



Short communication

Deiminated proteins in extracellular vesicles and plasma of nurse shark (*Ginglymostoma cirratum*) - Novel insights into shark immunityMichael F. Criscitiello^{a,b}, Igor Kraev^c, Sigrun Lange^{d,*}^a Comparative Immunogenetics Laboratory, Department of Veterinary Pathobiology, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX, 77843, USA^b Department of Microbial Pathogenesis and Immunology, College of Medicine, Texas A&M Health Science Center, Texas A&M University, College Station, TX, 77843, USA^c School of Life, Health and Chemical Sciences, The Open University, Walton Hall, MK7 6AA, UK^d Tissue Architecture and Regeneration Research Group, School of Life Sciences, University of Westminster, London, W1W 6UW, UK

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ABSTRACT

Peptidylarginine deiminases (PADs) are phylogenetically conserved calcium-dependent enzymes which post-translationally convert arginine into citrulline in target proteins in an irreversible manner, causing functional and structural changes in target proteins. Protein deimination causes generation of neo-epitopes, affects gene regulation and also allows for protein moonlighting. Extracellular vesicles are found in most body fluids and participate in cellular communication via transfer of cargo proteins and genetic material. In this study, post-translationally deiminated proteins and extracellular vesicles (EVs) are described for the first time in shark plasma. We report a poly-dispersed population of shark plasma EVs, positive for phylogenetically conserved EV-specific markers and characterised by TEM. In plasma, 6 deiminated proteins, including complement and immunoglobulin, were identified, whereof 3 proteins were found to be exported in plasma-derived EVs. A PAD homologue was identified in shark plasma by Western blotting and detected an expected 70 kDa size. Deiminated histone H3, a marker of neutrophil extracellular trap formation, was also detected in nurse shark plasma. This is the first report of deiminated proteins in plasma and EVs, highlighting a hitherto unrecognized post-translational modification in key immune proteins of innate and adaptive immunity in shark.

1. Introduction

Peptidylarginine deiminases (PADs) are phylogenetically conserved calcium-dependent enzymes which post-translationally convert arginine into citrulline in target proteins in an irreversible manner, causing functional and structural changes in target proteins [1–3,12]. Protein deimination affects gene regulation, causes generation of neoepitopes [4,5] and may also allow for protein moonlighting, an evolutionary acquired phenomenon facilitating proteins to exhibit several physiologically relevant functions within one polypeptide chain [6–8]. PADs are widely studied in cancer, autoimmune and neurodegenerative diseases [3–5] and crucial roles have also been described in CNS regeneration [9,10]. PADs have been identified throughout phylogeny from bacteria to mammals, with 5 tissue specific PAD isozymes in mammals, 3 in chicken, and 1 in bony fish [8,11,12,13]. In teleost fish, recent studies have identified novel roles for PADs and deiminated proteins during early teleost ontogeny and in mucosal and innate immunity [8,13–15]. In elasmobranchs, a PAD has been described in whale shark (*Rhincodon*

typus; XP_020374364) but no studies on protein deimination have hitherto been carried out in sharks.

Extracellular vesicles (EVs) are found in most body fluids and participate in cellular communication via transfer of cargo proteins and genetic material [4,16–19]. EVs in plasma can also be useful biomarkers to reflect health status [20,21]. Work on EVs has hitherto mainly been in the context of human pathologies while some studies on EVs and EV cargo have been recently performed in teleost fish, including in response to infection [90,92], in mucosal immunity [15] and cancer [22]. Roles for EVs in inter-organ communication have also been described in zebrafish (*Danio rerio*) [23].

Given the basal position cartilaginous fish maintain in jawed-vertebrate phylogeny, the genesis of immunoglobulin superfamily-based adaptive immune mechanisms in this group and the unusual lymphocyte antigen receptor biology that has been described in these animals [24], we felt that an analysis of deimination in a shark species was mandated. The nurse shark (*Ginglymostoma cirratum*) was identified as a shark species with a wealth of historical serological studies and

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reagents in which to launch the work.

In the current study we assessed deiminated proteins in shark plasma and plasma-derived EVs, and report for the first time deimination and EV-mediated export of key immune factors in an elasmobranch species.

2. Materials and methods

2.1. Fish and sampling

Nurse shark (*Ginglymostoma cirratum*) sample collection was conducted under Texas A&M Institutional Animal Care and Use Protocol #2015–0374. The animal was collected by Dynasty Marine (Marathon, FL) under Florida Fish and Wildlife Special Activity License #18-2013-SR to MFC, then shipped to College Station TX. The shark was euthanized and bled out after an overdose of MS222. Blood was collected in EDTA tubes from the caudal sinus of the two year old nurse shark and plasma was collected by centrifuging at 300 g for 10 min. Plasma was immediately frozen at -80°C until further use.

2.2. Extracellular vesicle isolation and NTA analysis

EVs were isolated by step-wise centrifugation according to established protocols using ultracentrifugation and the recommendations of MISEV2018 (the minimal information for studies of extracellular vesicles 2018 [25]). Shark plasma was diluted 1:4 in ultrafiltered (using a 0.22 μm filter) Dulbecco's PBS (DPBS) and then centrifuged at 4000 g for 30 min at 4°C for removal of cells and cell debris. The supernatant was collected and centrifuged at 100,000 g for 1 h at 4°C . The pellet was then resuspended in DPBS and washed again at 100,000 g for 1 h at 4°C . The resulting EV-enriched pellet was resuspended in 100 μl DPBS, diluted 1/100 in DPBS and analysed by nanoparticle tracking analysis (NTA), based on Brownian motion of particles in suspension, using the NanoSight NS300 system (Malvern, U.K.). The NanoSight was used in conjunction with a syringe pump to ensure continuous flow of the sample, with approximately 40–60 particles per frame and videos were recorded for 5×60 sec. The replicate histograms generated from these recordings were averaged.

2.3. Transmission electron microscopy

EVs were isolated from plasma as before, the EV pellets were fixed with 2.5% glutaraldehyde in 100 mM sodium cacodylate buffer (pH 7.0) for 1 h at 4°C , resuspended in 100 mM sodium cacodylate buffer (pH 7.0), placed on to a grid with a glow discharged carbon support film, stained with 2% aqueous Uranyl Acetate (Sigma-Aldrich) and thereafter viewed in TEM.

2.4. Western blotting

Shark plasma and plasma EV isolates (an EV pellet derived from 200 μl plasma, reconstituted in 100 μl DPBS after isolation and purification) were diluted 1:1 in 2 x Laemmli sample buffer, boiled for 5 min at 100°C and separated by SDS-PAGE on 4–20% TGX gels (BioRad U.K.) and transferred to nitrocellulose membranes using semi-dry Western blotting. Blocking of membranes was in 5% bovine serum albumin (BSA, Sigma-Aldrich, U.K.) in TBS-T (tris-buffered saline (TBS) containing 0.01% Tween-20, BioRad, U.K. (TBS-T)) for 1 h at room temperature (RT) and incubation with primary antibodies diluted in TBS-T was performed at 4°C overnight (F95 MABN328, Merck, 1/1000; PAD2 ab50257, Abcam, 1/1000; cith3 ab5103, 1/1000; CD63 (CD63 molecule, also known as LAMP-3 and TSPAN30) ab216130, 1/1000; Flotillin-1 (Flot-1) ab41927, 1/2000). The membranes were washed in TBS-T for 3×10 min at RT and thereafter incubated in the corresponding secondary antibody (anti-rabbit IgG BioRad or anti-mouse IgM BioRad, diluted 1/4000 in TBS-T) for 1 h, at RT. Membranes were

washed for 6×10 min in TBS-T and visualization performed using the UVP BioDoc-ITTM System (Thermo Fisher Scientific, U.K.).

2.5. Immunoprecipitation and protein identification

The Catch and Release immunoprecipitation kit (Merck, U.K.) was used together with the F95 pan-deimination antibody (MABN328, Merck), which has been developed against a deca-citrullinated peptide and specifically detects proteins modified by citrullination [26], to isolate total deiminated proteins from shark plasma and plasma derived EVs. For F95 enrichment, 100 μl plasma was used, according to the manufacturer's instructions (Merck), while for EVs, total protein was first extracted from EV pellets, using radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich, U.K.), supplemented with protease inhibitor cocktail P8340 (Sigma-Aldrich), on ice for 2 h followed by centrifugation at 16,000 g for 30 min to collect the supernatant containing the proteins. IP was carried out on a rotating platform overnight at 4°C , and F95 bound proteins were eluted using denaturing elution buffer according to the manufacturer's instructions (Merck). The F95 enriched eluates were then either analysed by Western blotting or by LC-MS/MS (Cambridge Proteomics, Cambridge, UK). Peak files were submitted to Mascot (Matrix Science).

3. Results

3.1. PAD and deiminated proteins in shark plasma

Total deiminated proteins in shark plasma were detected using the F95 pan-deimination antibody, revealing a range of proteins between 10 and 250 kDa (Fig. 1A). A PAD homologue was identified in shark plasma by Western blotting via cross reaction with human PAD2 and detected at an expected 70 kDa size (Fig. 1B). Deiminated histone H3 was also detected in shark plasma at the expected 20 kDa size (Fig. 1C). Deiminated protein candidates were further identified by F95 enrichment and LC-MS/MS analysis in shark plasma and 6 protein hits with nurse shark are listed in Table 1.

3.2. Extracellular vesicle analysis in shark plasma

EVs from shark plasma were characterised by size exclusion using

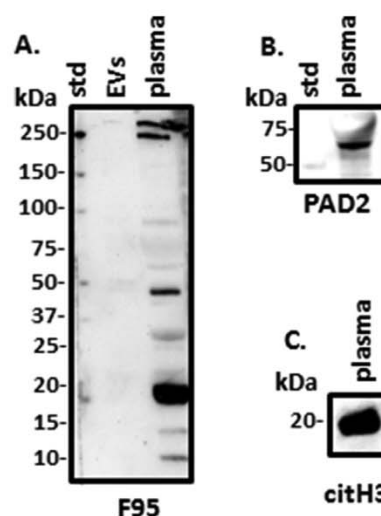


Fig. 1. Western blotting of deiminated proteins and PAD in shark plasma. A. Total deiminated proteins were identified in shark plasma and plasma EVs, using the F95 pan-deimination specific antibody. B. Shark PAD was identified at the expected size of approximately 70 kDa using the human PAD2 specific antibody. C. Deiminated histone H3 (cith3), representative of neutrophil extracellular traps (NETs) was verified in shark plasma.

Table 1

Deiminated proteins identified by F95 enrichment in total plasma of nurse shark (*Ginglymostoma cirratum*). Deiminated proteins were isolated by immunoprecipitation using the pan-deimination F95 antibody. The F95 enriched eluate was analysed by LC-MS/MS and peak list files were submitted to mascot. Only peptide sequence hits scoring with *G. cirratum* are included. Peptide sequences and *m/z* values are listed.

Protein name	<i>m/z</i>	Peptide sequence	Score (p < 0.05) ^a	Total score
H9LEQ0_GINCI <i>Haptoglobin</i>	386.7313	R.NDIALVK.L	64	639
	511.2690	R.VYVGIEDAR.E	76	
	539.7878	K.WIDGIIHPQ	29	
	787.3453	R.NTDLGYEFTCEK.V	90	
	567.6074	K.TGHVAGWGVETGETSR.A	77	
	852.8761	R.DFSDEGVYVCTIDGK.W	107	
	428.4166	R.EITAAHQVHVEDVHYHPR.M	62	
	752.8566	K.LKEDVHFSNHIMPACLPADHYAEEGK.T	53	
	929.6622	R.DGDDYYAAGVLSYDEGCAGEVYAVYTDVHHYLYK	80	
	544.7826	K.QDQSSPTPVK.R	73	
Q90544_GINCI <i>Novel antigen receptor</i>	544.8201	R.STSEIAVLLR.D	38	558
	617.8168	K.VFVQVNGVER.K	48	
	655.3514	K.TTSGFATTSPVK.T	63	
	709.8303	K.GVETQNPWESGSK.S	68	
	1080.9901	R.LTSSVEEWQSGVEYTCSAK.Q	106	
	805.0931	R.LLPPSPPEIQSTSSATLTCLIR.G	81	
	981.1190	K.VMASEWDSGTEYVCLVEDSELPTPVK.A	81	
	491.2474	R.GSLFNNAAMK.G	23	
	799.8751	R.LSSNNYQVYEPER.T	70	
	593.3202	K.LSHDVSVENQQAIIK.V	44	
A0A089YMI3_GINCI <i>Alpha 2-macroglobulin (Fragment)</i>	595.9613	R.NQVSLHFSEEEELPK.G	40	556
	697.0088	K.AEGVEIELTHSSFCPSGR.T	50	
	1228.0607	K.VYDYETGDSVVTQYEAPEPK.L	149	
	837.1073	R.APSAEVEMTSYVLLALLSGTEVTK.S	58	
	997.5215	K.IHLHLPETVVQGSAYAYLAVQGDILGSAMENLDGLLR.L	123	
	431.2510	K.NNVVIFR.A	42	
	506.3135	K.VGLIQPASVK.I	39	
	629.8041	R.NDNADVQNQIK.Y	33	
	640.8194	R.QASASMVAQPYK.T	84	
	480.5768	K.LIVSAANEHDENK.I	41	
A0A088MJF2_GINCI <i>C3 complement component</i>	944.9333	K.AVCSPIYESYEDSIQK.A	92	553
	985.8804	R.SDDDDYYSYPYEDIMSR.S	67	
	1117.0438	R.GEYGGGYSTTQATMVALQSLAK.Y	87	
	819.1269	R.EIGQIVVNMLIPITSDLPISFR.L	68	
	479.1939	R.ADFMSCVQ	37	
	595.8249	K.LEGQFLIQDK.F	39	
	532.6042	K.TWPGLPDHIDAAFR.I	36	
	790.4073	R.LLPPPAQETENQSR.V	54	
	688.3569	K.DQVDQTGVVLSK.R	44	
E7CQA0_GINCI <i>Hemopexin</i>	479.1939	R.ADFMSCVQ	37	112
	595.8249	K.LEGQFLIQDK.F	39	
	532.6042	K.TWPGLPDHIDAAFR.I	36	
U5NJD0_GINCI <i>Secreted IgW heavy chain</i>	790.4073	R.LLPPPAQETENQSR.V	54	54
U5NFT0_GINCI <i>Secreted IgW heavy chain</i>	688.3569	K.DQVDQTGVVLSK.R	44	44

^a Ions score is $-10 \cdot \log(P)$, 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. Cut-off was set at Ions score 20.

NTA, by morphological analysis using transmission electron microscopy (TEM) and by Western blotting using EV-specific markers (Fig. 2). A poly-dispersed population of EVs in the size range of 30–365 nm, with peaks at 31, 105, 145 and 280 nm was identified by NTA analysis (Fig. 2A), while Western blotting confirmed that the EVs were positive for the EV-specific markers CD63 and Flot-1, which have been established to be conserved in teleost fish but not shown in shark before, but did react with molecular weight bands at expected sizes (Fig. 2B). EVs were further characterised by morphology using TEM (Fig. 2C), confirming a poly-dispersed population.

3.3. Protein analysis of deiminated proteins in shark plasma derived EVs

Shark plasma EVs showed positive for deiminated proteins by Western blotting, using the pan-deimination F95 antibody (Fig. 3A). Deiminated proteins were further identified by F95 enrichment and LC-MS/MS analysis in EVs isolated from shark plasma revealed 3 protein hits with nurse shark, all of which were also identified in total plasma, namely novel antigen receptor (NAR), haptoglobin and hemopexin. Peptide sequences and *m/z* values are shown in Table 2. Overlap with deiminated proteins identified in shark plasma and plasma EVs are shown in Fig. 3B.

4. Discussion

For the first time, deiminated proteins are described in nurse shark (*Ginglymostoma cirratum*), unravelling novel aspects of post-translational deimination in key proteins of innate and adaptive immunity. A PAD homologue was identified for the first time in nurse shark plasma by Western blotting via cross reaction with human PAD2, which is the phylogenetically most conserved PAD form [8], at an expected 70 kDa size as in mammalian PADs and also seen in halibut (*Hippoglossus hippoglossus* L.) PAD [13]. In elasmobranchs, PAD has previously been identified in whaleshark (*Rhincodon typus*, XP_020374364). Deiminated histone H3, a marker of neutrophil extracellular trap formation (NETosis), was also detected here in shark plasma and at similar size as has previously described in teleost fish [8,13]. NETosis is driven by PADs [27], is conserved throughout phylogeny from fish to human, and is important in innate immune defences against a range of pathogens including bacteria, viruses and helminths [28–31]. NETosis has also been associated with clearance of apoptotic cells and during tissue remodelling in teleosts [8,13]. Deiminated histone H3 is described here for the first time in an elasmobranch species. Further deiminated proteins identified in shark plasma and plasma-derived EVs by F95 enrichment and LC-MS/MS analysis included key proteins of innate

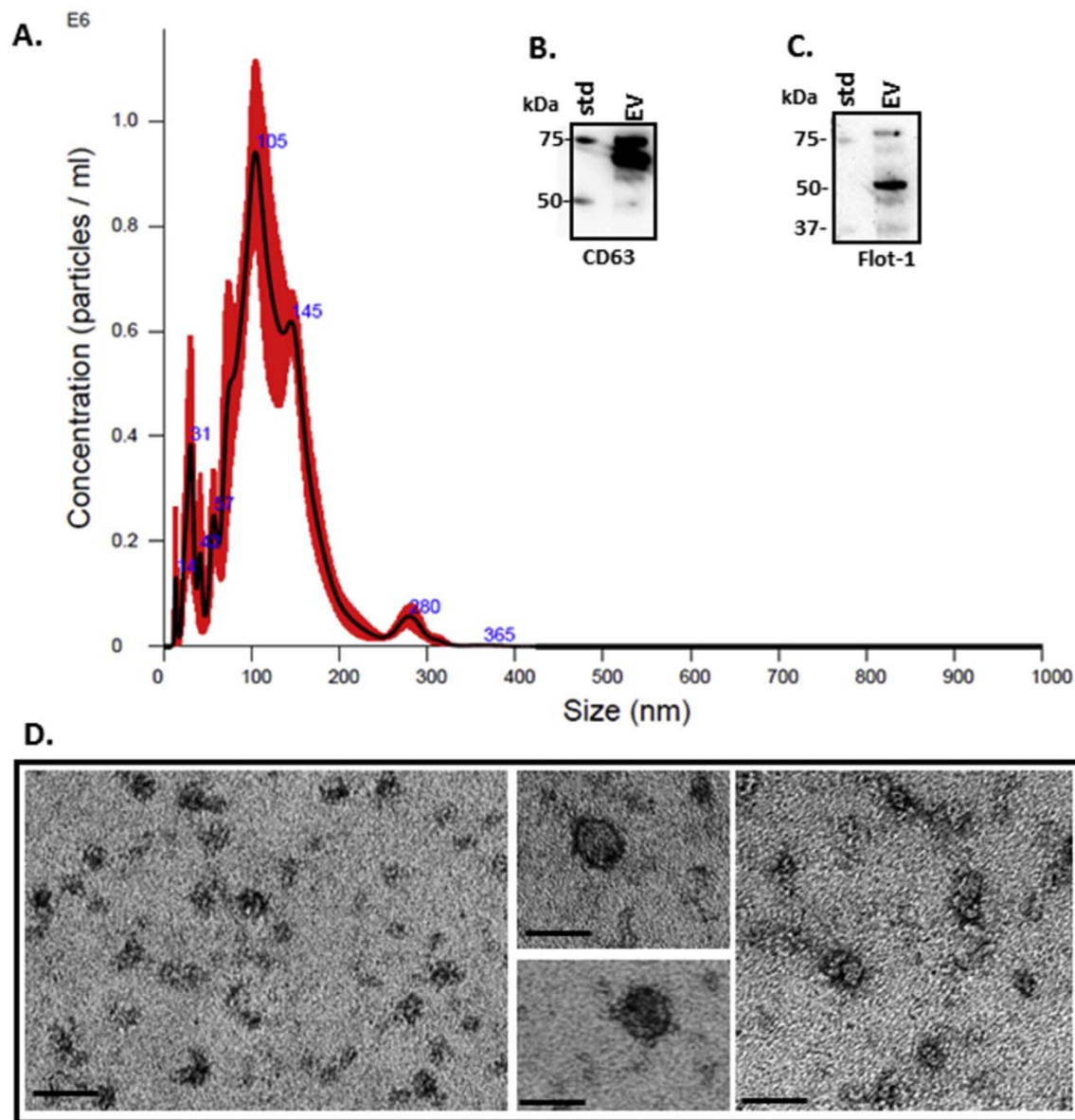


Fig. 2. Extracellular vesicles (EVs) isolated from shark plasma. **A.** Nanoparticle tracking analysis showing a poly-dispersed population of EVs in the size range of 30–365 nm, with peaks at 31, 105, 145 and 280 nm. **B.** Shark plasma EVs are positive for the EV-specific markers CD63 and Flot-1. **C.** Morphological analysis of EVs from shark plasma by transmission electron microscopy (TEM); scale bar is 50 nm.

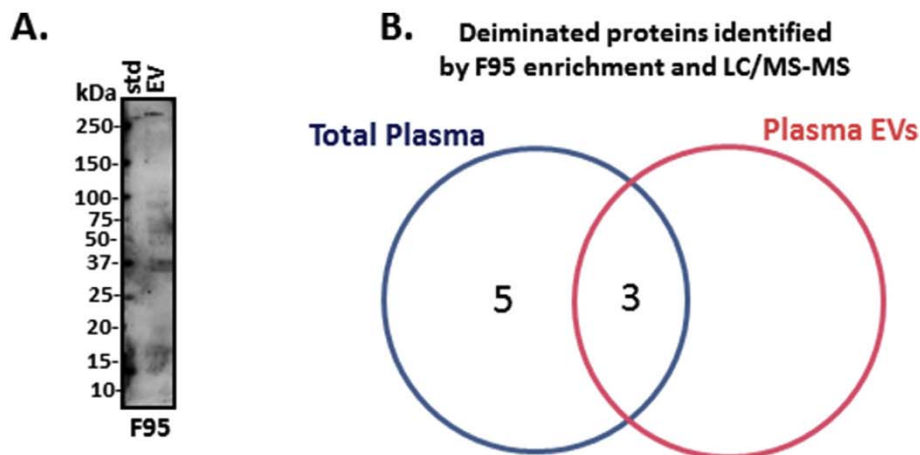


Fig. 3. LC-MS/MS analysis of deiminated protein profiles in shark total plasma and plasma-derived EVs. **A.** Western blotting confirms the presence of deiminated proteins in EVs from nurse shark plasma as assessed by the F95 pan-deimination antibody. **B.** Venn diagram representing deiminated proteins identified in total plasma and plasma-derived EVs by F95 enrichment; three proteins were identified in common with both samples, while 3 proteins were found deiminated in plasma only.

Table 2

Deiminated proteins identified by F95 enrichment in extracellular vesicles isolated from plasma of nurse shark (*Ginglymostoma cirratum*). Deiminated proteins were isolated by immunoprecipitation using the pan-deimination F95 antibody, the F95 enriched eluate was analysed by LC-MS/MS and peak list files were submitted to mascot. Only peptide sequence hits scoring with *G. cirratum* are included. Peptide sequences and *m/z* values are listed.

Protein name	<i>m/z</i>	Peptide sequence	Score (p < 0.05) ^a	Total score
Q90544_GINCI <i>Novel antigen receptor</i>	544.8186	R.STSEIAVLLR.D	48	164
	617.8140	K.VFWQVNGVER.K	50	
	652.2915	R.GSVYSCQVSHSATSSNQ.R	67	
H9LEQ0_GINCI <i>Haptoglobin</i>	539.7872	K.WIDGIIHPQ.-	35	35
E7CQA0_GINCI <i>Hemopexin</i>	532.6020	K.TWPGLPDHIDAAFR.I	20	20

^a Ions score is $-10 \times \log(P)$, 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. Cut-off was set at Ions score 20.

immunity and acute phase responses and are discussed below.

Hemopexin is a scavenger protein of haemoglobin and a pre-dominant heme binding protein, which contributes to heme homeostasis [32,33]. Hemopexin also associates with high density lipoproteins (HDL), influencing their inflammatory properties [34]. Hemopexin is a plasma glycoprotein and also named Warm temperature acclimation-associated 65-kDa protein (Wap65) in fish, and is associated with physiological stresses, including increased water temperature, immune response and heavy metal exposure. Wap65 has been identified in two different forms in teleosts, which differ in response to stress-factors and participate in inflammatory responses [35,36]. Hemopexin has also been described in various tissues in teleosts, but with a main expression in liver [37,38]. Hemopexin has been identified in nurse shark plasma as a Ni(2+)-binding serum glycoprotein, termed APP Hx, and found to bind heme, and to be present at unusually high levels in normal shark serum [39]. Hemopexin is a known glycoprotein and while post-translational deimination of hemopexin was recently described in Atlantic halibut (*Hippoglossus hippoglossus*) [13], this is the first report of hemopexin deimination in an elasmobranch species. Here, hemopexin was found deiminated both in shark whole plasma and plasma-derived EVs.

Haptoglobin is an acute phase plasma protein and in mammals it is involved in protection of oxidative damage by binding to haemoglobin [40]. In shark, haptoglobin has been identified to be a divergent MASP family member [41]. Haptoglobin has been identified in a range of teleost and cartilaginous fish [41–43] and has for example important against viral infections in teleosts [44]. Haptoglobin was here found to be deiminated both in shark whole plasma and plasma-derived EVs.

Alpha 2-macroglobulin was identified here to be deiminated for the first time in any species to our knowledge. It forms part of the innate immune system and clears active proteases from tissue fluids [45]. Alpha-2-M is phylogenetically conserved from arthropods to mammals and found at high levels in mammalian plasma and is closely related to other thioester containing proteins, complement proteins C3, C4 and C5 [46,47], which are phylogenetically conserved in shark [48–50]. Here, Alpha-2-M was found deiminated in whole plasma only and not identified to be exported in deiminated form in EVs.

Complement component C3 plays a central role in all pathways of complement activation and can also be directly activated by self- and non-self surfaces via the alternative pathway without a recognition molecule [48,49]. In nurse shark C3 has been characterised as a two-chain (α-chain and β-chain) thioester protein [50,51]. Contrary to teleost fish, where various isoforms of C3 have been identified, in shark only one C3 form has been so far described [51,52], although two complete C3 cDNA clones (GcC3-1 and GcC3-2) have been reported [53]. The complement system forms part of the first lines of immune defence against invading pathogens, in clearance of necrotic or apoptotic cells [49,54–56] as well as regeneration [57,58] and tissue remodelling [59–62]. This is the first report of deiminated complement C3 in an elasmobranch species, while C3 was recently identified for the

first time in deiminated form in halibut (*Hippoglossus hippoglossus* L.) [13]. Post-translational deimination of C3 may possibly influence its function including cleavage ability, binding, deposition and generation of the convertase. Studies using pharmacological PAD inhibitors to hinder protein deimination in a model of rheumatoid arthritis have for example shown a decrease in C3 deposition in synovium and cartilage and ameliorated collagen-induced arthritis [63,64]. It can be postulated that deimination of C3 may facilitate its functional diversity and this may be of importance in shark, where one C3 form (albeit two complete C3 cDNA clones- GcC3-1 and GcC3-2) has been reported [51–53]. Here, C3 was identified as deiminated in whole plasma only.

Novel antigen receptor (NAGR) is a heavy chain homodimer found in cartilaginous fish and lacks a light chain [65–67]. Cartilaginous fish are the oldest evolutionary animal group with adaptive immunity [68,69] and shark NAGR exhibits high target selectivity and affinity, making it a desirable natural compound for development of tools for therapeutics and immunotherapy [24,70–72]; most recently using VNARs for next-generation anti-TNF-α therapies [73]. As post-translational deimination has not been identified or studied in NAGR before, our current finding may provide novel insights into function of these immune proteins and be useful for refinement in therapeutic development using VNARs. NAGR was found here to be deiminated both in shark whole plasma and in plasma-derived EVs.

Secreted IgW heavy chain is part of the adaptive immune responses, and belongs to short forms of IgW [74]. IgW is one of the three immunoglobulin isotypes in shark, besides IgM and IgNAR [75–77,78,79,91]. In cartilaginous fish multiple forms of IgW have been described [77], including in sandbar shark (*Carcharhinus plumbeus*) [80] and banded houndshark (*Triakis scyllium*) [74]. IgW can perform isotype switching and several secreted isoforms have been identified in nurse shark [81]. Here, secreted IgW heavy chain was found deiminated in whole plasma only but not deiminated in plasma EVs.

Research on extracellular vesicles (EVs) is a relatively new field in fish immunology and to our knowledge this is the first description of EVs in an elasmobranch species. Studies on EVs have been carried out in some fish models, for example in zebrafish (*Danio rerio*) for studies of infection [82], cancer [22,83,84] and drug delivery [85]. Roles for EVs in zebrafish gut [86], in cod mucosal immunity [15] and for inter-organ communication [23] have also recently been described. As PADs have been identified to play major roles in the regulation of EV release [87–89], their contribution in EV-mediated communication in response to physiological and pathophysiological changes remains a field of further studies.

Here we identify for the first time deiminated proteins in shark plasma and EVs. Due to the fact that the nurse shark genome is not fully annotated, the hits identified here may underestimate the amount of deiminated proteins present in shark plasma and EVs. For the first time deimination of key immune factors of innate and adaptive immunity in shark is described, bringing a novel aspect on the possibility of protein moonlighting of these immune proteins via post-translational

deimination. In continuation of the current pilot study, the assessment of changes in deiminated proteins in shark plasma, and lateral transfer via EVs, will be of great interest in response to infection, environmental temperature and toxicology, as well as in the context of tissue remodelling and regeneration.

Credit author statement

MFC: Methodology; Resources; Validation; Writing - review & editing.

IK: Formal analysis; Methodology; Resources; Validation; Visualization; Writing - review & editing.

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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