Deiminated Proteins and Extracellular Vesicles as Novel Biomarkers in Pinnipeds: Grey seal (Halichoerus gryptus) and Harbour seal (Phoca vitulina)

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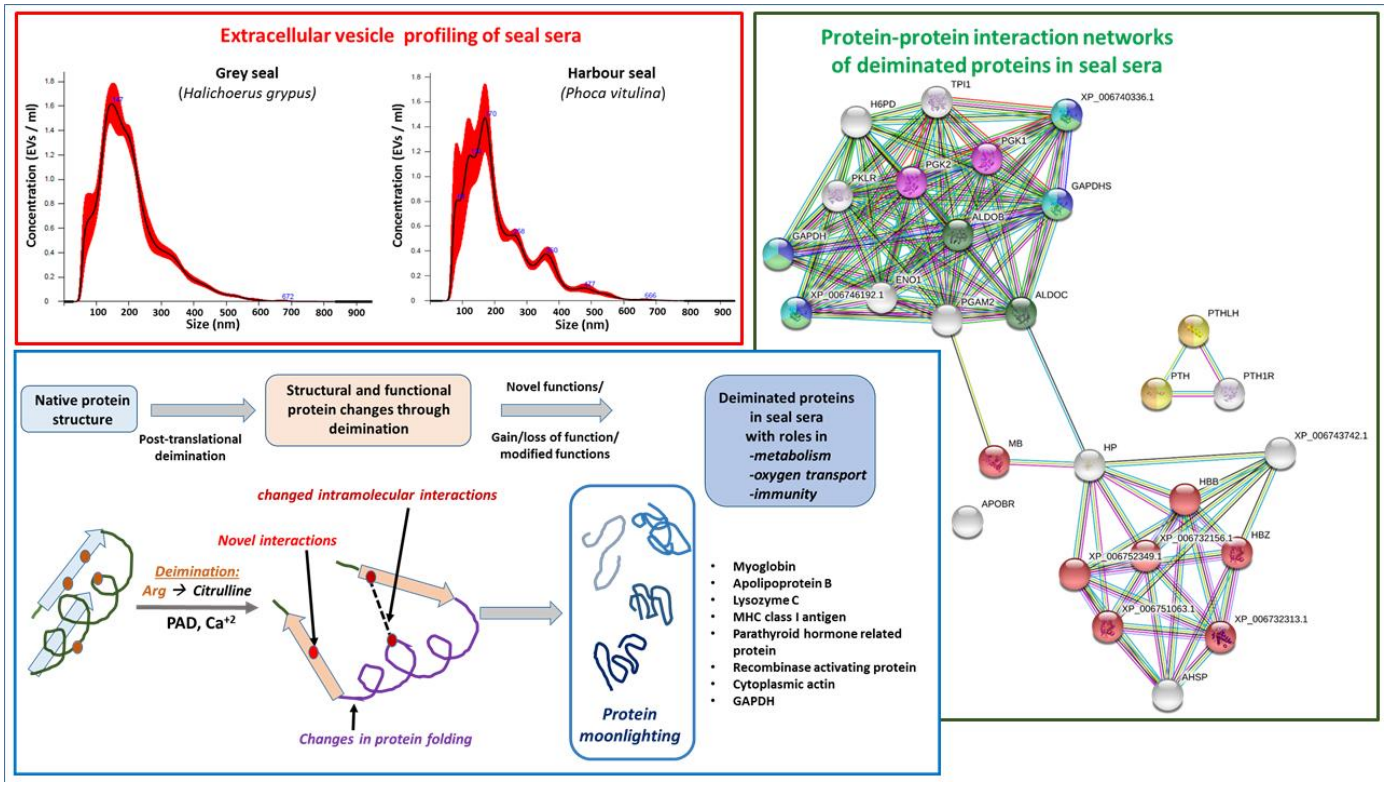
Abstract

Peptidylarginine deiminases (PADs) are phylogenetically conserved calcium-dependent enzymes which post-translationally convert arginine into citrulline in target proteins in an irreversible manner, leading to functional and structural changes in target proteins. Protein deimination can cause the generation of neo-epitopes, affect gene regulation and also allow for protein moonlighting and therefore facilitate multifaceted functions of the same protein. PADs are furthermore a key regulator of cellular release of extracellular vesicle (EVs), which 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 EVs were assessed in sera of two seal species, grey seal and harbour seal. We report a poly-dispersed population of serum-EVs, which were positive for phylogenetically conserved EV-specific markers and characterised by transmission electron microscopy. A number of deiminated proteins critical for immune and metabolic functions were identified in the seal sera and varied somewhat between the two species under study, while some targets were in common. EV profiles of the seal sera further revealed that key microRNAs for inflammation, immunity and hypoxia also vary between the two species. Protein deimination and EVs profiles may be useful biomarkers for assessing health status of sea mammals, which face environmental challenges, including opportunistic infection, pollution and shifting habitat due to global warming.

Key words: Peptidylarginine deiminases (PADs); protein deimination; Grey seal (*Halichoerus gryptus*); Harbour seal (*Phoca vitulina*); extracellular vesicles (EVs); immunity; metabolism; microRNA.

Graphic Abstract



Highlights

- Key immune and metabolic proteins were identified as deiminated in seal sera
- EV serum profiles varied somewhat between seal species
- EV microRNA cargo varied between seal species in relation to inflammatory makers miR155, miR21 and the hypoxia related miR210
- EV profiles and deiminated proteins are novel biomarkers in seal sera

Abbreviations:

BSA: Bovine Serum Albumin

CD63: CD63 antigen; granulophysin; lysosomal-associated membrane protein 3

ECL: Enhanced Chemiluminescence

EVs: Extracellular Vesicles

F95: Pan-deimination/citrullination antibody

FBS: Foetal Bovine Serum

Flot-1: Flotillin-1

KEGG: Kyoto Encyclopedia of Genes and Genomes

kDa: Kilodalton

LC-MS/MS:Liquid Chromatography Mass Spectrometry

miR: microRNA

NTA: Nanoparticle Tracking Analysis

PAD: Peptidylarginine Deiminase

SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

TBS: Tris Buffered Saline

TEM: Transmission Electron Microscopy

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 [1-4]. Protein structures which are most prone to deimination include beta-sheets and intrinsically disordered proteins, which are known to be heavily regulated by post-translational modifications [5]. A range of deiminated target proteins identified to date include nuclear, cytoplasmic and mitochondrial proteins [2,3;6-12]. Protein deimination can affect gene regulation and cause neo-epitope generation through which it can contribute to inflammatory, autoimmune and degenerative diseases [6,13]. Deimination-induced structural changes in proteins may though also allow for protein moonlighting, which is an evolutionary acquired phenomenon that can facilitate proteins to carry out a range of physiologically relevant functions within one polypeptide chain [14,15]. While some moonlighting proteins have been shown to exhibit multiple activities in different cellular locations, such as the extracellular matrix, nucleus, and cytoplasm, this ability can be a double-edged sword and besides physiological

functions, also contribute to a range of inflammatory diseases [16,17]. PADs have been studied extensively in recent years due to their key roles in mediating pathological processes in a range of chronic conditions including cancer, autoimmune and neurodegenerative diseases [3,6,13]. Crucial roles for PADs have also been described in CNS regeneration [18,19]. Furthermore, the roles for PADs in the regulation of extracellular vesicle (EVs) release, is becoming an increasingly acknowledged phylogenetically conserved pathway [20-24]. PADs are described throughout phylogeny from bacteria to mammals, with 5 tissue specific PAD isozymes in mammals, 3 in chicken, 1 in bony fish [1,7,8,25], in cartilaginous fish [10], and PAD homologues in parasites [23], fungi [26] and bacteria [24]. One of the key-mechanism in immunity which is mediated by PADs is the generation of extracellular trap formation from neutrophils and other white blood cells (NETosis/ETosis). In mammals, NETosis is known to be mainly PAD4 driven [27] and is found to be a phylogenetically conserved mechanism, recently also described in pinnipeds [28,29]. Besides assessment of NETosis/ETosis in pinnipeds, hitherto no further studies have been carried out regarding assessment of other PAD-driven mechanisms, including deiminated protein products or EVs, in pinnipeds.

EVs are membrane bound vesicles, released from cells and participate in cellular communication via transfer of cargo proteins and genetic material [6;30-33]. The main research carried out to date on EVs has been in the context of human pathologies, including via modulation of EV release using PAD-specific inhibitors in chronic disease and cancer [20-22], host-parasite interactions [23] and in bacterial antibiotic resistance [24]. Furthermore, EVs are important biomarkers that can be used to assess pathological processes and cellular communication [34-35]. Studies on EVs in relation to physiological processes have hitherto received less attention, while recent interest in the comparative immunology field has highlighted roles for EVs and EV cargo, including deiminated proteins and microRNAs, in a range of species throughout phylogeny [9-12;36-40].

Seals belong to pinnipeds, with the last common ancestor of humans and pinnipeds living about 65 MY ago [41]. Pinnipeds are a diverse group of semi-aquatic marine mammals comprised of walruses, eared seals and true seals. The family *Phocidae* is commonly referred to as true seals or earless seals. Harbour seal (*Phoca vitulina*), also called common seal, belongs to the *Phocidae* and is the most widely distributed species of pinniped, found along temperate and Arctic marine coastlines of the Northern hemisphere. Grey seal (*Halichoerus grypus (*Fabricius, 1791)), also known as Atlantic seal and the horsehead seal, is the only species classified in the genus *Halichoerus* of the *Phocidae* family. Grey seal has two subspecies, the Baltic grey seal (*Halichoerus grypus grypus*), which is an isolated population that exists in the Baltic Sea, and the Atlantic grey seal (*Halichoerus grypus atlantica*),

121 which is found in the North Atlantic including the United Kingdom, Orkney, the German Bight, the
122 coastal waters of Canada and south to Nantucket in the United States of America [42].

123 While seals have endured a range of long-standing threats due to hunting and other anthropogenic
124 causes of death, they are also exposed to accumulative effects of pollution and shift in habitat due
125 to global warming and changes in sea temperature, which also predisposes them to associated
126 changes in exposure to pathogens and opportunistic infection [43,44]. The identification of novel
127 health biomarkers markers, such as deiminated protein- and EV-profiles, may allow for the
128 development of novel tools to assess such impacts on seals' health status. Furthermore, marine
129 mammals are also highly valuable for comparative and evolutionary immunology studies, as they
130 represent three separate lineages of extant placental mammals, which arose from terrestrial
131 ancestors at different points in evolutionary history and recolonized the oceans [45]. They have
132 undergone a range of physiological adaptions to diving [46,47] and adapted their immune system to
133 aquatic ecosystems, including for host-parasite interactions [29].

134 In the current study, sera from two seal species, grey seal (*Halichoerus gryptus*) and harbour seal
135 (*Phoca vitulina*), were assessed for deiminated protein profiles and serum-derived EVs alongside EV-
136 associated microRNA cargo, indicative of immune status and hypoxia. We report for the first time
137 deimination of key immune and metabolic proteins in seal sera and EV profiles of the two seal
138 species under study.

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2. Materials and Methods

2.1 Sampling of seal sera

Two seal species, grey seal (*Halichoerus gryptus*) (2 adult individuals, one male and one female) and harbour seal (*Phoca vitulina*) (2 adult individuals, both female), in Icelandic waters of Western Iceland (Breiðafjörður and Faxaflói, for grey seal and harbour seal respectively), were utilised in this study. Sample collection had been conducted for use of these animals as negative healthy controls, in relation to research of the mass mortality in harbour seals in NW-Europe 1988-89 [48,49], and was in accordance with Icelandic laws and regulations on hunting of wild animals (64/1994). All seals used in this study were considered healthy adults, weighing from 80-100 kg for *P. vitullina* and 300-350 kg for *H. gryptus*. Following euthanasia, heart blood was collected from the seals and serum isolated by centrifuging at 2000 *g* for 20 min. The sera were immediately frozen at -20 °C and kept until further use.

2.2 Extracellular vesicle isolation and nanoparticle tracking (NTA) analysis

EVs were isolated according to established protocols, using step-wise centrifugation and ultracentrifugation according to the recommendations of MISEV2018 (the minimal information for studies of extracellular vesicles 2018; [50]). The seal sera were initially diluted 1:4 in ultrafiltered (using a 0.22 µm filter) Dulbecco's PBS (250 µl serum added to 750 µl DPBS) and centrifuged at 4,000 *g* for 30 min at 4 °C for the removal of cell debris and apoptotic bodies. The supernatant was collected and centrifuged again at 100,000 *g* for 1 h at 4 °C. The resulting EV-enriched pellet was next washed by resuspending it in 1 ml of DPBS and then ultracentrifuged again at 100,000 *g* for 1 h at 4 °C. The resulting final EV pellet was thereafter resuspended in 100 µl DPBS and diluted 1/100 in DPBS for nanoparticle tracking analysis (NTA), based on Brownian motion of particles in suspension, using the NanoSight NS300 system (Malvern, U.K.). The NanoSight NS300 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 x 60 sec. Replicate histograms generated from these recordings, using the NanoSight software (Malvern), were averaged.

2.3 Transmission Electron Microscopy

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

Visualisation by TEM was carried out using the Morada CCD camera system (EMSIS, Germany). Image processing was performed using iTEM (EMSIS).

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2.4 Western blotting

Seal sera and serum-EV isolates (each EV pellet derived from 250 µl serum, reconstituted in 100 µl PBS after isolation and purification) were diluted 1:1 in 2 x Laemmli reducing sample buffer (BioRad, U.K.), boiled for 5 min at 100 °C and separated by SDS-PAGE on 4-20 % TGX gels (BioRad U.K.). Following gel electrophoresis, proteins were transferred to nitrocellulose membranes using semi-dry Western blotting. Even protein transfer was assessed by PonceauS staining (Sigma, U.K.) and membranes were blocked in 5 % bovine serum albumin (BSA, Sigma, U.K.) in tris buffered saline (TBS) containing 0.1% Tween-20 (TBS-T) for 1 h at room temperature (RT). Primary antibodies were diluted in TBS-T and incubation was performed at 4 °C overnight as follows: F95 pan-deimination antibody (MABN328, Merck, 1/1000); anti-PAD2, (ab50257, Abcam, 1/1000); CD63 (ab216130, 1/1000); Flot-1 (ab41927, 1/2000). Following ON incubation, the membranes were washed in TBS-T for 3 x 10 min at RT and thereafter incubated in the corresponding secondary antibody (anti-rabbit IgG or anti-mouse IgM, BioRad, diluted 1/4000 in TBS-T) for 1 h, at RT. Membranes were thereafter washed for 6 x 10 min in TBS-T and visualisation was performed using enhanced chemiluminescence (ECL) and images captured using the UVP BioDoc-ITTM System (Thermo Fisher Scientific, U.K.).

2.5 Immunoprecipitation and Protein Identification

The Catch and Release® v2.0 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 [51], to isolate total deiminated proteins from seal sera and serum-derived EVs. For F95 enrichment, 50 µl serum was used per species (pooled from 2 individuals, 25 µl serum per individual animal), according to the manufacturer's instructions (Merck). For F95 enrichment from EVs, total protein was first extracted from EV pellets derived from 250 µl serum, of each of two individual seals per species, using 100 µl RIPA+ buffer per pellet on ice for 2 h, followed by centrifugation at 16,000 *g* for 30 min to collect the protein supernatants. Protein immunoprecipitation (F95-encrichment) was carried out on a rotating platform overnight at 4 °C, and the F95-bound proteins were eluted using denaturing elution buffer according to the manufacturer's instructions (Merck). The F95 enriched eluates were thereafter either analysed by Western blotting or by liquid chromatography with tandem mass spectrometry (LC-MS/MS; Cambridge Proteomics, Cambridge, U.K.). Following LC-MS/MS, peak files were submitted to Mascot (Matrix Science), using in-house databases (Cambridge Proteomics) for the

identification of species-specific protein hits with the following species-specific databases: CCP_*Halichoerus_grypus*_20190603 (143 sequences; 35525 residues) and CCP_*Phoca_vitulina*_20190603 (188 sequences; 46370 residues), respectively.

2.6 MicroRNA analysis of seal serum-EVs

EV isolates from the seal sera were assessed for relative changes in the expression of three key miRs related to inflammation (miR21), stress-response (miR155) and hypoxia and metabolic activity (miR210), respectively. RNA was extracted from EV preparations of the sera of two animals from each species (each EV pellet was prepared from 250 µl serum per sample as before), using Trizol (Sigma, U.K.). The purity and concentration of the isolated RNA were measured using the NanoDrop spectrophotometer at 260 nm and 280 nm absorbance. For cDNA production, the qScript microRNA cDNA Synthesis Kit (Quantabio, U.K.) was used according to the manufacturer's instructions. The cDNA was used to assess the expression of miR21, miR155 and miR210. U6-snRNA and has-let-7a-5p were used as reference RNAs for normalization of miR expression levels. The PerfeCTa SYBR Green SuperMix (Quantabio, U.K.) was used together with MystiCq microRNA qPCR primers for the miR21 (hsa-miR-21-5p), mir155 (hsa-miR-155-5p) and miR210 (hsa-miR-210-5p). All miR primers were obtained from Sigma (U.K.). Thermocycling conditions were used as follows: denaturation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 2 sec, 60 °C for 15 sec, and extension at 72 °C for 15 sec. The 2ΔΔCT method [52] was used for calculating relative miR expression levels and for normalisation. Each experiment was repeated in triplicates.

2.7 Statistical analysis

Histograms and graphs were prepared using the Nanosight NS300 software (Malvern, U.K.) and GraphPad Prism version 7 (GraphPad Software, San Diego, U.S.A.). Experiments were repeated in technical triplicates, using 2 animals per species; histograms represent mean of data and standard deviation (SD) or standard error of mean (SEM) are indicated by error bars. Significant differences were considered as $p \leq 0.05$, following one-way ANOVA or Student's t-test.

3. Results

3.1 PAD and deiminated proteins in seal sera and serum-derived EVs

A PAD homologue was identified in the seal sera by Western blotting via cross reaction with human PAD2 at an expected 70-75 kDa size (Fig 1A) and a human cancer cell line (glioblastoma multiforme LN18, ATCC® CRL-2610™, USA) was used as a positive control for PAD2 (Fig 1A.1) [22]. Total deiminated proteins in seal sera were detected by Western blotting, using the F95 pan-deimination

antibody, revealing deimination-positive proteins in the size range of 25-150 kDa (Fig. 1B). Deiminated proteins were also detected in the serum-derived EVs by Western blotting, mainly in the size range of 25-100 kDa (Fig. 1C). Deiminated protein candidates were further analysed by F95 enrichment in conjunction with LC-MS/MS analysis. Six protein hits were identified as species-specific with grey seal (Table 1), while 5 protein hits were identified to be species-specific for harbour seal (Table 2).

Table 1. Deiminated proteins identified by F95 enrichment in total serum of grey seal (*Halichoerus gryptus*). 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 *H. gryptus* are included. Peptide sequences and m/z values are listed.

Protein name	m/z	Peptide sequence	Score (p<0.05)[ǂ]	Total score
P68081|MYG_HALGR *Myoglobin*	470.7398 475.7399 697.4111 730.3347 818.9349 464.2458 397.2169	*K.SHPETLEK.F* *K.SHPETLEK.F* *K.HGNTVLTALGGILK.K* *K.HPAEFGADAQAAMK.K + Oxidation (M)* *K.VETDLAGHGQEVLIR.L* *K.GHHEAELKPLAQSHATK.H* *K.KGHHEAELKPLAQSHATK.H*	28 30 116 70 86 54 47	432
D2JZC7_HALGR *Apolipoprotein B*	419.7787 482.2584	*K.VQIPILR.M* *K.LEGTSSLTR.K*	34 25	59
Q659U5|LYSC_HALGR *Lysozyme C*	490.2334	*K.ATNYNPGSR.S*	42	42
K4JCP6_HALGR *MHC class I antigen*	388.2188	*R.GSLQTLR.G + Deamidated (NQ)*	28	28
Q659U3_HALGR *Parathyroid hormone related protein*	395.2078	*R.SVEELGR.R*	26	26
D2JZQ1_HALGR *Recombinase activating protein 1*	566.3133	*K.KMNLKPIMR.M + Deamidated (NQ)*	22	22

[ǂ]Ions score is -10*Log(P), where P is the probability that the observed match is a random event. Individual ions scores > 14 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.

Table 2. Deiminated proteins identified by F95 enrichment in serum of harbour seal (*Phoca vitulina*). 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 *P. vitulina* are included. Peptide sequences and m/z values are listed.

Protein name	m/z	Peptide sequence	Score (p<0.05)[ǂ]	Total score
P09909|HBB_PHOVI *Hemoglobin subunit beta*	456.7427 466.7635 501.2395 540.7820 549.7824 575.3399 403.5554 637.8665 657.8355 705.3566	*-.VHLTGEEK.S* *K.SAVTALWGK.V* *K.LSELHCDK.L* *K.VLNSFSDGLK.N + Deamidated (NQ)* *K.LHVDPENFK.L* *K.VVAGVANALAHK.Y* *K.KVLNSFSDGLK.N + Deamidated (NQ)* *R.LLVVYPWTQR.F* *K.VNVDEVGGEALGR.L* *K.EFTPQVQAAYQK.V*	38 55 45 71 39 80 43 52 76 61	560

P68080|MYG_PHOVI *Myoglobin*	438.7690 470.7403 475.7401 454.5851 465.2769 722.3373 546.2937 464.2458 496.2694	*K.KALELFR.N* *K.SHPETLEK.F* *K.YKELGFHG.-* *K.ALELFRNDIAAK.Y + Deamidated (R)* *K.HGNTVLTALGGILK.K* *K.HPAEFGADAQAAMK.K* *K.VETDLAGHGQEVLIR.L* *K.GHHEAELKPLAQSHATK.H* *K.KGHHEAELKPLAQSHATK.H*	31 30 40 25 45 94 60 44 45	413
B7NZX7_PHOVI *Cytoplasmic beta actin*	398.2396 507.7447 566.7689 581.3125 895.9495 652.0254 856.0606	*K.IIAPPER.K* *R.DLTDYLMK.I + Oxidation (M)* *R.GYSFTTTAER.E* *K.EITALAPSTMK.I* *K.SYELPDGQVITIGNER.F* *R.VAPEEHPVLLTEAPLNPK.A* *K.LCYVALDFEQEMATAASSSSLEK.S + Oxidation (M)*	39 38 45 46 93 23 74	357
P09908|HBA_PHOVI *Hemoglobin subunit alpha*	409.7240 507.2682 643.8529 505.9111 611.9684	*R.VDPVNFK.L* *R.TFTAFPTTK.T* *K.FFSAVSTVLTSK.Y* *K.IGGHAGEYGGEALER.T* *K.TYFPHFDLSHGSAQVK.A*	35 26 71 61 43	236
A0A1D6VJT2_PHOVI *Glyceraldehyde-3-phosphate dehydrogenase*	490.2639 685.3751 910.4588	*K.SISIFQER.D* *R.GAAQNIIPASTGAAK.A* *K.IVSNASCTTNCLAPLAK.V*	45 22 96	163

‡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. Cut-off was set at Ions score 20.

Deiminated protein hits were further assessed for disordered regions using FoldIndex© (https://fold.weizmann.ac.il/fldbin/findex) [53,54]. A summary for disordered regions identified in the protein hits, as well as number of arginines present in the corresponding seal Uniprot sequences identified here, are summarised in Table 3 and Table 4 for grey seal and harbour seal, respectively.

Table 3. FoldIndex analysis of deiminated proteins identified by F95 enrichment in serum of grey seal (*Halichoerus gryptus*). 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
P68081|MYG_HALGR *Myoglobin*	1	50	50	**5** (out of 154 residues)
D2JZC7_HALGR *Apolipoprotein B*	2	25	30	**6** (out of 313 residues)
Q659U5|LYSC_HALGR *Lysozyme C*	1	24	24	**10** (out of 148 residues)
K4JCP6_HALGR *MHC class I antigen*	7	81	207	**26** (out of 333

				residues)
Q659U3_HALGR *Parathyroid hormone related protein*	3	115	128	**18** (out of 177 residues)
D2JZQ1_HALGR *Recombinase activating protein 1*	4	47	123	**22** (out of 368 residues)

Table 4. FoldIndex analysis of deiminated proteins identified by F95 enrichment in serum of grey seal (*Phoca vitulina*). 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
P09909|HBB_PHOVI *Hemoglobin subunit beta*	None found	NA	NA	**2** (out of 146 residues)
P68080|MYG_PHOVI *Myoglobin*	1	*50*	50	**5** (out of 154 residues)
B7NZX7_PHOVI *Cytoplasmic beta actin (fragment)*	None found	*NA*	NA	**13** (out of 258 residues)
P09908|HBA_PHOVI *Hemoglobin subunit alpha*	None found	*NA*	NA	**3** (out of 141 residues)
A0A1D6VJT2_PHOVI *Glyceraldehyde-3-phosphate dehydrogenase*	None found	*NA*	NA	**5** (out of 184 residues)

3.2 Nanoparticle Tracking Analysis and Characterisation of Extracellular Vesicles in Seal Sera

EVs isolated from seal sera were characterised by size exclusion using nanoparticle tracking analysis (NTA; Fig 2A and 2B), by Western blotting (WB; Fig 2A.1 and 2B.1) and by morphological analysis using transmission electron microscopy (TEM; Fig 2A.2 and 2B.2). A poly-dispersed population of EVs in the size range of 50 to 600 nm was observed for both species, with some differences. In grey seal, one main peak EV was observed at 147 nm (Fig 2A), while for harbour seal several main EV peaks were observed at 64 nm, 124 nm 170 nm, 258 nm 350 nm and 477 nm, respectively (Fig. 2B). Western blotting confirmed that the EVs isolated from both species were positive for the EV-specific markers CD63 and Flot-1 (Fig 2A.1 and 2B.1). EVs were further characterised by TEM, confirming typical EV morphology (Fig. 2A.2 and 2B.2).

3.3 MicroRNA analysis of inflammatory and metabolic miRs in seal EVs

The inflammatory and stress related miR21, miR155 and the metabolic and hypoxia related miR210 were assessed in the EVs isolated from sera of the two seal species. Species-specific differences were

observed in the relative expression all three miRs, as shown in Fig. 3. Higher relative levels of miR21 (3.77-fold) were found in EVs from *P. vitulina* (Fig. 3A), while relative miR155 expression was 18.60-fold higher in EVs from *P. vitulina* (Fig. 3B) and miR210 relative levels were 1.73 fold higher in EVs of *H. gryptus* (Fig. 3C).

4. Discussion

In the current study, post-translationally deiminated serum protein profiles and EV profiles are described in pinnipeds as assessed in two seal species, the grey seal (*Halichoerus gryptus*) and harbour seal (*Phoca vitulina*), using two animals per species, respectively. The identification of post-translational deimination in key proteins of innate and adaptive immunity, as well as metabolic proteins, highlight putative novel modes of action for moonlighting functions of these proteins via such post-translational modifications. A PAD homologue was identified at an expected 70 – 75 kDa size in the seal sera by Western blotting, similar to as reported for other mammalian and human PADs, via cross reaction with human PAD2, which is the phylogenetically most conserved PAD form [1,7,8]. Deiminated proteins were detected in both whole sera and in serum-derived EVs, indicating EV-mediated export of deiminated proteins. The identity of specific protein hits in serum was assessed using F95 enrichment in conjunction with LC-MS/MS analysis. Identified species-specific protein hits included some key immune and metabolic related proteins, which are further discussed below and were also analysed for putative disordered regions to assess their proneness to post-translational modification, using FoldIndex© [53,54] (https://fold.weizmann.ac.il/fldbin/findex). Overall, deiminated protein hits identified in grey seal showed higher numbers of disordered regions and residues than proteins identified as deiminated in harbour seal and this furthermore correlated to a higher number of arginines in the deiminated protein hits identified in grey seal (Tables 3 and 4).

Myoglobin was identified as deiminated in sera of both seal species under study, while ***haemoglobin*** was identified as deiminated in harbour seal only. Both haemoglobin and myoglobin are key molecules in molecular oxygen transport in the bloodstream and for its storage in skeletal muscle. Myoglobin is found at higher concentrations in myocytes of deep diving animals compared to terrestrial animals and the diving capacity of mammals is related to the myoglobin concentration in their myocytes [55,56]. Myoglobin of diving animals have been found to have more positive net surface charges compared to myoglobin in terrestrial animals, possibly to cause electrostatic repulsion among myoglobin molecules and to prevent their aggregation and maintain high protein concentration [56,57]. In diving cetaceans myoglobin and haemoglobin have been reported to contribute to hypoxia tolerance [58,59] and haemoglobin has recently been studied in harbour seals

for the regulation of oxygen dynamics during diving [47]. Haemoglobin has furthermore been found to be a major binding protein for methylmercury in the liver of dolphins [60]. The post-translational deimination of myoglobin and haemoglobin identified here, in the seals under study, may be of considerable interest both in relation to defences to toxins as well as their physiological adaption to deep-diving, furthermore as harbour seal has been found to have greater aerobic capacity [61]. Myoblobin showed the same levels of disordered regions in both seal species, while haemoglobin was identified as deiminated in harbour seal only, with 3 and 2 arginines as putative deimination sites for haemoblobin subunit alpha and beta respectively, while disordered regions were not identified. This deimination of haemoglobin identified here may be of some importance for hitherto unrecognized effects on haemoglobin function and therefore also in relation to recent studies assessing haemoglobin for oxygen regulation during deep diving in harbour seals [47]. Such deimination-mediated functional effects will though require further in-depth investigation.

ApoB-100 is one of the lipoproteins found in pinnipeds and is conserved with other mammals [62]. ApoB-100 is synthesised by the liver and plays parts of innate immune responses [63]. ApoB-100 is also associated with insulin resistance and ER stress [64], as well as being linked to lipid metabolism disorders [65]. It was here identified as deiminated in grey seal only, and displays two disordered regions comprising 30 out of 313 residues and 6 arginines as putative deimination candidates. ApoB-100 has recently also been reported as deiminated in the naked mole-rat, an animal with unusual resistance to hypoxia [12], but the functional contribution of such post-translational deimination of ApoB-100 has yet to be assessed in further studies.

Lysozyme C was identified as deiminated in grey seal serum. Lysozymes are hydrolytic antimicrobial enzymes found in animals throughout phylogeny and play important roles in host defences [66]. Lysozyme c is a cornerstone of innate immunity, found in many body fluids and tissues, including respiratory, intestinal tract, in granules of neutrophils and in macrophages [67]. Lysozyme has immune-modulatory functions, including via regulation of the complement system [68] and provides protection against acute and chronic oxidant injury via suppression of ROS generation and OS response genes [69]. As post-translational deimination of lysozyme c has not been described before, it remains to be further explored how such deimination-mediated changes may contribute to moonlighting functions of the protein in immune modulation. In grey seal, one disordered region comprising 24 out of 148 residues of the protein hit was identified and 10 arginines, which could serve as putative deimination sites, were found within the 148 amino acid residues. Further investigation of the effects of deimination on functional effects of lysozyme c will be required to

assess the contribution of such post-translational changes on the multifaceted functions of lysozyme c.

MHC class I antigen was here identified as deiminated in serum of grey seal. The major histocompatibility complex (MHC) is involved with both adaptive and innate immune systems, including via antigen processing and presentation, and is found in vertebrates throughout phylogeny [70]. Roles for MHC class I can also serve as an inhibitory ligand for natural killer cells and this mechanism can be exploited by viruses, which can inhibit various stages of the MHC class I antigen presentation pathway for immune evasion [71-73] as well as by some cancer cells, which can down-regulate MHC I to avoid normal immune surveillance [74-75]. MHC I has been studied in harbour seal [76], with possible variation suggested in immune response genes in relation to susceptibility to phocine distemper virus [77] and with respect to morbillivirus infection in stranded cetaceans [78]. To what extent post-translational deimination may contribute to variable functions of MHC class I, remains to be further understood, as such post-translational changes have not been reported before. The interactions of MHC I and II with a range of deiminated proteins have previously been studied in relation to autoimmune disease [79-80], while deimination of MHC I itself has not been described. In grey seal, MHC I contains 26 arginines out of 333 residues, all of which can serve as putative deimination sites, and contains 207 disordered residues, with 7 disordered regions, whereof the longest one is 81 residues long. This indicates that MHC I is highly disordered in grey seal and therefore prone to post-translational modifications, possibly causing a range of structural protein changes which can facilitate MHC I protein moonlighting abilities. This will though require further investigation, including via site directed mutagenesis.

Parathyroid hormone related protein (PTHrP) was here identified as being deiminated in serum of grey seal. PTHrP is a member of the parathyroid hormone family and acts as an endocrine, autocrine, paracrine and intracrine hormone [81]. It regulates bone resorption and epithelial-mesenchymal interactions during the formation of mammary glands and is also involved in placental transfer of calcium and calcium transport to milk [82]. PTHrP is related to a range of metabolic disorders [81,83]. PTHrP can furthermore be secreted by cancer cells and in malignancies it can cause hypercalcemia [84-85]. As PTHrP has such multifaceted functions, the role for post-translational deimination for facilitating its functional diversity may be of considerable interest for physiological as well as pathological processes. In grey seal, PTHrP was found to contain 18 arginines out of 188 residues of this protein hit, with a total of 128 disordered residues found overall in 3 disordered regions, whereof the longest disordered region contained 115 residues. This indicates a

high level of disorder and therefore also the ability for protein moonlighting functions, including via post-translational deimination. This will though require further in depth study, also to assess which arginines would play the most critical function in relation to post-translational deimination and the resulting downstream structural and functional protein changes.

Recombinase activating protein 1 (RAG-1) is involved in antibody and T-cell receptor V(D)J recombination and is essential for the diversification and maturation of B- and T-cells [86-88]. Modifications in RAG-1 are linked to immunodeficiency and autoimmunity [89-91]. Antibody diversity is known to be achieved through a two stage process, firstly by gene arrangement, which is catalysed by the RAG1/2 recombinase, and thereafter via targeted removal of cytosines (deamination), which allows for further diversification and maturation by hypermutation, gene conversion and class-switch recombination [92]. Deimination of RAG-1 has not been reported before, but in the light of diversification of the immune system throughout phylogeny, such post-translational modifications may be of great interest for understanding multifaceted functions of RAG-1 via structural and functional changes caused by post-translational modifications. In grey seal, 22 arginines are found within the 368 amino acid sequence of RAG-1, which contains 4 disordered regions, with a total of 123 disordered residues, whereof the longest disordered region is 47 residues. Such a number of disordered regions, in addition to a high proportion of arginines, would in theory allow for a range of structural changes mediated by deimination, allowing for moonlighting functions. These putative changes will need further exploration and validation as deimination of RAG-1 has not been explored in any species so far.

Cytoplasmic beta actin was identified as deiminated in harbour seal serum. Cytoplasmic beta actin is ubiquitous, expressed in all cells in different ratios and plays key roles in cell adhesion, migration, polarisation and cytokinesis [93]. Beta actin maturation, which is important for effective interaction with myosin, is furthermore related to N-terminus arginylation [94]. Alterations in actin are related to several pathologies, including tumours [95], where deimination of action has been identified and also linked to regulation in EV release [20]. Besides EV release, beta actin participates in cytoskeletal rearrangement which is also necessary for successful phagocytosis and therefore effective immune responses [96]. Deimination of beta actin has previously been linked to EV release in cancer cells [20]. Structural changes in cytoplasmic actin, caused by post-translational changes, such as deimination identified here, may contribute to their multifaceted functions. While no disordered regions were identified in harbour seal serum using FoldIndex analysis, 13 arginines are present in the 258 amino acid sequence and can serve as targets for PAD-mediated conversion of arginine into

citrulline, leading to a range of structural changes via such post-translational deimination. Further studies on changes in deimination, including assessing the consequences of individual deiminated sites, possibly through site directed mutagenesis, will be required to understand the contribution of deimination to the diverse functions of beta-actin.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was identified as deiminated in harbour seal serum. Besides key metabolic functions in glycolysis, GAPDH is a moonlighting protein with pleiotropic non-metabolic functions including in apoptosis, transcription activation and axonal transport [97-100], as well as in iron metabolism [101]. GAPDH is associated to various pathologies [99] and has shown to be regulated via post-translational modifications [100,102,103]. GAPDH was recently identified as a deimination candidate in cancer [22], in EVs of the naked mole-rat, which also has unusual adaptions to hypoxia tolerance [12], as well as in EVs of teleost fish in a study assessing effects of water temperature changes on cod immunity and growth [39]. While GAPDH was not found to have disordered regions in harbour seal, using FoldIt analysis, 5 out of 184 residues of the protein sequence hit are arginines, which can serve as targets for post-translational deimination. These arginines, via deimination, may therefore contribute to its multifaceted physiological functions.

It must be noted that, while disordered proteins are the protein structures which are most prone to deimination, intrinsic disorder is though not a requirement for proteins to undergo the post-translational modification from arginine to citrulline [2].

The PPI enrichment *p*-value for the proteins above, identified as deiminated in the seals under study, was found to be 0.00212 by STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) analysis, based on sequence identifiers with the *Phocidae* family, which in STRING identifies sequences from the Weddell seal (*Leptonychotes weddellii*) (Fig. 4). MHC class-I which was not included in the analysis as a homologue with *Phocidae* was not found in STRING. Such an enrichment value indicates though that the identified network has significantly more interactions than expected. Therefore, these deiminated proteins have more interactions among themselves than what would be expected for a random set of proteins of similar size, drawn from the genome. Such enrichment indicates that the proteins are at least partially biologically connected, as a group. Pfam (https://pfam.xfam.org/) and SMART (Simple Modular Architecture Research Tool) protein domains identified in STRING are further highlighted in Fig. 4 and these belong to globin, glyceraldehyde-3 phosphate dehydrogenase C-terminal and NAD binding domains, the parathyroid hormone family, phosphoglycerate kinase and fructose-bisphosphate aldolase class-1 (Fig. 4).

MicroRNAs (miRs) are highly conserved small non-coding RNAs that control gene expression and regulate biological processes by targeting messenger RNAs (mRNAs). MiRs can inhibit post-transcriptional translation of mRNA or enhance mRNA degradation [104]. Hitherto, limited studies have been carried out on miRs in seals, while some expression profiling has been carried out in dolphins with the aim to identify health related biomarkers in relation to organ injury [105]. Diving animals, such as seals and cetaceans, undergo physiological and morphological changes needed for life in an aquatic environment, which are marked by resistance to physiological stresses caused by a lack of oxygen, increased amounts of reactive oxygen species and high salt levels [106]. MiR210 has previously been identified as a major miR induced under hypoxia and has important roles in mitochondrial metabolism, DNA damage response, cell proliferation and apoptosis [104]. MiR210 has a regulatory role in mitochondrial metabolism [107], as well as in cell glycolytic activity, and is also linked to inflammation [108]. MiR210 has been identified as a regulator of the hypoxia pathway and to have pro-apoptotic functions under normal oxygen conditions, while anti-apoptotic effects have been observed under hypoxic conditions [109,110]. In the current study, miR210 was found to be higher expressed in grey seal than in harbour seal and this may possibly reflect physiological differences between these species in relation to mitochondrial metabolism and oxygen transport.

MiR21 is strongly conserved throughout evolution, is a main immune regulatory and onco-related miR and is also associated to chronic diseases [111-113]. While many experimentally verified targets of miR21 are tumour suppressors, miR21 is also linked to cardiac disease and oxidative stress [114]. Less is known about physiological roles of miR21. In the current study miR21 was found to be higher expressed in harbour seal, compared to grey seal. Whether this difference species specific, or due to differences in immune and health status of the individual animals, remains to be further investigated as this could not be assessed in the current study due to only two individuals used per species. Roles for miR21 in immune responses of aquatic animals have previously been identified in cetaceans [38], as well as in teleost fish, where miR21 was found to regulate cytokine expression following immune stimulation [115].

In mammals, miR155 is known to be a major inflammatory related miR, linked to inflammatory and stress responses [116]. In the current study miR155 was higher expressed in grey seal than in harbour seal. As no previous studies have been carried out on these two miRs in seals, it remains to be fully understood which specific functions these have in seal physiology and immunity.

Both miR21 and miR155 have previously been associated to viral infections in fish [117] and found to be upregulated in fish exposed to chronic inflammation induced by [C_8mim]Br [118]. Temperature dependent effects on miR155 have also been observed in teleost fish [39]. The interspecies

differences in miR expression observed here in the two seal species under study may indicate that levels of these miRs vary between species, depending on their habitat and metabolic activity. This may though also reflect different health status of the individual animals used. As only two animals per species were assessed in the current study, such species specific differences need to be further evaluated. Effects of health status have indeed previously been observed on phocid immune function and seals dive duration has furthermore been shown to affect immune cell response [119].

This study reports deiminated proteins in seal sera and serum-derived EVs. Given that the animals were from similar demographic locations (waters of Western-Iceland), all adults and considered in good health, the findings presented in the current study should reflect baseline physiological parameters of these species. Due to the fact that the seal proteomes are not fully annotated, the hits identified here must underestimate the amount of deiminated proteins present in seal sera. Post-translational deimination of some specific key immune and metabolic factors in seals was though identified, therefore bringing a novel aspect of protein moonlighting functions of these proteins via such post-translational deimination. Research on EVs is a relatively new field in comparative immunology and to our knowledge this is the first description of EVs in pinnipeds. Recent comparative studies on EVs have focussed on some aquatic animal species, including teleost and cartilaginous fish [9,10,36,37,39;120- 125]. As PADs have been identified to be phylogenetically conserved regulators of EV release, including in pathologies and host-pathogen interactions [20-24], their contribution in EV-mediated communication in response to physiological and pathophysiological processes throughout phylogeny remains an emerging field of study, warranted further exploration.

In continuation of the current study, which provides the first baseline for these parameters in pinnipeds, the assessment of changes in deiminated proteins and EV profiles in seals, may be of interest for monitoring immunological responses to environmental changes, including infection, toxicology and shifts in habitat. This will require further development and refinement of these newly identified parameters presented in the current study. Furthermore, findings in deep-diving mammals, with adaptions to hypoxia, may be of considerable translatable value for informing studies on hypoxia-related human pathologies, cancer and neurodegenerative diseases.

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Credit Author Statement

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

PUO: Formal analysis; Methodology; Resources; Visualisation; Validation; Writing - review & editing.

IK: Formal analysis; Methodology; Resources; Visualization.

VS: resources; writing - review & editing.

KS: resources; 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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Figure legends

Fig. 1. PAD and deiminated proteins in seal sera and EVs. **A.** A seal PAD homologue was identified at the expected size of approximately 70-75 kDa for both seal species (*H.gryptus, P.vitulina*), using an anti-human PAD2 specific antibody. Figure **A.1** shows a human control sample (protein isolate from human glioblastoma cell line LN18), showing human PAD2 at a similar size, just below 75 kDa. The protein size standard (std) is indicated at the left of both blots. **B.** Total deiminated proteins were identified in seal sera, using the F95 pan-deimination specific antibody. **C.** Total deiminated proteins were identified in EVs isolated from the seal sera, using the F95 pan-deimination specific antibody.

Fig. 2. Extracellular vesicle profiling in seal sera. A. Nanoparticle tracking analysis shows a size distribution of EVs from grey seal (*Halichoerus grypus*) in the range of 30 to 600 nm, with one main peak at 147 nm. The black line represents the mean and the red lines represent the standard error of the mean (SEM), based on five 60 sec replicate video recordings of the same sample**. A.1** Western blotting analysis confirms that grey seal EVs are positive for the EV-specific markers CD63 and Flot-1. **A.2.** Transmission electron microscopy (TEM) analysis of grey seal serum-derived EVs shows typical

EV morphology; scale bar is 100 nm in all figures. **B.** Nanoparticle tracking analysis shows a poly-dispersed population of EVs from harbour seal (*Phoca vitulina*) in the size range of 50 to 600 nm, with peaks at 54, 124, 170, 258m 360 and 477 nm, respectively. **B.1** Harbour seal EVs are positive for the EV-specific markers CD63 and Flot-1, as confirmed by Western blotting. **B.2.** Transmission electron microscopy (TEM) analysis of harbour seal serum-derived EVs shows typical EV morphology; scale bar is 100 nm in all figures.

Fig. 3. MicroRNA analysis of seal serum-derived EVs. A. The inflammatory related miR21 relative expression varied between the two seal species and was significantly higher (3.77-fold) in EVs from *P. vitulina*. **B.** The inflammatory related miR155 varied significantly between the two seal species and was 18.60-fold higher in EVs from *P. vitulina*. **C.** The hypoxia related miR210 showed significant differences between seal species and was 1.73 fold higher in EVs of *H. gryptus*. Analysis for each microRNA was carried out using 2 animals per species (n=2) and performed in technical triplicates. The error bar represents the standard deviation (SD); exact *p*-values are indicated.

Fig. 4. Protein-protein interaction networks of the deiminated proteins identified in sera of both seal species under study (*P. vitulina* and *H. gryptus*), based on sequence identifiers with the *Phocidae* family, which in STRING identifies sequences from the Weddell seal (*Leptonychotes weddellii*). Coloured nodes represent query proteins and first shell of interactors; white nodes are second shell of interactors. 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). Identified PFAM protein domains are highlighted as follows: red=globin; dark blue=glyceraldehyde 3-phosphatase dehydrogenase C-terminal domain; light green=glyceraldehyde 3-phosphatase dehydrogenase NAD binding domain; yellow=parathyroid hormone family; pink=phosphoglycerate kinase; dark green=fructose-bisphosphate aldolase class-I. The following SMART protein domains are highlighted as follows: light blue=glyceraldehyde 3-phosphatase dehydrogenase NAD binding domain; orange=parathyroid hormone.

Figure 1.

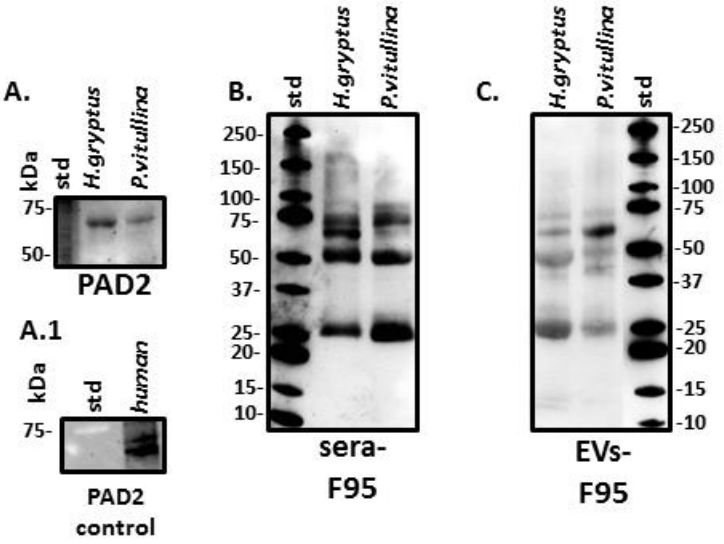

Figure 2.

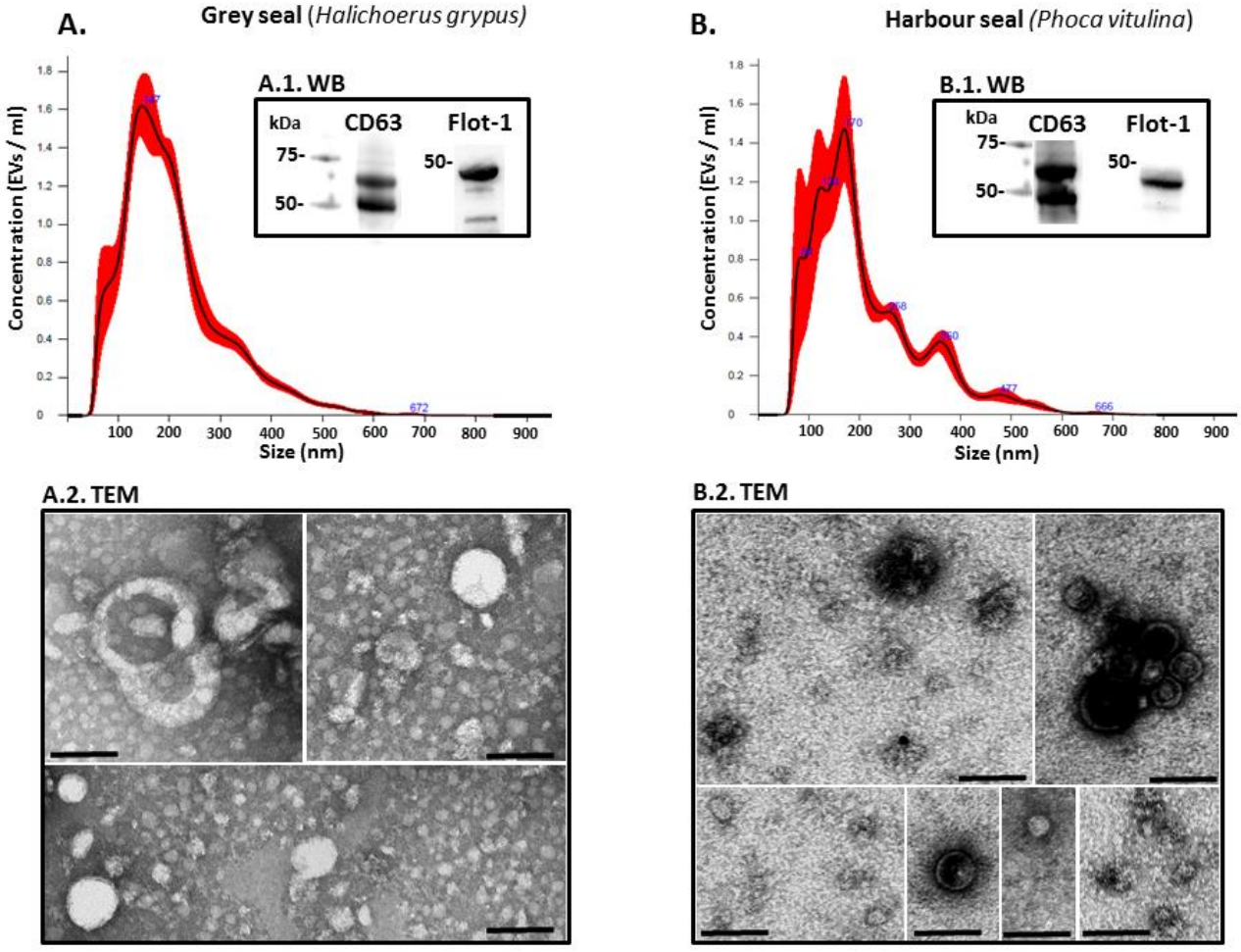

Figure 3.

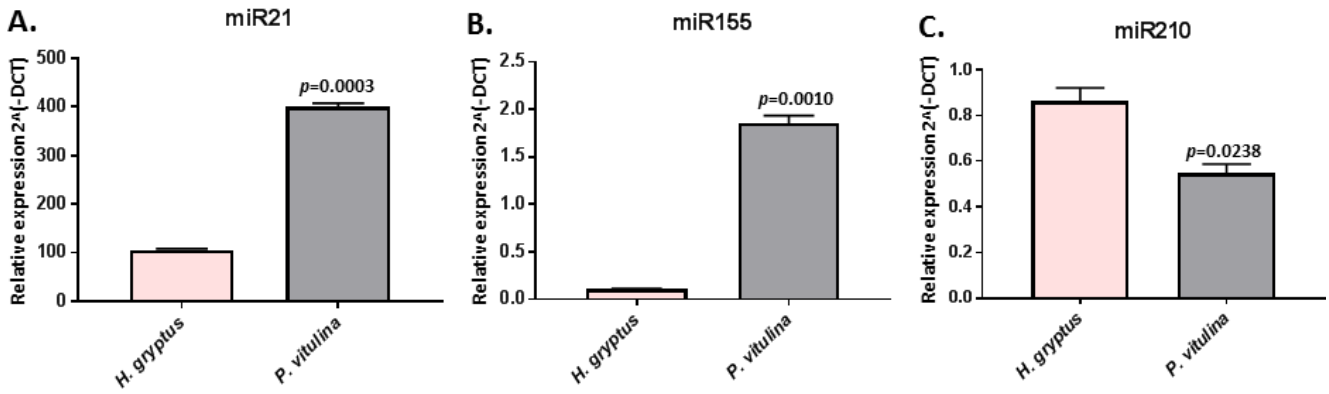

Figure 4.