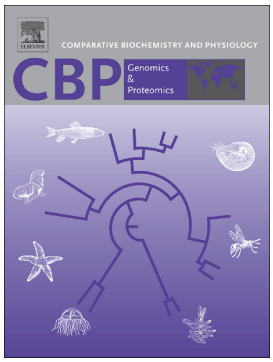
Deiminated proteins and extracellular vesicles - novel serum biomarkers in whales and Orca



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Deiminated Proteins and Extracellular Vesicles - Novel Serum Biomarkers in Whales and Orca

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Abstract

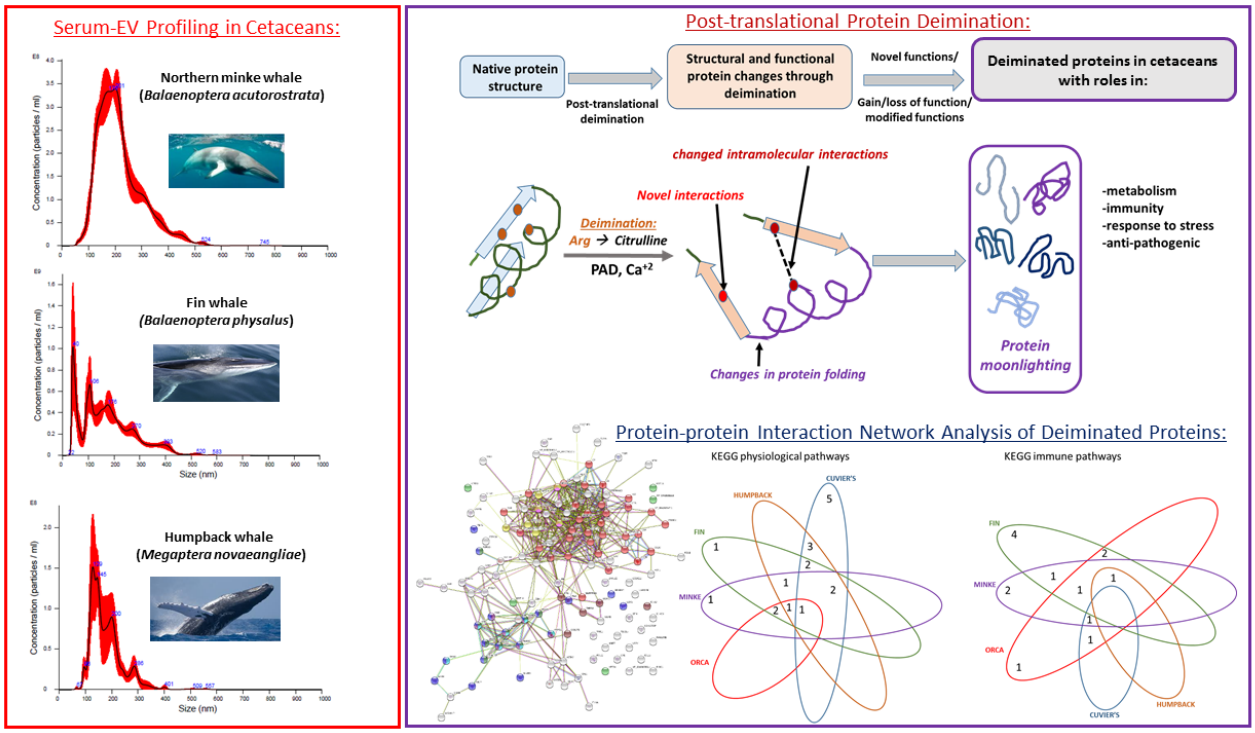
Peptidylarginine deiminases (PADs) are a family of phylogenetically conserved calcium-dependent enzymes which cause post-translational protein deimination. This can result in neo-epitope generation, affect gene regulation and allow for protein moonlighting via functional and structural changes in target proteins. Extracellular vesicles (EVs) carry cargo proteins and genetic material and are released from cells as part of cellular communication. EVs are found in most body fluids where they can be useful biomarkers for assessment of health status. Here, serum-derived EVs were profiled, and post-translationally deiminated proteins and EV-related microRNAs are described in 5 ceataceans: minke whale, fin whale, humpback whale, Cuvier's beaked whale and orca. EV-serum profiles were assessed by transmission electron microscopy and nanoparticle tracking analysis. EV profiles varied between the 5 species and were identified to contain deiminated proteins and selected key inflammatory and metabolic microRNAs. A range of proteins, critical for immune responses and metabolism were identified to be deiminated in cetacean sera, with some shared KEGG pathways of deiminated proteins relating to immunity and physiology, while some KEGG pathways were species-specific. This is the first study to characterise and profile EVs and to report deiminated proteins and putative effects of protein-protein interaction networks via such post-translationald deimination in cetaceans, revealing key immune and metabolic factors to undergo this post-translational modification. Deiminated proteins and EVs profiles may possibly be developed as new biomarkers for assessing health status of sea mammals.

Key words: Peptidylarginine deiminases (PADs); protein deimination; extracellular vesicles (EVs); microRNAs; cetaceans (*Balaenoptera acutorostrata; Balaenoptera physalus; Megaptera novaeangliae; Ziphius cavirostris; Orcinus orca*); immunity; metabolism.

Highlights

- Deiminated proteins were identified in whales and orca
- Key proteins of innate and adaptive immunity are deiminated in cetacean sera
- EV profiles were characterised in sera of 5 cetacean species
- Whale and orca serum-EVs are enriched in microRNAs compared to whole sera
- EV-cargo of immune related miR21, miR155 and metabolic miR210 expression varies between cetacean species
- Deiminated proteins and EV profiles are novel biomarkers in cetaceans

GRAPHIC ABSTRACT



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 protein changes (Vossenaar, 2003; György et al., 2006; Wang and Wang, 2013; Bicker and Thompson, 2013). Protein deimination can affect gene regulation, cause generation of neoepitopes (Witalison et al., 2015; Lange et al., 2017) and may also allow for protein moonlighting, an evolutionary acquired phenomenon facilitating proteins to exhibit several physiologically relevant functions within one polypeptide chain (Henderson and Martin, 2014; Jeffrey, 2018). Regulation of proteins through such post-translational changes and protein moonlighting of post-translationally modified proteins in health and disease are of great interest, also with regard to effects on function of protein networks in evolutionary conserved and adapted pathways. PADs have been widely studied in a range of human pathologies, including cancer, autoimmune and neurodegenerative diseases (Wang and Wang, 2013; Witalison et al., 2015; Lange et al., 2017). Crucial roles for PADs have also been described in CNS regeneration and hypoxia (Lange et al., 2011; Lange et al., 2014; Fan et al., 2018; Yu et al., 2018). PADs are phylogenetically conserved and have been identified in diverse taxa from bacteria to mammals, with 5 tissue specific PAD isozymes in mammals, 3 in birds, 1 in bony fish and arginine deiminase homologues in parasites and bacteria (Vossenaar et al., 2003; Rebl et al., 2010; Lange et al., 2011; Magnadottir 2018a, Magnadottir et al., 2019a; Gavinho et al., 2019; Kosgodage et al., 2019). In whales some PADs have been reported as gene sequences, for example PADI2 (Gene ID: 102988183) and PADI3 (Gene ID: 102986659) in sperm whale (*Physeter catodon*) (see Supplementary Fig. 1 for phylogenetic analysis of known PADs in ceataceans). Hitherto, PAD-related studies or investigations into their deiminated protein products are though almost non-existent, besides recent studies on cetacean extracellular trap formation (ETosis), which is a phylogenetically conserved innate defence mechanism that can be mediated via PADs (Li et al., 2010; Villagra-Blanco et al., 2019; Imlau et al., 2020). Research on PADs in normal vertebrate physiology has furthermore been limited compared to studies on pathophysiology. Putative control of physiological and immunological processes via post-translational deimination of proteins remains therefore a relatively unexplored field and has been a focus of recent ongoing comparative studies in our laboratory in a range of taxa throughout phylogeny (Criscitiello et al., 2019 and 2020; Kosgodage et al., 2019; Pamenter et al., 2019; Magnadottir et al., 2018a,b and 2019a,b; Kosgodage et al., 2019; Magnadottir et al., 2020a,b; Lange et al., 2020; Phillips et al., 2020). This also includes the identification of regulatory effects of PADs on extracellular vesicle (EV) release as a phylogenetically conserved mechanism (Kholia et al., 2015; Gavinho et al., 2019; Kosgodage et al., 2019).

EVs participate in cellular communication via transfer of cargo proteins and genetic material, including microRNAs (Inal et al., 2013; Colombo et al., 2014; Lange et al., 2017; Kosgodage et al., 2018; Turchinovich et al., 2019; Vagner et al., 2019). EVs can be isolated from most body fluids and be used as biomarkers for assessment of health status (Hessvik and Llorente, 2018; Ramirez et al., 2018). While work on EVs has hitherto largely focussed on human pathologies, roles for EVs in normal physiology and immunity also play important roles. Furthermore, key roles for PADs have been established in cellular release of EVs and EV biogenesis (Kholia et al., 2015; Kosgodage et al., 2017; Kosgodage et al., 2018; Kosgodage et al., 2019). Comparative studies assessing EVs and EV cargo have highlighted important roles for EVs in response to infection (Iliev et al., 2018; Yang et al., 2019), in mucosal immunity (Magnadottir et al., 2019b) and in host-pathogen interactions (Gavinho et al., 2019). Importantly, EVs were also recently identified by our group as novel biomarkers in teleost fish in response to environmental sea temperature changes (Magnadottir et al., 2020a). In elasmobranches, EVs have been characterised in shark, where deiminated proteins were also identified as part of EV cargo (Criscitiello et al., 2019), and a recent studies have been carried out on EV profiling and protein deimination in peglagic seabirds (Phipllips et al., 2020) and pinnipeds (Magnadottir et al., 2020b).

Whales belong to cetaceans, which also include dolphins and porpoises, and have undergone extensive underwater adaptions, both anatomically and immunologically, to physiological stress linked to their marine environments (Beineke et al., 2010; Yim et al., 2013; Tsagkogeorga et al., 2015). Whales are thought to have diverged from terrestrial mammals about 50 million years ago, with toothed wales and baleen whales separated about 30 million years ago (McGowen et al., 2009; Meredith et al., 2011; Gatesy et al., 2013). Marine mammals are of considerable interest for comparative and evolutionary immunology due to their shared linage with terrestrial mammals (Beineke et al., 2010; Meredith et al., 2011; Villagra-Blanco et al., 2019). Research into their immune systems may also further current understanding for resistance to cancer, insulin resistance and adaptions to hypoxia, highly relevant to a number of human pathologies (Tsagkogeorga et al., 2015; Tian et al., 2016; Seluanov et al., 2018). While cetacens have been studied for a range of immunological factors (Beineke et al., 2010; Zhou et al., 2018; Gelain and Bonsembiante, 2019) and assessed for conserved immunological and physiological mechanisms, including at the genetic level (Yim et al., 2014; McGowen et al., 2014; Braun et al., 2015; Lopes-Marques et al., 2018; Zhou et al., 2018), less emphasis has been on proteomic studies. No studies have hitherto assessed aspects of putative regulation via post-translational modifications, such as deimination, which may affect protein-protein interaction networks and therefore be critical for physiological and immunological functions. Furthermore, comparative studies on EVs are scarce, including basic EV characterisation

and identification, and have hitherto not been carried out in a cetacean species. While EVs are critical factors in cellular communication and acknowledged biomarkers in a range of human pathophysiologies, their potential for assessments of physiological status or the level of environmental or immunological challenges in other taxa remains a vastly underexplored area. In ongoing studies on deiminated protein pathways and EV profiling throughout phylogeny in our laboratory, including in species with unusual metabolic and immunological adaptions, as well as in human disease, we felt that a study on these parameters in ceataceans was warranted.

In order to identify putative PAD-mediated roles for regulation of physiological and immune pathways, the current study assessed deiminated proteins and serum-derived EV profiles, including microRNA (miR) markers, in five cetaceans. The species under study were northern minke whale (*Balaenoptera acutorostrata*), fin whale (*Balaenoptera physalus*), humpback whale (*Megaptera novaeangliae*), Cuvier's beaked whale (*Ziphius cavirostris*) and orca (*Orcinus orca*). The minke whale, fin whale and humpback whale are mysticetes, while the orca is an odontocete and belongs to the family Delphinidae. The Cuvier's beaked whale is most common beaked whale, an odontocete of the family Ziphiidae, and the only member of the genus *Ziphius*, believed to represent the remant of an ancient evolutinary lineage.

Whales are long-lived sea mammals and are, like other sea life, exposed to ongoing changes in sea temperatures due to global warming as well as a range of environmental contaminants. This can change sea animals' exposure to pathogens, some of which are adapted to certain temperatures, and therefore result in opportunistic infections, including due to shift in habitat. Effects of virus-induced immunosuppression, as well as increased bacterial and parasitic infections due to xenobiotic pollution, affect a range of marine mammals globally (Kennedy 1998; Martinau et al., 1999; Jepson et al., 1999, Wünschmann et al., 2001; Müller et al., 2004; Siebert et al., 2006; Siebert et al., 2009; Beineke et al., 2010). Such increased disease susceptibility due to environmental factors, alongside effects of chronic diseases and starvation, has received considerable attention although many aspects of aquatic mammal's immune functions still remain to be fully understood (Beineke et al., 2010). A range of critical knowledge gaps in cetacean host-pathogen interactions have furthermore recently been highlighted, urging research in the field of cetacean immunology (Di Guardo et al., 2018). As accumulative effects of pollution may impact long-living sea mammals, changes in inflammatory- and other parameters in their biofluids, such as EVs and deiminated proteins, may be novel biomarkers which could be used for assessment of environmental effects on their health status. Furthermore, studying long-lived mammals that display cancer resistance, such as cetaceans, may be of translational value for furthering current understanding of putative underlying pathophysiological mechanisms and for longevity (Ma and Gladyshev, 2017; Seluanov et al., 2018).

Here we report for the first time deimination of key immune and metabolic proteins in whale and orca sera, characterise serum-derived EVs and assess EV-mediated export of key miRs involved in immune responses and metabolism. Our findings indicate both shared KEGG pathways for immunological and physiological functions in these cetaceans, as well as some differences in these parameters between the species under study. Furthermore, our findings verify that EVs are a better source for assessment of miR expression, compared to whole sera. This report is a first base-line study, indicating the potential for the development of a health-index biomarker test, based on deiminated proteins and EV profiling, as a novel tool to assess sea mammal health.

2. Materials and Methods

2.1 Sampling of whale and orca sera

Blood utilised in this study was collected from one individual animal of each of the following cetacean species: Northern minke whale (*Balaenoptera acutorostrata*), fin whale (*Balaenoptera physalus*), humpback whale (*Megaptera novaeangliae*), Cuvier's beaked whale (*Ziphius cavirostris*) and orca (*Orcinus orca*). Protocols for blood collection were ethically approved and conducted under licences of the Chief Veterinary Officer, the Icelandic Government's Ministry of Industries and Innovation (98020047; 13-23-04; 13-08-02). *Balenoptera acutorostrata* and *Balaenoptera physalus* were free ranging (South-Western Iceland) and samples collected post-mortem following euthanasia; *Megaptera novaeangliae* and *Ziphius cavirostris* were free ranging (South-Eastern Iceland) and blood samples were collected following stranding. *Orcinus orca* was captive (The Whale Sanctuary Project, Klettsvik Bay, Iceland) and blood was collected during routine health checks. Blood was collected live from the dorsal fin *vena caudalis* of the orca, but post-mortem from an intrathoracic vessel of the minke whale, fin whale, humpback whale and beaked whale, using vacutainers. After collection, the blood was processed by storing at room temperature for 2 h and thereafter overnight at 4 °C. Serum was collected after centrifugation at 2000 *g* for 20 min, aliquoted and frozen at -20 °C until further use. The animals were considered healthy upon sampling (further information available on these individuals on age, sex and reproductive status/maturity is provided in Supplementary Table 1).

2.2 Extracellular vesicle isolation and NTA analysis

For isolation of EVs, step-wise centrifugation and ultracentrifugation was used, based on previously established protocols (Kosgodage et al., 2018; Magnadottir et al., 2019b) and according to the recommendations of MISEV2018 (the minimal information for studies of extracellular vesicles 2018;

Théry et al., 2018). Whale sera were diluted 1:4 in Dulbecco's PBS (DPBS which was ultrafiltered using a 0.22 µm filter) by adding 250 µl serum to 750 µl DPBS per EV isolation. The diluted sera were centrifuged at 4,000 *g* for 30 min at 4 °C for removal of cells and cell debris and thereafter the supernatant was collected and centrifuged at 100,000 *g* for 1 h at 4 °C for EV enrichment. The resulting EV pellet was then resuspended and washed in 1 ml DPBS and ultracentrifuged again at 100,000 *g* for 1 h at 4 °C. The final EV-enriched pellet was then resuspended in 100 µl DPBS for nanoparticle tracking analysis (NTA), based on Brownian motion of particles in suspension (Soo et al., 2012). The EV samples were diluted 1/100 in DPBS and applied to the NanoSight NS300 system (Malvern, U.K.) using a syringe pump to ensure continuous flow of the sample. Videos were recorded for 5 x 90 sec, with approximately 30-60 particles per frame. The replicate histograms generated from the recordings were averaged for assessment of EV size distribution profiles.

2.3 Transmission Electron Microscopy

For electron microscopic imaging of EVs, these were isolated from whale and orca sera as described above. EV pellets were then fixed with 2.5 % glutaraldehyde in 100 mM sodium cacodylate buffer (pH 7.0) at 4 °C for 1 h and resuspended in 100 mM sodium cacodylate buffer (pH 7.0). The EVs were placed on to a grid containing a glow discharged carbon support film and stained with 2 % aqueous uranyl acetate (Sigma-Aldrich). Imaging was performed using a JEOL JEM 1400 transmission electron microscope (JEOL, Japan) operated at 80 kV at a magnification of 80,000 to 100,000. Digital images were recorded using an AMT XR60 CCD camera (Deben, UK).

2.4 Western blotting

Sera and serum-derived EV isolates (with each EV pellet isolated from 250 µl serum and reconstituted in 100 µl PBS after isolation and purification) were diluted 1:1 in 2x Laemmli sample buffer (containing 5 % beta-mercaptoethanl) and boiled for 5 min at 100 °C before separation on 4-20 % gradient TGX gels (BioRad U.K.). Following SDS-PAGE, transfer to nitrocellulose membranes was performed using semi-dry Western blotting at 15 V for 1 h. The membranes were blocked in 5 % bovine serum albumin (BSA, Sigma-Aldrich, U.K.) diluted in TBS-T (tris-buffered saline containing 0.1 % Tween-20) at room temperature (RT) for 1 h, followed by overnight incubation at 4 °C with the primary antibodies, which were diluted in TBS-T as follows: F95 (MABN328, Merck, U.K.) 1/1000; anti-PAD2 (ab50257, Abcam) 1/1000; anti-PAD3 (ab50246) 1/1000; anti-citH3-r2-r8-r17 (ab5103) 1/1000; CD63 (ab216130) 1/1000; Flotillin-1 (ab41927) 1/2000. After incubation with the primary antibodies, the membranes were washed in TBS-T for 3 x 10 min at room temperature (RT) and thereafter incubated in the corresponding secondary antibody (anti-rabbit IgG BioRad or anti-mouse

IgM BioRad respectively, diluted 1/4000 in TBS-T) for 1 h, at RT. Membranes were then washed for 5 x 10 min in TBS-T, followed by one wash for 10 min in TBS. Visualisation was performed using electrochemiluminescence (ECL, Amersham, U.K.) and the UVP BioDoc-ITTM System (Thermo Fisher Scientific, U.K.).

2.5 Immunoprecipitation and protein identification

For isolation of deiminated proteins from whale and orca sera, the F95 pan-deimination antibody (MABN328, Merck), which is developed against a deca-citrullinated peptide and specifically detects proteins modified by citrullination (Nicholas and Whitaker, 2002), was used in conjunction with the Catch and Release immunoprecipitation kit (Merck, U.K.). According to the manufacturer's instructions (Merck), 50 µl of serum was used per sample for F95 enrichment and immunoprecipitation was carried out overnight on a rotating platform at 4 °C. The F95 bound proteins were eluted using denaturing elution buffer (according to the manufacturer's instructions, Merck) and the F95 enriched eluates were thereafter analysed both by Western blotting and by liquid chromatography mass spectrometry (LC-MS/MS) for identification of deiminated protein targets (Cambridge Proteomics, U.K.). For LC-MS/MS, peak files were submitted to in-house Mascot (Matrix Science, Cambridge Proteomics) using the following databases for identification of species-specific protein matches: *Balaenoptera_acutorostrata*_20190523 (32,118 sequences; 20,395,764 residues); *Balaenoptera_physalus*_20190523 (333 sequences; 92,646 residues); *Megaptera_novaeangliae*_20190523 (259 sequences; 50,653 residues); *Ziphius_cavirostris*_20190523 (176 sequences; 49,360 residues) and *Orcinus_orca*_20190523 (333 sequences; 105,106 residues). Furthermore, the LC-MS/MS peak files from all five species under study were also submitted to a larger cetacean UniProt database (CCP_Cetacea Cetacea_20191213; 252,001 sequences; 150,129,595 residues; Cambridge Proteomics).

2.6 Protein interaction network analysis

STRING analysis (Search Tool for the Retrieval of Interacting Genes/Proteins; https://string-db.org/) was used for the identification of putative protein-protein interaction networks for the deiminated proteins identified in northern minke whale, fin whale, humpback whale, Cuvier's beaked whale and orca, respectively. Species selection in the STRING database was set to "cetacean" and protein-interaction network analysis was based on hits with "minke whale" for minke whale, but on hits with "orca" for fine whale, humpback whale, Cuvier's beaked whale and orca (note that protein-interaction network analsyis for minke whale using corresponding orca hits revealed a similar analysis as when using the minke whale STRING database, therefore the minke whale database was

used for minke whale). Protein networks were built by using the function of "search multiple proteins" in STRING and applying basic settings and medium confidence, 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. Annotations for KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways identified were highlighted for physiological and immunological pathways identified by STRING (Fig. 4-8).

2.7. PAD sequence alignment and phylogenetic reconstruction

Pre-existing predicted PAD protein sequences for a number of cetaceans (Indo-Pacific humpback dolphin, *Sousa chinensis*; narwhal, *Monodon Monoceros*; sperm whale, *Physeter catodon*; common bottlenose dolphin, *Tursiops truncatus*; long-finned pilot whale, *Globicephala melas*; Pacific white-sided dolphin*, Lagenorhynchus obliquidens*; beluga *Delphinapterus leucas*; **narrow-ridged finless porpoise***, Neophoncaena asiaeorientalis*; baiji, *Lipotes vexillifer*; minke whale, *Balaenoptera acutorostrata*; fin whale, *Balaenoptera physalus*; killer whale, *Orcinus orca*) were retrieved from NCBI searches (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) or by searching specific genome resources (*Balaena mysticetus*; http://www.bowhead-whale.org/). For the remaining cetacea species (Indo-Pacific bottlenose dolphin, *Tursiops aduncus;* North Pacific right whale*, Eubalaena japonica*; Sowerby's beaked whale, *Mesoplodon bidens*; Amazon River dolphin, *Inia geoffrensis*; Antarctic minke whale, *Balaenoptera bonaerensis*; Indus river dolphin, *Platanista minor*; harbour porpoise, *Phocoena phocoena*; vaquita, *Phocena sinus*; La Plata dolphin, *Potoporia blainvillei*; humpback whale, *Megaptera novaeangliae*), and the common hippopotamus, *Hippopotamus amphibius* (included as the closest living terrestrial relative of the cetacea), which had no pre-existing predicted PAD sequences, tBLASTn searches (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=tblastn) searches using reference PAD protein sequences (*Orcinus orca*) were performed against available none annotated genome assemblies found NCBI. Where possible resultant scaffold matches were then used to predict PAD sequences using the FGENESH gene finder tool in Softberry (http://www.softberry.com). Resultant predicted PAD protein sequences were checked using BLASTp searches to ensure accuracy for the FGENESH protein prediction. Multiple sequence alignment was performed using ClustalW in Bioedit version 7 (Hall, 1999). Evolutionary analyses were conducted in MEGAX (Kumar et al., 2018). Phylogenetic relationships of the cetacean PAD proteins were inferred using the Neighbour-Joining method under the conditions of the Poisson correction distance model, with 1000 bootstrap replicates used to assess nodal support.

2.8 MicroRNA analysis in whale serum and EVs

Total whale and orca sera as well as the corresponding EV isolates, were assessed for relative changes in the expression of three key miRs related to inflammation (miR21), stress-response (miR155), hypoxia and metabolic activity (miR210), respectively. RNA was extracted from whole sera (50 µl of serum per isolation) and from EV preparations of the sera from each species (each EV pellet 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 (Livak and Schmittingen, 2001) was used for calculating relative miR expression levels and for normalisation. Each experiment was repeated three times.

2.8 Statistical Analysis

GraphPad Prism version 7 (GraphPad Software, San Diego, U.S.A.) was used to prepare graphs and NTA curves were generated using the NanoSight 3.0 software (Malvern Panalytical, UK). Significant differences were considered as $p \leq 0.05$, following one-way ANOVA.

3. Results

3.1 EV profile analysis of whale and orca sera

EVs from whale and orca sera were characterised by NTA for size profiling, by morphological analysis using transmission electron microscopy (TEM) and by Western blotting using EV-specific markers (Fig 1). NTA profiles of EVs revealed poly-dispersed EV populations which overall fell within a range of approximately 50 – 500 nm, albeit with some variation between the 5 cetaceans. For minke whale, main EV peaks were seen at 176 and 201 nm and a smaller peak at 524 nm (Fig. 1A); in fin whale main EV peaks were observed at 40, 108, 176, 270 and 393 nm; in humpback whale an EV profile between 67-401 nm, with main peaks at 129, 145, 200 and 286 nm was observed (Fig. 1C); Cuvier's

beaked whale had a more monodispersed profile with one main EV peak at 146 nm within a 50-400 nm overall EV distribution (Fig. D); orca showed main EV peaks at 51 and 123 nm, with a smaller peak at 470 nm (Fig. 1E). Western blotting confirmed that the EVs were positive for the EV-specific markers CD63 and Flot-1 (Fig. 1 A-E, see Western blot inserts for each species), which have been shown to be phylogenetically conserved from fish to mammals (Iliev et al., 2018; Criscitiello et al., 2019; Magnadottir et al., 2020a,b; Criscitiello et al., 2020). EVs were further characterised by morphology using TEM, confirming typical EV morphology and a poly-dispersed population (Fig. 1A-E, see inserted TEM images for each whale species). Overall yield of serum-derived EVs varied between the 5 species (Fig 2A), with 2.27 x 10^9 (+/- 4.21 x 10^8) EVs/ml for minke whale; 4.6 x 10^9 (+/- 2.14 x 10^8) EVs/ml for fin whale; 5.48 x 10^8 (+/- 4.85 x 10^7) EVs/ml for humpback whale; 7.52 x 10^9 (+/- 9.65 x 10^7) EVs/ml for Cuvier's beaked whale; and 8.84 x 10^9 (+/- 1.29 x 10^8) EVs/ml for orca (Fig. 2A). Some variation in modal size of EVs was also observed between the species (Fig. 2B).

3.2 PAD and deiminated proteins in whale and orca sera and serum-derived EVs

The presence of a PAD homologue was assessed in whale and orca sera by Western blotting via cross reaction with human PAD2 and PAD3 antibodies, showing expected 70-75 kDa size bands observed for PAD2 and PAD3, although more specifically for PAD2 (Fig. 3A), which is the phylogenetically most conserved PAD form. Deiminated histone H3 was detected in cetacean sera at the expected approximate 20 kDa size (Fig. 3B). Total deiminated proteins were detected in the whale and orca sera and in serum-derived EVs using the F95 pan-deimination antibody, with deimination positive bands detected in the range of 15-150 kDa (Fig. 3C-D), both in serum-derived EVs (Fig. 3C) and in whole sera (Fig. 3D).

3.3 LC-MS/MS analysis of deiminated proteins in whale and orca sera

Deiminated protein candidates were further identified in the sera of the wales and orca using F95 enrichment and LC-MS/MS analysis. Results for deiminated protein hits against species-specific databases for each species are listed below in Tables 1-5. It must be noted that differences in the number of peptide hits in this species-specific analysis is partly down to differences in the amount of annotated proteins available in the species-specific databases used for protein identification for the 5 species under study (see section 2.5). In Northern minke whale (*Balaenoptera acutorostrata*) over 90 deiminated protein candidates specific to minke whale were identified (Table 1); in fin whale (*Balaenoptera physalus*) 3 species specific proteins were identified to be deiminated (Table 2), but further hits were identified with other species (not shown); in humpback whale (*Megaptera novaeangliae*) 2 species-specific deiminated proteins (Table 3) and further hits (not shown) with

other species were identified to be deiminated; in Cuvier's beaked whale (*Ziphius cavirostris*) 2 species specific (Table 4) and also further hits with other species (not shown) were identified as deiminated protein candidates; in orca (*Orcinus orca*) 2 species specific hits (Table 5) and further hits with other species (not shown) were identified as deiminated protein candidates. Species-specific hits only, are listed in Tables 1-5. The protein hits identified by LC-MS/MS in all five cetacean species were furthermore assessed against a larger cetacean database (CCP_Cetacea Cetacea_20191213; 252,001 sequences; 150,129,595 residues), showing 137 cetacean hits for minke whale; 244 cetacean hits for fin whale, 155 cetacean hits for humpback whale, 88 cetacean hits for Cuvier's beaked whale and 106 cetacean hits for orca; these are represented in Supplementary Tables 2-6.

Table 1. Deiminated proteins identified by F95 enrichment in serum of Northern minke whale (*Balaenoptera acutorostrata*). 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 *B. acutorostrata* are included. Peptide sequences and m/z values are listed. For protein hits identified against the full cetacean database see Supplementary Table 2.

Protein name	m/z	Peptide sequence	Score (p<0.05)[ǂ]	Total score
A0A452CHV5_BALAS	393.2478	*R.LLLNGVR.T*	34	3805
Apolipoprotein B-100	394.2573	*K.FIIPGLK.L*	34	
	401.2408	*K.LVEEALK.K*	35	
	401.2451	*K.LNVGGTIK.G*	48	
	408.7322	*K.VGVELSGR.A*	53	
	419.7792	*R.VQIPLLR.M*	46	
	420.7687	*K.ITQLLPR.E*	53	
	423.2292	*K.FLDSHVK.F*	23	
	427.2443	*K.ALDFFIK.S*	33	
	430.2469	*K.TLQELQK.L*	31	
	448.7325	*K.HINIDER.M*	44	
	463.7402	*R.AFYELQR.D*	36	
	465.2867	*K.LVEEALKK.S*	41	
	472.2370	*K.HVSEAICK.E*	43	
	474.2468	*R.TMEQLTPK.L*	27	
	475.2409	*K.SKDFPEAR.A*	34	
	477.2687	*R.ASTALVYTK.N*	79	
	480.7716	*K.ALSDLQSVK.T*	49	
	482.2586	*K.LEGTSSLTR.K*	61	
	486.2612	*R.SISTALDHK.I*	41	
	508.7799	*R.TWLQEALR.N*	48	
	509.3010	*K.LATALSLSNK.L*	53	
	523.3055	*K.IPSVQINFK.K*	47	
	524.2568	*K.LDVTTNNDR.K*	50	
	526.7632	*R.VPQTGMTFR.H*	28	
	527.7561	*K.LDNIYSSDK.F*	69	
	528.3140	*K.LQDLQLLGR.L*	79	
	530.7622	*K.VQGTEFSHR.L*	29	
	539.7800	*R.EVTIDAQFR.D*	29	
	569.7801	*K.QGFFPDSVNK.A*	39	
	570.2720	*K.EVYGFNPEGK.A*	43	
	576.2888	*K.LDFSSQADLR.S*	91	
	386.2034	*K.SNTVAGLHTEK.N*	23	

	583.8014	*K.NSLFFSAQPR.A*	51	
	586.8195	*K.VDGIWNLEVK.E*	38	
	394.5385	*K.LHVSEQNAQR.A*	37	
	592.7663	*K.NSEEFAAAMSK.Y*	56	
	594.7802	*K.ATGASYDYVNK.Y*	59	
	604.3036	*R.DLSGMDTILAR.I*	54	
	607.7716	*K.FSALDMTNNGK.L*	76	
	609.8400	*K.TLVEQGFTVPK.I*	36	
	611.3148	*R.VNQNIVYESR.F*	73	
	633.3312	*R.IYAIWEQNTK.N*	35	
	633.8564	*K.TEVIPPLVENR.Q*	61	
	643.3612	*K.AGQLEFIVPSPK.R*	67	
	644.3610	*K.NTLELSNGVLVK.V*	21	
	649.8460	*R.VPELDDEIQIK.A*	66	
	437.2271	*K.SKPTVSSSMELK.Y*	46	
	439.5771	*R.KGNVATEISIER.N*	62	
	659.2654	*R.DFSAEYEEDGR.Y*	46	
	664.3650	*K.SISLPSLDPVSGR.I*	37	
	671.8826	*R.ALSNEAVTSLLPK.L*	35	
	673.8498	*K.VNNQLTLDSNTK.Y*	66	
	680.8447	*R.ALTASTNNEGNLR.V*	77	
	465.5612	*K.YHQEYTGLDLR.D*	30	
	701.8931	*K.IAELSTSAQEIIK.S*	49	
	709.8564	*K.ALYWVDGQVPDR.V*	64	
	474.5720	*K.FQETLEDIRDR.V*	35	
	482.9245	*R.LGGLFLTSGEHTSK.A*	50	
	736.8526	*R.DPATGQLNGESNLR.F*	26	
	491.6045	*K.QVLLYPEKEEPK.H*	22	
	748.8682	*K.AASSFPVDLSDYPK.S*	31	
	779.4096	*R.ITENDLQIALDNAK.I*	36	
	793.8498	*R.VYQMDMQQEVQR.Y*	97	
	530.2961	*R.AYLHILGEELGFVK.L*	52	
	535.6262	*R.LFSGSNTLHLVSTTK.T*	46	
	810.4105	*R.GLQNSAEQVYQGAVR.Q*	66	
	873.9498	*R.DAVDQPQEFTLVASVK.Y*	26	
	591.6442	*K.ADAVVDLLSYHVQGSAK.T*	72	
	607.6126	*R.NDCTGNEDHTYLILR.V*	83	
	630.3152	*K.TEHESEVLFVGNTIEGK.S*	68	
	476.7531	*K.FLHSIFQEIEEDLKR.L*	49	
	645.3337	*R.EVLLQTFLDDTSPGDKR.L*	97	
	666.0055	*R.FELELKPTGEVEQYSAR.A*	78	
	674.3291	*K.SLYQELLAQEDHSGFQR.L*	66	
	685.6516	*R.SGVQMNTNFFHETGLEAR.V*	71	
	894.4321	*K.SQVQVHSGSLQNNIQLSNDQEEAR.L*	47	
A0A384B912_BALAS ***Alpha-2-macroglobulin isoform X2***	383.2107	*R.GEAFTLK.A*	27	2013
	403.7056	*K.GPTQEFK.K*	28	
	408.7685	*K.RTTVLVK.N*	35	
	442.2842	*R.DLKPAIVK.V*	41	
	443.7246	*K.YGAATFTR.T*	57	
	445.7268	*K.SLDEEAVK.E*	40	
	447.2201	*R.VTINMCR.K*	33	
	452.2178	*K.DMYSFLK.D*	32	
	460.7271	*K.DLSGFPER.L*	33	
	467.7532	*K.GPTQEFKK.R*	39	
	484.2370	*K.RQEFEMK.I*	37	
	507.7535	*R.YLNTGYQR.Q*	47	
	509.8003	*K.ATVLNYLPK.C*	43	

	510.7849	*R.GIPFFGQVR.L*	29	
	516.2911	*K.TQQLTAEIK.S*	43	
	530.7842	*R.IMQWQNLK.V*	39	
	552.7985	*R.SSGSLLNNAIK.G + Deamidated (NQ)*	74	
	558.8060	*R.QTVSWAVTPK.S*	80	
	387.2121	*K.AHTSFQISLR.V*	43	
	581.2902	*K.ICPQPQQYK.I*	21	
	587.7799	*K.MLETSDHVSR.T*	72	
	398.8926	*K.SFVHLEPLPR.E*	44	
	605.8249	*K.LPPNVVEESAR.A*	63	
	620.7793	*K.YEVENCLANK.V + Deamidated (NQ)*	67	
	629.8445	*R.LLSSPVVAEMGR.G*	96	
	429.2349	*K.VFTNSNIHKPK.I + Deamidated (NQ)*	46	
	651.8457	*K.VTASPQSLCALR.A*	73	
	669.8295	*R.NALFCLESAWK.S*	51	
	472.9477	*K.MVSGFIPLKPTVK.M*	50	
	716.8622	*K.IQEEGTEVELTGK.G*	90	
	726.7958	*K.EQETQCICGNGR.Q + Deamidated (NQ)*	72	
	497.6242	*M.VLVPSLLHTGTPEK.G*	58	
	507.9207	*R.SGTHVLPVHQGDMK.G + Oxidation (M)*	31	
	763.3966	*K.AAQVTIQSSGTFSTK.F*	88	
	548.9582	*R.TEVSNNHVLIYLDK.V*	60	
	555.6386	*R.VHLEASPAFLAVPGEK.E*	56	
	861.4429	*R.APSNEEVMFLTIQVK.G + Oxidation (M)*	53	
	594.0087	*K.DTVIKPLLVEPEGLEK.E*	53	
	914.9600	*K.QLTFPLSSEPFQGSYK.V*	48	
	943.4475	*K.FSQQLNSQGCFSQQVK.T*	54	
	631.0072	*K.GHFPLSVPVESDIAPVAR.L*	64	
	477.7818	*R.KDTVIKPLLVEPEGLEK.E*	36	
	969.9594	*K.EATFNSLLCPSGAEVSEK.L*	58	
	494.2693	*K.VGLNFSPAQSLPASHAHLK.V*	51	
	681.6161	*K.YSNPSNCFGGESHAVCEK.F*	71	
	683.0209	*K.AGALCLSSDTGLGLSPTASLR.A*	96	
	713.7236	*R.AQTVQAHYVLNGQVLQELK.E*	63	
	724.6433	*R.KYSNPSNCFGGESHAVCEK.F + Deamidated (NQ)*	75	
	549.7866	*R.GHELMHIIHISEPPTETVR.K*	72	
	801.7352	*R.QQNSQGGFSSTQDTVVALHALSK.Y*	67	
	812.0687	*K.KEEFPFALEVQTLPQTCDGPK.A*	60	
	622.3431	*K.NEESLVIVQTDKPIYKPGQTVK.F*	63	
	845.3623	*K.VYDYYETDEFAVAEYNAPCSK.D*	49	
	649.3291	*K.SLDEEAVKEDNSVHWTRPQKPK.A + Deamidated (NQ)*	31	
	775.3732	*R.VHLEASPAFLAVPGEKEQETQCICGNGR.Q + Deamidated (NQ)*	89	
	793.8655	*R.HYDGSYSTFGEQHGNNEGNTWLTAFVLK.S*	85	
	824.3633	*K.EDNEDCISHHNIYLNGIMYSPVSNTNEK.D + Deamidated (NQ)*	46	
	863.6284	*K.DHSPCYGYQWLSEEYEEAYHTANLVFSR.S*	28	
A0A383Z2B4_BALAS ***Complement C3***	385.7113	*K.ENIPAAR.Q*	38	**2339**
	388.7367	*K.GVFVLNK.K*	39	
	400.7474	*R.VGLVAVDK.G*	52	
	402.2076	*K.VVPEGMR.V + Oxidation (M)*	34	
	417.2477	*R.LPYSVVR.N*	27	
	430.7455	*K.LLSTGVDR.Y*	41	
	437.7315	*R.DGTLELAR.S*	40	
	444.2327	*R.NEQVEIR.A*	47	
	451.7527	*K.IWDIVEK.A*	36	
	472.2767	*R.QEALELIK.K*	51	

	473.2628	*R.QPMTITVR.T*	54	
	476.2483	*K.FLNTATER.N*	37	
	479.7636	*K.LENDLLNK.F*	48	
	499.2950	*R.ISLAHSLTR.V*	63	
	539.2842	*R.DSCVGTLVVK.N*	42	
	546.8188	*K.NTLIIYLDK.V*	56	
	550.2806	*K.GYTQQLAYR.Q*	44	
	550.3271	*K.RQEALELIK.K*	39	
	551.2747	*R.VPITDGNGEAK.L + Deamidated (NQ)*	56	
	569.2968	*R.TLDPEHLGQK.G*	27	
	572.8036	*K.DFDTVPPVVR.W*	42	
	583.2883	*K.GSMILDICTR.Y*	62	
	587.3353	*R.IVWESASLLR.S*	67	
	587.8500	*R.HQQTLIIPPK.S*	32	
	597.7969	*R.YYTYLVMNK.G*	37	
	605.3170	*R.QPNSAYAAFLK.R*	36	
	409.8866	*K.KGYTQQLAYR.Q*	21	
	650.7977	*R.ACEPGVDYVYK.I*	30	
	673.3193	*K.FYYIDDPDGLK.V*	61	
	675.3480	*R.AQFILQGDACVK.A*	32	
	676.8200	*K.ENEDFTLTAQGK.G*	54	
	681.3184	*K.SGSDEVQVQQER.R*	88	
	685.8696	*K.TIYTPGSTVLYR.I*	57	
	687.3571	*R.EVVADSVWVDVR.D*	69	
	699.4239	*K.SSVPVPYVIVPLK.V*	33	
	473.9027	*K.VSHTLEDCLAFK.V*	42	
	526.5914	*K.AGDFLEDHYLELR.R*	58	
	829.8872	*K.AFLDCCEYIAQLR.L*	69	
	833.4464	*K.FVTVQADFGNVLVEK.V*	35	
	833.4814	*R.VLLNGVQPSQAAALVGK.S + Deamidated (NQ)*	22	
	852.9000	*K.AANLSDQVPDTESETK.I*	39	
	590.6614	*K.VHQYFNVGLIQPGAVK.V*	26	
	898.9664	*R.VELLYNPAFCSLATAK.K*	60	
	601.3332	*R.TGIPIVTSPYQIHFTK.T*	53	
	467.2613	*R.VELKPGETLNVNFHLR.T*	68	
	642.9693	*R.SEETKENEDFTLTAQGK.G*	36	
	704.3283	*K.AHYEDSPQQVFSAEFEVK.E*	51	
	715.0278	*K.ILLQGTPVAQMTEDAINGDR.L + Deamidated (NQ)*	89	
	1222.0769	*K.ADIGCTPGSGSDYAGVFTDAGLALK.T*	26	
	892.7664	*R.TMQALPYNTQDNSNNYLHLSVPR.V*	81	
A0A383ZXR0_BALAS ***Serum albumin***	395.2394	*K.IVTDLTK.V*	46	2314
	423.7320	*K.VTEEQLK.T*	22	
	433.7164	*K.ADFAEVSK.I*	49	
	445.2480	*K.LCAVASLR.E*	26	
	449.7443	*R.LCVLHEK.T*	28	
	457.2168	*K.DDNPDLPK.L*	34	
	457.2427	*K.YLYEIGR.R*	34	
	481.2510	*R.EQVLASSAR.Q + Deamidated (NQ)*	50	
	506.8237	*K.QIALVELVK.H*	50	
	509.2718	*K.SLHTLFGDK.L*	43	
	534.2449	*K.QNCELFEK.L*	61	
	536.2664	*K.SHCIAEVQK.D*	38	
	558.3192	*K.LVNEVTELAK.A*	57	
	569.7526	*K.CCTESLVNR.R*	58	
	570.8713	*K.KQIALVELVK.H*	72	
	576.2747	*K.ETCFALEGPK.L*	69	
	393.5316	*K.HKDDNPDLPK.L*	36	

	606.7807	*R.FNDLGEENFK.G*	77	
	646.3051	*K.ECCDKPLLEK.S*	25	
	657.3641	*R.HPEYSVSLLLR.I*	69	
	658.3209	*K.TVMGNFAAFVDK.C + Oxidation (M)*	79	
	678.2900	*K.GVFAECCQAADK.G*	56	
	699.3409	*K.YICENQATISAK.L*	66	
	705.7853	*K.ACVADESAANCDK.S*	84	
	717.7708	*R.ETYGEMADCCAK.Q*	74	
	490.6128	*R.RHPEYSVSLLLR.I*	63	
	753.9302	*K.LQPLVDEPQNLIK.Q*	38	
	756.4252	*K.VPQVSTPTLVEVSR.N*	83	
	756.8379	*K.DDPPACYATVFEK.L*	54	
	511.5983	*K.LKECCDKPLLEK.S*	62	
	768.4313	*K.LGEYLFQNALIVR.Y*	24	
	517.2570	*K.LKPDPDTLCSEFK.E*	55	
	547.3178	*K.KVPQVSTPTLVEVSR.N*	78	
	583.8923	*K.ECCHGDLLECADDR.A*	27	
	638.3090	*R.RPCFSALTVDETYEPK.A*	50	
	641.6367	*K.TFTFHADLCTLPENEK.Q*	43	
	980.4632	*K.DELPENLSPVAADFAEDK.E*	59	
	678.0515	*R.YTKKVPQVSTPTLVEVSR.N*	73	
	545.2658	*K.LKPDPDTLCSEFKENEQK.F*	42	
	735.0268	*R.HPYFYAPELLYYAHQYK.G*	35	
	562.7404	*K.ECCHGDLLECADDRADLAK.Y*	36	
	826.0563	*K.DELPENLSPVAADFAEDKEVCK.N*	55	
	753.8626	*K.SHCIAEVQKDELPENLSPVAADFAEDK.E*	61	
	706.5385	*K.SHCIAEVQKDELPENLSPVAADFAEDKEVCK.N*	76	
A0A384ALG4_BALAS	394.7163	*K.GSLLANGR.L + Deamidated (NQ)*	51	1968
Ceruloplasmin isoform X2	401.7099	*K.DPVCLAK.M*	20	
	458.7643	*R.KGSLLANGR.L + Deamidated (NQ)*	73	
	507.2159	*K.TYCSEPEK.V*	22	
	549.7883	*R.SYSIHAHGVK.T*	46	
	583.7699	*K.ANDEFIESNK.M*	76	
	587.7759	*K.MYYSAVDPTK.D*	45	
	595.2596	*R.EYTDGSFTNR.K*	49	
	403.5436	*R.IYHSHIDAPR.D*	43	
	629.8504	*K.HYLQVFNPIK.K*	48	
	637.3489	*R.SAPPPSASHVVPK.G*	28	
	664.8300	*K.GTFTYEWTVPK.E*	37	
	681.8541	*R.DTANLFPQTSLR.L*	59	
	456.2263	*K.AETGDTVYVHFK.N*	52	
	701.3909	*K.GTYPLSIEPIGVR.V*	75	
	746.3721	*K.ALYLQYTDETFK.T*	61	
	503.2819	*R.DIATGLIGPLIHCK.K*	60	
	756.3602	*R.QFTDSTFQVPGER.K*	57	
	506.9391	*R.RDTANLFPQTSLR.L*	43	
	785.8897	*K.TENPTVTPTAPGETR.T*	79	
	526.2732	*K.EVGPTYKDPVCLAK.M*	63	
	532.5693	*K.VDKDNEDFQESNR.M*	38	
	724.0344	*K.LISVDTEHSNIYLQNGPNR.I*	93	
	556.7984	*R.KPEEEHLGILGPQLHAGVGDK.V*	77	
	575.3015	*K.KLISVDTEHSNIYLQNGPNR.I*	37	
	777.3522	*R.SGAGIDDSPCIPWAYYSTVDR.V*	59	
	596.0428	*K.VRPGEQCMYILHANPEQGPGK.E*	69	
	796.7537	*R.GPEEEHLGILGPVISAEVGDTIR.V*	70	
	800.0380	*K.HYYIGIIETTWDYASDHGEK.K*	52	
	825.0328	*R.MFTTAPDQVDKENEDFQESNK.M*	50	

	648.2806	*K.TYCSEPEKVDKDNEDFQESNR.M*	94	
	866.0801	*K.AGLQAFFWVQDCQKPSSENDIR.G*	53	
	669.1016	*K.ERGPEEEHLGILGPVISAEVGDTIR.V*	69	
	744.3796	*K.ELHHLQELNLSNAFLDKEEFYIGSK.Y*	50	
	998.7731	*R.LFMQPDTEGTFDVECLTTDHYTGGMK.Q + Deamidated (NQ)*	37	
	669.3148	*K.VRPGEQCMYILHANPEQGPGKEDSNCVTR.I*	36	
A0A383Z5R5_BALAS	397.7057	*K.GTDFNLK.D*	23	1707
Serotransferrin	426.7085	*K.DSANGFLK.I + Deamidated (NQ)*	27	
	455.7112	*R.GSVDEFEK.C*	36	
	461.7534	*K.KGTDFNLK.D*	49	
	468.2747	*R.VPSHAVVAR.S*	59	
	475.2332	*K.LCQLCAGK.G*	45	
	500.2445	*K.TSYIDCIK.A*	26	
	503.7895	*K.YVTAVANLR.Q*	62	
	531.7373	*K.INHCEFDK.F*	24	
	542.2485	*R.YYGYSGAFR.C*	48	
	564.2920	*K.KTSYIDCIK.A*	38	
	575.8166	*K.THYYAVAVVK.K*	50	
	597.7642	*R.ECLPNNYER.Y*	47	
	426.9117	*K.THYYAVAVVKK.G*	48	
	431.1960	*R.WCTVSSHEASK.C*	39	
	656.2932	*K.FFSEGCAPGSPR.N*	81	
	678.3365	*K.VFDTGPFVSCVK.K*	69	
	696.8361	*R.EILDAQQDEFGK.H*	58	
	701.8334	*K.SSDPDLNWNNLK.D*	41	
	705.8417	*K.CLMDGVGDVAFVK.H*	79	
	723.3185	*K.ENTGGNNPEEWAK.T*	94	
	765.8958	*K.FTPESGYYAVAVVK.K*	42	
	550.2707	*K.GISEDFQLFSSPHGK.D*	44	
	878.3733	*K.IECESAESTEECIAK.I*	48	
	602.2895	*K.GEADAMSLDGGHIYIAGK.C*	40	
	956.9316	*K.TLQEDDFELLCTDGTR.K*	82	
	662.6082	*K.HGSDCSSSFCLFHSETK.D*	76	
	665.6106	*K.CACSNHEPYFGYSGAFK.C*	47	
	1028.0111	*K.CLAPLQNAITYESYLGNK.Y*	30	
	758.3589	*K.SVEEASDCFLAQGPNHAVVSR.E*	81	
	589.5130	*K.INHCEFDKFFSEGCAPGSPR.N*	51	
	601.0450	*R.KSVEEASDCFLAQGPNHAVVSR.E*	47	
	665.8179	*K.HTTVLENLPDEADKDEYELLCR.D*	77	
A0A384B6G0_BALAS	390.7158	*R.IASFSQK.C*	30	1145
Kininogen-1	423.2580	*K.AVDTALKK.Y*	40	
	436.7635	*K.ATVQVVAGK.K*	58	
	478.7635	*K.YSIVFTAR.E*	55	
	489.2745	*K.AYVDIQLR.I*	43	
	493.2605	*K.HSLILNCK.A + Deamidated (NQ)*	59	
	500.8110	*K.KATVQVVAGK.K*	86	
	530.7878	*K.RPPGFSPFR.S*	35	
	542.2828	*K.SGNQFVLYR.V*	78	
	542.8111	*K.KYSIVFTAR.E*	44	
	567.7400	*K.EGDCPVQSDK.T*	50	
	603.3133	*K.ADVYVVPWEK.K*	35	
	668.2909	*R.TDDPDTFYSFK.Y*	47	
	692.3344	*K.ENFLFLTPDCK.S*	72	
	698.3058	*K.SLSNGNIGECTDK.A + Deamidated (NQ)*	82	
	723.8487	*K.LNVENNGTFYFK.I + Deamidated (NQ)*	30	
	918.4359	*K.CDLYPVEDFVQPPTR.I*	36	

	967.5248	*K.IYPTVNCQSLGQISLLK.R*	64	
	688.0506	*K.KIYPTVNCQSLGQISLLK.R*	59	
	869.3638	*K.TWQDCDYGDSAQAATGECTATVAK.R*	64	
	725.8627	*R.ICAGCPRPIPVDSPELEEPLDHSIAK.L*	38	
	1190.0765	*K.FSVATQTCQITPAEGPVVTAQYDCLGCLHPISTESPDLEPVLR.H*	39	
A0A383ZCJ5_BALAS ***Hemopexin***	416.7208	*R.GPVDAAFR.H*	58	1094
	472.7288	*R.FWDFTTK.T*	46	
	495.7317	*K.LDPDVMER.C*	55	
	498.7498	*K.VNSLLGCPH.-*	73	
	503.7473	*K.VDAALCTEK.S*	52	
	524.2593	*K.VWEYPPEK.E*	40	
	551.3079	*R.QLWWLDLK.L*	28	
	579.7387	*R.DYFMPCPGR.G + Oxidation (M)*	43	
	669.3749	*K.LNVTEALPQPQK.V*	66	
	674.3301	*K.GDKVWEYPPEK.E*	31	
	727.3198	*R.SWQAVGNCSSAMR.W*	97	
	759.3982	*R.CSPDLVLSALLSDK.H*	82	
	547.2525	*K.HGATYAFSGSHYWR.L*	68	
	558.6348	*K.RCSPDLVLSALLSDK.H*	58	
	932.4734	*K.LGAQATWTELPWPHEK.V*	79	
	999.4752	*R.FNPVTGDMYPNYPLDVR.D*	95	
	814.8939	*K.EFGSPHGINLDTVDAAFTCPGSSLLHVMAGR.Q*	74	
	890.0217	*K.LLQEEFPGIPSPVDAAVECHHEECLHEGVLFFQGNHMR.F + Deamidated (NQ); Oxidation (M)*	52	
A0A383ZWG8_BALAS ***Keratin, type II cytoskeletal 5***	405.7086	*R.QSSVSFR.S*	21	1092
	414.2185	*R.FASFIDK.V*	39	
	442.7271	*R.TSFTSVSR.S*	57	
	453.7370	*R.FLEQQNK.V*	25	
	469.7508	*R.SLYNLGGSK.R*	52	
	473.2596	*R.GRLDSELR.N*	36	
	508.2349	*K.HEISEMNR.M*	39	
	513.7318	*K.DVDAAYMNK.V*	41	
	533.7618	*K.YEDEINKR.T*	41	
	541.8033	*R.FASFIDKVR.F*	64	
	547.2675	*K.AQYEEIANR.S*	50	
	567.2844	*R.KLLEGEECR.L*	64	
	571.2634	*K.DYQELMNTK.L*	58	
	576.7801	*K.NKYEDEINK.R*	41	
	597.7913	*K.YEELQQTAGR.H*	67	
	619.7900	*R.NMQDLVEDFK.N*	55	
	621.7853	*R.TEAESWYQTK.Y*	41	
	640.3489	*K.LSLDVEIATYR.K*	53	
	649.8187	*R.TTAENEFVMLK.K + Oxidation (M)*	42	
	436.8897	*K.NKYEDEINKR.T*	55	
	453.5542	*R.NTKHEISEMNR.M*	52	
	476.2464	*R.TTAENEFVMLKK.D + Oxidation (M)*	54	
	565.9468	*K.DVDAAYMNKVELEAK.V*	48	
A0A384ALK4_BALAS ***Fibronectin***	386.2217	*K.SEPLIGR.K*	32	1016
	397.2442	*R.ITGYVIK.Y*	23	
	555.7750	*R.STTPDITGYR.I*	49	
	569.2718	*R.FTNVGPDTMR.V*	40	
	585.2289	*R.YQCYCYGR.G*	22	
	611.2642	*R.TFYSCTTEGR.Q*	71	
	646.3669	*R.GATYNIIVEAIK.D*	49	
	441.9092	*K.LGVRPSQGGEAPR.E*	78	

	694.3301	*R.TFYQIGESWEK.F*	54	
	477.9213	*R.WSRPQAPITGYR.I*	22	
	716.3938	*R.VPGTSASATLTGLTR.G*	96	
	772.3859	*R.SYTITGLQPGTDYK.I*	32	
	515.2748	*K.EATIPGHLNSYTIK.G*	31	
	797.4046	*K.QYNVGPSATQYPLR.N*	60	
	542.5823	*K.LSCQCLGFGSGHFR.C*	71	
	622.6376	*R.HTTLQTTSAGSGSFTDVR.T*	78	
	691.9559	*R.GFNCESKPEPEETCFDK.Y*	39	
	727.7036	*K.NLHLETNPDTGVLTVSWER.S*	55	
	604.5263	*R.RPGAEPGHEGSTGHSYNQYSQR.Y*	47	
	830.7435	*R.TEIDKPSQMQVTDVQDNSISVR.W*	47	
	863.1167	*R.TKTETITGFQVDAIPANGQTPIQR.T + Deamidated (NQ)*	23	
A0A383YWT8_BALAS ***Complement factor H-like isoform X1***	433.2243	*K.SGEQVAFK.C*	65	929
	434.2106	*K.FSCIQGR.I*	40	
	457.7689	*K.VENAIIQK.E*	38	
	486.2400	*K.HTICINGR.W + Deamidated (NQ)*	66	
	508.2472	*R.DVSCVNPPK.V*	30	
	511.2604	*R.EAFTMIGPR.S*	41	
	547.7775	*K.EYLQGETVR.V*	58	
	554.2986	*R.TLGSIVMVCK.D*	21	
	603.2486	*K.CTSGFEYGER.G*	83	
	624.2720	*K.LSYTCEDGFR.I*	64	
	663.2846	*K.SCDMPVFENAR.A*	49	
	722.9049	*K.QPTILNGYPLSLK.E + Deamidated (NQ)*	25	
	741.8846	*K.SCAPPPQLLSGEVK.E*	48	
	499.9125	*R.CSFKPCDFPVIK.H*	27	
	556.2786	*K.LSCSQPPQVDHGTIK.S*	53	
	610.3134	*K.WTQPPQCIATEELKK.C*	33	
	955.9242	*R.GDAVCTEFGWTPVPSCK.E*	21	
	688.0054	*K.EEAQIQSCPPPPQIPNTR.D*	33	
	758.3656	*R.LGQQFTYHCDQYFVTPLR.T*	64	
	976.7631	*K.DSYQHGEEVIYNCDEGFGIDGPASIR.C*	74	
A0A383Z8T4_BALAS ***C4b-binding protein alpha chain isoform X7***	418.2499	*R.LALEVYK.L*	35	864
	489.3052	*K.LISSFLGLK.S*	61	
	556.2587	*K.QSIVFDCDK.G*	64	
	561.7481	*R.CTADGTWSPK.T*	27	
	585.7771	*K.CEPPPAISNGK.H + Deamidated (NQ)*	38	
	587.2454	*K.SGIDNSCTYR.Y + Deamidated (NQ)*	71	
	603.2824	*K.ADGPTTVTCQR.N*	65	
	426.8777	*R.YYCLSGYKPK.A*	23	
	434.2032	*R.KSGIDNSCTYR.Y*	41	
	668.2800	*R.YGDEVSYTCNK.K*	42	
	474.9221	*K.ALCLKPEIEHGR.L*	61	
	736.8660	*K.LMQCLPTPEEVR.L*	57	
	743.8723	*K.TISVWNPSPPTCK.K*	33	
	779.8806	*R.CGNPGELLNGQVTAK.T + Deamidated (NQ)*	57	
	866.9597	*K.LYVEIQQLELQNDK.A*	65	
	938.9174	*K.SGDAIYECDEGYTLVGK.N*	40	
	744.6987	*K.YGYQKPTEEEVYDIGTALR.Y*	46	
	580.0076	*K.TPECYPDCDSPPVIAHGHHK.L*	39	
P0DMA6|APOA1_BALAS ***Apolipoprotein A-I***	413.2325	*R.AHVETLR.Q*	26	856
	430.2295	*K.VQELQDK.L*	35	
	450.2508	*R.EQLGPVTR.E*	56	
	492.2794	*K.VAPLGEELR.E*	63	
	506.7929	*K.AKPALEDLR.Q*	59	

	530.2787	*K.LTPLAEEMR.D*	66	
	540.7534	*R.EFWDNLEK.E*	40	
	558.3066	*R.QKVQELQDK.L*	47	
	385.5595	*K.VREQLGPVTR.E*	39	
	608.8429	*K.VSILAAIDEASK.K*	74	
	612.3716	*R.QGLLPVLENLK.V*	54	
	416.2120	*K.EGGGSLVQYHAK.A + Deamidated (NQ)*	25	
	632.8199	*K.WQEELQIYR.Q*	48	
	633.8221	*K.VQPYLDEFQK.K*	26	
	464.9137	*K.KWQEELQIYR.Q*	40	
	714.8544	*R.DYVTQFEASALGK.Q*	79	
	490.2732	*R.VKDFATVYVDAIK.D*	48	
	599.2880	*R.EFWDNLEKETESLR.Q*	31	
A0A384BF87_BALAS ***Haptoglobin***	393.2293	*K.DIAPTLR.L*	36	722
	419.2086	*K.DYVEVGR.V*	27	
	429.7501	*K.QLVEIEK.V*	39	
	437.2558	*R.IIGGSLDAK.G*	68	
	460.7349	*K.GSFPWQAK.M*	27	
	490.7511	*R.VGYVSGWGR.N*	68	
	492.7956	*K.AKDIAPTLR.L*	42	
	573.7747	*K.HYEGSTVPEK.K*	33	
	587.8271	*K.VSSILDWVQK.T*	57	
	425.5507	*K.HYEGSTVPEKK.T*	40	
	667.3484	*R.NANFIFTEHLK.Y*	42	
	674.3165	*K.SCATAEYGVYVK.V*	53	
	843.9261	*K.VPTDETVMPICLPSK.D*	29	
	604.3366	*K.VLLHPDYSEVDIGLIK.L*	58	
	648.6652	*R.EKVPTDETVMPICLPSK.D*	34	
	718.7053	*K.SPVGVQPILNEHTFCAGLSK.Y*	69	
A0A383ZI56_BALAS ***Inter-alpha-trypsin inhibitor heavy chain H4 isoform X2***	420.7505	*K.ILGDLGPR.D*	46	584
	430.2478	*K.GSELVVAGK.L*	34	
	474.2815	*K.NVIFVIDK.S*	26	
	500.2831	*K.LALDNGGLAR.R*	46	
	571.3327	*K.AGLLLLSSPDR.V*	63	
	633.3223	*R.ITGGSSADPVFSK.R*	88	
	668.3364	*K.AAAQEQYSAAVAR.G*	26	
	452.5808	*K.LRDQNPDVLSAK.V*	48	
	695.8338	*R.VAEQEEAFQSPR.Y*	87	
	563.9771	*R.KTEQFQVSVSVAPAAK.V*	72	
	973.4864	*K.SPEQQQDTVLDGNFIVR.Y*	48	
A0A383ZCZ3_BALAS ***Hemoglobin subunit beta***	463.7501	*M.VHLTAEEK.S*	31	705
	473.7715	*K.SAVTALWAK.V*	58	
	518.7888	*K.VLASFSDGLK.H*	32	
	563.7844	*K.LHVDPENFR.L*	20	
	575.3408	*K.VVAGVANALAHK.Y*	73	
	637.8652	*R.LLVVYPWTQR.F*	55	
	640.4269	*R.LLGNVLVIVLAR.H*	76	
	664.8438	*K.VNVEEVGGEALGR.L*	88	
	712.8574	*K.EFTPELQAAYQK.V*	67	
	739.8492	*K.GTFATLSELHCDK.L*	87	
	882.9093	*R.FFEAFGDLSTADAVMK.N*	118	
A0A383ZRJ1_BALAS ***Keratin, type I cytoskeletal 14***	404.2033	*R.LAADDFR.T*	55	512
	405.2238	*R.LASYLDK.V*	46	
	515.3005	*R.VLDELTLAR.A*	54	
	532.8088	*R.LASYLDKVR.A*	53	
	553.7667	*K.VTMQNLNDR.L + Oxidation (M)*	92	

	572.7508	*K.DAEDWFFSK.T*	37	
	575.2972	*R.DVTSSSRQIR.T + Deamidated (R)*	28	
	629.7778	*K.NHEEEMNALR.G + Oxidation (M)*	42	
	701.3325	*R.GQVGGDVNVEMDAAPGVDLSR.I + Oxidation (M)*	81	
	538.7844	*K.VTMQNLNDRLASYLDKVR.A + Oxidation (M)*	26	
A0A383ZST7_BALAS	432.7446	*R.VFQTLEK.N*	40	**493**
Complement C5 isoform	447.7866	*R.LPLDLVPK.T*	34	
X1	456.2365	*R.IVACASYK.L*	38	
	474.2325	*K.WLSEEQR.Y*	41	
	549.3197	*K.LQGTLPIEAR.E*	65	
	555.7875	*R.VTFDSETAIK.E*	38	
	591.2880	*R.ESYAGTTLDPK.G*	31	
	668.8057	*K.TTCVNADLEEGK.Q*	62	
	674.3805	*K.IIAITEENAFVK.Y*	55	
	676.3415	*R.VDQQLTDYEIK.D*	47	
	513.5767	*K.TSTSEEICSFHLK.I*	22	
	814.4074	*R.GEQIQLSGTVYNYR.T*	23	
A0A384BAA9_BALAS	429.7251	*K.GNAAELQR.S*	33	**491**
Apolipoprotein A-IV	493.2884	*K.LAPLTESVR.G*	40	
	544.2851	*K.IDQNVEELK.A*	42	
	558.2883	*K.IDQNVEELR.R*	82	
	638.8320	*R.SLAPYAQDVQGK.L*	40	
	437.9069	*K.LVPFATELHER.L*	55	
	692.8688	*R.TQVNAQAQQLQR.Q*	74	
	613.9874	*K.LGPLAGDVEDHLSFLEK.D*	58	
	653.9971	*R.SLAKLSSHLDQQVETFR.H + Deamidated (NQ)*	68	
A0A383Z9Z9_BALAS	542.8118	*R.WGPETLLLR.L*	46	456
Alpha-mannosidase	558.8038	*R.ETTLAANQLR.A*	33	
	568.2965	*K.LATAQGQQYR.T*	79	
	597.8640	*K.EVLAPQVVLAR.G*	53	
	623.8615	*R.VLVIQNEYIR.A*	68	
	482.2321	*R.LEHQFAVGEDSGR.N*	29	
	910.9919	*R.IYITDGNVQLTVLTDR.S*	81	
	620.6356	*R.SQGGSSLSDGSLELMVHR.R*	67	
A0A383ZV20_BALAS	440.7245	*K.INDYVEK.G*	33	431
Alpha-1-antitrypsin	444.7557	*K.AVLTIDEK.G*	45	
	504.7717	*K.KINDYVEK.G*	48	
	548.7980	*K.LSISGTYDLK.T*	69	
	440.5420	*R.DFHVDEETTVK.V*	51	
	533.2636	*K.NLYHSEAFSINFR.D*	63	
	553.9299	*K.WEKPFEAEHTTER.D*	42	
	461.9787	*K.VFSNGADLSGITEEVPLK.L*	58	
	938.9814	*K.VFSNGADLSGITEEVPLK.L + Deamidated (NQ)*	23	
A0A383ZWF6_BALAS	414.2185	*R.FASFIDK.V*	39	388
Keratin, type II	453.7370	*R.FLEQQNK.V*	25	
cytoskeletal 6A-like	541.8033	*R.FASFIDKVR.F*	64	
isoform X2	567.2844	*R.KLLEGEECR.L*	64	
	570.2737	*K.DYQELMNVK.L*	41	
	590.3036	*K.YEELQLTAGR.H*	56	
	602.3222	*K.WTLLQEQGTK.T*	47	
	640.3489	*K.LSLDVEIATYR.K*	53	
A0A383ZRG8_BALAS	404.2033	*R.LAADDFR.L*	55	369
Keratin, type I	561.7931	*R.LEQEIATYR.S*	68	
cytoskeletal 15	601.8044	*R.QSVEADINGLR.R + Deamidated (NQ)*	81	
	651.3330	*R.ALEEANADLEVK.I*	78	
	453.5722	*R.QSVEADINGLRR.V + Deamidated (NQ)*	47	

	460.5807	*K.TRLEQEIATYR.S*	43	
A0A384A3E4_BALAS	380.2158	*M.VLSPTDK.S*	47	365
Hemoglobin subunit alpha	395.7083	*R.VDPANFK.L*	23	
isoform X1	559.7540	*R.MFMNFPSTK.T + Oxidation (M)*	43	
	626.8616	*K.FLASVSTVLTSK.Y*	86	
	539.5939	*K.IGNHSAEYGAEALER.M*	59	
	466.2274	*K.TYFPHFDLGHDSAQVK.G*	31	
	593.7971	*K.AVGHMDNLLDALSDLSDLHAHK.L*	57	
A0A384AZC9_BALAS	454.2658	*K.YINKEIK.N*	21	338
Clusterin	455.2451	*R.GSLFFNPK.S*	46	
	485.7313	*K.FMETVAEK.A*	42	
	523.7895	*K.SLLGSLEEAK.K*	46	
	616.8101	*K.TLIEQTNEER.K*	71	
	719.8353	*R.ASNIMDELFQDR.F*	80	
	666.6522	*R.KFQDTQYYSPFSSFPR.G*	32	
A0A452CBF7_BALAS	416.7299	*K.VTQLTDR.W*	47	334
Desmoplakin isoform X1	431.7552	*R.GLVGIEFK.E*	33	
	454.2658	*R.YIELLTR.S*	45	
	459.7684	*R.LPVDIAYK.R*	36	
	471.7719	*R.QVQNLVNK.S*	38	
	565.3095	*K.IEVLEEELR.L*	57	
	587.8157	*R.LLQLQEQMR.A + Oxidation (M)*	42	
	610.7909	*R.ETQSQLETER.S*	40	
A0A384B1Q0_BALAS	407.2576	*K.ILEPTLK.S*	41	328
Vitamin D-binding protein	469.7449	*R.DLSSFIEK.G*	35	
	489.7603	*K.ELPEYTVK.I*	24	
	588.7716	*R.ICSQYSAYGK.E*	70	
	433.5865	*R.KTHIPEVFLSK.I*	24	
	678.8436	*K.VLDQYTFDLSR.K*	64	
	614.9946	*R.GKLPDATETELEELVAK.R*	36	
	903.0740	*K.HQPQEFPTYVEPTNDEICEAFR.K*	35	
A0A384A7N6_BALAS	428.7480	*K.AGAVNPTVK.F*	52	323
Dipeptidyl peptidase 4	552.2830	*R.VLEDNSALDK.M*	79	
	604.7866	*K.MLQDVQMPSK.K + 2 Oxidation (M)*	41	
	755.8278	*K.WEYYDSVYTER.Y*	59	
	607.6143	*R.YMGLPTPEDNLDHYR.N*	34	
	858.4322	*R.AENFKQVEYLLIHGTADDNVHFQQSAQISK.A*	59	
A0A383ZRE5_BALAS	405.2238	*R.LASYLDK.V*	46	306
Keratin, type I	515.3005	*R.VLDELTLAR.A*	54	
cytoskeletal 17 isoform X1	531.7534	*K.ATMQNLNDR.L*	54	
	561.7931	*R.LEQEIATYR.R*	68	
	629.7778	*K.NHEEEMNALR.G + Oxidation (M)*	43	
	460.5807	*K.TRLEQEIATYR.R*	42	
A0A383ZRY6_BALAS	406.7840	*R.LVQLLVK.A*	26	275
Junction plakoglobin	412.8674	*R.ISEDKNPDYR.K*	55	
	618.8478	*R.VSVELTNSLFK.H*	36	
	684.8066	*R.TMQNTSDLDTAR.C + Oxidation (M)*	96	
	714.4056	*R.ALMGSPQLVAAVVR.T + Oxidation (M)*	36	
A0A452CBL8_BALAS	527.3082	*R.YLTVAAIFR.G*	66	272
Tubulin beta-2A chain	546.2818	*R.LHFFMPGFAPLTSR.G + Oxidation (M)*	76	
isoform X2	653.6653	*K.GHYTEGAELVDSVLDVVR.K*	94	
	776.3582	*K.FWEVISDEHGIDPTGSYHGDSDLQLER.I*	35	
A0A384AFQ0_BALAS	406.7473	*R.LPVIENK.V*	28	262
Plasminogen	425.2395	*R.DVVLFEK.R*	21	
	510.7323	*R.TPENFPCR.N*	26	
	549.2380	*R.NLEENYCR.N*	31	

	561.7552	*R.WEFCDIPR.C*	30	
	432.5549	*K.HNIFTPEANPR.A*	29	
	491.5804	*R.GTVAVTVSGHTCQR.W*	46	
	784.0802	*K.VVLGAHQETAVEDSVQEIQVAK.L*	51	
A0A383ZM34_BALAS	566.3240	*R.VGVIDLSPFGK.F*	106	257
Dimethylglycine	614.7959	*K.WTTTQYTEAK.A*	67	
dehydrogenase,	575.2853	*R.ISYTGELGWELYHR.R*	57	
mitochondrial isoform X1	779.4244	*K.KADIINIVNGPITYSPDILPMVGPHQGVR.N*	27	
A0A383ZRY3_BALAS	404.2033	*R.LAADDFR.T*	55	252
Keratin, type I	521.3062	*R.IVLQIDNAR.L*	37	
cytoskeletal 19	553.7667	*K.VTMQNLNDR.L + Oxidation (M)*	92	
	561.7931	*R.LEQEIATYR.N*	68	
A0A383YQI3_BALAS	467.7716	*K.AIYEAVLR.S*	34	192
Xaa-Pro dipeptidase	484.2400	*R.EASFEGISK.F*	59	
	557.3424	*K.VPLALFALNR.R*	27	
	489.2755	*R.LADRIHLEELTR.I*	31	
	493.9339	*R.VFKTDMELEVLR.Y*	41	
A0A384B2P9_BALAS	401.2265	*R.DLLDIGR.N*	41	191
Complement factor B	481.7398	*R.LPQSTTCR.Q*	52	
	497.2843	*K.ALFVSELSK.D*	39	
	501.2668	*K.DVSEVVTPR.F*	29	
	696.8091	*R.LEDSVTYYCSR.G*	31	
A0A384B2W1_BALAS	448.2590	*R.GQIVSVHR.E*	21	181
Complement C4	454.7454	*R.SGFLSIER.L*	42	
	585.3170	*K.LQDAPSGQVVR.G*	41	
	769.3969	*K.VENSISSANTFLGAK.V*	28	
	778.4198	*K.ILSLAQEQVGGSPEK.L*	50	
A0A383Z8T4_BALAS	868.8886	*K.LSCTSSGWSPAAPQCK.A*	87	176
C4b-binding protein alpha	938.9143	*K.SGDAIYECDEGYTLVGK.N*	88	
chain isoform X7				
A0A384B7A3_BALAS	463.7584	*K.QPPAWSIK.D*	23	160
selenoprotein P	528.7536	*K.DDFLIYDR.C*	39	
	549.7421	*K.TLEDEGFCK.N*	49	
	623.0826	*R.LVYHLGLPYSFLTSSHVEDAIK.T*	50	
A0A383ZWH5_BALAS	404.2033	*R.LAADDFR.V*	55	155
Keratin, type I	521.3062	*R.IVLQIDNAR.L*	37	
cytoskeletal 18	533.2643	*R.AQYDELAQK.N*	25	
	553.8142	*R.LASYLERVR.S*	38	
A0A452C4G6_BALAS	490.2336	*R.ATNYNPGSR.S*	47	154
lysozyme C	492.2796	*R.VVQDPQGIK.A*	21	
	700.8442	*R.STDYGIFQINSR.Y*	86	
A0A383ZW41_BALAS	543.3140	*K.EIIDLVLDR.I*	57	153
Tubulin alpha chain	470.9295	*R.QLFHPEQLITGK.E*	23	
	573.6323	*R.NLDIERPTYTNLNR.L*	36	
	777.3443	*R.AFVHWYVGEGMEEGEFSEAR.E*	37	
A0A452C7H9_BALAS	438.7630	*R.IFLQDIK.K*	41	144
Ferritin heavy chain-like	449.2144	*K.YFLHQSHEER.E*	31	
	515.9064	*R.QNYHQDSEAAINR.Q*	72	
A0A384AF15_BALAS	488.7278	*K.AGFAGDDAPR.A*	64	141
Actin, cytoplasmic 2	566.7672	*R.GYSFTTTAER.E*	55	
	652.0265	*R.VAPEEHPVLLTEAPLNPK.A*	22	
A0A383YRP6_BALAS	420.2108	*K.FDTISEK.T*	45	136
Antithrombin-III	456.2691	*R.LPGIVAEGR.N*	59	
	764.3883	*K.AFLEVNEEGSEAAASTVIGIAGR.S*	32	
A0A383ZRJ1_BALAS	404.2033	*R.LAADDFR.T*	36	135
Keratin, type I	405.2238	*R.LASYLDK.V*	23	

cytoskeletal 14	1043.4943	*R.GQVGGDVNVEMDAAPGVDLSR.I*	78	
A0A384AFN8_BALAS	845.1037	*K.LTAVSVGVQGSGWGWLGFNKEQGR.L*	80	130
Superoxide dismutase	698.3638	*K.EKLTAVSVGVQGSGWGWLGFNKEQGR.L*	50	
A0A452CDN2_BALAS	405.2238	*R.LASYLDK.V*	46	120
Keratin, type I	532.8088	*R.LASYLDKVR.A*	53	
cytoskeletal 12	561.7629	*K.MTMKNLNDR.L*	21	
A0A383Z527_BALAS	489.5794	*K.TATPQQAQEVHEK.L*	42	118
Triosephosphate	801.9481	*K.VVLAYEPVWAIGTGK.T*	30	
isomerase	434.7322	*K.TATPQQAQEVHEKLR.G*	46	
A0A452C549_BALAS	830.9147	*K.YAASSYLALTASEWK.S*	117	117
Immunoglobulin lambda-				
1 light chain				
A0A384AEC5_BALAS	505.2635	*R.ATVIYEGEK.V*	40	111
Beta-2-glycoprotein 1	477.5748	*R.TCPKPDELPFAR.V*	37	
	872.9594	*K.LPVCAPTTCPPPPIPK.F*	34	
A0A384AU56_BALAS	474.7794	*K.DIVYIGLR.D*	33	109
Arginase	557.2958	*R.GGVEEGPTVLR.K*	76	
A0A384B6M8_BALAS	500.3157	*K.LVVLPFPSK.E*	73	108
Fetuin-B	595.2817	*K.SVSVTCDFFK.S*	35	
A0A383ZV15_BALAS	408.2605	*R.ALLFVPR.R*	41	108
Heat shock protein HSP	757.3957	*R.GVVDSEDLPLNISR.E*	67	
90-alpha				
A0A384B2D0_BALAS	415.2681	*R.ALLFIPR.R*	36	102
Heat shock protein HSP	757.3957	*R.GVVDSEDLPLNISR.E*	67	
90-beta				
A0A384AGF6_BALAS	401.2451	*R.ALQASALK.A*	33	101
Fructose-bisphosphate	566.7927	*R.ALANSLACQGK.Y*	35	
aldolase	745.8580	*R.LQSIGTENTEENR.R*	32	
A0A383ZNS6_BALAS	490.7239	*R.QDGSVDFGR.K*	30	99
Fibrinogen beta chain	891.4093	*K.DNENVINEYSSQLEK.H*	69	
A0A383YX88_BALAS	465.7194	*K.HADPDFTK.K*	25	93
Inter-alpha-trypsin	494.9440	*K.AHVTFKPTVAQQR.K*	26	
inhibitor heavy chain H2	791.9318	*K.IQPSGGTNINEALLR.A*	42	
A0A384A960_BALAS	574.7888	*R.NPNPGAQPEPK.E*	27	90
Alpha-2-antiplasmin	648.8311	*K.LGNQEPGGQTAPK.K*	34	
	761.7364	*R.GISDQSLVVSSVQHQSTLELR.E*	30	
A0A384BAB1_BALAS	865.9330	*K.DALTSVQESQVAQQAR.G*	87	87
Apolipoprotein C-III				
A0A452C549_BALAS	830.9166	*K.YAASSYLALTASEWK.S*	89	89
Immunoglobulin lambda-				
1 light chain				
A0A383ZWG2_BALAS	453.7370	*R.FLEQQNK.L*	25	82
Keratin, type II cuticular	611.8196	*R.ATAENEFVTLK.K*	32	
Hb6	632.3506	*K.LGLDIEIATYR.R*	25	
A0A384BC72_BALAS	396.2076	*R.IIGANMR.T + Deamidated (NQ); Oxidation (M)*	31	82
Unconventional myosin-	429.7501	*K.LQVQLEK.K + Deamidated (NQ)*	22	
Vb isoform X4	444.2327	*K.NELNELR.K*	30	
A0A384A9E5_BALAS	645.2896	*R.GLYCYELDEK.A*	31	80
Vitronectin	675.3448	*K.GIPDNVDAALALPAHSYSGR.E*	50	
A0A383ZQR0_BALAS	416.2502	*K.STELLIR.K*	28	77
Histone H3.1-like	495.2926	*K.VFLENVIR.D*	50	
A0A384BAE9_BALAS	565.8066	*R.VLQEAGADISK.T*	76	76
Phosphotriesterase-				
related protein				
A0A452CPB6_BALAS	516.2723	*K.VVEESELAR.T*	50	76
Complement component	699.3558	*R.AIADYINEFSVR.K*	25	

C9				
A0A452CB89_BALAS ***Unconventional myosin-XV***	422.2686 523.3142	*K.IILLQSR.A + Deamidated (NQ)* *K.TEATKLILR.Y + Deamidated (R)*	46 30	75
A0A383ZIC9_BALAS ***L-lactate dehydrogenase***	624.8040	*R.VIGSGCNLDSAR.F*	74	74
A0A384A978_BALAS ***14-3-3 protein epsilon isoform X1***	628.7988	*R.YLAEFATGNDR.K*	68	68
A0A384A061_BALAS ***Cathepsin G-like***	451.9123 849.7634	*R.RTDTLHDVQIR.V* *R.AIPHPGYNPQNNENDIMLLQLR.S*	28 39	66
A0A383ZVX9_BALAS ***Protein kinase C-binding protein NELL2***	572.2315	*R.LDQCYCER.T*	64	64
A0A384AG73_BALAS ***Histone H2A***	425.7666 458.2725	*R.HLQLAIR.N* *K.KMRIIPR.H + 2 Deamidated (R)*	31 31	62
A0A384BA01_BALAS ***Heparin cofactor 2 isoform X1***	449.2447 544.7905	*K.FALDLYR.A* *K.QVPVLDDFR.A*	40 22	62
A0A384B0X4_BALAS ***Glutathione synthetase***	436.7455	*K.ILSNNPSK.G*	60	60
A0A452CCD2_BALAS ***Alpha-1B-glycoprotein***	567.3012	*R.FPLGAVTGDTR.G*	60	60
A0A383YT12_BALAS ***Annexin***	622.8151	*R.TNQELQEINR.V*		
A0A383ZP87_BALAS ***Charged multivesicular body protein 5***	466.7456 572.8304	*K.KMREGPAK.N + Oxidation (M)* *K.ISRLDAELVK.Y + Deamidated (R)*	21 36	57
A0A383Z9P7_BALAS ***Polymeric immunoglobulin receptor***	486.2736 758.7378	*K.IVEGEPSLK.V* *K.SNADLQVLKPETELIYGDLR.G*	32 24	56
A0A383Z8Q9_BALAS ***Heterogeneous nuclear ribonucleoproteins A2/B1 isoform X1***	594.8271	*K.IDTIEIITDR.Q*	56	56
A0A452CGT3_BALAS ***Periplakin***	473.2628 487.2586	*R.QSLQLEAR.R + Deamidated (NQ)* *R.GELQQLQR.R + 2 Deamidated (NQ)*	21 37	
A0A383ZLI1_BALAS ***Microtubule-associated protein 1B***	528.3140 626.8099	*R.AIGNIELGIR.S* *K.ETKNAANTSTSK.S + Deamidated (NQ)*	25 30	54
A0A383ZSV7_BALAS ***Tenascin isoform X1***	568.2903	*K.APTTQVESFR.V*	51	51
A0A383YYI6_BALAS ***Serine/threonine-protein phosphatase***	466.2614 473.2596	*K.QTIETAIR.G* *R.AAARAESLR.A + Deamidated (R)*	21 29	50
A0A384B0M0_BALAS ***E3 ubiquitin-protein ligase SH3RF1***	452.2299	*K.EDELELR.K*	47	47
A0A384A314_BALAS ***Hydroxyacylglutathione hydrolase, mitochondrial isoform X1***	435.7740	*K.ALLEVLGR.L*	46	46
A0A383ZYA9_BALAS ***F-box only protein 50***	410.2362	*R.TVIAQHHVAPR.T*	46	46
A0A383YRQ8_BALAS ***Histone H2B***	408.7324	*R.EIQTAVR.L*	45	45

A0A383ZNS6_BALAS ***Fibrinogen beta chain***	474.7266	*R.ECEEIIR.N*	45	45
A0A384ATV8_BALAS ***Glycerol-3-phosphate dehydrogenase [NAD(+)]***	436.2840	*K.GALGISLIK.G*	44	44
A0A383Z1U4_BALAS ***Complement C3-like isoform X1***	652.8309	*R.VFVSNPDGSPASK.V*	43	43
A0A383ZQ81_BALAS ***Trypsin-like***	588.3214	*R.TLNNDIMLIK.L + Deamidated (NQ)*	43	43
A0A383YW83_BALAS ***Desmoglein-1***	513.7371	*R.TMNNFLDR.E + Oxidation (M)*	43	43
A0A383ZIZ4_BALAS ***Rootletin***	453.7302 487.2586	*R.TLSEEATR.L* *K.GQLQQELR.R + 2 Deamidated (NQ)*	21 22	43
A0A383ZUP7_BALAS ***Protein S100***	494.7593	*K.LLQTECPK.F*	42	42
A0A383ZJG1_BALAS ***Basement membrane-specific heparan sulfate proteoglycan core protein***	549.7988	*R.IGAPTNLEQR.A*	42	42
A0A384AJC0_BALAS ***protrudin isoform X1***	610.8038	*R.IGAPTNLEQR.A*	42	42
A0A452C841_BALAS ***Ankyrin repeat domain-containing protein 24***	539.5939	*R.ARQAQSRAQEALER.A + Deamidated (NQ); 2 Deamidated (R)*	42	42
A0A452C9S2_BALAS ***DnaJ homolog subfamily C member 14-like***	492.2611 398.8752	*R.RKEYIMK.R + Oxidation (M)* *R.SPGRHQLGGKR.S + Deamidated (NQ); Deamidated (R)*	22 22	40
A0A383ZSX3_BALAS ***Protein AMBP***	483.7103	*K.ECLQTCR.T*	39	39
A0A383YSS2_BALAS ***Thioredoxin reductase 1***	624.3392	*R.QFVPIKVEQIEAGTPGR.L + 2 Deamidated (NQ)*	39	39
A0A452CBX0_BALAS ***Myosin-1-like***	468.2629	*K.CASLKKTK.Q*	38	38
A0A383YQ74_BALAS ***60S ribosomal protein L30***	879.4589	*R.VCTLAIIDPGDSDIIR.S*	36	36
A0A383ZYL4_BALAS ***Carbonic anhydrase 2***	527.9437	*K.YAAELHLVHWNTK.Y*		
A0A383ZZQ3_BALAS ***N6-adenosine-methyltransferase catalytic subunit***	508.7721	*K.QLDSLRER.L*	36	36
A0A384ANP8|A0A384ANP8_BALAS ***HERV-H LTR-associating protein 1***	460.5570	*K.QKCLENICKSV.- + Deamidated (NQ)*	35	35
A0A384A168|A0A384A168_BALAS ***Catalase***	487.7490	*K.NLSVEDAAR.L*	35	35
A0A384AEC5_BALAS ***Beta-2-glycoprotein 1***	759.6873	*K.TSYAPGEEIVYTCQPGYVSR.G*	34	34
A0A384A9V8|A0A384A9V8_BALAS ***SWI/SNF-related matrix-associated actin-dependent regulator of***	424.7348	*K.KNVFNPK.R + 2 Deamidated (NQ)*	34	34

chromatin subfamily A containing DEAD/H box 1 isoform X2				
A0A384BAV9_BALAS ***Collagen alpha-1(IV) chain isoform X1***	642.3357	*K.ILYHGYSLLYVQGNER.A*	34	34
A0A452CIN7_BALAS ***Pleckstrin homology domain-containing family G member 3***	483.2744	*K.SKVYQLAR.Q + Deamidated (NQ)*	34	34
A0A383YWG2_BALAS ***Kinesin-like protein KIF21A***	483.2744	*R.ASQQINALR.S + 2 Deamidated (NQ)*	33	33
A0A384ACX2_BALAS ***Charged multivesicular body protein 4c***	514.8088	*K.RAALQALKR.K + Deamidated (NQ); Deamidated (R)*	33	33

ǂIons score is -10*Log(P), where P is the probability that the observed match is a random event. Individual ions scores > 32 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 fin whale (*Balaenoptera physalus*). 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 *B. physalus* are included. Peptide sequences and m/z values are listed. For protein hits against the full cetacean database see Supplementary Table 3.

Protein name	m/z	Peptide sequence	Score (p<0.05)ǂ	Total score
Q0QES7_BALPH ***Glyceraldehyde-3-phosphate dehydrogenase***	406.2101 435.2576 685.3741 778.9081 539.2985 910.4540	*K.LTGMAFR.V* *K.VIPELNGK.L* *R.GAAQNIIPASTGAAK.A* *R.VPTPNVSVVDLTCR.L* *K.AITIFQERDPANIK.W* *K.IVSNASCTTNCLAPLAK.V*	40 42 77 97 33 92	381
O02673_BALPH ***Gamma fibrinogen***	486.5787	*R.SDGPAKPNGIDSATK.I*	46	46
Q0QEQ3_BALPH ***Isocitrate dehydrogenase 1***	690.3438	*K.VEISYTPSDGSPK.T*	22	22

ǂIons score is -10*Log(P), where P is the probability that the observed match is a random event. Individual ions scores > 15 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 3. Deiminated proteins identified by F95 enrichment in serum of humpback whale (*Megaptera novaeangliae*). 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 *M. novaeangliae* are included. Peptide sequences and m/z values are listed. For protein hits against the full cetacean database see Supplementary Table 4.

Protein name	m/z	Peptide sequence	Score (p<0.05)ǂ	Total score
A1E0X3_MEGNO ***Beta-actin***	488.7276 652.0251	*K.AGFAGDDAPR.A* *R.VAPEEHPVLLTEAPLNPK.A*	51 27	78
R9S009_MEGNO ***Myoglobin***	479.0133	*K.YLEFISDAIIHVLHSR.H*	47	47

ǂIons score is -10*Log(P), where P is the probability that the observed match is a random event. Individual ions scores > 13 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 4. Deiminated proteins identified by F95 enrichment in serum of Cuvier's beaked whale (*Ziphius cavirostris*). 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 *Z. cavirostris* are included. Peptide sequences and m/z values are listed. For protein hits against the full cetacean database see Supplementary Table 5.

Protein name	m/z	Peptide sequence	Score (p<0.05)[ǂ]	Total score
MYG_ZIPCA	395.7186	*K.ASEDLKK.H*	31	696
Myoglobin	455.7348	*K.GHPETLEK.F*	31	
	475.7403	*K.YKELGFHG.-*	46	
	434.2224	*K.GHPETLEKFDK.F*	37	
	708.9197	*K.HGHTVLTALGGILK.K*	89	
	773.8487	*R.HPSDFGADAQAAMTK.A*	126	
	818.9368	*K.VEADLSGHGQEILIR.L*	102	
	464.2471	*K.GHHEAELKPLAQSHATK.H*	51	
	933.5031	*M.GLSEAEWQLVLHVWAK.V*	85	
	638.3455	*K.YLEFISDAIIHVLHSR.H*	57	
	397.2168	*K.KGHHEAELKPLAQSHATK.H*	42	
A8IY74_ZIPCA	612.8331	*R.KGHQPSTQLTK.K*	23	23
Recombination activating protein 1				

[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 5. Deiminated proteins identified by F95 enrichment in serum of orca (*Orcinus orca*). 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 *O. orca* are included. Peptide sequences and m/z values are listed. For protein hits against the full cetacean database see Supplementary Table 6.

Protein name	m/z	Peptide sequence	Score (p<0.05)[ǂ]	Total score
G8Z0F2_ORCOR	533.7796	*R.SAFSVGLETR.V*	42	42
Adiponectin				
G8Z0E8_ORCOR	398.2398	*K.IIAPPER.K*	33	101
Cytoplasmic beta-actin	895.9500	*K.SYELPDGQVITIGNER.F*	68	

[ǂ]Ions score is -10*Log(P), where P is the probability that the observed match is a random event. Individual ions scores > 15 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.

3.4 Protein interaction network analysis

STRING analysis (Search Tool for the Retrieval of Interacting Genes/Proteins; https://string-db.org/) was used for the identification of putative protein-protein interaction networks for the deiminated proteins identified in northern minke whale, fin whale, humpback whale, Cuvier's beaked whale and orca, respectively – based on identification of protein hits using the larger cetacean database following LC-MS/MS analysis. These networks were based on the STRING cetacean database for minke whale (constructed using minke whale hits), while hits with orca were used for constructing protein-interaction networks for fin whale, humpback whale, Cuvier's beaked whale and orca. As protein-interaction network analysis for minke whale, using the corresponding orca STRING

database, revealed a similar analysis as when using the minke whale STRING database, the minke whale STRING database was used for the analysis of protein-interaction networks in minke whale (Figure 4). The PPI enrichment *p*-value for all protein networks identified in the 5 ceataceans under study was $p < 1.0 \times 10^{-16}$, indicating that the deiminated protein hits have more interactions among themselves than what would be expected for a random set of proteins of similar size. Annotations for KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways identified for the deiminated protein hits, which relate to physiological and immunological pathways, are represented in Fig. 4-8, with some shared pathways common between all or some of the species, while other pathways identified were species-specific (see Venn Diagram in Fig. 9). Physiological KEGG pathways identified for deiminated proteins included the complement and coagulation cascades, renin-angiotensin system, oestrogen signalling pathway, cholesterol metabolism, vitamin digestion and absorption, glycolysis/gluconoegenesis, biosynthesis of amino acids, fat digestion and absorption, metabolic pathways, carbon metabolism, ECM-receptor interaction, nitrogen metabolism, pentose phosphate pathway, HIF-1 signalling, thyroid hormone synthesis and proximal tubule bicarbonate reclamation (Fig. 4A, 5A, 6A, 7A, 8A). KEGG pathways for immune defences/host-pathogen interactions included *Staphylococcus aureus* infection, systemic lupus erythematosus (SLE), amoebiasis, ferroptosis, phagosome, necroptosis, prion diseases, pertussis, metabolism of xenobiotics, cancer, prion diseases and tryptamosomiasis (Fig. 4B, 5B, 6B, 7B, 8B). Figures 4-8 highlight the different KEGG pathways for deiminated proteins identified in the cetaceans under study, while the Venn diagrams in Fig. 9 show the number of common and species-specific physiological (Fig. 9A) and immune related (Fig. 9B) pathways identified for the deiminated protein hits identified. Relevance of these KEGG pathways for physiology and immunity of the cetacean species under study are further discussed in the discussion.

3.5 Phylogenetic reconstruction of PAD sequences.

Five well supported and distinct clades representing each PAD were formed within the Neighbour-joining phylogeny (Supp. Fig. 1). The PAD1 clade appeared to resolve the best supported phylogenetic topology with the cetacean sequences falling into two subclades, one for the Odontoceti and one for the Misticety, with La Planta dolphin (*Pontoporia blainvillei*) as a paraphyletic group. The first PAD1 “Odontoceti” subclade was further split into two clades, one representing members of the Lipotidae and Iniideae, and the other further split into a Delphinidae clade and another clade comprising Montodontidae and Phocoenidae lineages. The second PAD1 “Misticety” subclade also formed two further clades, one representing members of the Ziphiidae and the other representing members of the Physeteridae, Balaenidae and Balaenopteridae.

The PAD2, PAD3, PAD4 and PAD6 clades do not show the same differentiation into such distinct groups as for PAD1 (Supp. Fig. 1). Interestingly, the PAD sequences for the common hippopotamus, *Hippopotamus amphibius*, were intermingled with the cetacean sequences within each of the respective PAD clades, with the exception of the *H. amphibius* PAD3 which formed the expected paraphyletic clade (Supp. Fig. 1).

3.6 Analysis of inflammatory and metabolic microRNAs in whale sera and serum derived EVs

The inflammatory and stress related miR21, miR155 and the metabolic and hypoxia related miR210 were assessed both in whole sera and in EVs isolated from sera of the 5 cetaceans. Species-specific differences were observed in the relative expression all three miRs as shown in Fig. 10. Furthermore, EVs were found to be a better source of miRs, compared to whole sera (Fig. 10). The highest relative levels of miR21 were found in humpback whale EVs, followed by minke whale (Fig. 10A), while relative miR155 expression was highest in minke whale EVs (Fig. 10B) and miR210 relative levels were highest in orca, followed by humpback whale (Fig. 10C).

4. Discussion

This is the first study to characterise deiminated protein networks, extracellular vesicles (EVs) and microRNA (miR) EV-cargo in cetacean sera. Deiminated proteins were identified and compared in the 5 different cetacean species under study: Northern minke whale (*Balaenoptera acutorostrata*), fin whale (*Balaenoptera physalus*), humpback whale (*Megaptera novaeangliae*), Cuvier's beaked whale (*Ziphius cavirostris*) and orca (*Orcinus orca*). The findings presented here unravel novel aspects of post-translational deimination in key proteins of metabolism, innate and adaptive immunity in these sea mammals.

PAD homologues were identified in whale sera by Western blotting via cross reaction with human PAD2 and PAD3. PAD2 is the phylogenetically most conserved PAD form (Vossenaar et al., 2003; Magnadottir et al., 2018a), and in cetacean sera PAD-positive bands were seen at an expected 70 - 75 kDa size, similar to as seen for other mammalian PADs.

Deiminated histone H3, a marker of neutrophil extracellular trap formation (NETosis), was detected in the whale and orca sera. NETosis is partly driven by PADs (Li et al., 2010), is conserved throughout phylogeny (Magnadottir et al., 2018a; Magnadottir et al., 2019a; Criscitiello et al., 2019), and is important in innate immune defences against a range of pathogens including bacteria, viruses and helminths (Brinkmann et al., 2004; Palić et al., 2007; Branzk et al., 2014; Schönrich and Raftery, 2016). Indeed, NET/ETosis has recently been related to parasitic defence mechanisms in cetaceans (Villagra-Blanco et al., 2019). NETosis has furthermore been associated with clearance of apoptotic

cells and tissue remodelling (Magnadottir et al., 2018a; Magnadottir et al., 2019a) as well as being associated with chronic pathologies (Lee et al., 2017; O'Neil and Kaplan, 2019), neurodegenerative diseases (Pietronigro et al., 2017) and cancer (Gonzalez-Aparicio and Alfaro, 2019). Histones undergo various post-translational modifications that affect gene regulation and can also act in concert (Bird, 2007; Latham et al., 2007). In addition to acetylation, phosphorylation and ubiquitination, histones are indeed known to undergo deimination, including H2B (Sohn et al., 2015) and H3 as identified here in minke whale. Other histones known to undergo deimination are H2A (Hagiwara et al., 2005) and H4 (Chen et al., 2014; Kosgodage et al., 2018), both of which were also identified here as deimination targets based on the larger cetacean database search (Supplementary Tables 2-5).

Further deiminated proteins identified here in whale and orca sera by F95 enrichment and LC-MS/MS analysis included key immune, nuclear and metabolic proteins. Most species-specific protein hits were found for minke whale which, has to be noted, also had the largest searchable species-specific database and therefore the most annotations for identification of species-specific proteins. Furthermore, all protein hits from the LC-MS/MS analysis for deiminated proteins isolated from the individual cetaceans were also assessed against a larger common cetacean database (Supplementary Tables 2-6). The protein list for species-specific deiminated proteins identified in minke whale was submitted to STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) analysis (https://string-db.org/) to predict putative protein-protein interaction networks. For deiminated proteins identified in minke whale serum, PPI enrichment value for 85 of the identified deiminated proteins was found to be $p< 1x10^{-16}$, which indicates that these 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. KEGG pathways involved in immunity and metabolism are highlighted for the minke whale species-speficic derived STRING protein network analysis in Supplementary Fig 2.

In addition to the species-specific analysis of protein interaction networks, based on the species-specific UniProt databases for each of the cetaceans under study, a wider analysis of deiminated protein hits based on a larger cetacean UniProt database was used to create protein-protein interaction networks in STRING (Figures 4-8). These revealed both common/shared deiminated protein pathways related to physiology and immunity as well as highlighting some differences in deiminated KEGG pathways between the species under study (Fig. 9). KEGG pathways for the complement and coagulation cascades were identified in all 5 cetacean species, and form part of the acute phase response and first line of immune defences against invading pathogens as well as in the clearance of necrotic or apoptotic cells (Dodds and Law, 1998; Fishelson et al., 2001; Gelain and

Bonsembiante, 2019). Pathways for glycolysis/gluconeogenesis were identified in minke, fin, humpback and Cuvier's beaked whale. Recent studies in dolphins have identified low activity of the glycolysis metabolic pathway (Suzuki et al., 2018) and changes in glycolysis pathways has been assessed in diving vertebrates (Hochachka et al., 1975). Post-translationally deiminated proteins in glycolysis pathways have been recently identified in the naked mole-rat (*Heterocephelus glaber*) (Pamenter et al., 2019), where changes in glycolysis has for example been related to anoxia resistance (Park et al., 2017). How deimination of glycoloysis pathway related proteins play roles in hypoxia tolerance and cancer-resistance remains to be further investigated. Oestrogen signalling patways were identified as deiminated in minke whale, fin whale and humpback whale. Oestrogen has been studied in a range of cetaceans, including during pregnancy (Robeck et al., 2016) and as a marker for environmental pollution in orca at the mRNA expression level (Buckman et al., 2011), while post-translational modification and putative effects of deimination on oestrogen signalling remains to be investigated. KEGG pathways relating to cholesterol metabolism were identified in minke whale, fin whale, humpback whale and orca, while KEGG pathways for fat digestion and absorption were identified to be deiminated in minke whale, fin whale and orca. Cholesterol has been studied in whales in relation to lifespan (Borchman et al., 2017) and lipidomics are being developed as a diagnostic tool for metabolic and physiological state in whales, including cholesterol (Tang et al., 2018). Roles for post-translational modifications, including deimination, in cholesterol metabolism remain to be fully understood. KEGG pathways relating to vitamin digestion and absorption were identified to be deiminated in minke whale, finwhale and orca. Disruption of vitamin profiles has been identified in cetaceans in response to exposure to environmental pollutants (Desforges et al., 2013; Pedro et al., 2019), but to what extend deimination plays a role in vitamin processing is not known. KEGG pathways for biosynthesis of amino acids were identified to be deiminated in minke whale, fin whale, humpback whale and Cuvier's beaked whale. Such deimination may be of considerable interest as amino acid assessment for mammalian metabolism is being developed for health management of cetaceans (Suzuki et al, 2018) and for research into ageing and disease, including in cetacean samples (Fry and Carter, 2019). Pathways for carbon metabolism were identified in fin whale, humpback whale and Cuvier's beaked whale, while nitrogen metabolism pathways were identified in humpback whale and Cuvier's beaked whale. Both pathways have been untilised to assess environmental contamination in whales (Borrell et al., 2018; Pinzone et al., 2019). Pathways relating to HIF-1 signalling were identified in Cuvier's beaked whale and humpback whale, which correlates with these two cetaceans being the most deep diving of the species under study; for example gas and fat embolic syndrome is a pathologic condition of *Ziphiidae* and mirrors decompression sickness identified in human divers (Fernández et al., 2005; Di Guardo et

al., 2019). Deiminated protein pathways related to HIF-1 signalling have also recently been identified in the naked mole-rat, which is known for unusual resistance to hypoxia (Pamenter et al., 2019), while studies on hypoxia in the CNS have revealed roles for PAD-mediated pathways (Lange et al., 2014; Fan et al., 2018; Yu et al., 2018).

Other deiminated KEGG physiological pathways identified to be species-specific were renin-angoistensin system (minke whale), proximal tubule biocarbonate reclamation (Cuvier's beaked whale), ECM-receptor interaction (fin whale), and in Cuvier's beaked whale KEGG pathways for thyroid hormone synthesis, fructose, mannose and pyruvate metabolism, as well as mineral absorption were identified to be deiminated. The renio-angiotensin system plays pivotal roles in maintaining blood pressure and extracellular volume homeostasis (Yang and Xu, 2017). It is critical for osmoregulation in cetaceans and has been studied in relation to adaptions due to their evolutionary transition from terrestrial to hyperosmotic envornments (Oritz, 2001; Zu et al., 2013). Cetacean kidneys have several evolutionary adaptions, including larger size and relatively large ratio of medulla to renal cortex, which allows for production of highly concentrated urine (Zu et al., 2013). Furthermore, hormonal regulation of salt and water balance is of great importance, while overall molecular mechanisms underlying these specialised pathways still remain to be fully explored. Proximal tubule bicarbonate reclamation pathways werehere identified to be deiminated in Cuvier's beaked whale, and these are critical for kidney function in whales (Maluf and Gassman, 1998) and are primary target of kidney injury in human and animal models (Chevalier, 2016). Therefore post-translationally mediated regulation of proteins involved in these pathways, including deimination identified here, may be of some interest for osmoregulation and specialised kidney function in ceatceans as well as for andvancing therapeutic strategies in kidney disease (Chevalier, 2016). ECM-receptor interactinos play direct and indirect roles in control of a range of cellular activities including adhesion, migration, differentiation, proliferation and apoptosis and are widely studied in cancer, including at the transcriptome level (Bao et al., 2019). KEGG pathways for both ECM-receptor interaction and focal adhesion have been identified to be enriched in EVs of mesenchymal stem cells (Mardpour et al., 2019), and both pathways were here found enriched in deiminated proteins in fin whale only. Roles for regulation of these pathways via post-translational deimination have not been investigated. Thyroid hormones have been studied in a range of whales and dolphins, including for evaluation of energetics and stress (Suzuki et al., 2018; Hunt et al., 2019), as well as environmental pollution (Buckmann et al., 2011; Villanger et al., 2011; Hunt et al., 2017; Simond et al., 2019). PADs have been related to thyroid cancer (Guo et al., 2017) and deimination of histone H3 has been linked to autoimmune thyroid disease (Morshed et al., 2019), while physiological effects of deimination on thyroid hormone synthesis remain to be investigated. Fructose, mannose and pyruvate metabolism

are associated with glycolytic pathways, which have been found to be of low activity in ceataceans (Suzuki et al., 2018) and therefore regulatory mechanisms via deimination may be of relevance for understanding of cancer and insulin resistance pathways (Brown et al., 2016; Guzmán-Flores et al., 2018). In cetaceans, ATP-mediated control of pyruvate is associated with aerobic-anaerobic transition during diving (Storey and Hochachka, 1974), but to what extent post-translational modifications are involved in regulating proteins involved in these processes remains to be investigated.

Notably, KEGG pathways for host-pathogen interactions were enriched in all five cetacean species under study, with pathways relating to bacterial infection in all five species, amoebiasis in minke whale and fin whale and trypanosomiasis in Cuvier's beaked whale and orca. KEGG immune pathways for viral infection were identified in fin whale only, as well as cancer related pathways and metabolism of xenobiotics by cytochrome P450, a detoxification system in a range of marine mammals (Goksøyr et al., 1995) and a biomarker for environmental stress (Waugh et al., 2011; Bachman et al., 2015; Righetti et al., 2019). KEGG pathways relating to autoimmunity were identified in minke whale, fin whale and orca. Pathways relating to ferroptosis, a form of regulated cell death and implicated in multiple physiological and pathological processes (Xie et al., 2016; Shi et al., 2019), were furthermore identified in minke whale, Cuvier's beaked whale and orca, while pathways relating to phagosome and necroptosis, critical for necrotic and infectious diseases (Xia et al., 2020), were identified in minke whale. These findings highligth novel roles for protein deimination in infection, chronic diseases and host-pathogen interactions in ceataceans. While exact mechanistic pathways will need to be followed up and validated, the current findings of post-translational deimination in these KEGG pathways add to the acknowledged lack of information for host-pathogen interactions in ceataceans (Di Guardo et al., 2018). Transmission of marine morbilliviruses for example requires further research into host-specific factors (Jo et al., 2018; Di Guardo and Mazzariol, 2019; Ohishi et al., 2019) and research into immune mechanisms in relation to bacterial infections, including multidrug-resistant bacteria and opportunistic infection, are of pivotal importance (Reif et al., 2017; Mazzariol et al., 2018). In addition, specific roles for deiminated protein cargo exported in EVs will also need to be assessed as deiminated protein enrichment has been shown to differ in EVs compared to whole plasma and serum (Criscitiello et al., 2019 and 2020; Pameneter et al., 2019; Magnadottir et al., 2020a). Further to previously identified roles for PAD-mediated NETosis/ETosis in cetaceans in response to zoonotic diseases (Villagara-Blance et al., 2019; Imlau et al., 2020), also verified in the present study, newly recognized targets of deimination in cetaceans are identified here and highlight putative roles for regulation of immunological pathways via such post-translational deimination. Selected deiminated protein candidates, based on

identification from the species-specific search for the individual cetaceans under study, which are involved in immune, nuclear and metabolic functions are discussed below in relation to roles in physiology and pathology:

Adiponectin was identified to be deiminated in orca only and is in human the most abundant secreted adipokine with pleiotropic roles in physiological and pathophysiological processes (Fiaschi, 2019). It has received considerable interest in the field of metabolic and obesity research (Frankenberg et al., 2017; Spracklen et al., 2019), as well as in diabetes (Yamauchi et al., 2003), due to its key function in regulating glucose (Yamauchi et al., 2002; Kadowaki & Yamauchi, 2005; Almabouada et al., 2013). Adiponectin is furthermore linked to longevity (Chen et al., 2019), regenerative functions (Fiaschi et al., 2014), myopathies (Gamberi et al., 2019) and cancer (Parida et al., 2019). Adiponectin also plays roles in reproduction, embryo pre-implantation and embryonic development (Barbe et al., 2019). Due to the range of functions in relation to key pathophysiologies, there is a great interest in drug development to modulate adiponectin signalling (Fiaschi, 2019). Recent studies in rheumatoid arthritis made a correlation between inflammation, autoantibodies and adiponectin levels (Hughes-Austin et al., 2018; Liu et al., 2019). Post-translational deimination may be a hitherto unrecognized mechanism for adiponectin and of relevance for metabolic adaptions, as adiponectin was recently identified as a deimination candidate in naked mole-rat and llama (*Lama glama*), both which display unusual metabolism and adaption to extreme environments (Pamenter et al, 2019; Criscitiello et al., 2020). Deimination may allow for protein moonlighting functions via changes in protein folding and therefore interaction with other proteins. Adiponectin is a small 244 aa protein (NP_001171271.1) in humans and contains 2 unfolded regions and 7 arginine sites, while orca adiponectin (XP_004278522.1) has also 2 unfolded regions and 7 arginine sites, that could be subjected to PAD-mediated deimination and therefore modulate adiponectin folding and function, depending on which arginine is deiminated. STRING analysis for orca adiponectin and relevant KEGG pathways are shown in Supplementary Fig. 3 (Supplementary Fig. 3A and 3B). Given that orca will have some unique physiological characteristics, the identification of post-translational deimination of this key metabolic protein may be of great interest for comparative metabolic studies.

Albumin is a major acidic plasma protein in vertebrates and serves as a transport molecule for fatty acids, bilirubin, steroids, amino acids and copper, as well as having roles in maintaining the colloid osmotic pressure of blood (Peters, 1996; Metcalf et al., 2007). In aquatic animals, albumin has been identified as a putative health marker in response to environmental conditions in porpoises (Nabi et

al., 2017) and for assessment of serum chemistry in wild beluga whales (*Delphinapterus leucas*) (Norman et al., 2012). In teleost fish, albumin levels were shown to be raised upon heavy metal exposure in water (Firat and Kargin, 2010). While albumin is known to be a glycoprotein in some species (Metcalf et al., 1998; Tao et al., 2019), roles for post-translational deimination remain to be further understood. Indeed, albumin was recently found to be deiminated in teleost fish (Magnadottir et al., 2019a).

Alpha-2-macroglobulin was found deiminated in minke whale serum. It forms part of the innate immune system and clears active proteases from tissue fluids (Armstrong and Quigley, 1999). Alpha-2-M is phylogenetically conserved from arthropods to mammals, is found at high levels in mammalian plasma and is closely related to other thioester containing proteins, complement proteins C3, C4 and C5 (Sottrup-Jensen et al., 1987; Davies and Sim, 1981). Alpha-2-M was recently found to be deiminated in shark (Criscitiello et al., 2019), camelid (Criscitiello et al., 2020), naked mole-rat and Antarctic seabirds (Phillips et al., 2020) and this may contribute to phylogenetically conserved and adapted functions in immune responses.

Alpha-1-microglobulin/bikunin precursor (AMBP protein) was here found deiminated in minke whale serum. It has been linked to oxidative stress and altered protein composition of subcutaneous adipose tissue in chronic disease (Gertow et al., 2017) but has not been identified as deiminated in any species before to our knowledge.

Antithrombin-III was here found to be deiminated in minke whale serum. It is a phylogenetically conserved hepatic glycoprotein and found in blood plasma (Jordan, 1983). Antithrombin forms part of the coagulation system and in whales, intestinal heparin has been found to bind with high affinity for antithrombin III (Uchiyama et al., 1990) and studied in coagulation (Oshima, 1990). Roles in disease are linked to thrombosis, pulmonary embolism (Amiral and Seghatchian, 2018) and angiogenesis in cancer (O'Reilly et al., 1999). Antithrombin-III furthermore has anti-inflammatory action and is linked to kidney diseases (Lu et al., 2017). Glycosylation has been found to affect the activity of antithrombin (McCoy al., 2003). Post-translational deimination has also been shown to affect its function by converting antithrombin to a form with a four-fold higher affinity for heparin pentasaccharide (Pike et al., 1997) and its deimination was recently identified also in seabirds (Phillips et al., 2020). Therefore post-translational modifications, including deimination, seem to play important roles in the functional diversity of antithrombin-III.

Apolipoproteins A-I, A-IV, B-100 and C-III were here identified as deiminated in minke whale serum. Apolipoprotein A-I is primarily involved in lipid metabolism where conformational plasticity and flexibility are of importance (Arciello et al., 2016). Apo A-I is also associated with regulation of mitochondrial function and bioenergetics (White et al., 2017). Furthermore, Apo A-I has been shown to have a regulatory role in the complement system by affecting membrane attach complex (MAC) assembly (Hamilton et al., 1993; Jenne et al., 1991; French et al., 1994). ApoB-100 is conserved between cetacean and other mammals (Amrine-Madsen et al., 2003), synthesised by the liver and plays parts of innate immune responses (Peterson et al., 2008). ApoB-100 is furthermore associated with ER stress and insulin resistance (Su et al., 2009) as well as lipid metabolism disorders (Andersen et al., 2016). ApoA-IV is a lipid binding protein, primarily synthesized in the small intestine and involved in a range of physiological proteins including lipid absorption and metabolism, glucose homeostasis, platelet aggregation and thrombosis (Qu et al., 2019). Apo-CIII is a glycoprotein secreted by liver and small intestine and has roles in secretion of triglyceride-rich VLDL particles from hepatic cells under lipid rich conditions (Sundaram et al., 2010). Apo-CIII is also produced within pancreatic islets and linked to diabetes (Juntti-Berggren and Berggren, 2017). The roles for post-translational deimination in protein moonlighting of these apolipoproteins in whale physiology may be of considerable interest. Deimination of some apolipoproteins has previously been also identified for in other vertebrates, including camelids (Criscitiello et al., 2020), naked mole-rat (Pamenter et al., 2019) and teleost fish (Magnadottir et al., 2019a).

Beta-2-glycoprotein 1 (apolipoprotein H) was here identified as deiminated in minke whale. It is a 38 kDa multifunctional plasma protein which binds cardiolipin. It has roles in agglutination and has anti-coagulation activity in serum (Rikarni et al., 2015). Furthermore it is implicated in anti-phospholipid syndrome (Radic and Pattanaik, 2018; Yin et al, 2018). Its post-translational deimination has recently been identified also in naked mole-rat (Pamenter et al., 2019) and may be of some interest in relation to moonlighting functions in physiology, in addition to roles in autoimmunity.

Ceruloplasmin is a serum ferroxidase with antioxidative function and roles in iron homeostasis and carries over 90 % of the copper in plasma (Liu et al., 2011). In aquatic animals, ceruloplasmin has been shown to contribute to acute immune responses in teleost fish (Lü et al., 2013). It is also upregulated as an acute phase protein in response to growth hormone (Yada, 2007) and upon heavy metal exposure (Firat and Kargin, 2010), as well as being upregulated in response to bacterial challenge (Liu et al., 2011). Ceruloplasmin is furthermore related to bacterial resistance (Sahoo et al.,

2013) and parasitic infection (Kovacevic et al., 2015; Henry et al., 2015). As fish have been shown to use iron deprivation as a nutritional immunity mechanism, by withholding iron from iron-requiring pathogens (Lange et al., 2001; Martínez et al., 2017), such uses in other aquatic animals, including whales, may be possible. Ceruloplasmin has been identified to be deiminated in teleost fish (Magnadottir et al., 2019a) as well as in the naked mole-rat (Pameneter et al., 2019) and it may be postulated that post-translational deimination may facilitate the diverse functions of ceruloplasmin.

Clusterin (apolipoprotein J) was identified here as deiminated in minke whale serum. It is a glycoprotein and a stress-activated ATP-independent molecular chaperone for protein folding and involved in clearance of cellular debris and apoptosis (Jones and Jomary, 2002; Wilson and Zoubeidi, 2017). Furthermore, clusterin is involved in a range of diseases related to oxidative stress, including inflammatory diseases, neurodegeneration, cancer and ageing (Yao et al., 2018; Foster et al., 2019). In seal brains, clusterin has been found to be four-fold higher expressed than in any other mammalian brain transcriptome. It has been postulated that elevation of this stress related gene may contribute to the hypoxia tolerance of diving mammals (Fabrizius et al., 2016). Roles for post-translational deimination in the function of clusterin in whale physiology may therefore be of interest.

A range of **complement components** was here identified as deiminated in minke whale serum. This included complement component C3, C5, C9, C1q, complement component C4-binding protein, complement factor B and factor H. The complement system forms part of the first line of immune defences against invading pathogens and also participates in the clearance of necrotic or apoptotic cells (Dodds and Law, 1998; Sunyer and Lambris, 1998; Fishelson et al., 2001; Carrol and Sim, 2011). The complements system is furthermore implicated in regeneration (Del-Rio-Tsonis et al., 1998; Haynes et al., 2013) and tissue remodelling (Lange et al., 2004a; 2004b; Lange et al., 2005; Lange et al., 2006; Lange et al., 2019). ***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 (Dodds and Law, 1998; Dodds, 2002). This is to our knowledge the first report of deiminated complement C3 in a mammalian species, while C3 was recently identified by our group in deiminated form in teleost fish (Magnadottir et al., 2019a and 2019b) as well as in elasmobranch shark (Criscitiello et al., 2019). Post-translational deimination of C3 may possibly influence its function including in the generation of the convertase, its cleavage ability, binding and deposition. ***Complement component C5*** plays important roles in inflammation and apoptosis. It is cleaved into C5a, which has pleiotropic biologic functions including as

anaphylotoxin (Klos et al., 2009) while C5b forms the basis of the membrane attach complex (MAC) (Morgan et al., 2016). In cellular *in vitro* models, C5 has previously been reported to undergo deimination by *Porphyromonas gingivalis*, which evades complement-mediated killing by disabling anaphylatoxin C5a protein function via deimination of a critical C-terminal arginine (Bielecka et al., 2014). ***Complement component C9*** participates in the formation of the membrane attack complex, leading to lysis of the pathogen. C9 has previously been found to be deiminated in teleost fish (Magnadottir et al., 2019a). ***Complement C1q*** can activate the classical complement system by binding to the Fc region of immunoglobulins that are bound to antigen (Reid et al., 2002; Reid, 2018). Interestingly, an essential role for arginine in C1q has been suggested for C1q-IgG interaction (Kojouharova et al., 2004). C1q also serves as a potent pattern recognition molecule which recognises self, non-self and altered self-signals (Nayak et al., 2012; Reid, 2018). ***Complement component C4-binding protein*** (C4BP) is a large glycoprotein, synthesised in the liver. It acts as an inhibitor of the classical and lectin pathways of the complement system and has multifaceted roles in immunity and homeostasis. C4BP is a cofactor for serine protease factor I, and besides C4-binding functions, it can also bind to C3b and accelerate decay of the C3 convertase. C4BP binds necrotic and apoptotic cells, as well as DNA, and therefore is involved in tissue clearance post injury (Ermert and Bloom, 2016). ***Complement factor B*** is a phylogenetically conserved activation protease of the complement system (Nonaka, 2014). ***Complement factor H*** is a major regulator of the alternative complement pathway and factor H family proteins are involved in modulating a range of cellular functions (Józsi et al., 2019). The deimination of the various complement components identified here may have diverse effects on complement activity and add to moonlighting functions of the complement system in homeostasis and immune defences. Recent comparative studies have indeed highlighted deimination of a range of complement proteins, and this is of considerable importance for understanding the diversity of complement function throughout phylogeny (Magnadottir et al., 2018a; Magnadottir et al., 2019a; Lange et al., 2019; Pameneter et al., 2019; Criscitiello et al., 2019 and 2020).

Charged multivesicular body protein 4c was identified as deiminated in minke whale serum. It is a core component of the endosomal sorting required for transport complex III (ESCRT-III) which is involved in multivesicular bodies (MVBs) formation and sorting of endosomal cargo proteins into MVBs and is furthermore involved in cell division (Carlton et al., 2012). It is also associated to cancer (Sadler et al., 2018) and to the regulation of viral replication (Li et al., 2013).

Desmoplakin was here identified to be deiminated in minke whale serum. Desmoplakin is a unique and critical component of desmosomal cell-cell junctions and involved in integrity of the cytoskeletal intermediate filament network (Bendrick et al., 2019). It has been shown to be required for epidermal integrity and embryo morphogenesis (Bharathan and Dickinson, 2019), as well as in the coordination of cell migration (Bendrick et al., 2019). Mutations in desmoplakin have been linked to multiple allergies, severe dermatitis and metabolic wasting (SAM) syndrome (Liang et al., 2019). It is also linked to Carvajal syndrome, involving altered skin and hair abnormalities, and heart diseases (Reichl et al., 2018; Yermakovich et al., 2018; Chen et al., 2019). Desmosomal proteins have been shown to have both tumour-promoting and tumour-suppressive functions, depending of cancer types and can regulate cell proliferation, differentiation, migration, apoptosis, and impact treatment sensitivity in different types of cancers (Zhou et al., 2017). Desmoplakin was recently identified to be also deiminated in camelid serum EVs, but not whole plasma (Criscitiello et al., 2020). As the roles of desmosomal proteins in cancer and metastasis are not fully understood, the identification of deiminated desmoplakin in minke whale serum may be of some interest and add to understanding of pleiotropic functions throughout phylogeny via such post-translational modification.

Dimethylglycine dehydrogenase (DMGDH), mitochondrial isoform X1 was here identified as deiminated in minke whale serum. It is a mitochondrial matrix enzyme with roles in choline degradation, one-carbon metabolism and electron transfer to the respiratory chain (Augustin et al., 2016). DMGDH is a key metabolic enzyme, linked to diabetes, kidney disease (Zhu et al., 2018; Magnusson et al., 2015), carcinoma and metastasis (Liu et al., 2016).

Dipeptidylpeptidase 4 (DPP4, also known as CD26) was here identified to be deiminated in minke whale serum. DPP4 controls glucose homeostasis and has complex roles in inflammation and homeostasis, including in liver cytokine expression, while its activity in plasma has been shown to correlate with body weight and fat mass (Varin et al., 2019). Furthermore, roles for DDP4 in cancer have been found to relate to its post-translational processing of chemokines, thereby limiting lymphocyte migration to sites of inflammation and tumours (Barreira da Silva et al., 2015). DPP4 inhibitors have therefore been suggested as a strategy to enhance tumour immunotherapy (Barreira da Silva et al., 2015). Furthermore, serum DDP4 activity levels in primary HIV infection were found to be significantly decreased and to correlate with inflammation and HIV-induced intestinal damage (Ploquin et al., 2018). The identification of DPP4 as a deimination candidate has previously been verified in camelids (Criscitiello et al., 2020) and may be of some relevance as such post-translational

modification can affect DPP4 structure and function, allowing for moonlighting functions in pathological and pathophysiological milieus throughout phylogeny.

Fetuin-B was identified as deiminated in minke whale. It is a member of the fetuin family and part of the cystatin superfamily of cysteine protease inhibitors (Lee et al., 2009). Fetuin-B is a hepatokine, secreted by hepatocytes and linked to the regulation of the insulin and hepatocyte growth factor receptors, to metabolism as well as metabolic dysfunction, including insulin resistance and chronic kidney disease (Meex and Watt, 2017; Lin et al., 2019). Fetuins have further functions in osteogenesis and bone resorption, response to systemic inflammation and infection (Qu et al., 2018; Li et al., 2017) as well as in fertilisation (Stöcker et al., 2014; Fang et al., 2019). The structure of mammalian plasma fetuin-B has been extensively studied and fetuin-B is described throughout phylogeny from cartilaginous fishes to mammals (Cuppari et al., 2019). Post-translational deimination of fetuin-B has not been reported before.

Fibrinogen is a glycoprotein, synthesised in liver (Tennent et al., 2007) and forms part of the acute phase response as part of the coagulation cascade (Tiscia and Margaglione, 2018). Fibriongen is well described in cetaceans (Gatesy, 1997; Terasawa et al., 2008). In humans, impaired mechanism of fibrinogen formation and fibrin polymerization are implicated in various pathologies including coagulopathies and ischemic stroke (Weisel and Litvinov, 2013). Acquired fibrinogen disorders are also associated with cancer, liver disease or post-translational modifications (Besser and MacDonald, 2016). Fibrinogen is indeed a known deimination candidate and this post-translational modification contributes for example to its antigenicity in autoimmune diseases (Hida et al., 2004; Okumura et al., 2009; Muller and Radic, 2015; Blachère et al., 2017), and deimination has also bee identified in other taxa, including camelids (Criscitiello et al., 2020) and naked mole-rat (Pamenter et al., 2019). In aquatic animals, fibrinogen has been associated with teleost host defence against pathogens (Blanco-Abad et al., 2018), in acute phase and stress responses during temperature acclimation (Dietrich et al., 2018) and upon exposure to tetrodotoxin (Kiriake et al., 2016). Fibrinogen and gamma-fibrinogen were here found to be deiminated in minke whale and fin whale respectively.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was identified as deiminated in fin whale (*Balaenoptera physalus*). GAPDH has key metabolic functions in glycolysis but also has pleiotropic non-metabolic functions including in apoptosis, transcription activation and axonal transport (Tarze et al., 2007; Zala et al., 2013; Sirover 2018; Butera et al., 2019). GAPDH has been identified to have a range of moonlighting functions, including in iron metabolism (Boradia et al., 2014) and is associated

to various pathologies (Sirover 2018). GAPDH has been shown to be regulated via post-translational modifications (Tristan et al., 2011; White and Garcin, 2017; Butera et al., 2019) and was recently identified as a deimination candidate in brain-cancer (Kosgodage et al., 2018), as well as in plasma-EVs of naked mole-rat and in harbour seal (Magnadottir et al., 2020b). Deimination of GAPDH identified here in fin whale may contribute to its multifaceted physiological functions.

Haptoglobin is an acute phase plasma protein and is in mammals involved in protection of oxidative damage by binding to haemoglobin (Andersen et al., 2017; Redmond et al., 2018). Haptoglobin has been described in a range of whales and dolphins, with some specific features identified (Travis et al., 1971; Yim et al., 2014). In aquatic animals it has been found to have roles in anti-viral immunity (Cordero et al., 2017). Haptoglobin was here found to be deiminated in minke whale serum and has previously been identified as deiminated in nurse shark (Criscitiello et al., 2019), possibly adding to some of its functional diversity throughout phylogeny.

Heat shock protein 90 (Hsp90) was here found to be deiminated in minke whale. Hsp90 is a phylogenetically highly conserved chaperone protein involved in protein folding, the stabilisation of proteins against heat stress and aids in protein degradation (Buchner 1999; Picard, 2002). Hsp90 also stabilizes a number of proteins required for tumour growth and is therefore important in anti-cancer drug investigations (Goetz et al., 2003). Hsp90 is responsible for most of the ATPase activity of the proteasome (Imai et al., 2003) and has an ATP binding region, which also is the main binding site of drugs, including anti-tumour drugs, that can be used to target Hsp90 (Chiosis et al., 2006). Hsp90 has previously been described to be post-translationally deiminated in rheumatoid arthritis, allowing deimination-induced shifts in protein structure to generate cryptic epitopes capable of bypassing B cell tolerance (Travers et al., 2016). HSP90 has also been verified as a deimination candidate in camelids (Criscitiello et al., 2020). Finding post-translational deimination of this protein throughout phylogeny supports translational investigations between species to further current understanding of HSP90 function, both in physiology and pathologies.

Hemopexin is a plasma glycoprotein and scavenger protein of haemoglobin and a predominant heme binding protein, which contributes to heme homeostasis (Smith and McCulloh, 2015; Immenschuh et al., 2017). Hemopexin also associates with high density lipoproteins (HDL), influencing their inflammatory properties (Mehta and Reddy, 2015). In relation to aquatic animals, hemopexin has been associated with physiological stresses, including increased water temperature, immune response and heavy metal exposure in fish (Kwon and Ghil, 2017; Diaz-Rosales et al., 2014).

Here, hemopexin was found deiminated in minke whale serum. Previously, deimination of hemopexin has been found in teleost fish (Magnadottir et al., 2019a), in elasmobranch shark (Criscitiello et al., 2019), in naked mole-rat (Pamenter et al., 2019) and in camelid (Criscitiello et al., 2020). While hemopexin is a known glycoprotein, little is known regarding post-translational deimination for its function.

Immunoglobulin (Ig) proteins were identified here as being deiminated in minke whale serum. Ig's are key molecules in adaptive immunity and have been studied in whales (Andrésdóttir et al., 1987; Nollens et al., 2008). Furthermore, serum immunoglobulin levels have been associated with more active immune defences in free ranging bottlenose dolphins (*Tursiops truncatus*) compared to managed dolpins (Ruiz et al., 2009). Ig quantification assays have also been developed to assess immune function in orca (Taylor et al., 2002). Post-translational deimination of Ig's and roles in Ig function have hitherto received little attention. In patients with bronchiectasis and RA, post-translational deimination of the IgG Fc region has been identified (Hutchinson et al., 2017). Deimination of Ig's in a range of taxa has recently been described by our group including teleost fish (Magnadottir et al., 2019a), elasmobranchs (Criscitiello et al., 2019), camelid (Criscitiello et al., 2020) and peglagic seabirds (Phillips et al., 2020). Given the increased interest in furthering understanding of Ig diversity throughout phylogeny (Dooley and Flajnik, 2006; Smith et al., 2012; Zhang et al., 2013; de los Rios et al., 2015; Zhang et al., 2016; Zhang et al., 2017; Stanfield et al., 2018) our current finding of deimination of whale Ig's highlights a novel concept of diversification of their function via post-translational deimination.

Isocitrate dehydrogenase 1 (IDH1) was here identified to be deiminated in fin whale only. It is found in the cytosol and in peroxisomes and involved in a major pathway for cellular NADPH generation (Golub et al., 2019). Mutations in IDH have been associated with cancer, leading to development of targeted cancer therapeutics (Golub et al., 2019), while post-translational deimination has, to our knowledge, not been described in any species before.

Keratins were here identified as deiminated in minke whale serum. Keratin cytoskeleton evolution has been extensively studied, including in marine mammals and whales (Sun et al., 2017; Ehrlich et al., 2019), with cetaceans displaying impaired terminal keratinocyte differentiation (Lopes-Marques et al., 2018). Baleen keratin is furthermore of some interested as a bioengineering material (Wang et al., 2019). In aquatic animals, roles for keratin in anti-bacterial defences have been shown in skin mucus, as keratin has pore-forming abilities. Keratin also serves as a first barrier to injury as a

cytoskeletal protein (Molle et al., 2011). Downregulation of keratin II has for example been observed in *vibrio* infected fish (Rajan et al., 2013), while deiminated keratin was recently identified in teleost fish mucus (Magnadottir et al., 2018a), in birds (Phillips et al., 2020), naked mole-rat (Pamenter et al., 2019) and camelids (Criscitiello et al., 2020). In mammals, deimination of keratin is important including in skin physiology associated to cutaneous diseases (Chavanas et al., 2006; Ying et al., 2009); deimination indeed seems a phylogenetically conserved mechanism for faclilitation of moonlighting functions of keratin.

Kininogen forms part of the acute phase response, has been described in whales (Semba et al., 2000) and was here identified to be deiminated in minke whale serum. Kininogen has been previously found to be deiminated in teleost fish (Magadottir et al., 2019a), Antarctic birds (Phillips et al., 2020), camelid (Criscitiello et al., 2020) and naked mole-rat (Pamenter et al., 2019). In mammals, elevated levels of kininogen are linked to sepsis (Hofman et al., 2018) and inflammatory and oxidative stress pathways in type I diabetes (Al Hariri et al., 2017). To what extent kininogen function is dependent on post-translational deimination remains to be further investigated.

Myoglobin was identified as deiminated in humpback whale (*Megaptera novaeangliae*) and Cuvier's beaked whale (*Ziphius cavirostris*), while ***hemoglobin*** was identified as deiminated in minke whale. Both haemoglobin and myoglobin are key molecules in molecular oxygen transport in the bloodstream and for its storage in skeletal muscle. In diving cetaceans they contribute to hypoxia tolerance (Tian et al., 2016; Fago et al., 2017). Hemoglobin has furthermore been found to be a major binding protein for methylmercury in the liver of dolphins (Zayas et al., 2014). Myoglobin is found at higher concentrations in myocytes of deep diving animals compared to terrestrial animals and has been described in a range of whales (Isogai et al., 2018; Iwanami et al., 2006; Jones et al., 1979), including in humpback whale (Lehman et al., 1978), finback whale (DiMarchi et al., 1978) and minke whale (Lehman et al., 1977). The diving capacity of mammals is related to the myoblobin concentration in their myocytes. A more positive net surface charges of myoglobin are seen in diving animals compared to terrestrial animals, possibly to cause electrostatic repulsion among myoglobin molecules and to prevent their aggregation and maintain high protein concentration (Mirceta et al., 2013; Isogai et al., 2018). Therefore, post-translational deimination of myoglobin identified here in whales may be of considerable interest in relation to their physiological adaption to deep-diving. This correlates also with our recent findings of deiminated myoglobin and hemoglobin in pinnipeds, possibly playing roles in their adaption to hypoxic conditions (Magnadottir et al., 2020b).

Plakoglobin (γ-catenin) was here found to be deiminated in minke whale serum. It forms part of the Wnt signalling pathway, is a component of adherens junctions and desmosomes and plays therefore a vital role in the regulation of cell-cell adhesion (Aktary et al., 2017). Plakoglobin is also involved in the regulation of tomourigenesis and metastasis (Aktary et al., 2017). Plakoglobin is important in heart development and regeneration (Piven and Winata, 2017). Mutations in plakoglobin are for example linked to cardiomyopathies and Naxos disease (Li et al., 2018). Phosphorylation of plakoglobin has been described, through which it associates with cadherins (Shibata et al., 1994), and deimination of plakoglobin (junction plakoglobin) was recently identified in the naked mole-rat (Pamenter et al., 2019). Therefore its deimination identified here in whales, also long-lived and cancer-resisitant animals, may be of some interest and further understanding of such post-translational changes contributing to plakoglobin functional diversity.

Protein S100 was here identified as deiminated in minke whale. The S100 family of proteins in vertebrates participate in a variety of intracellular and extracellular functions. This includes regulation of protein phosphorylation and transcription factors, regulation of enzyme activities, cell growth and differentiation and inflammatory responses, including as damage associated molecular patterns (DAMPs) (Donato, 2003; Marenholz et al., 2004). In whales, S100B, a calcium-binding stress protein with pleiotropic function, has been found to be significantly enriched in the brain transcriptome compared to terrestrial mammals, possibly contributing to hypoxia tolerance (Fabrizius et al., 2016). Deimination of S100 proteins, as identified here, may indeed contribute to their pleiotropic functions throughout phylogeny.

14-3-3 protein epsilon isoform X1 was here identified as deiminated in minke whale serum. 14-3-3 protein are a conserved family of regulatory molecules, involved in signalling via binding to a range of kinases, transmembrane receptors and phosphatases (Bridges and Moorhead., 2005). 14-3-3 proteins are involved in a range of pathologies and linked to cancer, age-related neurodegenerative diseases and ageing (Fan et al., 2019). Furthermore, 14-3-3 proteins play roles in immunoglobulin class switch recombination and are therefore important for the immune response (Xu et al., 2012; Lam et al., 2013; Li et al., 2017). 14-3-3 proteins act also via conformational change (Bridges and Moorhead, 2005) and therefore the identification of post-translational deimination here may be of considerable interest.

Recombination activating protein 1 (RAG1) was here identified as deiminated in Cuvier's beaked whale (*Ziphius cavirostris*) only. It is involved in immunoglobulin V-D-J recombination, which

facilitates the generation of diverse repertoires of antigen receptors (Rodgers, 2017). RAG proteins are therefore critical for adaptive immune responses and are believed to have evolved from a mobile DNA element into a tightly controlled DNA recombinase in lymphocytes (Fugmann, 2010). In humans, mutation in RAG genes are related to a range of pathologies related to immune dysregulation (Notarangelo et al., 2016). In cetaceans, RAG1 has been identified in a range of species (McGowen et al., 2009). Post-translational deimination of RAG1 has not been identified in any species before to our knowledge and may possibly contribute to the diversification of adaptive immunity throughout phylogeny.

Rootletin, also known as ciliary rootlet coiled-coil protein (CROCC), was here identified as deiminated in minke whale. It is a protein that is required for centrosome cohesion and therefore plays important roles in mitosis (Bahe et al., 2005; Graser et al., 2007). Rootletin has been shown to be phosphorylated and to have the ability to form centriole-associated fibers, suggesting a dynamic model for centrosome cohesion based on entangling filaments (Bahe et al., 2005). Deletion of rootletin in mouse models causes photoreceptor degeneration and impaired mucociliary clearance, supporting its key function in rootlet structures (Yang et al., 2005). Rootlets have been studied in the development of the nervous terminalis in toothed whales (Oelschläger et al., 1987) and baleen whales (Oelschläger, 1989). Rootletin was recently identified to be deiminated in camelid serum (Criscitiello et al., 2020) and its deimination also identified here in minke whale may provide novel insights into its dynamic functions via such post-translational modification.

Selenoprotein P (Sepp1) is a plasma glycoprotein, mainly secreted from liver but also other tissues and contains most of the selenium in plasma (Mosert, 2000; Persson-Mochos, 2000; Burk and Hill, 2009). It has antioxidant properties (Mosert, 2000) and serves in homeostasis and distribution of selenium (Burk and Hill, 2009). Phylogenetically Sepp1 is believed to have appeared in early metazoan species and terrestrial animals have fewer selenoproteins than marine animals, which may be reflected in different functions (Lobanov et al., 2008). While Sepp1 is known to be glycosylated, other post-translational changes have not been studied. Besides its identification here as a protein candidate for post-translational deimination, deimination was recently reported in Sepp1 in Antarctic seabirds (Phillips et al., 2020).

Serotransferrin was here identified as deiminated in minke whale serum. It acts as an antimicrobial agent and is at the frontier in innate immune mechanisms in some aquatic animals (Stafford and Belosevic, 2003; Mohd-Padil et al., 2013). In bottlenose dolphins (*Tursiops truncatus*), differences in

transferrin levels of managed versus wild dolphins has been observed (Mazzaro et al., 2012), while in fish increased serotransferrin is found in response to toxins (Kiriake et al., 2016). Serotransferrin was recently identified to be deiminated in teleost fish (Magnadottir et al., 2018a), as well as in the naked mole-rat (Pamenter et al., 2019).

STE20-like serine/threonine-protein kinase isoform X2 was here found deiminated in minke whale serum. STE20-like kinases form part of the Hippo signalling pathway which regulates the balance between cell proliferation and apoptosis and is therefore involved in controlling organ size and tissue homeostasis (Bae and Luo, 2018). It is for example involved in liver size control and regeneration, as well as in tumourigenesis (Hong et al., 2015). Its deimination is here described for the first time to our knowledge.

Triose phosphate isomerase (TPI) was here identified as deiminated in minke whale serum. TPI plays an important role in glycolysis and is essential for efficient energy production. Besides roles in glycolysis, TPI is linked to lipid metabolism and is involved in ageing, metabolism and a range of human diseases (Olivares-Illana et al., 2017). Other moonlighting functions identified for TPI are in sperm-egg interactions (Petit et al., 2014). Deimination of TPI has not been described before to our knowledge.

Vitamin D-binding protein (VDBP) is a multifaceted protein mainly produced in the liver, where its regulation is influenced by estrogen, glucocorticoids and inflammatory cytokines (Bikle and Schwartz, 2019). It is secreted into the blood circulation and is able to bind the various forms of vitamin D (Verboven et al., 2002; Norman, 2008). It transports vitamin D metabolites between skin, liver and kidney, as well as various target tissues (Norman, 2008). In minke whale, VDBP has been identified in the liver transcriptome, is negatively correlated with polychlorinated biphenyl levels and therefore may be a putative toxicology marker (Niimi et al., 2014). In humans, VDBP has been tested as an anti-cancer agent via activation of macrophages against cancer cells (Yamamoto et al., 2008). Some association has also been made between polymorphisms of VDBP and the risk of coronary artery disease (Tarighi et al., 2017). Post-translational modifications of VDBP have been associated with multiple sclerosis (MS) (Perga et al., 2015), although it remains to be exactly identified which these post-translational modifications are. On the other hand, protein deimination is well known to be associated with MS (Moscarello et al., 2013), while a link to VDBP has not been made in MS. VDBP has previously been identified to be glycosylated (Kilpatrick and Phinney, 2017) and was found to be deiminated in camelids (Criscitiello et al., 2020). Here it was identified as a deiminiation

candidate in minke whale. Post-translational deimination may contribute to various functions of VDBP in physiological as well as pathophysiological processes.

Vitronectin (VTN) is a glycoprotein of the hemopexin family which is abundantly found in serum, the extracellular matrix and in bone. VTN is identified as a key controller of mammalian tissue repair and remodelling activity (Leavesley et al., 2013). It promotes cell adhesion and spreading. It also inhibits the membrane-damaging effect of the terminal cytolytic complement pathway and binds to several serine protease inhibitors (Felding-Habermann and Cheresh, 1993). VTN is also involved in haemostasis and tumour malignancy (Preissner and Seiffert, 1998; Hurt et al., 2009). Deimination of VTN has recently been desribed in wandering albatross (*Diomedea exulans*) plasma (Phillips et al., 2020) and was here identified in minke whale, possibly contributing to some of its pleiotropic functions throughout phylogeny.

Xaa-Pro dipeptidase, also known as prolidase, was here identified as deiminated in minke whale serum. Post-translational modifications of prolidase have been shown to regulate its enzymatic abilities and in humans, deficiency in prolidase can cause a range of chronic, debilitating health conditions (Viglio et al., 2006; Kitchener et al., 2012). Increased serum proloidase activity has also been associated with oxidative stress in humans in relation to obesity (Aslan et al., 2017). Increased levels of prolidase activity are associated to some cancers, leading to the development of proline prodrugs (Mittal et al., 2005). Serum prolidase enzyme activity is also currently being explored as a biomarker for diseases including chronic hepatitis B and liver fibrosis (Duygu et al., 2013; Şen et al., 2014; Stanfliet et al., 2015). Phosphorylation of prolidase has been shown to increase its activity while dephosphorylation leads to a decrease in enzyme activity. Post-translational deimination of prolidase has recently been described in the llama (Criscitiello et al., 2020), a species adapted to high altitude and therefore tolerant of low oxygen levels. Studies into post-translational deimination of Xaa-Pro dipeptidase may add to current understanding of how this enzyme is regulated.

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 (Bavelloni et al., 2017). Hitherto no studies have been carried out on miRs in whales, while some expression profiling has been carried out in dolphins with the aim to identify health related biomarkers in relation to organ injury (Segawa et al., 2016). Diving animals, such as whales and orca, 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 (Yim et al., 2014). 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 apopotosis (Bavelloni et al., 2017). MiR210 has a role in regulating mitochondrial metabolism (Chen et al., 2010) and cell glycolytic activity and is also linked to inflammation (Volobоueva et al., 2017). MiR210 is also involved in angiogenesis and vascular remodelling (Fasanaro et al., 2008); for example, mesenchymal stem cell derived EVs have been identified to be enriched in miR210 and to promote angiogenesis in ischemic myocardium (Wang et al., 2017). MiR210 has been identified as a regulator of the hypoxia pathway and to have pro-apoptotic functions under normal oxygen conditions, but anti-apoptotic effects under hypoxic conditions (Favaro et al., 2010; Huang et al., 2010). In the current study, miR210 was found to be highest expressed in orca, followed by humpback whale, Cuvier's beaked whale, fin whale and minke whale 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 immunoregulatory and onco-related miR and is also associated to chronic diseases (Musso et al., 2016, Juźwik et al., 2019; Li et al., 2019). While many experimentally verified targets of miR21 are tumour suppressors, miR21 is also linked to cardiac disease and oxidative stress (Xu et al., 2019). In the current study miR21 was found to be highly expressed in humpback EVs, followed by minke whale EVs, but at lower levels in the other three species, with lowest levels detected in Cuvier's beaked whale. Whether this difference is innate to the whale's and orca's normal physiology, 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 one individual used per species. Roles for miR21 in immune responses of aquatic animals have previously been identified in teleost fish, where miR21 was found to be up-regulated after immune stimulation and to inhibit the expression of cytokines via regulation of Toll-like receptor signalling (Bi et al., 2017).

In mammals, miR155 is known to be a major inflammatory related miR, linked to inflammatory and stress responses (Xiaoyan et al., 2017). In the current study miR155 was by far most highly expressed in minke whale EVs, while fin whale, humpback whale and orca showed similar levels of miR155 and lowest levels were seen in Cuvier's beaked whale. As no previous studies have been carried out on these two miRs in cetaceans it remains to be fully understood which specific functions these have in whale physiology. Furthermore, both miR21 and miR155 have been associated to viral infections in fish (Andreassen and Høyheim, 2017), been found to be upregulated in fish exposed to chronic [C_8mim]Br induced inflammation (Ma et al., 2019) and related to changes in sea temperature in

teleost fish (Magnadottir et al., 2020a). Interspecies differences in miR expression observed here 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 5 animals used. As only one animal per species was assessed in this pilot study, such species-specific differences need to be further evaluated in larger sample cohorts.

This is the first study to assess EV profiles and PAD-mediated protein deimination in cetaceans, while recent studies on other pelagic animals such as teleost and elasmobranch fish as well as pinnipeds and seabirds have recently been carried out (Iliev et al., 2018; Magnadottir et al., 2019b; 2020a; 2020b; Criscitiello et al., 2019; Phillips et al., 2020), as well as on other mammals with unusual metabolism (Pamenter et al., 2019; Criscitiello et al., 2020). Roles for PADs and EVs in have also been described in parasite host-pathogen interactions (Gavinho et al., 2019), but remain to be further investigated for parasite infections and other zoonotic diseases in aquatic mammals. Furthermore, we identified here post-translational deimination of key immune factors of innate and adaptive immunity, as well as in metabolism of the cetaceans assessed in the current study. These findings highlight novel aspects of protein moonlighting functions of these immune proteins in sea mammals via post-translational deimination. Due to the limited annotation of the whale and orca genomes, the hits identified in this study may though underestimate the amount of deiminated protein targets present in their sera, although this was compensated for to some extent using a wider search by assessing protein targets against a larger cetacean database, revealing a number of common KEGG pathways. Roles for miRs in gene regulation, including in stress responses due to environmental changes, toxicology and infection, are increasingly acknowledged and as miRs are known to be exported via EVs, changes in such EV cargo may be of considerable interest. An important finding of the current study is that EVs were a better source for miR analysis compared to whole sera, with EVs posing as better diagnostic markers than whole serum.

Findings of the current study touch upon a hugely understudied and emerging field of EV research in diverse taxa, in relation to sea mammal health and for the development of EV-related biomarkers to assess health status of wild sea animals in response to pollution, opportunistic infections as well as in response to changing sea temperatures and shift in habitat due to global warming. Orcas are for example among the world's most PCB-contaminated marine mammals, raising concerns about implications for their health (Buckman et al., 2011). Furthermore, findings in long-lived mammals that display cancer resistance, including cetaceams, may be of considerable translational value for furthering understanding of mechanisms underlying cancer resistance for improved development of human cancer therapies (Seluanov et al., 2018) and novel insights into potentially unique non-age-

related mechanisms of carcinogenesis across species (Pesavento et al., 2018). Such comparative studies furthermore provide translational value for mechanisms involved insulin resistance (Tsagkogeorga et al., 2015), as well as revealing molecular signatures of longevity (Ma and Gladyshev, 2017).

In continuation of the current pilot study, further assessment of changes in deiminated proteins, EV profile and miR expression will be of great interest to assess health status of wild sea mammals in response to infection, environmental temperature and toxicology. In addition, wider sampling within and between populations would enable comparisons with normal physiological protein deimination status and EV-profiles. This would be particularly valuable for assessing natural and anthropogenic stresses in cetaceans in general, many of which face increasing threats related to changing climate. While the current study lays a base-line for these novel biomarkers, future studies will need to further refine and develop these markers as an applicable tool in the evaluation of cetacean health status.

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Credit Author Statement

BM: Resources; Validation; Writing - review & editing

PU-O: Formal analysis; Resources; Validation; Visualisation, Writing - review & editing.

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

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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. Extracellular vesicles (EVs) isolated from sera of whales and orca. Nanoparticle tracking analysis (NTA), Western blotting (WB) and transmission electron microscopy (TEM) for characterisation of EVs from: **A.** Minke whale (*Balaenoptera acutorostrata*); **B.** Fin whale (*Balaenoptera physalus*); **C.** Humpback whale (*Megaptera novaeangliae*); **D.** Cuvier's beaked whale (*Ziphius cavirostris*); **E.** Orca (*Orcinus orca*). For all profiles, a poly-dispersed population of EVs in the size range of mainly 50-400 nm is seen, with main peaks of EV sizes differing between species, as seen in the individual NTA profiles. Analysis of EVs from whale serum by TEM confirms size and typical morphology of EVs; scale bar (100 nm) is indicated in all figures.

Fig. 2. EV yield and modal size of EVs from whale and orca sera. **A.** Total yield of EVs from whale sera varied between the sera from the 5 species under study. Highest levels of EV were seen in orca (*O. orca*) and Cuvier's beaked whale (*Z. cavi*), while the lowest EV serum yield was found in humpback whale (*M. Nova*). Similar EV yield was observed in minke (*B. acut*) and fin whale (*B. phys*). **B.** Modal size of EVs isolated from sera of the five cetacean species varied somewhat within an overall range of 100-170 nm. Minke whale showed larger EV modal size compared to the other whale species.

Fig. 3. Western blotting of deiminated proteins and PAD in whale and orca sera. **A.** PAD homologues were identified in whale sera at the expected size of approximately 70-75 kDa, using human PAD2 and PAD3 specific antibodies. The presence of deiminated histone H3 (citH3), representative of (neutrophil) extracellular traps (NET/ETs) was also identified, at approximately 20 kDa. **B.** Total deiminated proteins were identified in EVs from whale sera, using the F95 pan-deimination specific antibody. **C.** Western blotting confirmed the presence of deiminated proteins in total sera from whales and orca, as assessed by immunoprecipition of deiminated proteins from whole sera using the F95 pan-deimination antibody.

Fig. 4 Protein-protein interaction networks of deiminated protein hits identified in serum of minke whale (*Balaenoptera acutorostrata*). Reconstruction of protein-protein interactions based on known and predicted interactions using STRING analysis and the minke whale protein database in STRING. **A. KEGG pathways relating physiological pathways** are highlighted as follows: red=complement and coagulation cascade; blue=renin-angiotensin system; light green=oestrogen signalling; yellow=cholesterol metabolism; pink=vitamin digestion and absorption; dark

green=glycolysis/gluconeogenesis; light blue=biosynthesis of amino acids; orange=fat digestion and absorption. **B. KEGG pathways relating to immunity**: red=*Staphylococcus aureus* infection; blue=SLE; light green=amoebiasis; yellow=ferroptosis; pink=phagosome; dark green=necroptosis. 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). PPI enrichment p-value: $p < 1.0 \times 10\text{-}16$

Fig. 5 Protein-protein interaction networks of deiminated protein hits identified in serum of fin whale (*Balaenoptera physalus*). Reconstruction of protein-protein interactions based on known and predicted interactions using STRING analysis and the orca protein database in STRING. **A. KEGG pathways relating physiological pathways** are highlighted as follows: red=complement and coagulation cascade; blue=metabolic pathways; light green=oestrogen signalling; yellow=cholesterol metabolism; pink=vitamin digestion and absorption; dark green=glycolysis/gluconeogenesis; light blue=biosynthesis of amino acids; orange=fat digestion and absorption; purple=carbon metabolism; brown=ECM-receptor interaction. **B. KEGG pathways relating to immunity**: red=*Staphylococcus aureus* infection; blue=SLE; light green=amoebiasis; yellow=prion diseases; pink=pertussis; dark green=proteoglycans in cancer; orange=metabolism of xenobiotics by cytochrome P450; light blue=human papillomavirus infection; purple=focal adhesion. 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). PPI enrichment p-value: $p < 1.0 \times 10\text{-}16$

Fig. 6 Protein-protein interaction networks of deiminated protein hits identified in serum of humpback whale (*Megaptera novaeangliae*). Reconstruction of protein-protein interactions based on known and predicted interactions using STRING analysis and the orca protein database in STRING. **A. KEGG pathways relating physiological pathways** are highlighted as follows: red=complement and coagulation cascade; blue=metabolic pathways; light green=oestrogen signalling; yellow=cholesterol metabolism; pink=HIF-1 signalling pathway; dark green=glycolysis/gluconeogenesis; light blue=biosynthesis of amino acids; orange=carbon metabolsim; purple=nitrogen metabolism; brown=pentose phosphate pathway. **B. KEGG pathways relating to immunity**: red=complement and coagulation cascade; blue=*Staphylococcus aureus* infection. 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). PPI enrichment p-value: $p < 1.0 \times 10\text{-}16$.

Fig. 7 Protein-protein interaction networks of deiminated protein hits identified in serum of Cuvier's beaked whale (*Ziphius cavirostris*). Reconstruction of protein-protein interactions based on known and predicted interactions using STRING analysis and the orca protein database in STRING. **A. KEGG pathways relating physiological pathways** are highlighted as follows: red=complement and coagulation cascade; blue=metabolic pathways; light green=mineral absorption; yellow=thyroid hormone synthesis; pink=HIF-1 signalling pathway; dark green=glycolysis/gluconeogenesis; light blue=biosynthesis of amino acids; orange=carbon metabolism; purple=nitrogen metabolism; brown=proximal tubule bicarbonate reclamation. **B. KEGG pathways relating to immunity (and metabolism)**: red=African trypanosomiasis; pink= ferroptosis; blue=pentose phosphate pathway; light green=fructose and mannose metabolism; yellow=pyruvate metabolism. 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). PPI enrichment p-value: $p < 1.0 \times 10\text{-}16$

Fig. 8 Protein-protein interaction networks of deiminated protein hits identified in serum of orca (*Orcinus orca*). Reconstruction of protein-protein interactions based on known and predicted interactions using STRING analysis and the orca protein database in STRING. **A. KEGG pathways relating physiological pathways** are highlighted as follows: red=complement and coagulation cascade; blue=fat digestion and absorption; light green=cholesterol metabolism; yellow=vitamin digestion and absorption. **B. KEGG pathways relating to immunity**: red=*Staphylococcus aureus* infection; blue=SLE; light green=pertussis; yellow=prion diseases; pink=Chagas disease (American trypanosomiasis); dark green=African trypanosomiasis; light blue=ferroptosis. 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). PPI enrichment p-value: $p < 1.0 \times 10\text{-}16$

Fig. 9. Venn diagram for physiological and immunological KEGG pathways in cetaceans. A. Deiminated KEGG protein pathways identified in STRING for the 5 cetaceans under study reveal a

number of common/shared and species-specific pathways relating to physiological functions. **B.** Deiminated KEGG protein pathways immune functions, identified in STRING for the 5 cetaceans under study, reveal a number of common/shared and species-specific pathways.

Fig. 10. MicroRNA expression in whale and orca sera and serum-derived EVs. A-B. MiR21, miR155 and miR210 relative expression was assessed for key inflammatory and metabolic miRs respectively, in whale and orca sera and serum derived EVs. **A.** MiR21 expression was compared in EVs and sera of the 5 species under study, with highest levels found in EVs of humpback whale and minke whale; **B.** MiR155 expression was compared in EVs and sera of the 4 whales and orca, with the highest levels observed in minke whale; **C.** MiR210 expression was compared in EVs and sera of whales and orca, showing highest relative levels in orca EVs.

Fig. 1

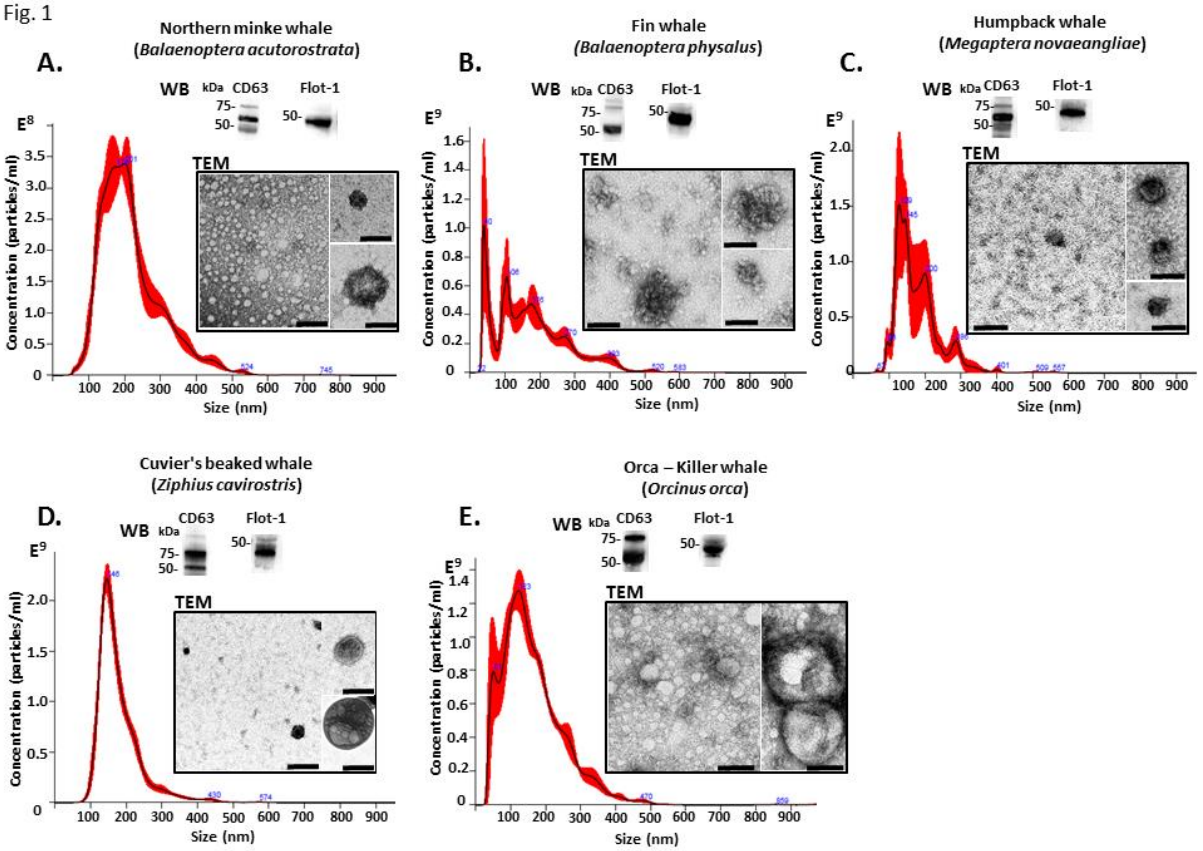

A.
Northern minke whale
(Balaenoptera acutorostrata)
WB kDa CD63 Flot-1
75-
50-
50-
TEM
E8
Concentration (particles/ml)
Size (nm)
B.
Fin whale
(Balaenoptera physalus)
WB kDa CD63 Flot-1
75-
50-
50-
TEM
E9
Concentration (particles/ml)
Size (nm)
C.
Humpback whale
(Megaptera novaeangliae)
WB kDa CD63 Flot-1
75-
50-
50-
TEM
E9
Concentration (particles/ml)
Size (nm)
D.
Cuvier's beaked whale
(Ziphius cavirostris)
WB kDa CD63 Flot-1
75-
50-
50-
TEM
E9
Concentration (particles/ml)
Size (nm)
E.
Orca – Killer whale
(Orcinus orca)
WB kDa CD63 Flot-1
75-
50-
50-
TEM
E9
Concentration (particles/ml)
Size (nm)

Fig. 2

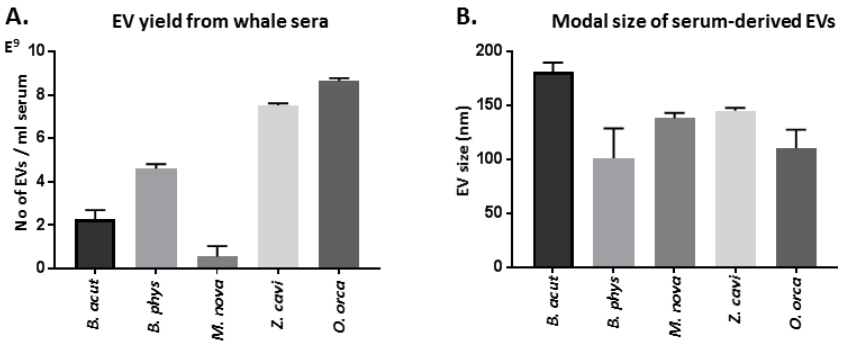

A.
EV yield from whale sera
E9
No of EVs / ml serum
B. acut
B. phys
M. nova
Z. cavi
O. orca
B.
Modal size of serum-derived EVs
EV size (nm)
B. acut
B. phys
M. nova
Z. cavi
O. orca

Fig. 3

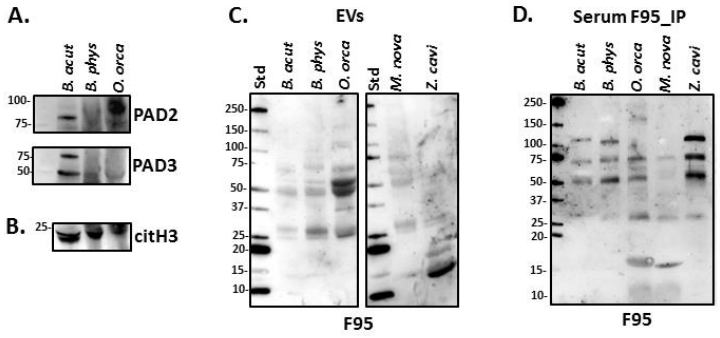


Fig. 4. Minke whale (*Balaenoptera acutorostrata*)

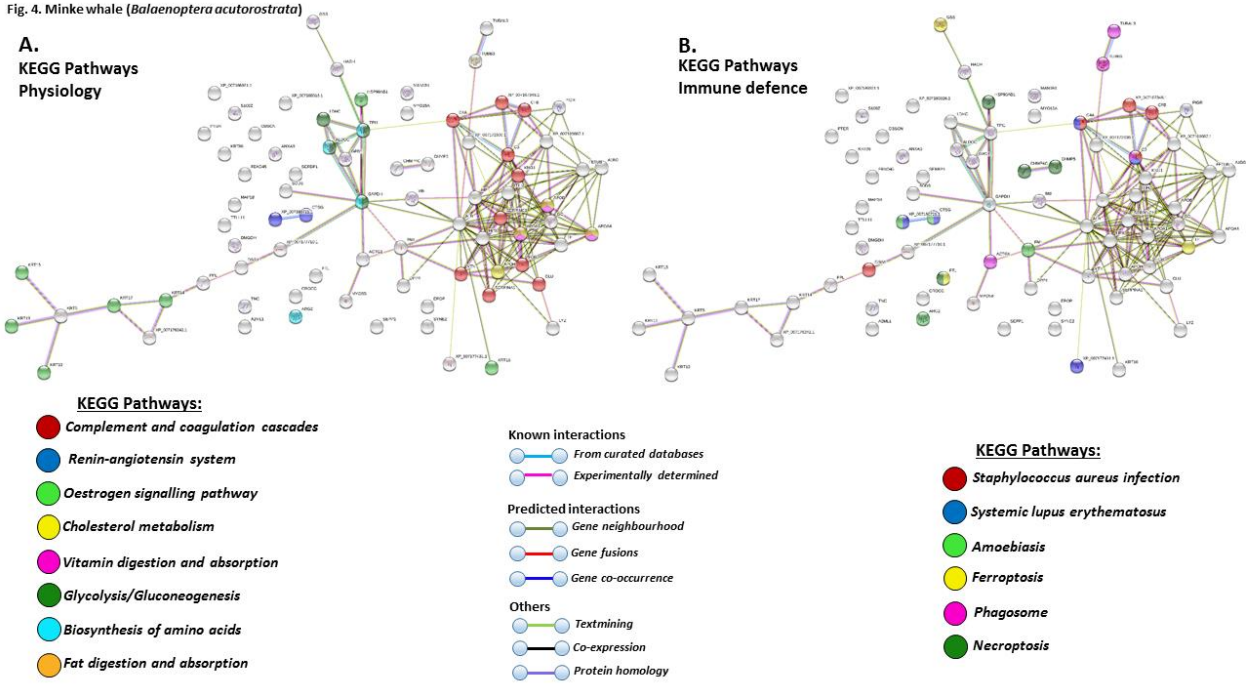


Fig. 5. Fin whale (*Balaenoptera physalus*)

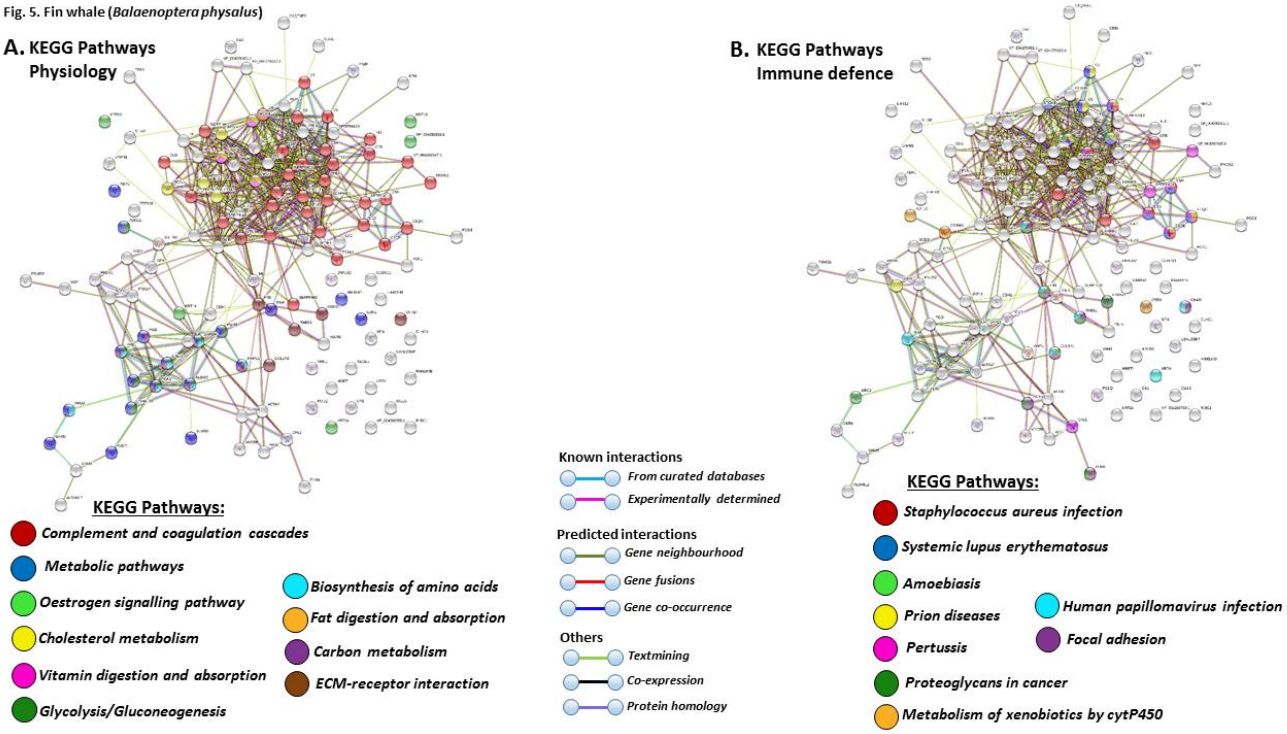


Fig. 6. Humpback whale (*Megaptera novaeangliae*)

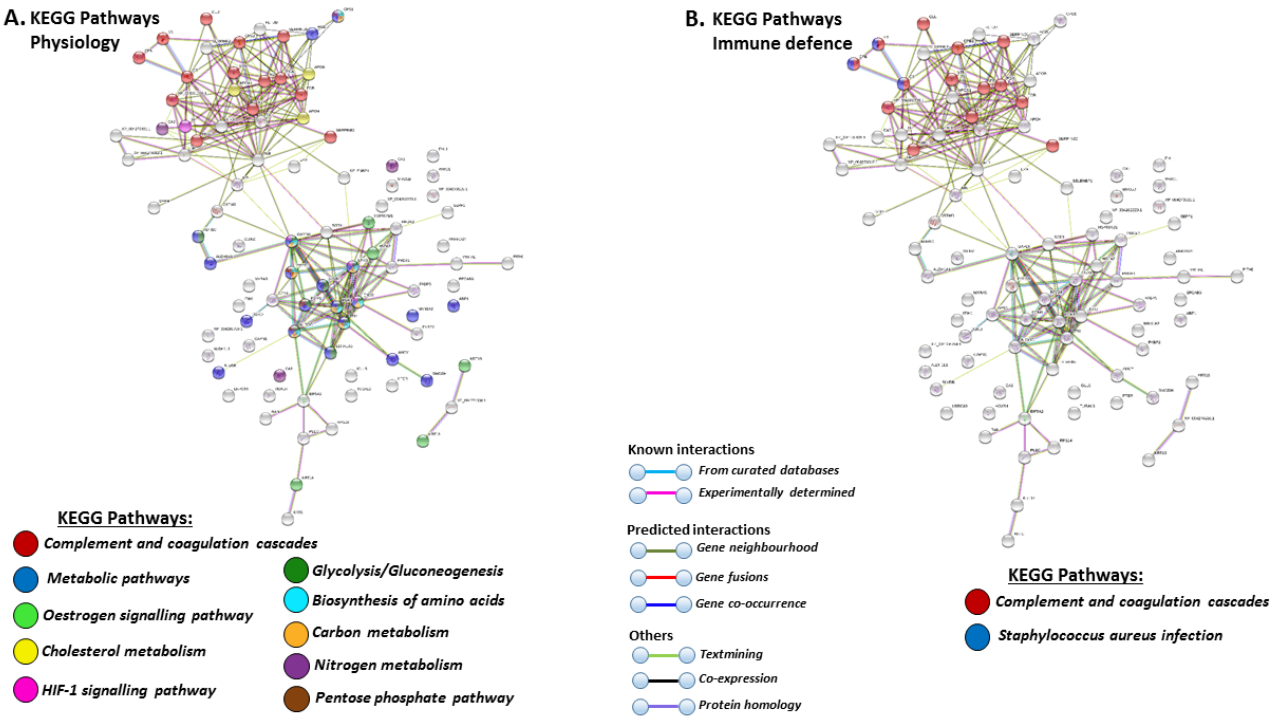


Fig. 7. Cuvier's beaked whale (*Ziphius cavirostris*)

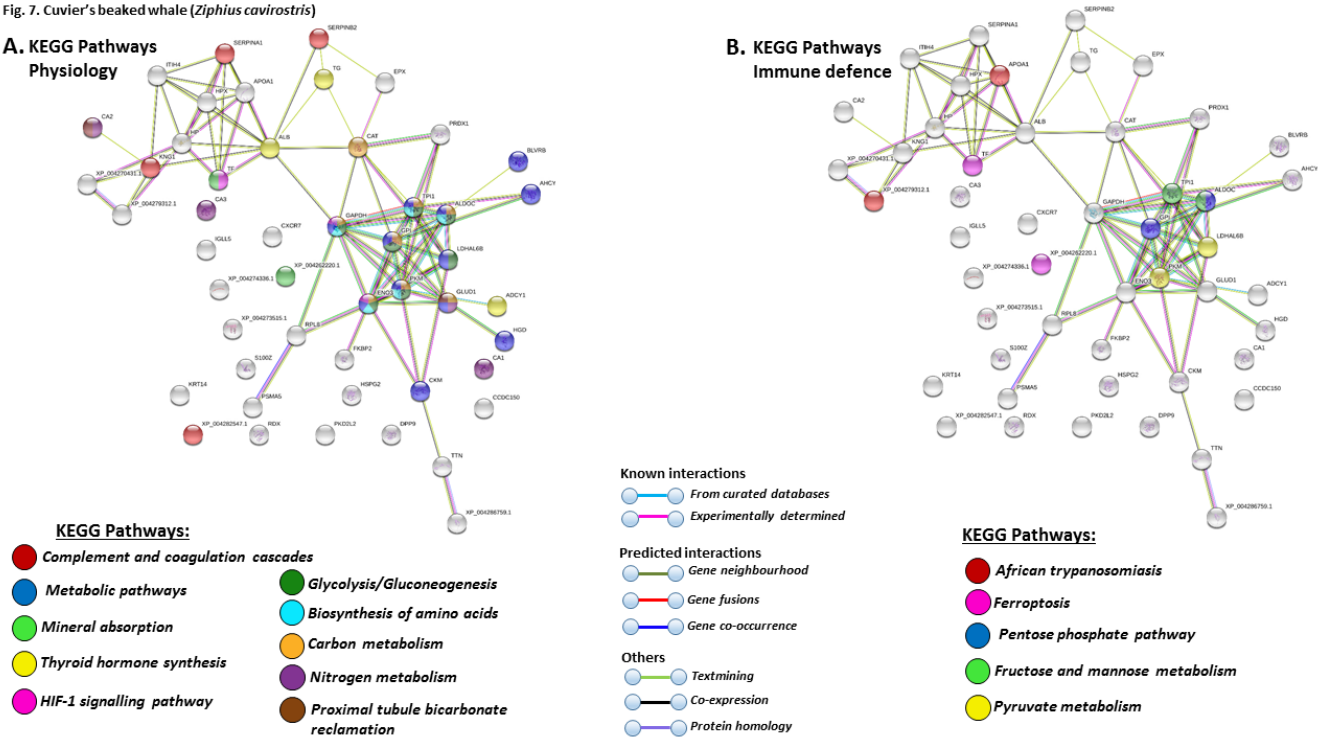


Fig. 8. Orca (*Orcinus orca*)

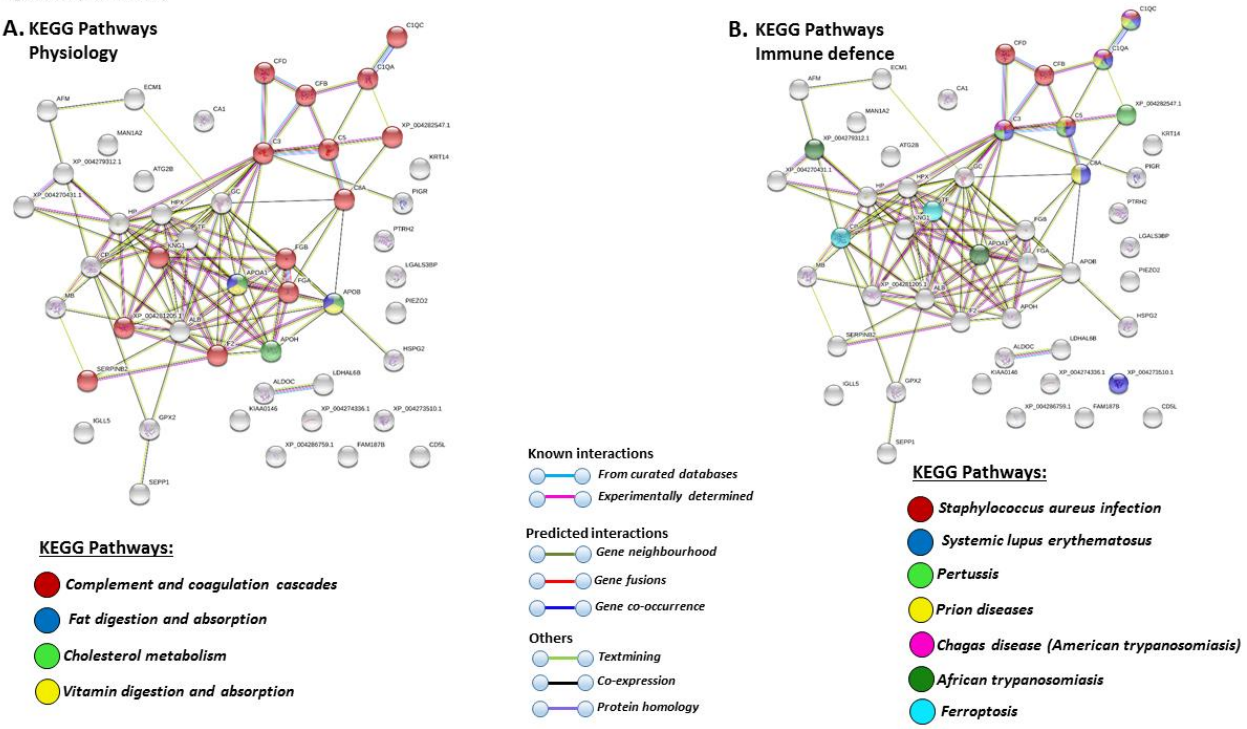


Fig. 9.

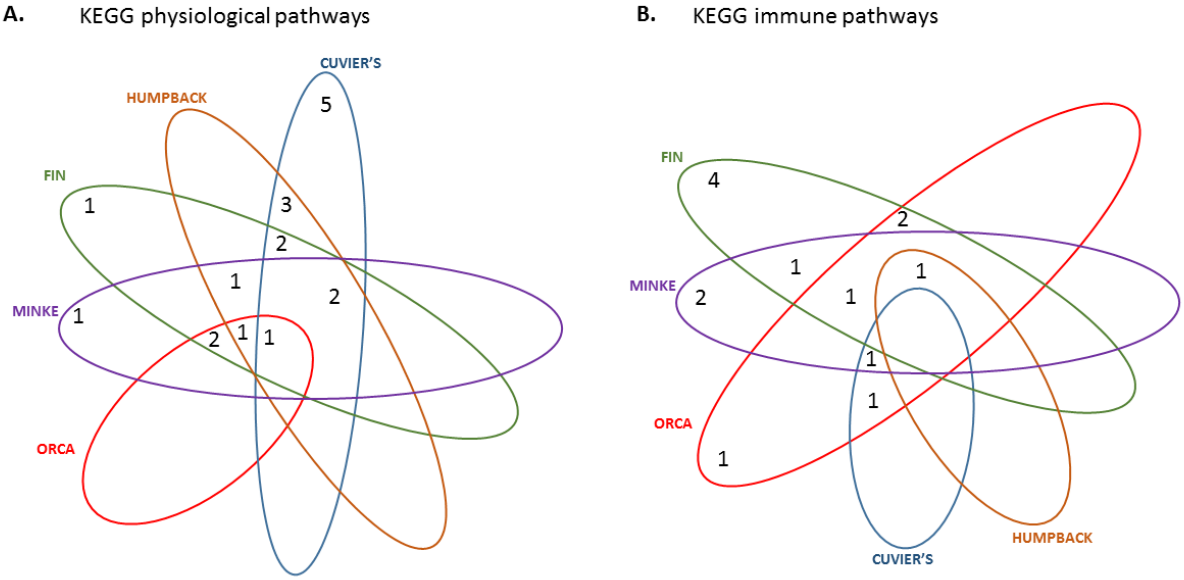
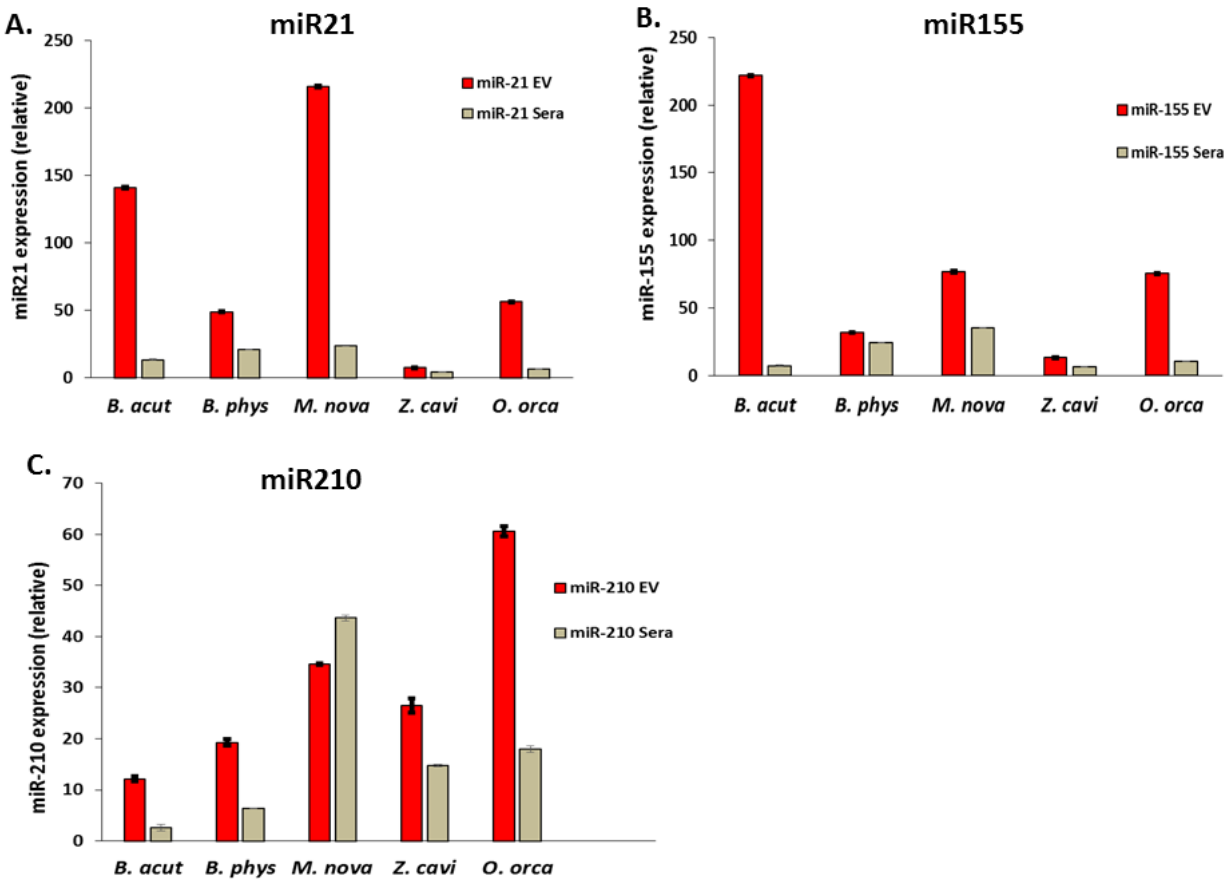

A.
KEGG physiological pathways
CUVIER'S
HUMPBACK
FIN
MINKE
ORCA
5
3
2
1
2
1
1
2
1
1
2
B.
KEGG immune pathways
FIN
MINKE
ORCA
CUVIER'S
HUMPBACK
4
2
1
2
1
1
1
1
1

Fig. 10


A.
miR21
miR21 expression (relative)
miR-21 EV
miR-21 Sera
B. acut
B. phys
M. nova
Z. cavi
O. orca
B.
miR155
miR-155 expression (relative)
miR-155 EV
miR-155 Sera
C.
miR210
miR-210 expression (relative)
miR-210 EV
miR-210 Sera

Conflict of interest statement

The authors declare no conflicting interest.

Graphical abstract