Lange, S., 2018b. Pentraxins CRP-I and CRP-II are post-translationally deiminated and differ in tissue specificity in cod (*Gadus morhua* L.) ontogeny. Dev. Comp. Immunol. 87, 1-11.

Magnadottir, B., Bragason, B.T., Bricknell, I.R., Bowden, T., Nicholas, A.P., Hristova, M., Guðmundsdóttir, S., Dodds, A.W., Lange, S., 2019a. Peptidylarginine deiminase and deiminated proteins are detected throughout early halibut ontogeny - complement components C3 and C4 are post-translationally deiminated in halibut (*Hippoglossus hippoglossus* L.). Dev. Comp. Immunol. 92, 1-19.

Magnadottir, B., Kraev, I., Guðmundsdóttir, S., Dodds, A.W., Lange, S., 2019b. Extracellular vesicles from cod (*Gadus morhua* L.) mucus contain innate immune factors and deiminated protein cargo. Dev. Comp. Immunol. 99, 103397.

Magnadottir, B., Uysal-Onganer, P., Kraev, I., Dodds, A.W., Gudmundsdottir, S., Lange, S, 2020a. Extracellular vesicles, deiminated protein cargo and microRNAs are novel serum biomarkers for environmental rearing temperature in Atlantic cod (*Gadus morhua* L.). Aquaculture Rep. 16, 100245.

Magnadottir, B, Uysal-Onganer, P, Kraev, I, Svansson, V, Hayes, P, Lange, S., 2020b. Deiminated proteins and extracellular vesicles - novel serum biomarkers in whales and orca. Comp. Biochem. Physiol. Part D Genomics Proteomics. 34, 100676.

Magnadottir, B., Uysal-Onganer, P., Kraev, I., Svansson, V., Skírnisson, K., Lange, S., 2020c. Deiminated proteins and extracellular vesicles as novel biomarkers in pinnipeds: Grey seal (*Halichoerus gryptus*) and harbour seal (*Phoca vitulina*).Biochimie. 171-172, 79-90.

Martinez-Prat, L., Palterer, B., Vitiello, G., Parronchi, P., Robinson, W.H., Mahler, M., 2019. Autoantibodies to protein-arginine deiminase (PAD) 4 in rheumatoid arthritis: immunological and clinical significance, and potential for precision medicine. Expert Rev. Clin. Immunol. 15 (10), 1073-1087.

Martins, E., Santos, R.S., Bettencourt, R., 2015. *Vibrio diabolicus* challenge in *Bathymodiolus azoricus* populations from Menez Gwen and Lucky Strike hydrothermal vent sites. Fish Shellfish Immunol. 47 (2), 962-77

Mastronardi, F.G., Wood, D.D., Mei, J., Raijmakers, R., Tseveleki, V., Dosch, H.M., Probert, L., Casaccia-Bonnefil, P., Moscarello, M.A., 2006. Increased citrullination of histone H3 in multiple sclerosis brain and animal models of demyelination: a role for tumor necrosis factor-induced peptidylarginine deiminase 4 translocation. J. Neurosci. 26 (44), 11387-96.

Matsumoto, I., Kurata, I., Ohyama, A., Kawaguchi, H., Ebe, H., Osada, A., Kondo, Y., Tsuboi, H., Sumida, T., 2020. Revisit of autoimmunity to glucose-6-phosphate isomerase in experimental and rheumatoid arthritis. Mod. Rheumatol. 30 (2), 232-238.

Méchin, M.C., Takahara, H., Simon, M., 2020. Deimination and peptidylarginine deiminases in skin physiology and diseases. Int. J. Mol. Sci. 21 (2), pii E566.

Minetti, C.A., Lin, Y.A., Cislo, T., Liu, T.Y., 1991. Purification and characterization of an endotoxin-binding protein with protease inhibitory activity from *Limulus* amebocytes. J. Biol. Chem. 266 (31), 20773-80.

Miranda, M., 1976. Specific activities of 6-phosphogluconate dehydrogenase, glucose-6-phosphate dehydrogenase and glucose-6-phosphate isomerase during Bufo bufo development. Biochim. Biophys. Acta. 422 (2), 249-53.

Mohanan, S., Cherrington, B.D., Horibata, S., McElwee, J.L., Thompson, P.R., Coonrod, S.A., 2012. Potential role of peptidylarginine deiminase enzymes and protein citrullination in cancer pathogenesis. Biochem. Res. Int. 2012, 895343.

Mondal, S., Thompson, P.R., 2019. Protein arginine deiminases (PADs): Biochemistry and chemical biology of protein citrullination. Acc. Chem. Res. 52 (3), 818-832.

Montaner-Tarbes, S., Pujol, M., Jabbar, T., Hawes, P., Chapman, D., Portillo, H.D., Fraile, L., Sánchez-Cordón, P.J., Dixon, L., Montoya, M., 2019. Serum-derived extracellular vesicles from African swine fever virus-infected pigs selectively recruit viral and porcine proteins. Viruses. 11 (10), pii E882.

Moscarello, M.A., Lei H., Mastronardi, F.G., Winer, S., Tsui, H., Li, Z., Ackerley, C., Zhang, L., Raijmakers, R., Wood, D.D., 2013. Inhibition of peptidyl-arginine deiminases reverses protein-hypercitrullination and disease in mouse models of multiple sclerosis. Dis. Model Mech. 6 (2), 467-78.

Muraro, S.P., De Souza, G.F., Gallo, S.W., Da Silva, B.K., De Oliveira, S.D., Vinolo, M.A.R., Saraiva, E.M., Porto, B.N., 2018. Respiratory Syncytial Virus induces the classical ROS-dependent NETosis through PAD-4 and necroptosis pathways activation. Sci. Rep. 8 (1), 14166.

Nagai, T., Kawabata, S., Shishikura, F., Sugita, H., 1999. Purification, characterization, and amino acid sequence of an embryonic lectin in perivitelline fluid of the horseshoe crab. J. Biol. Chem. 274 (53), 37673-8.

Nakao, M., Kato-Unoki, Y., Nakahara, M., Mutsuro, J., Somamoto, T., 2006. Diversified components of the bony fish complement system: more genes for robuster innate defense? Adv. Exp. Med. Biol. 586, 121-138.

Nakao, M., Tsujikura, M., Ichiki, S., Vo, T.K., Somamoto, T., 2011. The complement system in teleost fish: progress of post-homolog-hunting researches. Dev. Comp. Immunol. 35 (12), 1296-308.

Ng, P.M., Le Saux, A., Lee, C.M., Tan, N.S., Lu, J., Thiel, S., Ho, B., Ding, J.L., 2007. C-reactive protein collaborates with plasma lectins to boost immune response against bacteria. EMBO J. 26 (14), 3431-40.

Nicholas, A.P., Whitaker, J.N., 2002. Preparation of a monoclonal antibody to citrullinated epitopes: its characterization and some applications to immunohistochemistry in human brain. Glia. 37(4), 328-36.

Nomura, K., 1992. Specificity and mode of action of the muscle-type protein-arginine deiminase. Arch. Biochem. Biophys. 293 (2), 362-9.

Novák, L., Zubáčová, Z., Karnkowska, A., Kolisko, M., Hroudová, M., Stairs, C.W., Simpson, A.G., Keeling, P.J., Roger, A.J., Čepička, I., Hampl, V., 2016. Arginine deiminase pathway enzymes: evolutionary history in metamonads and other eukaryotes. BMC Evol. Biol. 16(1), 197.

Novitsky, T. J., 1994. *Limulus* amebocyte lysate (LAL) detection of endotoxin in human blood. J. Endotox. Res. 1 (4), 253-263.

Ogawa, M., Yoshikawa, Y., Kobayashi, T., Mimuro, H., Fukumatsu, M., Kiga, K., Piao, Z., Ashida, H., Yoshida, M., Kakuta, S., Koyama, T., Goto, Y., Nagatake, T., Nagai, S., Kiyono, H., Kawalec, M., Reichhart, J.M., Sasakawa, C. A ., 2011.Tecpr1-dependent selective autophagy pathway targets bacterial pathogens. Cell Host Microbe. 9 (5), 376-89.

Olmos, G., López-Ongil, S., Ruiz Torres, M.P., 2017. Integrin-linked kinase: A new actor in the ageing process? Exp. Gerontol. 100, 87-90.

1087 Osaki, T., Kawabata, S., 2004. Structure and function of coagulogen, a clottable protein in horseshoe crabs.
1088 Cell. Mol. Life Sci. 61 (11), 1257-65.

1089 Pamenter, M.E., Uysal-Onganer, P., Huynh, K.W., Kraev, I., Lange, S., 2019. Post-translational deimination of
1090 immunological and metabolic protein markers in plasma and extracellular vesicles of naked mole-Rat
1091 (*Heterocephalus glaber*). Int. J Mol. Sci. 20 (21), pii E5378

1092 Pan, B., Alam, H.B., Chong, W., Mobley, J., Liu, B., Deng, Q., Liang, Y., Wang, Y., Chen, E., Wang, T., Tewari, M.,
1093 Li, Y., 2017. CitH3: a reliable blood biomarker for diagnosis and treatment of endotoxic shock. Sci. Rep. 7.(1),
1094 8972.

1095 Pasupuleti, M., Walse, B., Nordahl, E.A., Mörgelin, M., Malmsten, M., Schmidtchen, A., 2007. Preservation of
1096 antimicrobial properties of complement peptide C3a, from invertebrates to humans. J. Biol. Chem. 282 (4),
1097 2520-8.

1098 Pathak, A, Agrawal, A., 2019. Evolution of C-Reactive Protein. Front. Immunol. 10, 943.

1099 Paul, I., Mandal, C., Allen, A.K., Mandal, C., 2001. Glycosylated molecular variants of C-reactive proteins from
1100 the major carp *Catla catla* in fresh and polluted aquatic environments. Glycoconj. J. 18, 547-56.

1101 Paupe, V., Prudent, J., 2018. New insights into the role of mitochondrial calcium homeostasis in cell migration.
1102 Biochem. Biophys. Res. Commun. 500 (1), 75-86.

1103 Phillips, R.A., Kraev, I., Lange, S., 2020. Protein deimination and extracellular vesicle profiles in Antarctic
1104 seabirds. Biology (Basel). 9 (1), pii: E15.

1105 Pierrakakis, S., Xepapadakis, G., Mega, A.M., Megas, T., Katergiannakis, V.G., Perpirakis, G., Filippakis, M.,
1106 1990. Blood endotoxin in acute pancreatitis using *limulus* amebocyte lysate test (LAL test). Minerva Chir. 45
1107 (5), 295-301.

1108 Prilusky, J., Felder, C.E., Zeev-Ben-Mordehai, T., Rydberg, E.H., Man, O., Beckmann, J.S., Silman, I, Sussman, J.L.,
1109 2005. FoldIndex: a simple tool to predict whether a given protein sequence is intrinsically unfolded.
1110 Bioinformatics. 21 (16), 3435-8.

1111 Puri, B.K., 2020. Calcium Signaling and Gene Expression. Adv. Exp. Med. Biol. 1131, 537-545.

1112 Raman, A., Reif, G.A., Dai, Y., Khanna, A., Li, X., Astleford, L., Parnell, S.C., Calvet, J.P., Wallace, D.P., 2017.
1113 Integrin-Linked Kinase Signaling Promotes Cyst Growth and Fibrosis in Polycystic Kidney Disease. J. Am. Soc.
1114 Nephrol. 28 (9), 2708-2719.

1115 Ramirez, S.H., Andrews, A.M., Paul, D., Pachter, J.S., 2018. Extracellular vesicles: mediators and biomarkers of
1116 pathology along CNS barriers. Fluids Barriers CNS. 15(1), 19.

1117 Rebl, A, Köllner, B, Anders, E, Wimmers, K, Goldammer, T., 2010. Peptidylarginine deiminase gene is
1118 differentially expressed in freshwater and brackish water rainbow trout. Mol. Biol. Rep. 37 (5), 2333-9.

1119 Romero, V., Darrah, E., Andrade, F., 2019. Peptidylarginine deiminase type 2 and 4 generate distinct patterns
1120 of rheumatoid arthritis autoantigens during perforin-induced cell damage. Arthritis Rheumatol. doi:
1121 10.1002/art.41196.

1122 Ruiz-Romero, C., Lam, M.P.Y., Nilsson, P., Önnerfjord, P., Utz, P.J., Van Eyk, J.E., Venkatraman, V., Fert-Bober,
1123 J., Watt, F.E., Blanco, F.J., 2019. Mining the Proteome Associated with Rheumatic and Autoimmune Diseases. J.
1124 Proteome Res. 18(12), 4231-4239.

1125 Saha, P., Yeoh, B.S., Xiao, X., Golonka, R.M., Singh, V., Wang, Y., Vijay-Kumar, M., 2019. PAD4-dependent NETs
1126 generation are indispensable for intestinal clearance of Citrobacter rodentium. Mucosal Immunol. 12 (3), 761-
1127 771.

Sase, T., Arito, M., Onodera, H., Omoteyama, K., Kurokawa, M.S., Kagami, Y., Ishigami, A., Tanaka, Y., Kato, T., 2017. Hypoxia-induced production of peptidylarginine deiminases and citrullinated proteins in malignant glioma cells. Biochem. Biophys. Res. Commun. 482 (1), 50-56.

Seri, Y., Shoda, H., Suzuki, A., Matsumoto, I., Sumida, T., Fujio, K., Yamamoto, K., 2015. Peptidylarginine deiminase type 4 deficiency reduced arthritis severity in a glucose-6-phosphate isomerase-induced arthritis model. Sci. Rep. 5, 13041.

Sharma, M., Damgaard, D., Senolt, L., Svensson, B., Bay-Jensen, A.C., Nielsen, C.H., Hägglund, P., 2019. Expanding the citrullinome of synovial fibrinogen from rheumatoid arthritis patients. J. Proteomics. 208, 103484.

Simpson, S.D., Ramsdell, J.S., Watson, Iii W.H., Chabot, C.C., 2017. The Draft Genome and Transcriptome of the Atlantic Horseshoe Crab, *Limulus polyphemus*. Int. J. Genomics. 2017, 7636513.

Šimundić, M., Švara, T., Štukelj, R., Krek, J.L., Gombač, M., Kralj-Iglič, V., Tozon, N., 2019. Concentration of extracellular vesicles isolated from blood relative to the clinical pathological status of dogs with mast cell tumours. Vet. Comp. Oncol. 17 (4), 456-464.

Singh, D., Arora, R., Kaur, P., Singh, B., Mannan, R., Arora, S., 2017. Overexpression of hypoxia-inducible factor and metabolic pathways: possible targets of cancer. Cell. Biosci. 7, 62.

Sottrup-Jensen, L., Borth, W., Hall, M., Quigley, J.P., Armstrong, P.B., 1990. Sequence similarity between alpha 2-macroglobulin from the horseshoe crab, *Limulus polyphemus*, and proteins of the alpha 2-macroglobulin family from mammals. Comp. Biochem. Physiol. B. 96 (3), 621-5.

Sottrup-Jensen, L., Stepanik, T.M., Kristensen, T., Lønblad, P.B., Jones, C.M., Wierzbicki, D.M., Magnusson, S., Domdey, H., Wetsel, R.A., Lundwall,A., et al., 1985. Common evolutionary origin of alpha 2-macroglobulin and complement components C3 and C4. Proc. Natl. Acad. Sci. USA. 82 (1), 9-13.

Stobernack,T., du Teil Espina, M., Mulder, L.M., Palma Medina, L.M., Piebenga, D.R. et al., 2018. A secreted bacterial peptidylarginine deiminase can neutralize human innate immune defenses. mBio. 9 (5), pii, e01704-18.

Sudhakar, P., Jacomin, A.C., Hautefort, .I, Samavedam, S., Fatemian, K., Ari, E., Gul, L., Demeter, A., Jones, E., Korcsmaros, T., Nezis, I.P., 2019.Targeted interplay between bacterial pathogens and host autophagy. Autophagy. 15 (9), 1620-1633.

Sunyer, J.O., Lambris, J.D, 1998. Evolution and diversity of the complement system of poikilothermic vertebrates. Immunol. Rev. 166, 39-57.

Svärd, A., Renvert, S., Sanmartin Berglund, J., Persson, R.G., Söderlin, M., 2019. Antibodies to citrullinated peptides in serum and saliva in patients with rheumatoid arthritis and their association to periodontitis. Clin. Exp. Rheumatol. 2019 Nov 16. [Epub ahead of print]

Swarnakar, S., Quigley, J.P., Armstrong, P.B., 1996. The Plasma-Based Cytolytic System of the American Horseshoe Crab, *Limulus polyphemus*: Cooperative Interaction of the Sialic Acid-Binding Lectin Limulin and Thiol Ester-Reacted α2-Macroglobulin. Biol. Bull. 191 (2), 298.

Swarnakar, S., Asokan, R., Quigley, J.P., Armstrong, P.B., 2000. Binding of alpha2-macroglobulin and limulin: regulation of the plasma haemolytic system of the American horseshoe crab, *Limulus*. Biochem J. 347 (3), 679-85.

Tagawa, K., Yoshihara, T., Shibata, T., Kitazaki, K., Endo, Y., Fujita, T., Koshiba, T., Kawabata, S., 2012. Microbe-specific C3b deposition in the horseshoe crab complement system in a C2/factor B-dependent or -independent manner. PLoS One. 7 (5), e36783.

Tarcsa, E., Marekov, L.N., Mei, G., Melino, G., Lee, S.C., Steinert, P.M., 1996 Protein unfolding by peptidylarginine deiminase. Substrate specificity and structural relationships of the natural substrates trichohyalin and filaggrin. J. Biol. Chem. 271 (48), 30709-16.

Théry, C., Witwer, K.W., Aikawa, E., Alcaraz, M.J., Anderson, J.D., Andriantsitohaina, R., et al., 2018. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): A position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. J. Extracell. Vesicles. 7, 1535750.

Tilvawala, R., Nguyen, S.H., Maurais, A.J., Nemmara, V.V., Nagar, M., Salinger, A.J., Nagpal, S., Weerapana, E., Thompson, P.R, 2018. The Rheumatoid Arthritis-Associated Citrullinome. Cell. Chem. Biol. 25 (6), 691-704.e6.

Turchinovich, A., Drapkina, O., Tonevitsky, A., 2019. Transcriptome of extracellular vesicles: State-of-the-art. Front. Immunol. 10, 202.

Umeda, N., Matsumoto, I., Ito, I., Kawasaki, A., Tanaka, Y., Inoue, A., Tsuboi, H., Suzuki, T., Hayashi, T., Ito, S., Tsuchiya, N., Sumida, T., 2013. Anti-citrullinated glucose-6-phosphate isomerase peptide antibodies in patients with rheumatoid arthritis are associated with HLA-DRB1 shared epitope alleles and disease activity. Clin. Exp. Immunol. 172 (1), 44-53.

Uversky, V.N., Gillespie, J.R., Fink, A.L., 2000. Why are "natively unfolded" proteins unstructured under physiologic conditions? Proteins. 41 (3), 415-27.

Uysal-Onganer, P., MacLatchy, A., Mahmoud, R., Kraev, I., Thompson, P.R., Inal, J., Lange, S., 2020. Peptidylarginine deiminase isozyme-specific PAD2, PAD3 and PAD4 inhibitors differentially modulate extracellular vesicle signatures and cell invasion in two glioblastoma multiforme cell lines. Int. J. Mol. Sci. 21 (4), pii E1495.

Vagner, T., Chin, A., Mariscal, J., Bannykh, S., Engman, D.M., Di Vizio, D., 2019. Protein composition reflects extracellular vesicle heterogeneity. Proteomics. 19 (8), e1800167.

Vossenaar, E.R., Zendman, A.J., van Venrooij, W.J., Pruijn, G.J., 2003. PAD, a growing family of citrullinating enzymes: genes, features and involvement in disease. Bioessays. 25 (11), 1106-18.

Wang, S., Wang, Y., 2013. Peptidylarginine deiminases in citrullination, gene regulation, health and pathogenesis. Biochim. Biophys. Acta. 1829 (10), 1126-35.

Wang, D., Li, S., Li, F., 2013. Screening of genes regulated by Relish in Chinese shrimp *Fenneropenaeus chinensis*. Dev. Comp. Immunol. 41 (2), 209-16.

Witalison, E.E., Thompson, P.R., Hofseth, L.J., 2015. Protein arginine deiminases and associated citrullination: physiological functions and diseases associated with dysregulation. Curr. Drug Targets. 16(7), 700-10.

Watson, W.H. 3rd., Bedford L., Chabot C.C., 2008. Rhythms of locomotion expressed by *Limulus polyphemus*, the American horseshoe crab: II. relationship to circadian rhythms of visual sensitivity. Biol Bull. 215 (1), 46-56.

Wu, D., Sun, L., Li, C.H., Yang, L., Zhao, J.X., Liu, X.Y. , 2016. Significance of antibodies to the citrullinated glucose-6-phosphate isomerase peptides in rheumatoid arthritis. Beijing Da Xue Xue Bao Yi Xue Ban. 48 (6), 937-941.

Wong, S.L., Wagner, D.D., 2018. Peptidylarginine deiminase 4: a nuclear button triggering neutrophil extracellular traps in inflammatory diseases and aging. FASEB. J., fj201800691R.

Wong, S.L., Demers, M., Martinod, K., Gallant, M., Wang, Y., Goldfine, A.B., Kahn, C.R., Wagner, D.D., 2015. Diabetes primes neutrophils to undergo NETosis, which impairs wound healing. Nat. Med. 21 (7), 815-9.

Xia, X., 2000. Phylogenetic relationship among horseshoe crab species: effect of substitution models on phylogenetic analyses. Syst. Biol. 49 (1), 87-100.

Yang, L., Tan, D., Piao, H., 2016. Myelin Basic Protein Citrullination in Multiple Sclerosis: A potential therapeutic target for the pathology. Neurochem, Res. 41 (8), 1845-56.

Yang, X.M., Duan, C.G., Zhang, J., Qu, X.J., Wang, Y.S., 2018. Integrin-Linked Kinase Controls Choroidal Neovascularization by Recruitment of Endothelial Progenitor Cells. Invest. Ophthalmol. Vis. Sci. 59 (5), 1779-1789.

Ying, S.C., Marchalonis, J.J., Gewurz, A.T., Siegel, J.N., Jiang, H., Gewurz, B.E., Gewurz, H., 1992. Reactivity of anti-human C-reactive protein (CRP) and serum amyloid P component (SAP) monoclonal antibodies with limulin and pentraxins of other species. Immunology. 76 (2), 324-30.

Yu, R., Li, C., Sun, L., Jian, L., Ma, Z., Zhao, J., Liu, X., 2018. Hypoxia induces production of citrullinated proteins in human fibroblast-like synoviocytes through regulating HIF1α. Scand. J. Immunol. 87 (4), e12654.

Ždralević, M., Vučetić, M., Daher, B., Marchiq, I., Parks, S.K., Pouysségur, J., 2018. Disrupting the 'Warburg effect' re-routes cancer cells to OXPHOS offering a vulnerability point via 'ferroptosis'-induced cell death. Adv. Biol. Regul. 68,55-63.

Zheng, C.C., Hu, H.F., Hong, P., Zhang, Q.H., Xu, W.W., He, Q.Y., Li, B., 2019. Significance of integrin-linked kinase (ILK) in tumorigenesis and its potential implication as a biomarker and therapeutic target for human cancer. Am. J. Cancer Res. 9(1), 186-197.

Zhu, Y., Thangamani, S., Ho, B., Ding, J.L. , 2005. The ancient origin of the complement system. EMBO J. 24 (2), 382-94.

Zimmer, J., Hobkirk, J., Mohamed, F., Browning, M.J., Stover, C.M., 2015. On the Functional Overlap between Complement and Anti-Microbial Peptides. Front. Immunol. 5, 689.

Figure legends

Figure 1. Extracellular vesicle profiling in horseshoe crab haemolymph serum. A. Nanoparticle tracking analysis shows size distribution of serum-EVs from *Limulus polyphemus* in the size range of mainly 40 to 300 nm, with main peak at approximately 123 nm. **B.** Western blotting analysis confirms that horseshoe crab serum-EVs are positive for the phylogenetically conserved EV-specific marker CD63, while cross-reaction with Flot-1 was low. **C.** Transmission electron microscopy (TEM) analysis of horseshoe crab haemolymph serum-derived EVs; scale bar is 20 nm in all figures.

Figure 2. PAD and Deiminated proteins in horseshoe crab haemolymph serum. **A.** A PAD homologue was identified in horseshoe crab serm at an expected ≈70 kDa size, via cross-reaction with the anti-human PAD2 antibody. **B.** Total deiminated proteins were identified in horseshoe crab serum, using the pan-deimination specific F95 antibody. **C.** F95-enriched IP fractions from horseshoe crab serum, shown by silver-staining, reveal multiple protein bands in the size range of 15-200 kDa.

Figure 3. Deiminated (F95-enriched) protein hits identified in horseshoe crab haemolymph serum. The Venn diagram represents species-specific hits identified for deiminated proteins in horseshoe

crab (*Limulus polyphemus*) serum and overlap with protein candidates identified for other Merostomata, the mangrove horseshoe crab (*Carcinoscorpius rotundicauda*) and the tri-spine horseshoe crab (*Tachypleus tridentatus*).

Figure 4. Protein-protein interaction networks of all deiminated proteins identified in horseshoe crab haemolymph serum. Reconstruction of protein-protein interaction networks for identified deiminated protein candidates in horseshoe crab, based on known and predicted interactions, using mouse homologue identifiers and STRING analysis. **A. KEGG pathways** relating to the identified deiminated proteins (see colour code for identified pathways highlighted in the figure). **B. GO biological pathways** relating to the identified proteins are highlighted (see colour code included in the figure). **C. GO MOLECULAR pathways** relating to the identified proteins are highlighted (see colour code included in the figure). **D. UniProt keywords** relating to the identified proteins are highlighted (see colour code included in the figure). **E. Reactome pathways** relating to the identified proteins are highlighted (see colour code included in the figure). **F. SMART protein domains** relating to the identified proteins are highlighted (see colour code included in the figure). **G. PFAM protein domains** relating to the identified proteins are highlighted (see colour code included in the figure). **H. INTERPRO protein domains and features** relating to the identified proteins are highlighted (see colour code included in the figure). Coloured lines indicate whether protein interactions are identified via known interactions (curated databases, experimentally determined), predicted interactions (gene neighbourhood, gene fusion, gene co-occurrence) or via text mining, co-expression or protein homology (see the colour key for connective lines included in the figure).

Figure 1

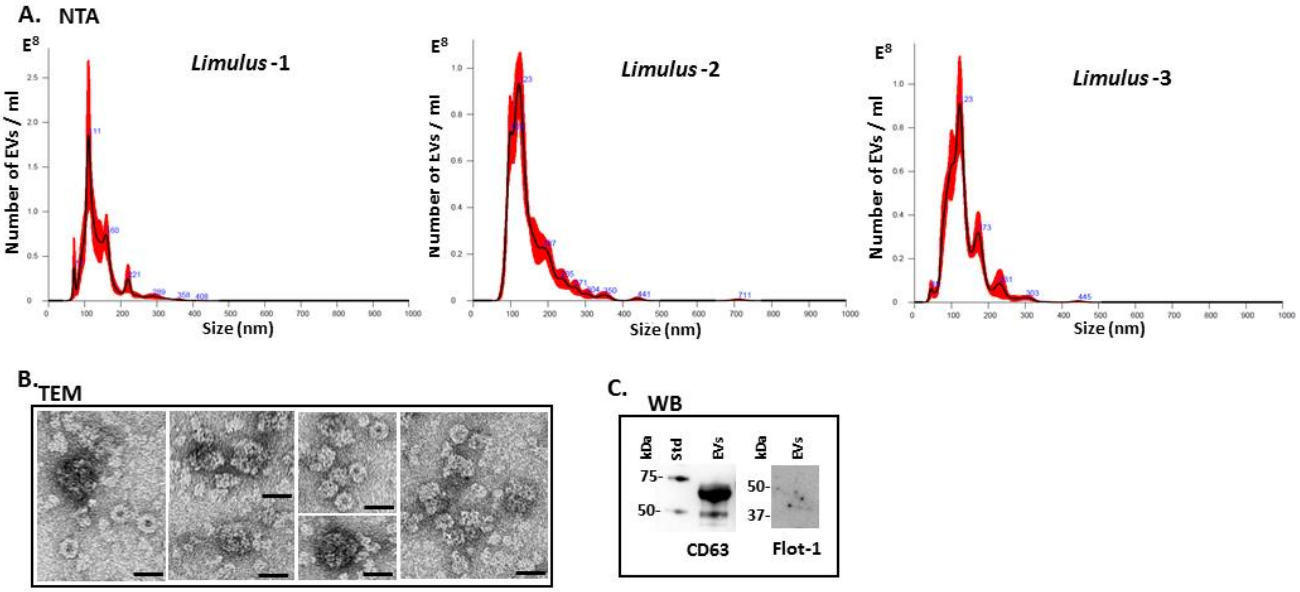


Figure 2.

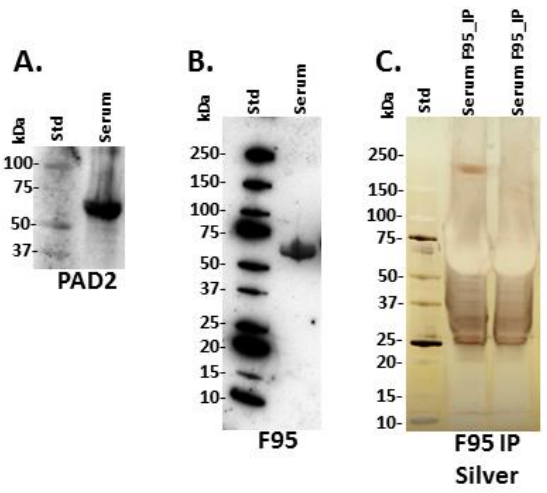


Figure 3.

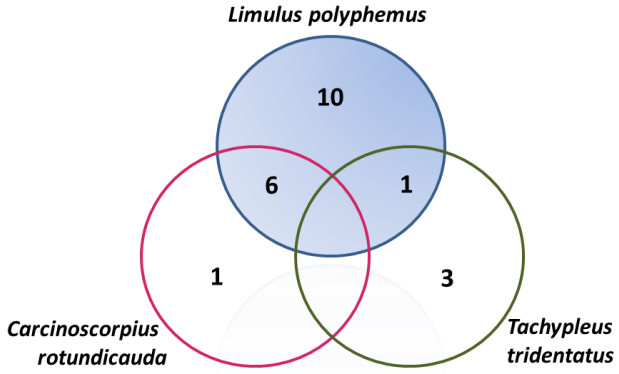
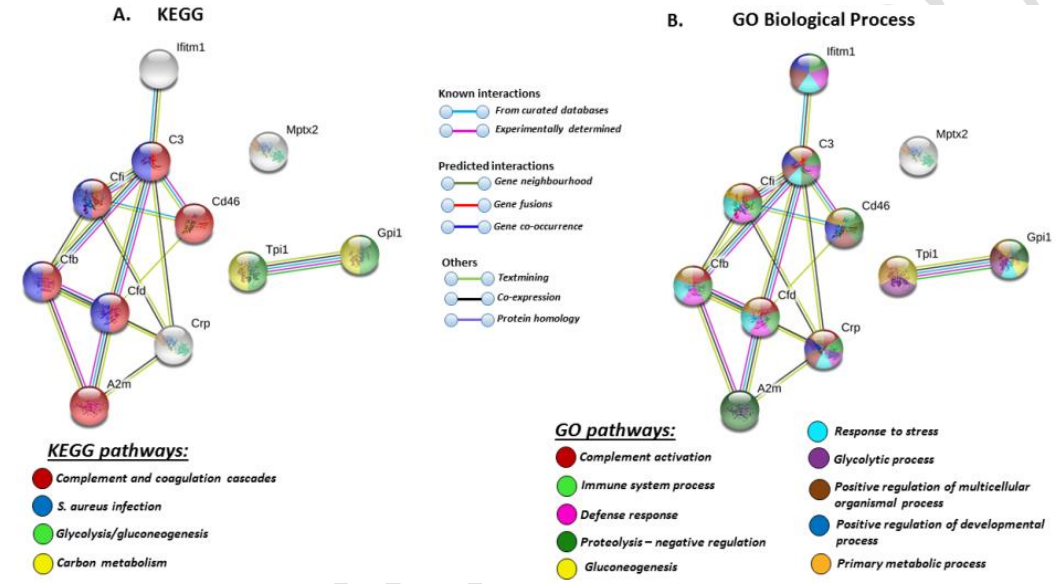
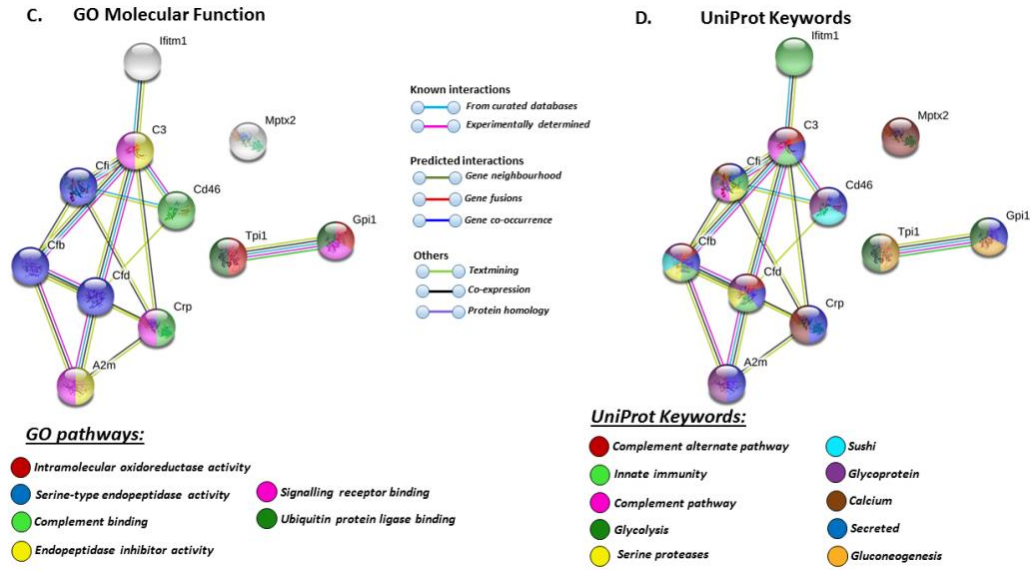
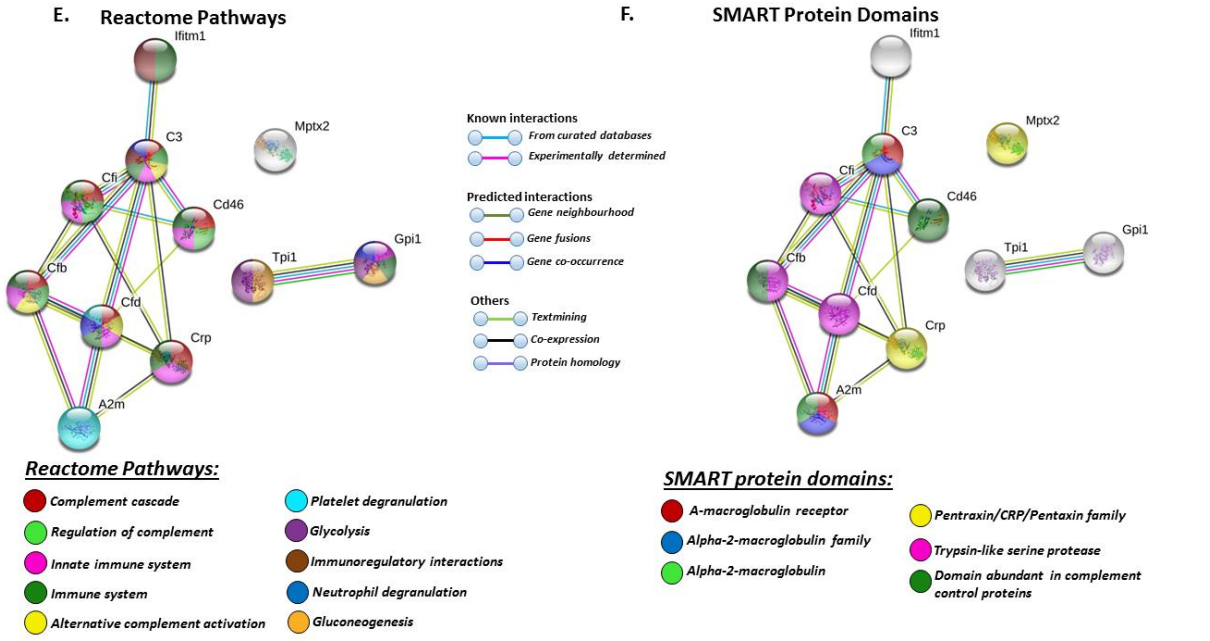


Figure 4.







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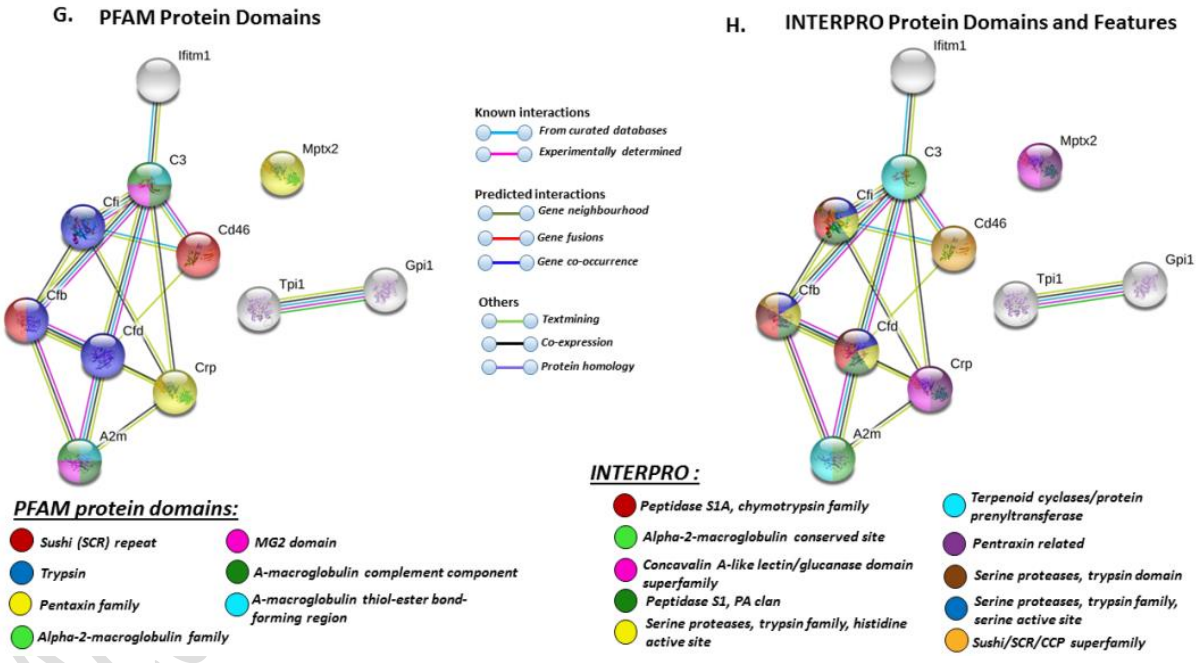

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