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# Deiminated proteins in extracellular vesicles and serum of llama (*Lama glama*)—Novel insights into camelid immunity



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#### ABSTRACT

Peptidylarginine deiminases (PADs) are phylogenetically conserved calcium-dependent enzymes which posttranslationally convert arginine into citrulline in target proteins in an irreversible manner, causing functional and structural changes in target proteins. Protein deimination causes generation of neo-epitopes, affects gene regulation and also allows for protein moonlighting. Furthermore, PADs have been found to be a phylogenetically conserved regulator for extracellular vesicle (EVs) release. EVs are found in most body fluids and participate in cellular communication via transfer of cargo proteins and genetic material. In this study, post-translationally deiminated proteins in serum and serum-EVs are described for the first time in camelids, using the llama (Lama glama L. 1758) as a model animal. We report a poly-dispersed population of llama serum EVs, positive for phylogenetically conserved EV-specific markers and characterised by TEM. In serum, 103 deiminated proteins were overall identified, including key immune and metabolic mediators including complement components, immunoglobulin-based nanobodies, adiponectin and heat shock proteins. In serum, 60 deiminated proteins were identified that were not in EVs, and 25 deiminated proteins were found to be unique to EVs, with 43 shared deiminated protein hits between both serum and EVs. Deiminated histone H3, a marker of neutrophil extracellular trap formation, was also detected in llama serum. PAD homologues were identified in llama serum by Western blotting, via cross reaction with human PAD antibodies, and detected at an expected 70 kDa size. This is the first report of deiminated proteins in serum and EVs of a camelid species, highlighting a hitherto unrecognized post-translational modification in key immune and metabolic proteins in camelids, which may be translatable to and inform a range of human metabolic and inflammatory pathologies.

#### 1. Introduction

Lamoids, or llamas, belong to a family of camelids which are economically important livestock and have developed complex features and immunological traits related to their habitat (Wu et al., 2014; Saadeldin et al., 2018a). The llama (*Lama glama*), Bactrian camel (*Camelus bactrianus*), dromedary (*Camelus dromedarius*) and alpaca (*Vicugna pacos*) differ in their habitat. The Bactrian camel and dromedary are adapted to arid-desert-adapted environments, alpacas to plateaus, and the llama to higher altitudes. Llamas are historically found in the Andean highlands, specifically the Altiplano of southeast Perú and western Bolivia, as well as in Chile and Argentina, which has the third highest population of llamas (Wu et al., 2014). The domesticated llama is closely related to two extant wild South American camelids, the vicuña (*Vicugna vicugna*) and guanaco (*Lama guanicoe*). Previous genomic studies have revealed a range of specific adaptions in camelids relating to fat and water metabolism, osmoregulation, blood glucose level regulation, stress responses to heat, aridity, as well as to intense ultraviolet radiation and dust (Wu et al., 2014). Furthermore, a particularly important feature in camelid immunity is the production of small, homodimeric heavy chain-only, antibodies (HCAbs) which are of great value for the biomedical industry (Henry et al., 2019).

Peptidylarginine deiminases (PADs) are phylogenetically conserved calcium-dependent enzymes which post-translationally convert arginine into citrulline in target proteins in an irreversible manner. This can cause functional and structural changes in target proteins (Vossenaar, 2003; György et al., 2006; Wang and Wang, 2013; Bicker and Thompson, 2013). Structures most prone to deimination are beta-sheets

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Received 3 July 2019; Received in revised form 5 October 2019; Accepted 23 October 2019 Available online 13 November 2019 0161-5890/ © 2019 Elsevier Ltd. All rights reserved. and intrinsically disordered proteins, while the position of the arginine is also important; arginines sitting next to aspartic acid residues are most prone to citrullination, arginines next to glutamic acid residues are rarely citrullinated and those flanked by proline are poorly citrullinated (Nomura, 1992; Tarcsa et al., 1996; György et al., 2006). 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; Magnadóttir et al., 2018a). PADs have been identified throughout phylogeny from bacteria to mammals, with 5 tissue specific PAD isozymes in mammals. 3 in chicken, 1 in bony fish and arginine deiminase homologues in bacteria (Vossenaar et al., 2003; Rebl et al., 2010; Magnadóttir et al., 2018a, a; Kosgodage et al., 2019). While studies on PADs in relation to human pathophysiology, including cancer, autoimmune and neurodegenerative diseases (Wang and Wang, 2013; Witalison et al., 2015; Lange et al., 2017; Kosgodage et al., 2017 & 2018) and CNS regeneration (Lange et al., 2011 and 2014) exist, relatively little phylogenetic research has been carried out on PADs in relation to normal physiology and evolutionary acquired adaptions of the immune system. Recent comparative studies focussing on roles for PADs in teleost fish have identified post-translational deimination in key proteins of innate, adaptive and mucosal immunity (Magnadóttir et al., 2018a, b; Magnadottir et al., 2019a; Magnadóttir et al., 2019b). A recent study in shark also revealed novel insights into this post-translational modification in relation to key immune factors, including shark immunoglobulins (Criscitiello et al., 2019). As the camelid family has developed unusual small immunoglobulins similar as to shark through convergent evolution, indicating common factors between shark and camelid immunity, we felt that an investigation of post-translationally deiminated proteins in camelids was warranted.

As PADs have been identified to be a key regulator of extracellular (EV)-release, a mechanism that has been found to be phylogenetically conserved from bacteria to mammals (Kholia et al., 2015; Kosgodage et al., 2017, 2018; Gavinho et al., 2019; Kosgodage et al., 2019), the characterisation of EVs in camelids is of further interest. Extracellular vesicles (EVs) are found in most body fluids and participate in cellular communication via transfer of cargo proteins and genetic material (Inal et al., 2013; Colombo et al., 2014; Lange et al., 2017; Turchinovich et al., 2019; Vagner et al., 2019). EVs in body fluids, including serum, can also be useful biomarkers to reflect health status (Hessvik and Llorente, 2018; Ramirez et al., 2018). Previous work on EVs has hitherto mainly been in the context of human pathologies, while recently comparative studies are growing (Iliev et al., 2018; Yang et al., 2015; Magnadóttir et al., 2019b; Criscitiello et al., 2019; Gavinho et al., 2019; Kosgodage et al., 2019). Few studies have been performed on EVs in camelids but therapeutic effects of EVs isolated from camel milk have been identified in halting cancer progression (Badawy et al., 2018). A recent study in shark identified for the first time deiminated small immunoglobulin proteins as part of EV cargo (Criscitiello et al., 2019). Due to the link between camelid and shark immunity through convergent evolution including the unusual immunoglobulin structure of small heavy chain-only Ig's, a comparative study on EVs and deiminated EV cargo in a camelid species may provide further insights into shared immunological traits. Camelids have an unusual Ig repertoire and a large diversity of functional nanobodies has been identified in the llama (Harmsen and De Haard, 2007; Deschaght et al., 2017). This has made camelids an important source for small immunoglobulins that can be used for immunotherapy purposes, including for tumour targeting (van Lith et al., 2016), as well as for assessing cancer metastasis (Ramos-Gomes et al., 2018). As these nanobodies can also penetrate the blood-brain barrier (Širochmanová et al., 2018) they are of great value for a range of therapeutic treatment applications, including for brain cancers (Iqbal et al., 2010). Furthermore, as the camelid family has acquired unique metabolic features, they are also of interest as a model

species for informing metabolic diseases.

In the current study we assessed post-translationally deiminated proteins in llama serum and serum-derived EVs, and report for the first time EV-mediated export of deiminated key immune, metabolic and nuclear proteins in serum of a camelid species.

#### 2. Materials and methods

#### 2.1. Animals and sampling

Llama (*Lama glama* L. 1758) serum was shared from excess blood collected in routine health checks of a resident male llama at the Texas A&M Winnie Carter Wildlife Center. Blood collected from the jugular vein of this 21 year old llama was allowed to clot at room temperature for 2 h before serum was collected by centrifuging at 300 g for 10 min. Serum was aliquoted and immediately frozen at -80 °C until further use.

### 2.2. Extracellular vesicle (EV) isolation and nanoparticle tracking analysis (NTA)

EVs were isolated by step-wise centrifugation according to established protocols using ultracentrifugation and the recommendations of MISEV2018 (the minimal information for studies of extracellular vesicles 2018; Théry et al., 2018). Llama serum was diluted 1:5 in ultrafiltered (using a  $0.22\,\mu m$  filter) Dulbecco's PBS (DPBS,  $100\,\mu l$  serum added to 400  $\mu$ l DPBS) and then centrifuged at 4000 g for 30 min at 4 °C for removal of cells and cell debris. The supernatant was collected and centrifuged at 100,000 g for 1 h at 4 °C. The pellet was then resuspended in DPBS and washed again at 100,000 g for 1 h at 4 °C. The resulting EVenriched pellet was resuspended in 100  $\mu$ l DPBS, diluted 1/100 in DPBS and analysed by NTA, based on Brownian motion of particles in suspension, using the NanoSight NS300 system (Malvern, UK). The NanoSight was used in conjunction with a syringe pump to ensure continuous flow of the sample, with approximately 40-60 particles per frame and videos were recorded for 5 x 90 s. The replicate histograms generated from the recordings were averaged.

#### 2.3. Transmission electron microscopy (TEM)

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

#### 2.4. Western blotting

Llama serum and EV isolates (an EV pellet derived from 100 µl serum, reconstituted in 100 µl DPBS after isolation and purification) were diluted 1:1 in 2x Laemmli sample buffer, boiled for 5 min at 100 °C and separated by SDS-PAGE on 4–20 % gradient TGX gels (BioRad UK). Approximately 5 µg protein was loaded per lane and transferred to nitrocellulose membranes using semi-dry Western blotting. Blocking of membranes was performed in 5 % BSA in TBS-T for 1 h at room temperature (RT) and incubation with primary antibodies, diluted in TBS-T, was carried out at 4 °C overnight (F95 MABN328, Merck, 1/1000; PAD2 ab50257, Abcam, 1/1000; PAD3 ab50246, 1/1000; PAD4 ab50247, 1/1000; citH3 ab5103, 1/1000; CD63 ab216130, 1/1000; Flot-1 ab41927, 1/2000). The membranes were washed in TBS-T for  $3 \times 10$  min at RT and thereafter incubated in the corresponding secondary antibody (anti-rabbit IgG BioRad or anti-mouse IgM BioRad,

diluted 1/4000 in TBS-T) for 1 h at RT. Membranes were washed for  $6 \times 10$  min in TBS-T and visualisation performed using electrochemiluminescence (ECL) and the UVP BioDoc-ITTM System (Thermo Fisher Scientific, UK).

## 2.5. Immunoprecipitation and identification of deiminated proteins in llama serum and EVs

For isolation of total deiminated proteins from llama serum and serum derived EVs, the Catch and Release immunoprecipitation kit (Merck, UK) was used together with the F95 pan-deimination antibody (MABN328, Merck), which has been developed against a deca-citrullinated peptide and specifically detects proteins modified by citrullination (Nicholas and Whitaker, 2002). For F95 enrichment, 50 µl serum was used according to the manufacturer's instructions (Merck). For EVs, total protein was first extracted from EV-enriched pellets derived from 100 µl serum, using 100 µl radioimmunoprecipitation assay (RIPA) buffer, containing protease inhibitor cocktail (P8340, Sigma, UK), and shaken gently on ice for 2 h. Thereafter proteins were isolated from the EVs by centrifugation at 16,000 g for 30 min, collecting the supernatant containing the proteins. Immunoprecipitation was carried out according to the manufacturer's instructions (Merck), using a rotating platform overnight at 4 °C. The F95 bound proteins were eluted using denaturing elution buffer, according to the manufacturer's instructions (Merck). The F95 enriched eluates were then either analysed by Western blotting or by LC-MS/MS (Cambridge Proteomics, Cambridge, UK). For LC-MS/MS, the F95-enriched eluates were run 1 cm into a SDS-PAGE gel and the whole F95-enriched eluate was cut out as one band, whereafter it was processed for proteomic analysis (carried out by Cambridge Proteomics). Peak files were submitted to in-house Mascot (Matrix Science; Cambridge Proteomics). Databases used for protein identification (in house, Cambridge Proteomics) were as follows: Camelidae\_family\_20190613 (21429 sequences; 9086806 residues) and also specifically for llama: Lama glama 20190613 (234 sequences; 52757 residues).

#### 3. Results

#### 3.1. EV analysis in llama serum

EVs from llama serum were characterised, following step-wise ultracentrifugation, by size exclusion using NTA, by morphological analysis using TEM and by Western blotting using EV-specific markers (Fig. 1). A poly-dispersed population of EVs in the size range of 30–576 nm, with main peaks at 38, 119, 167, 237, 323 and 403 nm was identified by NTA analysis (Fig. 1A). Western blotting confirmed that the llama serum EVs were positive for the EV-specific markers CD63 and Flotillin-1 (Fig. 1B). TEM analysis confirmed a poly-dispersed EVs population (Fig. 1C).

#### 3.2. PAD and deiminated proteins in llama serum

A cross-reaction with human PAD2, 3 and 4 isozyme-specific antibodies was observed in llama serum by Western blotting, at an approximate 70–75 kDa size range as expected for mammalian PADs (Fig. 2A). Deiminated histone H3 was also detected in llama serum by Western blotting at the expected approximate 20 kDa size (Fig. 2A). Total deiminated proteins in llama serum-EVs were detected by Western blotting using the F95 pan-deimination antibody, revealing a range of proteins between 25–100 kDa in size (Fig. 2B). Deiminated proteins were also assessed by Western blotting after F95 enrichment, using immunoprecipitation (IP) from llama serum and serum-derived EVs (Fig. 2C). Deiminated proteins from the F95 enriched eluates were further identified by LC–MS/MS analysis. In llama serum, 103 hits for camelid proteins were identified, as listed in Table 1 and Supplementary Table 1. Overall, 43 of these deiminated protein hits were common to whole serum and serum-derived EVs, while 60 hits were specific for whole serum (Fig. 2D).

#### 3.3. Identification of deiminated proteins in EVs from llama serum

Llama serum-derived EVs showed positive for deiminated proteins by Western blotting, using the pan-deimination F95 antibody (Fig. 2B). Deiminated proteins were further identified by F95 enrichment and LC–MS/MS analysis, revealing 68 deiminated protein hits in total for EVs, with 25 hits unique to EVs (not identified from serum). Peptide sequences of hits with camelid proteins and m/z values are listed in Table 2 and Supplementary Table 2. Overlap with deiminated proteins identified in whole llama serum and EVs is represented in the Venn diagram in Fig. 2D.

#### 4. Discussion

For the first time deiminated proteins are described in a camelid, using the llama (Lama glama) as a model species. Post-translational deimination was identified in key immune, nuclear and metabolic proteins. A llama PAD homologue was identified at an expected 70-75 kDa size similar as for human PADs by Western blotting via cross reaction with anti-human PAD2, PAD3 and PAD4 antibodies. PAD2 is known to be the phylogenetically most conserved PAD form (Vossenaar et al., 2003; Magnadóttir et al., 2018a) and has also been seen in shark (Criscitiello et al., 2019). Deiminated histone H3, a marker of neutrophil extracellular trap formation (NETosis), was also detected in llama serum by Western blotting and is described for the first time in a camelid species but was recently described in shark (Criscitiello et al., 2019). NETosis is driven by PADs (Li et al., 2010), is conserved throughout phylogeny and is important in innate immune defences against a range of pathogens including bacteria, viruses and helminths (Brinkmann et al., 2004; Branzk et al., 2014; Schönrich and Raftery, 2016). NETosis has also been associated with clearance of apoptotic cells and during tissue remodelling in teleosts (Magnadóttir et al., 2018a, 2019a). Furthermore, NETosis is linked to a range of autoimmune diseases, due to NET activation via neo-epitopes (O'Neil and Kaplan, 2019), which can also lead to organ damage (Lee et al., 2017). NETosis is also associated with cancer (Gonzalez-Aparicio and Alfaro, 2019) and neurodegenerative diseases (Pietronigro et al., 2017).

Further deiminated proteins identified in llama serum and serumderived EVs by F95 enrichment and LC-MS/MS analysis included key proteins of camelid innate and adaptive immunity, nuclear proteins, as well as proteins involved in metabolic function. Using STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) analysis (https:// string-db.org/) for protein-protein interaction, the PPI enrichment value for deiminated proteins in whole llama serum was found to be p < 1.0e-16 for 43 deiminated proteins out of 103 identified in serum, 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 (STRING analysis, see Fig. 3A). For deiminated proteins in 29 out of the 68 deiminated EV cargo proteins, the PPI enrichment value was found to be p = 0.0193 (STRING analysis, see Fig. 4A). Such enrichment indicates that the proteins are at least partially biologically connected, as a group. As the camelid proteins are not present in the STRING database, corresponding protein homologues for human were chosen to create the protein-protein interaction networks shown in Figs. 3 and 4. Out of the 103 camelid proteins identified as deiminated in serum, protein IDs for 43 were present for Homo sapiens to perform the assessment of protein-protein interactions and identification of main biological GO pathways (response to stress, response to wounding, oxygen transport, small molecule metabolic process, vesicle mediated transport and regulated exocytosis; Fig. 3B). For llama EVs, out of 68 proteins identified as deiminated, 29 corresponding protein homologues were found in the STRING database for Homo sapiens and were analysed highlighting

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**Fig. 1.** Extracellular vesicles (EVs) isolated from llama serum. **A.** Nanoparticle tracking analysis shows a poly-dispersed population of EVs in the size range of 30–576 nm, with main peaks at 38, 119, 167, 237, 323 and 403 nm. **B.** Llama serum EVs are positive for the EV-specific markers CD63 and Flotillin-1 (Flot-1). **C.** Transmission electron microscopy (TEM) imaging of EVs isolated from llama serum shows a polydispersed population; scale bar represents 100 nm.

main biological GO pathways (response to stress, cytoskeleton organisation and vesicle-mediated transport; Fig. 4B).

Deimination protein candidates identified here in llama serum and EVs, which are involved in immune, nuclear and metabolic functions, are further discussed below, including where appropriate in a comparative context with relevant human diseases. Proteins that have previously been identified as deiminated in other species are listed first.

Nanobodies are based on immunoglobulin single variable domains, derived from the variable domains of heavy chain-only antibodies, which occur naturally in camelids (Deschaght et al., 2017). In the llama the heavy chain-only antibodies are comprised of at least two subclasses (Henry et al., 2019). These types of homodimeric, light chain-less antibodies have evolved through convergent evolution (Brooks et al., 2018), in at least two groups (camelids and cartilaginous fish), and their variable binding domains (V<sub>H</sub>H<sub>S</sub>) are of great value for therapeutic and diagnostic applications (Muyldermans, 2013; Criscitiello, 2014; Steeland et al., 2016; Könning et al., 2017; Henry et al., 2019). Nanobodies can act against challenging targets such as small molecules and toxins (Wesolowski et al., 2009; Bever et al., 2016), viruses (Wei et al., 2011; Hassiki et al., 2016; Vanlandschoot et al., 2011; Cohen, 2018), enzymes (Muyldermans, 2013), ion channels (Wei et al., 2011; Danquah et al., 2016) and G protein-coupled receptors GPCRs (Cromie et al., 2015). Llama nanobodies have been shown to tether to early endosomes and to mitochondria (Traub, 2019), be used for diagnostics (Shriver-Lake et al., 2018), be used for design of cancer immunotherapeutics (Hussack et al., 2018; Bannas and Koch-Nolte, 2018; Rossotti et al., 2019) and have been approved for passive immunotherapy (Sheridan, 2019). Our current finding, that llama nanobodies can be post-translationally deiminated may shed some light on their observed structural variation which still remains to be fully explained as sequence alignment does not fully elucidate their diversity (Mitchell and Colwell, 2018). As a structurally analogous immunoglobulin in shark, new antigen receptor (NAR) (Greenberg et al., 1995; Barelle et al., 2009; Flajnik and Dooley, 2009; De Silva et al., 2019) was recently also found to be deiminated (Criscitiello et al., 2019), our current finding may provide novel insights into function of these immune proteins and be useful for refinement in therapeutic nanobody development. Llama single-chain antibodies were here found to be deimination candidates both in llama whole serum and in serum derived EVs, highlighting also their EV-mediated export.

Ig proteins were identified here as being deiminated in llama serum and in serum-derived EVs, scoring with Ig components from other camelids. Immunoglobulins (Ig) are key molecules in adaptive immunity but post-translational deimination of Ig's has hitherto received little attention. Deimination of the IgG Fc region in patients with bronchiectasis and RA has been identified (Hutchinson et al., 2017). Furthermore, deimination of Ig's in teleost fish was recently described (Magnadóttir et al., 2019a), as well as in shark (Criscitiello et al., 2019). Given the increased focus on understanding of Ig diversity throughout phylogeny (Smith et al., 2012; Zhang et al., 2013; de los Rios et al., 2015; Zhang et al., 2016, 2017; Stanfield et al., 2018) and the unique features of camelid immunoglobulins (Plasil et al., 2019), our current finding of deimination of llama Ig's highlights a novel concept that may further understanding of Ig diversity throughout evolution. Of additional interest is the finding that some deiminated Ig proteins were also found to be exported in EVs.

Complement components identified to be deiminated in llama serum included C3, C4 and C1q, while only C3 was identified to be deiminated in serum derived EVs. Complement component C3 plays a central role in all pathways of complement activation and can also be directly activated by self- and non-self surfaces via the alternative pathway without a recognition molecule (Dodds and Law, 1998; Dodds, 2002). In camelids, the alternative and classical pathway haemolytic activity of serum has been assessed in the dromedary camel with respect to age and gender (Olaho-Mukani et al., 1995a; and 1995b). Hitherto little is known about roles for post-translational deimination of complement components throughout phylogeny. In teleost fish, C3 has been identified in serum in deiminated form (Magnadóttir et al., 2019a) and also found to be deiminated in mucosal and serum EVs of teleost fish (Magnadóttir et al., 2019b, 2019c), while post-translationally deiminated C3 was identified in shark total serum but not EVs (Criscitiello et al., 2019). Other complement components, including C4 and C1q, which belong to the classical pathway of complement activation were here identified as deiminated in llama whole serum and some of those



**Fig. 2.** Western blotting of deiminated proteins and PAD in llama serum. **A.** Llama PAD homologues were identified at the expected size of approximately 70–75 kDa using the anti-human PAD2, PAD3 and PAD4 isozyme specific antibodies respectively. Deiminated histone H3 (citH3), representative of neutrophil extracellular traps (NETs), was verified in llama serum. **B.** Total deiminated proteins were assessed by Western blotting in llama serum EVs, using the F95 pan-deimination specific antibody. **C.** Immunoprecipitated (IP) deiminated proteins after F95-enrichment were assessed both in serum-EVs and whole serum of llama by Western blotting. **D.** The Venn diagram represents deiminated proteins identified in total serum and serum-derived EVs by F95 enrichment and LC–MS/MS analysis. Overall, 43 proteins were identified in common with both samples, while 60 proteins were found deiminated in serum only and 25 proteins were identified as deiminated in EVs only.

have also recently been reported to be deiminated in teleost fish (Magnadóttir et al., 2019a). The C1q subcomponent can bind to the Fc region of immunoglobulins that are bound to antigen and activate the classical part of the complement pathway (Reid et al., 2002; Reid, 2018). Interestingly, an essential role for arginine in C1q has previously 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) and may therefore also bind to deiminated neo-epitopes (Magnadóttir et al., 2019a). The complement system has multifaceted roles. It forms part of the first line of immune defence against invading pathogens, acting in clearance of necrotic or apoptotic cells (Dodds and Law, 1998; Sunyer and Lambris, 1998; Fishelson et al., 2001; Carroll and Sim, 2011). Complement also has roles in regeneration (Del Rio-Tsonis et al., 1998; Haynes et al., 2013) and tissue remodelling (Lange et al., 2004a, 2004b; Lange et al., 2005, 2006, 2019). Furthermore, C1 is also implicated in multiple non-complement functions including binding of apoptotic cells, cleavage of nuclear antigens and cleavage of MHC class I (Lu and Kishore, 2017). Post-translational deimination of complement components may possibly influence their function including cleavage ability, binding, deposition and generation of the convertase.

**Apolipoprotein A–I** is primarily involved in lipid metabolism where conformational plasticity and flexibility are regarded as key structural features (Arciello et al., 2016). Apo A–I is associated with regulation of

#### Table 1

Deiminated proteins identified by F95 enrichment and LC–MS/MS in total serum of llama (*Lama glama*). 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 inhouse Mascot (Cambridge Proteomics). Peptide sequence hits scoring with *Lama glama* (LAMGL) are included as well as hits with other camelids (CAMFR = *Camelus ferus*; CAMDR = *Camelus dromedaries*; LAMGU = *Lama guanicoe*; VICPA = *Vicugna pacos* (Alpaca)). Hits with uncharacterized proteins are omitted in the list. For a full list of peptide sequences and *m/z* values see Supplementary Table 1. An asterix (\*) indicates that the protein hit is specific to whole serum only.

Protein name (*unique for serum)	Number of peptide sequences identified	Total score $(p < 0.05)^{\dagger}$
*097643_LAMGL	6	282
Fibrinogen A-alpha chain *P01973 HBA_LAMGL	5	189
Hemoglobin subunit alpha P68226 HBB_LAMGL	4	166
Hemoglobin subunit beta Q865W8_LAMGL	4	109
Beta actin A0A1W5VKM5_LAMGL	2	117
Anti-RON nanobody *A0A1W5VKM7_LAMGL	2	111
Anti-RON nanobody *A0A1W5VKR8_LAMGL	2	96
Anti-RON nanobody *A0A1W5VKQ9_LAMGL	1	66
Anti-RON nanobody S9XDK9_CAMFR	34	1575
Complement C3-like protein S9WI87_CAMFR	20	972
Serum albumin S9XAP9_CAMFR Keratin, type I cytoskeletal 14-like	9	522
protein S9Y6J1_CAMFR Keratin, type II cytoskeletal 5 isoform 13-	11	502
*S9 × 688_CAMFR	10	462
Keratin 6A-like protein *S9YD43_CAMFR	9	443
Complement component 4A-like protein *S9Y253_CAMFR	8	380
Kininogen-2 isoform I *TOMII3_CAMFR	7	371
Alpha-2-macroglobulin-like protein S9XI90_CAMFR Keratin, type II cytoskeletal 75-like	8	357
isoform S9 $\times$ 494_CAMFR	6	285
Keratin, type I cytoskeletal 42 S9XBS9_CAMFR	6	263
Ig gamma-3 chain C region *S9XYY2_CAMFR	5	246
Hemopexin A0A075T9L1_CAMDR	4	174
Dipeptidylpeptidase 4 S9XXW2_CAMFR	3	172
Fibrinogen beta chain *S9WDV3_CAMFR	3	169
Fibrinogen gamma chain isoform gamma-B		
*A0A1K0GY87_VICPA Globin A1	4	166
S9WB99_CAMFR Histone H2B	3	146
S9XNF8_CAMFR Xaa-Pro dipeptidase	3	142
*S9YS49_CAMFR Putative E3 ubiquitin-protein ligase	3	132
Koquin *S9YGW7_CAMFR Heparin cofactor 2	3	123

(continued on next page)

#### Table 1 (continued)

#### Table 1 (continued)

Protein name (*unique for serum)	Number of peptide sequences identified	Total score $(p < 0.05)^{\frac{1}{2}}$
S9WPM4_CAMFR	3	115
Adiponectin A2V743 CAMDR	4	109
Beta actin		107
*S9XP08_CAMFR Inter-alpha-trypsin inhibitor heavy chain	1 2	109
H1	2	
S9XM15_CAMFR Ferritin	2	
T0NNK2_CAMFR	1	93
L-lactate dehydrogenase	1	90
Ig kappa chain V-II region RPMI 6410-	-	20
like protein A0A0A0PAR2 CAMDR	2	88
Heat shock protein 90	-	
*S9XYF2_CAMFR Heat shock cognate protein HSP 90-beta-	2	87
like isoform 3		
S9XHZ4_CAMFR Phosphotriesterase-related protein	1	83
S9XM68_CAMFR	2	73
Xaa-Pro dipeptidase isoform 3	2	73
Tubulin beta chain	2	/5
*S9YV02_CAMFR	1	69
*S9XC57_CAMFR	1	63
Plasminogen *SQVI 21 CAMER	2	62
Apolipoprotein A-I	2	02
*S9WKZ8_CAMFR	1	62
H4		
S9WIA5_CAMFR	1	61
T0MHN9_CAMFR	1	60
Pyruvate kinase	1	50
Complement C1q subcomponent subunit	1	36
C isoform 2	2	F7
Unconventional myosin-Va isoform 2	2	5/
S9WVY1_CAMFR	1	56
S9XA40_CAMFR	1	55
Heat shock cognate protein		50
Keratin, type II cytoskeletal 71	1	52
A0A0U2KTX5_CAMDR	1	52
S9XR87_CAMFR	1	50
Ig lambda chain C regions isoform 19-like		
protein S9WGH8_CAMFR	1	49
Lysozyme	2	40
Dystonin	2	49
*S9 × 8K9_CAMFR	1	46
Transaldolase *T0NM23_CAMFR	2	45
Rootletin		
S9W6I0_CAMFR Ferritin	1	45
S9WF34_CAMFR	1	43
*S9YSI7_CAMFR	1	43
Triosephosphate isomerase	1	40
~59A5Q6_CAMFR Vitamin D-binding protein-like protein	1	40
*S9YMC0_CAMFR	2	40
Transcription factor 20 isoform 1 *S9Y636 CAMFR	1	39
-		

Protein name (*unique for serum)	Number of peptide sequences identified	Total score $(p < 0.05)^{\dagger}$
Receptor-type tyrosine-protein		
phosphatase-like N		
$*S9 \times 6M4$ _CAMFR	1	37
Dyslexia-associated protein		
T0MH94_CAMFR	1	36
Rabenosyn-5-like protein		
S9WJW3_CAMFR	1	35
N6-adenosine-methyltransferase subunit		
*A8IY99_LAMGU	1	35
Gamma-fibrinogen	_	
S9WRI7_CAMFR	1	35
Nuclear receptor coactivator 5 isoform 3-		
like protein	1	25
S9W421_CAMFR	1	35
*SOW711 CAMER	1	24
Charged multivesiever body protein 4e	1	54
$\times$ 080 CAMEP	1	24
Ig lambda chain V-III region I OI-like	1	34
protein		
SOWLICS CAMER	1	32
Ig kappa chain V-II region RPMI 6410-	1	52
like protein		
*S9WVI6 CAMFR	1	32
Complement C1q subcomponent subunit		
Â		
*S9Y5S1_CAMFR	1	32
Transcriptional repressor NF-X1		
*S9YC53_CAMFR	1	32
Alpha-1-antitrypsin-like protein		
*S9Y3F6_CAMFR	1	31
Dual specificity testis-specific protein		
kinase 1		
S9WKI8_CAMFR	1	31
HEAT repeat-containing protein 7B1		
*S9WVS9_CAMFR	1	29
Peroxisome proliferator-activated		
receptor gamma coactivator-related		
protein 1	1	20
^S9XVK5_CAMFR	1	29
	1	20
"S91907_CAMFR General transcription factor IL i isoform 4	1	28
icoform 1 like protein		
*TOMC04 CAMER	1	28
Spermatogenesis associated protein 2	1	20
like protein		
*S9VS76 CAMER	1	28
Centromere protein J	1	20
controllere protein v		

<sup>1</sup> Ions score is -10\*Log(P), where P is the probability that the observed match is a random event. Individual ions scores > 20 indicated identity or extensive homology (p < 0.05). Protein scores were derived from ions scores as a nonprobabilistic basis for ranking protein hits. Cut-off was set at Ions score 20.

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 and thus the final lytic pathway (Hamilton et al., 1993; Jenne et al., 1991; French et al., 1994). Given the diverse roles of Apo A–I, the current finding of deiminated forms in serum may be of quite some relevance and has previously been identified in teleost fish (Magnadóttir et al., 2019a). Apo A–I was here found to be deiminated in whole llama serum only.

*Serum 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). In camelids, total albumin levels have been assessed, for example in relation to reproductive efficiency (El-Malky et al., 2018) and as biomarkers of oxidative stress

#### Table 2

Deiminated proteins identified by F95 enrichment and LC–MS/MS in EVs isolated from serum of llama (*Lama glama*). 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. Peptide sequence hits scoring with L. *Lama glama* (LAMGL) are presented as well as hits with other camelids (CAMFR=*Camelus ferus*; CAMDR=*Camelus dromedaries*; LAMGU=*Lama guanicoe*). Hits with uncharacterised proteins are not listed. For a full list of peptide sequences and m/zvalues see Supplementary Table 2. An asterix (\*) indicates that the protein hit is unique for EVs only.

Protein name (*unique for EVs)	Number of peptide sequences identified	Total score $(p < 0.05)^{\frac{1}{2}}$
A0A1W5VKM5_LAMGL	2	164
Q865W8_LAMGL	2	85
*S9XAP9_CAMFR	9	554
Keratin, type I cytoskeletal 14-like protein		
*S9 × 688_CAMFR Keratin 6A-like protein	10	496
S9Y6J1_CAMFR Keratin, type II cytoskeletal 5 isoform	10	438
13-like protein S9WI87_CAMFR	9	430
Serum albumin *S9YN99 CAMER	6	417
Keratin, type I cytoskeletal 17-like	0	417
*S9XI90_CAMFR	6	318
isoform	_	
S9 $\times$ 494_CAMFR Keratin, type I cytoskeletal 42	5	269
S9XBS9_CAMFR Ig gamma-3 chain C region	4	162
A0A075T9L1_CAMDR	3	153
S9 $\times$ 684_CAMFR	2	136
Keratin, type II cytoskeletal 8 S9WB99_CAMFR	3	133
Histone H2B S9YQ51_CAMFR	3	114
Tubulin beta chain	2	00
Histone 1, H2ai isoform 3-like protein	3	89
*S9 × 8G9_CAMFR Desmoplakin	2	88
A2V743_CAMDR Beta actin	2	85
A0A0A0PAR2_CAMDR	2	85
S9XA40_CAMFR	1	85
Heat shock cognate protein T0NNK2_CAMFR	1	81
L-lactate dehydrogenase S9XNF8_CAMFR	2	74
Xaa-Pro dipeptidase T0MHN9_CAMFR	1	71
Pyruvate kinase S9WVY1 CAMFR	1	60
Actinin, alpha 1 isoform 6-like protein *A0A0E3Z5I3 CAMDR	1	59
Superoxide dismutase	1	57
Phosphotriesterase-related protein	1	37
S9W9Y4_CAMFR Ferritin	1	57
S9XR87_CAMFR Ig lambda chain C regions isoform 19-	1	56
like protein		50
S9 × 3E8_CAMFR Ig kappa chain V-II region RPMI 6410-	1	50
like protein S9WAX5_CAMFR	2	49

#### Table 2 (continued)

Protein name (*unique for EVs)	Number of peptide sequences identified	Total score $(p < 0.05)^{\dagger}$
Unconventional myosin-Va isoform 2 S9WF34_CAMFR	1	44
Tubulin alpha chain *S9W806_CAMFR	2	43
Filamin-A isoform 1	2	42
Scaffold attachment factor B-like	2	72
protein TOMH94_CAMFR	1	40
Rabenosyn-5-like protein *S9Y0S0_CAMFR	1	39
DNA-directed RNA polymerase subunit beta		
S9WJW3_CAMFR	1	38
subunit		
S9XM68_CAMFR Xaa-Pro dipeptidase isoform 3	1	38
*S9YV02_CAMFR Non-specific protein-tyrosine kinase	1	38
S9WGH8_CAMFR	1	32
Lysozyme S9WRI7_CAMFR	1	32
Nuclear receptor coactivator 5 isoform 3-like protein		
S9W421_CAMFR Hemoglobin_ensilon_1	1	32
*S9WI71_CAMFR	1	32
*S9WB50_CAMFR	1	31
TSC22 domain family protein 3-like protein		
S9WMX2_CAMFR	1	31
S9WKI8_CAMFR	1	31
HEAT repeat-containing protein 7B1 S9WIA5_CAMFR	1	31
Glutathione synthetase *S9XC05 CAMFR	1	31
Telomere-associated protein RIF1		
A0A0U2KTX5_CAMDR	1	30
VHH5 *T0MGG7_CAMFR	1	30
Nucleoredoxin *SOXET3_CAMER	1	30
Rac GTPase-activating protein 1	1	
S9XDK9_CAMFR Complement C3-like protein	1	30
*S9XMI2_CAMFR Pseudopodium-enriched atypical	1	29
kinase 1	1	20
Core histone macro-H2A.1 isoform 2	1	25
S9XXW2_CAMFR Fibrinogen beta chain	1	29
*S9YGX6_CAMFR PAS_domain_containing_serine/	1	29
threonine-protein kinase		00
*TONMUT_CAMFR SH2 domain-containing protein 7	1	28
*TOMIT6_CAMFR Serine-tRNA ligase. mitochondrial	1	28
*S9W449_CAMFR	1	28
re receptor-like protein 5		

<sup>1</sup> Ions score is -10\*Log(P), where P is the probability that the observed match is a random event. Individual ions scores > 22 indicated identity or extensive homology (p < 0.05). Protein scores were derived from ions scores as a nonprobabilistic basis for ranking protein hits. Cut-off was set at Ions score 20.





(caption on next page)

Α.

DST

Fig. 3. Protein-protein interaction networks of deiminated protein hits identified in whole llama (Lama glama) serum. Reconstruction of protein-protein interactions based on known and predicted interactions using STRING analysis. Due to annotations for camelids not being present in STRING, proteins are based on corresponding human protein identifiers. A. Coloured nodes represent query proteins and first shell of interactors; white nodes are second shell of interactors. B. Biological GO processes are highlighted for the same protein network as follows: red = response to stress; blue = response to wounding; green = vesicle mediated transport; yellow = oxygen transport; purple = regulated exocytosis; dark green = small molecule metabolic process. 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 colour key for connective lines in A).

HBE1

FGB

NXN

PEAK1

(El-Deeb and Buczinski, 2015). While albumin has been identified as a glycoprotein in some species (Metcalf et al., 2007) investigation of posttranslational deimination has been limited, but was recently identified in teleost fish (Magnadóttir et al., 2019a). Serum albumin was identified as deiminated in both whole llama serum and EVs.

Hemopexin is a 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). Hemopexin is a plasma glycoprotein that has been previously identified as a deimination candidate in teleost fish (Magnadóttir et al., 2019a) and shark (Criscitiello et al., 2019). Here, hemopexin was found deiminated in whole llama serum only.

Inter-alpha-trypsin inhibitor (heavy chain H1) belongs to the serpin

SH2D7

family of proteins, which have protease-inhibitory functions and are involved in diverse physiological and pathological processes including fertilisation, ovulation, coagulation, inflammation, as well as tumorigenesis, metastasis and dementia (Zhuo and Kimata, 2008; Weidle et al., 2018). Inter-alpha-trypsin inhibitor is synthesised in the liver, circulates in the blood and has two chains, a light and heavy chain, whereof the heavy-chain (ITIH) includes a von Willebrand domain and can interact with the extracellular matrix (Bost et al., 1998). ITIH is downregulated in tumours via methylation and ITIH2 is strongly reduced in invasive cancers (Hamm et al., 2008). While ITIH was recently identified as a deimination candidate in teleost fish (Magnadóttir et al., 2019a) further studies on the regulation of ITIH2 via post-translational deimination have not been carried out. ITIH was here identified as deiminated in whole llama serum only.

Fibrinogen is a glycoprotein, synthesised in liver (Tennent et al.,

Fig. 4. Protein-protein interaction networks of deiminated protein hits identified in EVs of llama (Lama glama) serum. Reconstruction of protein-protein interactions based on known and predicted interactions using STRING analysis. Due to annotations for camelids not being present in STRING, proteins are based on corresponding human protein identifiers. A. Coloured nodes represent query proteins and first shell of interactors. B. Biological GO processes are highlighted as follows: red = response to stress; blue = cytoskeleton organisation; green = vesicle mediated transport. Coloured lines indicate whether protein interactions are identified via known interactions databases, experimentally (curated determined), predicted interactions (gene neighbourhood, gene fusion, gene co-occurrence) or via text mining, co-expression or protein homology (see colour key for connective lines in A).



45

METTL3 湖 LDHAL6A ALB PKLR KRT8 FCRL5 GRM3 1 YZ Known interactions From curated databases GSS RACGAP1 Experimentally determined CAPZB SCARA5 HSPA8 Predicted interactions TUBB Gene neighbourh PEPD ccs Gene fusions Gene co-occurrence  $\bigcirc$ MROH2A PRKCI TRAP Others TUBA8 Textmining PTER Co-expression Protein homology

2007) and forms part of the acute phase response as part of the coagulation cascade (Tiscia and Margaglione, 2018). In camelids, fibrinogen is a biomarker for stress and infection (Greunz et al., 2018; El-Bahr and El-Deeb, 2016; El-Deeb and Buczinski, 2015). Impaired mechanism of fibrinogen formation and fibrin polymerization are implicated with various pathologies including coagulopathies and ischemic stroke (Weisel and Litvinov, 2013), while acquired fibrinogen disorders can be 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 to its antigenicity in autoimmune diseases (Hida et al., 2004; Muller and Radic, 2015; Blachère et al., 2017). Fibrinogen was here identified as deiminated both in whole llama serum and EVs.

**Tubulin beta-chain** participates in cytoskeletal rearrangement and its deimination has previously been linked to EV release (Kholia et al., 2015). Deimination of tubulin may therefore be crucial for facilitating EV-mediated processes in homeostasis, immune responses and in relation to pathologies. Here, tubulin was identified as deiminated in both whole llama serum and EVs.

*Histone H2B* was identified as being deiminated in both llama serum and EVs and in addition, Histone 1 (H2ai isoform 3-like protein) and core histone macro-H2A.1 isoform 2 were identified as deiminated in EVs only. Histones undergo various posttranslational modifications that affect gene regulation and can also act in concert (Latham and Dent, 2007; Bird, 2007). In addition to acetylation, phosphorylation and ubiquitination, histones are known to undergo deimination, including H2B (Sohn et al., 2015) and H2A (Hagiwara et al., 2005), as identified in this study in llama. Other histones that are known to undergo deimination include H3 and H4 (Chen et al., 2014; Kosgodage et al., 2018).

Heat shock protein 90 (Hsp90) was here found to be deiminated both in llama whole serum and EVs. HSP90 has been described in camelid (Saeed et al., 2015). Hsp90 is a phylogenetically highly conserved chaperone protein involved in protein folding, 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 (Chiosis et al., 2006). In camelids, Hsp90 has been related to adaptive tolerance of camel somatic cells to acute and chronic heat shock (up to 20 h at 45 degrees Celsius), which is lethal to many mammalian cells (Saadeldin et al., 2018b). Hsp90 has previously been described to be post-translationally deiminated in rheumatoid arthritis, allowing deiminationinduced shifts in protein structure to generate cryptic epitopes capable of bypassing B cell tolerance (Travers et al., 2016). It is of some interest to find that Hsp90 is also found deiminated in llama serum, further highlighting protein moonlighting functions of Hsp90 in physiological and pathophysiological context via post-translational deimination. In addition, finding post-translational deimination of the same protein throughout phylogeny also supports translational value between species to further understanding of this post-translational modification in human pathologies.

The following deimination protein candidates identified in the current study in llama serum and serum-EVs have to our knowledge not been previously reported as deiminated:

**Alpha 2-macroglobulin** is closely related to other thioester containing proteins, such as complement proteins C3, C4 and C5 (Sottrup-Jensen et al., 1985; Davies and Sim, 1981). Alpha-2-M is phylogenetically conserved from arthropods to mammals and found at high levels in mammalian plasma. Alpha-2-M forms part of the innate immune system and clears active proteases from tissue fluids (Armstrong and Quigley, 1999). Here, Alpha-2-M was found deiminated in whole llama serum only and has not reported as deiminated before, to our knowledge.

Adiponectin is the most abundant secreted adipokine with pleiotrophic 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). Adiponectin is furthermore linked to longevity (Chen et al., 2019a, 2019b), regenerative functions (Fiaschi et al., 2014) and has roles in myopathies, such as Duchenne muscular dystrophy and collagen VI-related myopathies (Gamberi et al., 2019). Adiponectin is also implicated in a range of cancers, often in relation to obesity (Parida et al., 2019). Furthermore, adiponectin 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 great interest in drug development for modulating adiponectin signalling (Fiaschi, 2019). Given the unique metabolic adaptive features of camelids, the identification of post-translational deimination of this key metabolic protein may be of some interest. Recent studies in rheumatoid arthritis made a correlation between inflammation, autoantibodies and adiponectin levels (Hughes-Austin et al., 2018; Liu et al., 2019), while adiponectin itself has not been previously identified to be deiminated to our knowledge. Post-translational deimination may be a hitherto unrecognized mechanism for adiponectin, also in humans, to adapt 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 camel adiponectin has 8 arginine sites. These could be subjected to PAD-mediated deimination and therefore modulate adiponectin folding and function, depending on which arginine is deiminated. Here, deiminated adiponectin was identified in whole llama serum only.

*Dystonin* is a plakin-family adhesion junction plaque protein and was here identified as deiminated in both llama serum and EVs. Dystonin, along with XVII collagen, form hemidesmosomes and both proteins are autoantigens believed to be responsible for the Type II hypersensitivy pathologic in the pruritic skin disease bullous pemphigoid (Bağcı et al., 2017; Basseri et al., 2018). Dystonin has also been linked to Sjögren's syndrome and linked to a hypermethylation status (Gonzalez et al., 2011), but post-translational deimination of dystonin has not been previously described to our knowledge, although deimination is associated with a range of autoimmune diseases, including Sjögren's syndrome (Konsta et al., 2014; Selmi et al., 2015).

Xaa-Pro dipeptidase, also known as prolidase, is an enzyme that in humans is encoded by the PEPD gene. Post-translational modifications of prolidase regulate its enzymatic abilities. Deficiency in prolidase leads to a rare, severe autosomal recessive disorder (prolidase deficiency) that causes many chronic, debilitating health conditions in humans (Viglio et al., 2006). These phenotypic symptoms vary and may include skin ulcerations, mental retardation, splenomegaly, recurrent infections, photosensitivity, hyperkeratosis, and unusual facial appearance. Furthermore, prolidase activity was found to be abnormal compared to healthy levels in various medical conditions including: bipolar disorder, breast cancer, endometrial cancer, keloid scar formation, erectile dysfunction, liver disease, lung cancer, hypertension, melanoma, and chronic pancreatitis (Kitchener and Grunden, 2012). In some cancers with increased levels of prolidase activity, such as melanoma, the differential expression of prolidase and its substrate specificity for dipeptides with proline at the carboxyl end suggests the potential of prolidase in becoming a viable, selective endogenous enzyme target for 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; Sen 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 not been described before to our knowledge and may add

to understanding of how this enzyme is regulated. Prolidase was here identified as deiminated in both llama serum and EVs.

Dipeptidylpeptidase 4 (DPP4, also known as CD26) was here identified to be deiminated in both llama whole serum and EVs. 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). Interestingly, in camel milk, DDP4 inhibitory peptides have been identified and suggested to play roles in the regulation of glycaemia in humans (Nongonierma et al., 2018). Furthermore, roles for DDP4 in cancer have been found to relate to its posttranslational processing of chemokines, thereby limiting lymphocyte migration to sites of inflammation and tumours (Barreira da Silva et al., 2015). As the success of antitumour immune responses depends on the infiltration of solid tumours by effector T cells, a process which is guided by chemokines, DPP4 inhibitors have 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). Middle East respiratory syndrome coronavirus (MERS-CoV) has been found to utilize dipeptidyl peptidase 4 (DPP4) as an entry receptor, via glycosylated sites (Peck et al., 2017). Therefore the identification of DPP4 as a deimination candidate may be of some relevance as such post-translational modification can affect DPP4 structure and function, allowing for moonlighting functions which may vary in pathological compared to pathophysiological milieus.

*E3 ubiquitin-protein ligase Roquin*, belongs to the Roquins which are a family of highly conserved RNA-binding proteins involved in ubiquination, are crucial for T-cell-dependent B-cell responses (Athanasopoulos et al., 2016) and play important roles in modulating T-cell activity (Akef and Muljo, 2018). Roquins repress constitutive decay elements containing mRNAs and play a critical role in RNA homeostasis and immunological self-tolerance (Zhang et al., 2015; Essig et al., 2018; Mino and Takeuchi, 2018). Roquin plays multifaceted roles both in the generation of a homeostatic immune response, as well as during chronic inflammation and autoimmunity (Schaefer and Klein, 2016; Lee et al., 2019). While roquin causes post-translational ubiquination of target proteins, post-translational deimination of roquin itself may modulate is function and is described here for the first time. Roquin was here identified as deiminated in whole llama serum only.

*Serine-tRNA ligase, mitochondrial* was here identified as a deimination candidate protein in llama EVs only. It has been linked to HUPRA syndrome which is a rare mitochondrial disease characterized by hyperuricemia, pulmonary hypertension, renal failure in infancy and alkalosis (Rivera et al., 2013). It has furthermore been linked to progressive spastic paresis (Linnankivi et al., 2016). Post-translational deimination of this mitochondrial protein has not been described before to our knowledge.

**Nucleoredoxin** (Nrx) was here identified to be deiminated in EVs only. It is an oxidoreductase of the thioredoxin family of proteins with numerous functions in the redox regulation of metabolic pathways, cellular morphology, and signal transduction (Urbainsky et al., 2018). Nrx has been shown to inhibit Wnt-beta-catenin signalling (Funato et al., 2006) and is linked to  $Ca^{2+}$ -mediated mitochondrial reactive oxygen species metabolism (Rharass et al., 2014). Nrx has furthermore been identified as an epigenetic cancer marker related to the oxidative status of human blood (Schöttker et al., 2015), but hitherto not been described as deiminated.

*Dyslexia-associated protein* was here identified to be deiminated in total serum of llama. It is associated with developmental dyslexia (Levecque et al., 2009) and has been shown to have roles in cell-cell interactions and signalling and neuronal migration (Velayos-Baeza et al., 2008), as well as in axon guidance (Poon et al., 2011). Dyslexia-associated protein has previously been found to follow the classical clathrin-mediated endocytosis pathway and its surface expression

seems regulated by endocytosis, indicating that the internalization and recycling of the protein may be involved in fine-tuning its role in neuronal migration (Levecque et al., 2009). It has been descried to be highly glycosylated in different mammalian cell lines (Velayos-Baeza et al., 2008), while deimination has not been described before.

Metabotropic glutamate receptor 3 was identified here as deiminated in llama EVs only. It has, like other components of the glutamatergic system, a widespread distribution outside the central nervous system (CNS) and has been linked to regulation of the brain-gut axis (Julio-Pieper et al., 2013). It has also been linked to pain (Acher and Goudet, 2015) and psychotic disorders (Matrisciano et al., 2016). The group III mGlu receptors have been described in human stomach and colon, revealing a huge potential for these receptors in the treatment of peripheral disorders, including gastrointestinal dysfunction (Julio-Pieper et al., 2013). As post-translational deimination has not been described before in this receptor, but may well affect is tertiary structure, our current finding may be of some relevance in relation to strategies for developing antagonistic probes (Wenthur et al., 2019). Such pharmacological tools originally designed for mGlu receptors in the CNS may also be directed towards new disease targets in the periphery. Ulcerative colitis and Crohn's disease are potential targets, as irritable bowel diseases can be co-morbid with anxiety and depression (Julio-Pieper et al., 2013).

Glutathione synthetase (GSS) was here identified to be deiminated in both llama whole serum and EVs. GSS is the second enzyme in the glutathione (GSH) biosynthesis pathway involved in homeostasis and cellular maintenance and also acts as a potent antioxidant (Njålsson and Norgren, 2005). In camelids, glutathione peroxidase, which also belongs to the GSH biosynthesis pathway and is a potent anti-oxidant, has been identified as a seminal plasma fertility biomarker (Waheed et al., 2015). Furthermore, camel milk has been shown to boost glutathione and total anti-oxidant capacity in sera and exudates in animal studies (Arab et al., 2017). In a diabetic mouse model, the activities of GSH, alongside glucose insulin and ROS levels, were restored after camel whey protein treatment (Sayed et al., 2017). In humans, GSS deficiency is an autosomal recessive disorder with varyingly severe clinical manifestations that include metabolic acidosis, hemolytic anemia, hyperbilirubinemia, neurological disorders and sepsis (Guney Varal et al., 2019). Deimination of GSS identified here has not been assessed before and may possibly affect the GSH biosynthesis pathway and such posttranslational regulation remains to be further investigated.

**Rootletin**, also known as ciliary rootlet coiled-coil protein (CROCC), is a protein that is required for centrosome cohesion and is therefore important in mitosis (Bahe et al., 2005; Graser et al., 2007). It was identified here as deiminated in whole llama serum only. 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). Post-translational deimination of rootletin, as identified here, may possibly facilitate its dynamic functions.

*Centromere protein J* is is a highly conserved and ubiquitiously expressed centrosomal protein, involved in microtubule disassembly and plays a structural role in the maintenance of centrosome integrity, genome stability and normal spindle morphology during cell division (Tang et al., 2009; McIntyre, 2012). It was here identified as deiminated in whole llama serum only. Knockout mouse models targeting the gene encoding this protein have phenotypes of impaired glucose tolerance, hypoalbuminemia and increased micronuclei, indicative of genomic instability (Gerdin, 2010). In humans, it is associated with primary autosomal recessive microcephaly (Gul et al., 2006) and the microcephalic primordial dwarfism disorder Seckel syndrome (Al-Dosari et al., 2010; McIntyre et al., 2012). Deimination has not bee described before in this protein to our knowledge.

Telomere-associated protein RIF1 isoform 1 is involved in the

repair of double-strand DNA breaks in response to DNA damage (Silverman et al., 2004; Escribano-Díaz et al., 2013; Drané et al., 2017) and protects teleomeres (Fontana et al., 2018). It was identified here as deiminated in EVs only. RIF1 has been associated with cancer via activation of human telomerase reverse transcriptase expression (Liu et al., 2018). It is also required for immunoglobulin class-switch recombination during the germinal centre reaction for humoral antibody immunity (Di Virgilio et al., 2013). RIF1 is involved in telomere homeostasis and was recently found to be post-translationally S-acylated, identifying a novel posttranslational modification regulating DNA repair (Fontana et al., 2019). Post-translational deimination has hitherto not been described.

Rabenosyn-5-like protein was here identified as a deimination protein candidate in both llama whole serum and EVs. It acts in early endocytic membrane fusion and membrane trafficking of recycling endosomes (Naslavsky et al., 2004; Rai et al., 2017). It plays a role in the lysosomal trafficking of CTSD/cathepsin D from the Golgi to lysosomes and also promotes the recycling of transferrin directly from early endosomes to the plasma membrane (Navaroli et al., 2012). Rabenosyn-5-like protein also binds phospholipid vesicles and plays roles in regulating protein sorting and recycling to the plasma membrane (Nielsen et al., 2000; de Renzis et al., 2002; Naslavsky et al., 2004). Rabenosyn-5 was recently identified as an upregulated urinary biomarker associated with malignant upper gastrointestinal cancer (Husi et al., 2019) and found to be increased in diabetic kidney disease (Dumont et al., 2017). Rabenosyn-5 has been shown to be phosphorylated, regulating its recruitment to membranes (Macé et al., 2005). Post-translational deimination has hitherto not been described ..

*Spermatogenesis-associated protein 2 (SPATA2)* was here identified as deiminated in whole llama serum only. It is expressed in testis and to a lesser extent in spleen, thymus, and prostate (Graziotto et al., 1999). SPATA2 has been identified as a bridging factor that regulates TNF-alphA–Induced necroptosis and is instrumental for TNF-induced cell death (Elliott et al., 2016; Kupka et al., 2016; Schlicher et al., 2016; Wagner et al., 2016; Schlicher et al., 2017). SPATA2 ensures normal secretory function of Sertoli cells (Zhao et al., 2017) and has recently been identified as a novel predictor in ovarian cancer outcome (Wieser et al., 2019). While SPATA2 has been linked to ubiquination (Schlicher et al., 2016), post-translational deimination has not been invesigated.

Vitamin D-binding protein (VDBP) belongs to the albumin gene family, together with human serum albumin and alpha-fetoprotein. It 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 including ergocalciferol (vitamin D2) and cholecalciferol (vitamin D3), the 25hydroxylated forms (calcifediol), and the active hormonal product 1,25dihydroxyvitamin D (calcitriol) (Verboven et al., 2002; Norman, 2008). It transports vitamin D metabolites between skin, liver and kidney, and then on to various target tissues (Norman, 2008). VDBP is a macrophage activating factor (MAF) and 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 (which still remain to be identified) of VDBP have been associated with multiple sclerosis (MS) (Perga et al., 2015), while protein deimination is well known to be associated with MS (Moscarello et al., 2013). VDBP has previously been identified to be glycosylated (Kilpatrick and Phinney, 2017) but was here identified as a deimination candidate in whole llama serum only. Post-translational deimination may contribute to various functions of VDBP in physiological as well as pathophysiological processes.

*Pseudopodium-enriched atypical kinase 1* (PEAK1) was here identified as deiminated in EVs only. It is a tyrosine kinase and scaffold protein that transmits integrin-mediated extracellular matrix (ECM) signals to facilitate cell movement and growth. While aberrant

expression of PEAK1 has been linked to cancer progression, its normal physiological role in vertebrate biology is still relatively unknown. Deletion of the PEAK1 gene in zebrafish, mice and human endothelial cells (ECs) induced severe defects in new blood vessel formation due to deficiencies in EC proliferation, survival and migration. PEAK1 seems therefore to play roles in modulation of cell adhesion and growth factor cues from the extracellular environment necessary for new vessel formation during vertebrate development and cancer (Wang et al., 2018). PEAK1 has not been described as deiminated before.

Desmoplakin was here identified to be deiminated in llama EVs only. 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 morphogenesis in the Xenopus laevis embryo (Bharathan and Dickinson, 2019) and novel roles in coordination of cell migration were recently established (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 (Yermakovich et al., 2018), including cardiomyopathies (Reichl et al., 2018; Chen et al., 2019a, 2019b). Desmosomal proteins have been shown to have both tumourpromoting 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). As the roles of desmosomal proteins in cancer and metastasis are not fully understood, the identification of deiminated desmoplakin in llama EVs here, and not described before, may be of some interest and add to understanding of diverse functional ability via such post-translational modification.

TSC22 domain family protein 3, also called glucocorticoid-induced leucine zipper protein (GILZ), was here identified as a deimination candidate in llama EVs only. It is a glucocorticoid-responsive molecule involved in immune regulation and glucocorticoid actions. Its interactions with signal transduction pathways, many of which are operative in RA and other inflammatory diseases, suggest that it is a key endogenous regulator of the immune response including a key glucocorticoid-induced regulator of inflammation in rheumatoid arthritis (RA) (Beaulieu and Morand, 2011). GILTZ is a small, 135-amino acid protein with anti-inflammatory properties and has been shown to inhibit NF-kB and MAPK pathways (Bereshchenko et al., 2019; Ricci et al., 2019). It has also been shown to be induced in response to hypoxia by a HIF1\alpha-dependent mechanism and in response to cholesterol starvation, leading to downstream shedding of procoagulant EVs in ovarian cancer (Koizume et al., 2019). Post-translational deimination of GILZ has not been described before but may indeed affect its multifaceted functions, including in inflammation and cancer.

In the current study we report deiminated proteins in llama serum and serum-derived EVs. Due to the fact that the llama genome is not yet fully annotated, the hits identified here may underestimate the amount of deiminated proteins present in llama serum and EVs. Therefore a wider protein-hit analysis was carried out including other members of the camelid family, using known sequences from *Camelus ferus, Camelus dromedaries, Lama guanicoe* and *Vicugna pacos*. Deimination of key immune factors of innate and adaptive immunity and key metabolic proteins is identified here for the first time in a camelid species, highlighting putative protein moonlighting functions via post-translational deimination. It must be noted that in relation to previously observed increases of deiminated proteins with age (Ding et al., 2017), the llama used in this study would be considered relatively old at 21 years of age, as typical llama lifespans are 15–25 years, with some individuals surviving 30 years or more.

Our findings presented here furthermore complement expanding research in the comparative EV research field. Previous studies on the camel urinary proteome revealed enriched proteins from EVs and relevance to stress and immune responses as well as antimicrobial activities (Alhaider et al., 2012). Research on EVs is a relatively new field in comparative immunology and to our knowledge; this is the first description of EVs in serum of a camelid species. Previous studies on EVs in camelids focussed on EVs in camel milk, which were shown to have anti-cancerous properties (Badawy et al., 2018). As PADs have been identified to play major roles in the regulation of EV release (Kholia et al., 2015; Kosgodage et al., 2017 and 2018), including in host-pathogen interactions (Gavinho et al., 2019), such EV-mediated communication may be of great relevance also for addressing diverse zoonotic diseases identified in camels (El-Alfy et al., 2019; Zhu et al., 2019).

In continuation of the current pilot study, the assessment of changes in deiminated proteins in camelid serum, and their lateral transfer via EVs, will be of great interest to assess animal health in response to infection and environmental stress. Our findings will further current understanding of the roles for EVs, PADs and posttranslational deimination throughout phylogeny and in relation to adaption to a range of, including extreme, environments. Furthermore, novel identification of post-translational deimination in key proteins of metabolism and immunity may reveal hitherto unrecognized moonlighting function of these proteins throughout phylogeny, in relation to physiological and pathological processes, as well as being translational to and informing inflammatory and metabolic diseases. PAD-mediated contribution to protein moonlighting and in EV-mediated communication in response to physiological and pathophysiological changes remains therefore a field of further studies.

#### 5. Conclusion

This is the first report of deiminated proteins in serum and serum-EVs of a camelid species, using the llama as a model animal. Our findings highlight a hitherto unrecognized post-translational modification in key immune and metabolic proteins in camelids, which may be translatable to and inform a range of human metabolic and inflammatory pathologies.

#### CRediT authorship contribution statement

Michael F. Criscitiello: Resources, Funding acquisition, Validation, Writing - review & editing. Igor Kraev: Formal analysis, Resources, Validation, Visualization. Sigrun Lange: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing.

#### **Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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#### Appendix A. Supplementary data

Supplementary material related to this article can be found, in the

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Supplementary Table 1. Deiminated proteins identified, including full list of all peptide sequences, by F95 enrichment and LC-MS/MS in total serum of Ilama (*Lama glama*). 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 in-house Mascot. Peptide sequence hits scoring with *Lama glama* (LAMGL) are included as well as hits with other camelids (CAMFR=*Camelus ferus*; CAMDR=*Camelus dromedaries*; LAMGU=*Lama guanicoe*; VICPA=*Vicugna pacos* (Alpaca)). Hits with uncharacterized proteins are omitted in the list. Peptide sequences and m/z values are listed. An asterix (\*) indicates that the protein hit is specific to whole serum only.

Protein name	m/z	Peptide sequence	Score	Total
(*unique for serum)			(p<0.05) <sup>‡</sup>	score
*097643   AMGI	437,7734	R.OYI PLIK.M	26	282
Fibrinoaen A-alpha	524,2692	K.EVSGSVSPGTK.K	62	
chain	415.8943	K.GDKFI I ISNEK.V	38	
	513.5782	R.GDSVSHGAGSVPESPR.K	36	
	790.4611	K.OLEOVIGINI LPSR.D	69	
	697.8077	K.EVINSEDGSDCGDTSLDLHHTFPSR.G	50	
*P01973 HBA_LAMGI	409.7237	R. VDPVNEK I	25	189
Hemoalobin subunit	521,2757	R.MFI GEPTTK.T	21	
alpha	640.3666	K.FLANVSTVITSK.Y	57	
	510,5830	K.IGGHAADYGAFAI FR.M	43	
	575 0433	K AADHI DDI PSALSAI SAI SDI HAHK I	43	
P68226 HBB LAMG	573 8375	R I I VVYPW/TR R	31	166
Hemoalobin subunit	589 3436	K VVAGVANALAHR Y	52	100
heta	664 8632	K VKVDEVGGEALGR I	45	
Setu	705 8497	Κ ΕΕΤΡΟΙ ΟΔΑΥΟΚ V	38	
	566 7665	R GYSETTTAER E	38	109
Beta actin	895 9506	K SYELPDGOVITIGNER E	22	105
	652 0263	R VΔΡΕΕΗΡΥΙΙΤΕΔΡΙΝΡΚ Δ	22	
	796 6590	R TTGIVMDSGDGVTHTVPIVEGVALPHALLR I	22	
	653 7847	K SEDTAVVVCAK D	11	117
Anti-RON nanobody	941 5050	- FVOLVESGGGLVOPGGSLR I	73	11/
*A0A1W5VKM7 LAMGI	608 7926	R I SCAASGETER A	38	111
Anti-RON nanobody	941.5050	FVOLVESGGGLVOPGGSLR.I	73	
*A0A1W5VKR8 LAMGI	423 7271	K GI FWVSR I	23	96
Anti-RON nanobody	941.5050	FVOLVESGGGLVOPGGSLR.I	73	50
*A0A1W5VK09 LAMGI	425 2160	RISCVASGRA	66	66
Anti-RON nanobody	12512200			00
S9XDK9 CAMFR	386.2036	K.EGIPEAR.Q	42	1575
Complement C3-like	388.7369	K.GVFVLNK.K	21	
protein	400.7478	R.VGLVAVDK.G	39	
-	417.2477	R.LPYSVVR.N	41	
	444.2330	R.NEQVEIR.A	47	
	449.7427	R.AVLYNYR.E	25	
	476.2484	K.FLNTATER.T	42	
	516.7754	K.LSINTQNSR.Q	53	
	531.7480	K.ADIGCTPGSGK.D	48	
	534.3140	K.VLLDGVQAPR.A	79	
	546.2920	K.DTCVGTLVVK.G	38	
	546.8186	K.NTLIIYLDK.I	56	
	567.8348	R.HQQTLVIPAK.S	40	
	584.2801	K.QNEDFTLTAK.G	39	
	673.3544	R.EVVADSVWVDVK.D	64	
	680.3649	K.QVLSSENTVLNR.A	92	
	685.8693	K.TIYTPGSTVLYR.I	60	

	720 0725	KOVACVETDACLALKT	74	
	/20.8/25	K.DYAGVFTDAGLALK.T	74	
	488.8977	K.ISHTQEDCLSFK.V	40	
	498.2757	K.ELNLDVSIHLPSR.S	56	
	/8/.91/5	K.FDLTVSLTPAPEPVK.K	68	
	807.4440	K.FDLTVSLTPAPEPVK.K	58	
	842.3702	K.VYSYYNLDETCTR.F	59	
	844.8922	K.AFLDCCEYITQLR.Q	22	
	567.9807	R.LPYSVVRNEQVEIR.A	22	
	867.3949	K.AADLSDQVPDTESETR.I	25	
	581.2754	R.SEETKQNEDFTLTAK.G	45	
	581.3295	K.KFDLTVSLTPAPEPVK.K	65	
	898.9662	R.VELLYNPAFCSLATAK.K	38	
	601.3332	R.TGIPIVTSPYQIHFTK.T	44	
	932.4729	K.EYVLPSFEVQVEPAEK.F	36	
	936.9587	R.SDLEDEIIPEEDIISR.S	22	
	765.0651	R.HIPVVTQGSNVQSLTQDDGVAK.L	44	
	639.8195	K.QKPDGVFQEDGPVIHQEMIGGFK.N	32	
S9WI87 CAMFR	386,7229	K.AACLLPK.A	34	972
Serum albumin	449.7441	R.LCVLHEK.T	28	
	460.2552	K.LCTVASLR.E	46	
	464 2503	K YI YEIAR R	32	
	469 7087	K DI GEDDEK G	38	
	528 2522	R NECELOHK S	27	
	550.2352		27 12	
	554.7510	K.HVFLLCCK.D	45 F0	
	509.7529	K.CCTESLVINK.K	50	
	375.3115	R.LVNEVIEFAR.I	02	
	405.1956	K.FKDLGEDDFK.G	42	
	435.8773	K.ECCERPLLER.S	30	
	/21.2823	K. ICVADESAADCDK.S	93	
	744.8028	K.EYEATLEDCCAK.D	71	
	746.3249	K.YFCDNQETISSK.L	59	
	746.7734	R.ETYGEMADCCEK.Q	60	
	516.2707	K.LKECCEKPLLEK.S	54	
	538.5977	K.DVFLGMFLHEYAR.R	46	
	623.2894	K.TFTFHADLCSVSEPEK.Q	66	
	668.6628	K.LKPEPEALCTAFQENEK.R	34	
	540.7744	K.LKPEPEALCTAFQENEKR.F	52	
S9XAP9_CAMFR	404.2031	R.LAADDFR.T	55	522
Keratin, type I	515.3008	R.VLDELTLAR.A	55	
cytoskeletal 14-like	546.2613	K.VTMQNLNDR.L + Deamidated (NQ)	72	
protein	561.7933	R.LEQEIATYR.R	45	
	651.3333	R.ALEEANADLEVK.I	78	
	454.2382	R.MSVEADINGLRR.V	23	
	681.3488	R.EVATNSELVQSGK.T	93	
	685.3802	K.ILTATVDNANIVLQIDNAR.L	52	
	770.3600	R.LLEGEDAHLSSSQFSSGSQSSR.D	52	
S9Y6J1 CAMFR	405.7086	R.QSSVSFR.S	48	502
Keratin, tvpe II	453.7376	R.FLEQQNK.V	21	-
cytoskeletal 5 isoform	473.2593	R.GRLDSELR.N	41	
13-like protein	508,2349	K.HEISEMNR.M	41	
	533.7618	K.YEDEINKR.T	57	
	602 3223	K WTI OFOGTK T	46	
	621 7855	R TEAESWYOTK I	35	
	632 3512	KIAIDVELATVR K	76	
	6/0 2100	R TTAENEEVMIKK + Ovidation (M)	70 21	
	651 9621	R SI DI DSILAEVK A	21 6/	
	001.0021	N.JLULUJIIAL VN.A	04	

	436.8894	K.NKYEDEINKR.T	55	
*S9X688_CAMFR	453.7376	R.FLEQQNK.V	21	462
Keratin 6A-like protein	469.7505	R.SLYNLGGSK.S	29	
	473.2593	R.GRLDSELR.N	41	
	503.2372	K.LLEGEECR.L	26	
	533.7618	K.YEDEINKR.T	57	
	578.2714	R.DYQELMNVK.L + Oxidation (M)	22	
	398.8752	K.KYEDEINKR.T	55	
	604.8117	R.TAAENDFVTLK.K	73	
	632.3512	K.LALDVEIATYR.K	76	
	651.8621	R.SLDLDSIIAEVK.A	64	
*S9YD43_CAMER	485 2559	B VEYGEOVK V	31	443
Complement	532,7665	R.FGLIGEDGEK.T	30	
component 4A-like	557 8146	K VGDTINI NI R A	63	
protein	566 7924	K STGI (VATPAR V	84	
protein	577 8012	R OGVNI FESSR R	61	
	633 2790		44	
	653 3385	R GSLEEPVGDAVSK V	42	
	663 8513	R EVSSPESI DI SK T	51	
	670 3705	K I SININDI PGOR I	30	
*S9Y253 CAMFR	0.0.0,00	R.KAI DI INK.G	31	380
Kininogen-2 isoform I		$K_{ATAOVVAGMK,Y} + Oxidation (M)$	30	000
		K FSDCPVI SR K	74	
		K ENSDEASER V	52	
		K SGNOEVLYR V	78	
		K I NAENNGNEYEK I	45	
		K DSAOAATGECTVTVAK R	48	
		K.CNLYPGEDFVQPPGK.I	21	
*TOMII3 CAMFR	401.2163	R.HVFSPSK.S	41	371
Alpha-2-macroglobulin-	498.2343	K.IENCFANK.V	35	-
like protein	595.3289	K.ELTFYYLIK.A	35	
	654.8455	R.AFEVNEYVLPK.F	50	
	442.5714	K.VTATPHSLCALR.A	59	
	1092.0768	K.LQGGLNQQSFPLSEEPILGR.Y	62	
	772.3862	K.SYVHLEPVAGTLACGQTQEVR.A	91	
S9XI90 CAMFR	453.7376	R.FLEQQNK.V	21	357
Keratin, type II	503.2372	K.LLEGEECR.L	26	
cytoskeletal 75-like	508.2349	R.HEISEMNR.V	41	
isoform	597.7917	K.YEELQQTAGR.H	68	
	604.8117	R.TAAENEFVSLK.K	32	
	632.3512	K.LALDVEIATYR.K	76	
	651.8621	R.SLDLDSIIAEVK.A	64	
	436.8894	K.VRYDDEINKR.T + Deamidated (R)	29	
S9X494_CAMFR	404.2031	R.LAADDFR.T	55	285
Keratin, type I	515.3008	R.VLDELTLAR.A	55	
cytoskeletal 42	561.7933	R.LEQEIATYR.R	45	
	651.3333	R.ALEEANADLEVK.I	78	
	703.3495	R.EVATNTEALQSSR.T	22	
	1043.4957	R.GQVGGDVNVEMDAAPGVDLSR.I	32	
S9XBS9_CAMFR	433.7584	K.ALPAPIER.T	37	263
lg gamma-3 chain C	537.8010	K.APSVYPLTAR.C	49	
region	561.2972	K.DTVSVTCLVK.G	30	
	511.9165	K.TFICDVAHPASSTK.V	29	
	810.8932	K.GFYPPDINVEWQR.N	54	
	607.3409	R.VVSVLPIQHQDWLTGK.E	64	
*S9XYY2 CAMFR	482.7420	K.VDGALCTTK.F	61	246

Hemopexin	540.7608	K.FLGPNSCSAK.G	50	
	546.7894	K.KVDGALCTTK.F	53	
	603.8037	R.EDPVTGEVOSK.Y	45	
	620.8141	K.GGYTI VENYPK.R	36	
A0A075T9L1_CAMDR	428,7481	K.AGAVNPTVK.F	50	174
Dipeptidvlpeptidase 4	458,2740	R.ISI OWIR.R	36	
	755 8279	K WEYYDSVYTER Y	56	
	493 9853	R ERPAEPHETSDGSSEYK I	34	
S9XXW2 CAMER	490.7253	R.ODGSVDFGR.K	68	172
Fibrinogen betg chain	620 2627	K EDGGGWWYNR C	45	1/2
normogen seta cham	646 8159	R OGEGNIATNADGK K	58	
*S9W/DV/3_CAMER	597 7473		70	169
Fibringgen gamma	704 3240	K TSTADYSTESVGPESDKYR M	56	105
chain isoform aamma-B	740 6835	K EGEGHI SPTGNTEEWI GNEK I	43	
	573 8375	R I I M/VDW/TR R	31	166
Globin A1	580 3/36	K WAGVANALAHR V	52	100
GIODIN AI	664 8632		JZ 45	
	705 8407		45	
	103.8497	R.EFTFDEQAATQK.V	30	146
Jestono H2P	406.7522		45 21	140
HISTOILE HZB	477.3030	K. AAGINANSEVADJEER L 1 2 Ovidation (M)	51	
	888.4080	K.AMGIMINSEVINDIFER.1 + 2 OXIUULION (IVI)	69	140
S9XNF8_CAMFR	410.2396	K.SILFVPR.L	42	142
Xaa-Pro alpeptiaase	557.3425	K.VPLALFALNK.Q	45	
*****	493.9340	R.VFKIDMELEVLR.Y	56	
*S9YS49_CAMFR	400.7165	K.IPEATNR.R	42	132
Putative E3 ubiquitin-	420.2108	K.FDTISEK.T	31	
protein ligase Roquin	496.2475	K.LGACDNILK.Q	59	
*S9YGW7_CAMFR	418.2495	R.VIIDLFK.H	30	123
Heparin cofactor 2	546.7880	K.DYNLVEALR.S	52	
	/2/.4034	K.ALEAQLIPQVVER.W	41	
S9WPM4_CAMFR	4/8.7579	K.AVLFTYDK.Y	32	115
Adiponectin	491.7873	R.STVPNVPIR.F	31	
	533.7802	R.SAFSVGLETR.S	52	
A2V743_CAMDR	566.7665	R.GYSFITTAER.E	38	109
Beta actin	895.9506	K.SYELPDGQVIIIGNER.F	22	
	652.0263	R.VAPEEHPVLLTEAPLNPK.A	22	
	796.6590	R.TIGIVMDSGDGVTHTVPIYEGYALPHAILR.L	27	
*S9XP08_CAMFR	511.7794	R.LTYEEVLR.R	38	109
Inter-alpha-trypsin	540.2900	K.LDAQASFLSK.E	48	
inhibitor heavy chain H1	579.3171	K.AAISGENAGLVR.A	23	
S9XM15_CAMFR	472.9072	R.ELAEEKREGAER.L	37	
Ferritin	569.3168	K.NLNQALLDLHALGSAR.A	69	
TONNK2_CAMFR	624.8058	R.VIGSGCNLDSAR.F	93	93
L-lactate				
dehydrogenase				
S9X3E8_CAMFR	659.3199	R.FTGSGSGTDFTLK.I	90	90
Ig kappa chain V-II				
region RPMI 6410-like				
protein				
A0A0A0PAR2_CAMDR	408.2604	R.ALLFVPR.R	38	88
Heat shock protein 90	757.3968	R.GVVDSEDLPLNISR.E	50	
*S9XYF2_CAMFR	415.2682	R.ALLFIPR.R	38	87
Heat shock cognate	757.3968	R.GVVDSEDLPLNISR.E	50	
protein HSP 90-beta-like				
isoform 3				
S9XHZ4 CAMFR	565.8065	R.VLQEAGADISK.T	83	83

Phosphotriesterase-				
related protein				
S9XM68_CAMFR	467.7714	K.AIYEAVLR.S	33	73
Xaa-Pro dipeptidase	489.2769	R.LADRIHLEELTR.I	41	
isoform 3				
S9WT57_CAMFR	527.3079	R.YLTVAAIFR.G	27	73
Tubulin beta chain	623.3002	R.ISEQFTAMFR.R + Oxidation (M)	46	
*S9YV02_CAMFR	513.3091	K.IGGIGTVPVGR.V	69	69
Non-specific protein-				
tyrosine kinase				
*S9XC57_CAMFR	631.7936	K.QLGAGSVDECAR.K	63	63
Plasminogen				
*S9YL21 CAMFR	463.2769	K.VAPLGAELR.E	34	62
Apolipoprotein A-I	633.8224	K.VQPYLEDFQK.K	28	
*S9WKZ8 CAMFR	573.2996	R.GESAGLVQATGR.K	62	62
Inter-alpha-trypsin				
inhibitor heavy chain H4				
S9WIA5 CAMFR	436.7454	K.ILSNNPSK.G	61	61
Glutathione synthetase				
TOMHN9 CAMFR	680.3563	R.NTGIICTIGPASR.S	60	60
Pyruvate kinase				
*S9Y4U4 CAMFR	798.3819	R.VITNPQGDYDTSTGK.F	58	58
Complement C1a				
subcomponent subunit				
C isoform 2				
S9WAX5 CAMFR	396.2074	R.IIGANMR.T + Deamidated (NQ): Oxidation (M)	29	57
Unconventional myosin-	444.2330	K.NELNELR.K	30	
Va isoform 2				
S9WVY1 CAMFR	715.3859	R.TINEVENQILTR.D	56	56
Actinin. alpha 1 isoform		······		
6-like protein				
S9XA40 CAMFR	627.3116	R.FEELNADLFR.G	55	55
Heat shock cognate				
protein				
*S9YFM0 CAMFR	617.8373	R.TAAENEFVLLK.K	52	52
Keratin, type II			-	-
cvtoskeletal 71				
A0A0U2KTX5 CAMDR	498.7536	R.FTISTDNAK.N	52	52
VHH5 (Fraament)			-	-
S9XR87 CAMFR	592.9709	K.QDGTTVTQGVETTKPSK.Q	50	50
la lambda chain C				
regions isoform 19-like				
protein				
S9WGH8 CAMFR	700.8439	R.STDYGIFQINSR.Y	49	49
Lysozyme				
S9WMX2 CAMFR	772.4020	R.ILTGENAVGELRNR.T + 2 Deamidated (NQ)	30	49
Dvstonin	1048.0221	R.VGQSLSSLTCSTEQGVLAEK.L + Deamidated (NQ)	22	-
*S9X8K9 CAMFR	438.7245	R.VSTEVDAR.L	46	46
Transaldolase				
*TONM23 CAMFR	544.3185	K.AGTLQLTVER.L	26	45
Rootletin	673.3367	$K_{RI} OFOARDIGR.O + 2 Deamidated (NO): 2$	21	
		Deamidated (R)		
S9W6I0 CAMFR	438.7629	R.IFLODIK.Q	45	45
Ferritin				
S9WF34 CAMFR	543.3137	K.EIIDLVLDR.I	43	43
Tubulin alpha chain			-	-

*S9YSI7_CAMFR	489.5794	K.TATPQQAQEVHEK.L	43	43
Triosephosphate				
isomerase				
*S9XSQ6 CAMFR	903.0750	K.HQPQEFPTYVEPTNDEICEAFR.K	40	40
Vitamin D-binding				
protein-like protein				
*S9YMC0_CAMFR	416.2516	K.TVGVIVSR.E + Deamidated (R)	20	40
Transcription factor 20	516.7796	K.LKMSPGRSR.G + Deamidated (R)	20	
isoform 1				
*S9Y636_CAMER	402 7397	R LI OAGER F	39	39
Recentor-type typosine-	1021/03/		55	55
nrotein nhosnhatase-				
like N				
*SOXEMA CAMER	758 3907	K GVRDSSVSLESSIELLOKDVVOLHAPR V + 2	37	37
Dyslevia_associated	738.3507	Deamidated (NO): 2 Deamidated (R)	57	57
protain				
	727 2070		26	26
Diving4_CAMFR	/3/.39/0	R. IDEVRILQENLR.Q	30	30
Rabenosyn-3-like				
	500 7704		25	25
S9WJW3_CAMFR	508.7724	K.QLDSLRER.L	35	35
Nb-adenosine-				
methyltransferase				
subunit				
*A8IY99_LAMGU	590.9761	K.AIQVSYNPAEPSKPNR.I	35	35
Gamma-fibrinogen				
S9WRI7_CAMFR	450.2690	R.RDRSPIR.G	35	35
Nuclear receptor				
coactivator 5 isoform 3-				
like protein				
S9W421_CAMFR	664.8442	K.VNVEEAGGEVLGR.L	35	35
Hemoglobin, epsilon 1				
*S9W711_CAMFR	514.8088	K.RAALQALKR.K + Deamidated (NQ); Deamidated	34	34
Charged multivesicular		(R)		
body protein 4c				
*S9X089_CAMFR	550.3109	K.DSERPSGIPER.F	34	34
Ig lambda chain V-III				
region LOI-like protein				
S9WUC8_CAMFR	550.3109	R.LLIYYASTR.E	32	32
lg kappa chain V-II				
region RPMI 6410-like				
protein				
*S9WVI6_CAMFR	667.3889	K.GLFQVVSGGTVLR.L + Deamidated (NQ)	32	32
Complement C1q				
subcomponent subunit				
Α				
*S9Y5S1_CAMFR	572.8298	K.ISRLDAELVK.Y + Deamidated (R)	32	32
Transcriptional				
repressor NF-X1				
*S9YC53_CAMFR	454.2354	R.YPSSANLR.F	32	32
Alpha-1-antitrypsin-like				
protein				
*S9Y3F6_CAMFR	564.8041	R.LPSNRGNTLR.E + Deamidated (R)	31	31
Dual specificity testis-				
specific protein kinase 1				
S9WKI8_CAMFR	421.7685	R.VGTLALIR.A	31	31
HEAT repeat-containing				

protein 7B1				
*S9WVS9_CAMFR	430.2352	K.QAQKNLR.R + 2 Deamidated (NQ)	29	29
Peroxisome				
proliferator-activated				
receptor gamma				
coactivator-related				
protein 1				
*S9XVK5_CAMFR	704.8226	K.AADETWEPFASGK.T	29	29
Transthyretin				
*S9Y967_CAMFR	550.3373	K.INTKALQSPK.R	28	28
General transcription				
factor II, i isoform 4				
isoform 1-like protein				
*T0MC04_CAMFR	486.7591	R.QELLSQPR.D + Deamidated (NQ); Deamidated	28	28
Spermatogenesis-		(R)		
associated protein 2-				
like protein				
*S9YSZ6_CAMFR	714.9224	K.AENTSLAKLRIGR.E	28	28
Centromere protein J				

<sup>†</sup>lons score is -10\*Log(P), where P is the probability that the observed match is a random event. Individual ions scores > 20 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 lons score 20.

Supplementary Table 2. Deiminated proteins, including all peptide sequences, identified by F95 enrichment and LC-MS/MS in extracellular vesicles isolated from serum of Ilama (*Lama glama*). 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. Peptide sequence hits scoring with *L. glama* (LAMGL) are presented as well as hits with other camelids (CAMFR=Camelus ferus; CAMDR=Camelus dromedaries; LAMGU=Lama guanicoe). Hits with uncharacterised proteins are not listed. Peptide sequences and m/z values are listed. An asterix (\*) indicates that the protein hit is unique for EVs only.

Protein name	m/z	Peptide sequence	Score	Total
(*unique for EVs)			(p<0.05) <sup>‡</sup>	score
A0A1W5VKM5 LAMGL	653.7846	K.SEDTAVYYCAK.D	22	164
Anti-RON nanobody	941.5044	EVQLVESGGGLVQPGGSLR.L	74	
Q865W8 LAMGL	566.7667	R.GYSFTTTAER.E	85	85
Beta actin	895.9502	K.SYELPDGQVITIGNER.F		
*S9XAP9 CAMFR	404.2033	R.LAADDFR.T	55	554
Keratin, type I	515.3006	R.VLDELTLAR.A	51	
cytoskeletal 14-like	546.2614	K.VTMQNLNDR.L + Deamidated (NQ)	74	
protein	553.7849	R.ISSVLAGGSCR.A	54	
-	561.7932	R.LEQEIATYR.R	56	
	651.3332	R.ALEEANADLEVK.I	78	
	681.3492	R.EVATNSELVQSGK.T	100	
	685.3798	K.ILTATVDNANIVLQIDNAR.L	41	
	770.3588	R.LLEGEDAHLSSSQFSSGSQSSR.D	46	
*S9X688_CAMFR	469.7508	R.SLYNLGGSK.S	37	496
Keratin 6A-like protein	473.2593	R.GRLDSELR.N	31	
	503.2371	K.LLEGEECR.L	42	
	533.7617	K.YEDEINKR.T	53	
	578.2716	R.DYQELMNVK.L + Oxidation (M)	35	
	398.8752	K.KYEDEINKR.T	61	
	604.8117	R.TAAENDFVTLK.K	79	
	619.7895	R.NMQDLVEDFK.K	21	
	632.3508	K.LALDVEIATYR.K	76	
	651.8625	R.SLDLDSIIAEVK.A	63	
S9Y6J1_CAMFR	405.7087	R.QSSVSFR.S	23	438
Keratin, type II	473.2593	R.GRLDSELR.N	31	
cytoskeletal 5 isoform	533.7617	K.YEDEINKR.T	53	
13-like protein	576.7803	K.NKYEDEINK.R	27	
	602.3220	K.WTLLQEQGTK.T	44	
	619.7895	R.NMQDLVEDFK.N	21	
	621.7851	R.TEAESWYQTK.L	43	
	632.3508	K.LALDVEIATYR.K	76	
	651.8625	R.SLDLDSIIAEVK.A	63	
	436.8895	K.NKYEDEINKR.I	58	
S9WI87_CAMFR	449.7441	R.LCVLHEK.I	27	430
Serum albumin	460.2554	K.LCTVASLR.E	43	
	464.2504	K.YLYEIAR.R	34	
	554.7310	K.HVFEECCK.D	46	
	509.7525		58	
	575.3110	K.LVINEVIEFAK.I	48	
	744 8020	K. ICVADESAADCDK.S	19	
	744.0029		22	
*SOVNOG CAMER	140.3243		52	/17
Keratin tyne I	515 3006	R VIDEITIAR A	55	41/
cvtoskeletal 17-like	561 7932	R I FOFIATYR R	56	
isoform	651 3332	RAIFFANADIEVKI	78	
	681.3492	R.EVATNSELVQSGK.T	100	

	690.0522	K.ILTATVDNANILLQIDNAR.L	77	
*S9XI90_CAMFR	503.2371	K.LLEGEECR.L	42	318
Keratin, type II	597.7917	K.YEELQQTAGR.H	69	
cytoskeletal 75-like	604.8117	R.TAAENEFVSLK.K	36	
isoform	632.3508	K.LALDVEIATYR.K	76	
-	651.8625	R.SLDLDSIIAEVK.A	63	
	436.8895	K.VRYDDEINKR.T + Deamidated (R)	32	
S9X494 CAMFR	404.2033	R.LAADDFR.T	55	269
Keratin, type I	515.3006	R.VLDELTLAR.A	51	
cytoskeletal 42	561.7932	R.LEQEIATYR.R	56	
	651.3332	R.ALEEANADLEVK.I	78	
	1043.4962	R.GQVGGDVNVEMDAAPGVDLSR.I	28	
S9XBS9 CAMFR	433.7584	K.ALPAPIER.T	34	162
Ig gamma-3 chain C	537.8009	K.APSVYPLTAR.C	50	
region	561.2974	K.DTVSVTCLVK.G	32	
_	607.3406	R.VVSVLPIQHQDWLTGK.E	47	
A0A075T9L1_CAMDR	428.7482	K.AGAVNPTVK.F	52	153
Dipeptidylpeptidase 4	458.2738	R.ISLQWIR.R	42	
	755.8281	K.WEYYDSVYTER.Y	59	
S9X684_CAMFR	632.3508	K.LALDVEIATYR.K	76	136
Keratin, type II	672.8415	R.ASLEAAIADAEQR.G	61	
cytoskeletal 8				
S9WB99_CAMFR	408.7321	R.EIQTAVR.L	45	133
Histone H2B	477.3053	R.LLLPGELAK.H	33	
	888.4087	K.AMGIMNSFVNDIFER.I + 2 Oxidation	55	
		(M)		
S9YQ51 CAMFR	527.3073	R.YLTVAAIFR.G	29	114
Tubulin beta chain	623.3008	R.ISEQFTAMFR.R + Oxidation (M)	62	
	546.2816	R.LHFFMPGFAPLTSR.G + Oxidation (M)	24	
*S9WX81_CAMFR	416.2502	K.STELLIR.K	34	89
Histone 1, H2ai isoform	425.7669	R.HLQLAIR.N	25	
3-like protein	472.7691	R.AGLQFPVGR.V	31	
*S9X8G9_CAMFR	565.3085	K.IEVLEEELR.L	56	88
Desmoplakin	636.3563	R.QLQNIIQATSR.E	33	
A2V743_CAMDR	566.7667	R.GYSFTTTAER.E	48	85
Beta actin	895.9502	K.SYELPDGQVITIGNER.F	37	
A0A0A0PAR2_CAMDR	408.2603	R.ALLFVPR.R	34	85
Heat shock protein 90	757.3967	R.GVVDSEDLPLNISR.E	51	
S9XA40_CAMFR	627.3118	R.FEELNADLFR.G	85	85
Heat shock cognate				
protein				
T0NNK2_CAMFR	624.8045	R.VIGSGCNLDSAR.F	81	81
L-lactate				
dehydrogenase				
S9XNF8_CAMFR	410.2398	K.STLFVPR.L	38	74
Xaa-Pro dipeptidase	493.9347	R.VFKTDMELEVLR.Y	36	
T0MHN9_CAMFR	680.3557	R.NTGIICTIGPASR.S	71	71
Pyruvate kinase				
S9WVY1_CAMFR	715.3860	R.TINEVENQILTR.D	60	60
Actinin, alpha 1 isoform				
6-like protein				
*A0A0E3Z5I3_CAMDR	845.1036	K.LTAVSVGVQGSGWGWLGFNKEQGR.L	59	59
Superoxide dismutase				
S9XHZ4_CAMFR	565.8064	R.VLQEAGADISK.T	57	57
Phosphotriesterase-				
related protein				

S9W9Y4_CAMFR	569.3167	K.NLNQALLDLHALGSAR.A	57	57
Ferritin				
S9XR87_CAMFR	592.9710	K.QDGTTVTQGVETTKPSK.Q	56	56
Ig lambda chain C				
regions isoform 19-like				
protein				
S9X3E8_CAMFR	659.3194	R.FTGSGSGTDFTLK.I	50	50
lg kappa chain V-II				
region RPMI 6410-like				
protein				
S9WAX5_CAMFR	396.2075	R.IIGANMR.T + Deamidated (NQ);	30	49
Unconventional myosin-		Oxidation (M)		
Va isoform 2	565.3085	K.LKNELNELR.K + Deamidated (NQ)	22	
S9WF34_CAMFR	543.3137	K.EIIDLVLDR.I	44	44
Tubulin alpha chain				
*S9W806_CAMFR	529.7776	K.VAQPAITDNK.D + 2 Deamidated (NQ)	23	43
Filamin-A isoform 1		K.GEITGEVRMPSGK.V + Deamidated		
	681.3492	(R)	20	
*S9X6X3_CAMFR	474.2696	R.LSKEEKGR.S + Deamidated (R)	21	42
Scaffold attachment	543.3296	K.ADTLLAVVKR.E	21	
factor B-like protein				
T0MH94_CAMFR	737.3968	R.TDEVRTLQENLR.Q	40	40
Rabenosyn-5-like				
protein				
*S9Y0S0_CAMFR	408.7425	K.TQISLVR.M	39	39
DNA-directed RNA				
polymerase subunit				
beta				
S9WJW3 CAMFR	508.7720	K.QLDSLRER.L	38	38
N6-adenosine-				
methyltransferase				
subunit				
S9XM68_CAMFR	467.7713	K.AIYEAVLR.S	38	38
Xaa-Pro dipeptidase				
isoform 3				
*S9YV02 CAMFR	513.3086	K.IGGIGTVPVGR.V	38	38
Non-specific protein-				
tyrosine kinase				
S9WGH8 CAMFR	700.8445	R.STDYGIFQINSR.Y	32	32
Lysozyme				
S9WRI7_CAMFR	450.2691	R.RDRSPIR.G	32	32
Nuclear receptor				
coactivator 5 isoform 3-				
like protein				
S9W421 CAMFR	664.8441	K.VNVEEAGGEVLGR.L	32	32
Hemoglobin, epsilon 1				
*S9WI71 CAMFR	552.2800	R.INEDRGIQR.L + 2 Deamidated (NQ);	32	32
Metabotropic		Deamidated (R)		
glutamate receptor 3		. ,		
*S9WB50 CAMFR	693.8827	R.EEVEILKEQIR.E + Deamidated (R)	31	31
TSC22 domain family				
protein 3-like protein				
S9WMX2 CAMFR	772.4021	R.ILTGENAVGELRNR.T + 2 Deamidated	31	31
Dystonin		(NQ)	-	-
S9WKI8 CAMFR	421.7683	R.VGTLALIR.A	31	31
HEAT repeat-containing				

protein 7B1				
S9WIA5_CAMFR	436.7452	K.ILSNNPSK.G	31	31
Glutathione synthetase				
*S9XC05_CAMFR	503.7640	K.SSEKSVRGR.T + Deamidated (R)	31	31
Telomere-associated				
protein RIF1 isoform 1				
A0A0U2KTX5_CAMDR	498.7538	R.FTISTDNAK.N	30	30
VHH5				
*T0MGG7_CAMFR	650.3872	K.VVCRNGLLVIR.D + Deamidated (R)	30	30
Nucleoredoxin				
*S9XET3_CAMFR	652.3622	R.VRSTLTRNTPR.R + Deamidated (NQ);	30	30
Rac GTPase-activating		2 Deamidated (R)		
protein 1				
S9XDK9_CAMFR	417.2478	R.LPYSVVR.N	30	30
Complement C3-like				
protein				
*S9XMI2_CAMFR	913.8976	K.ENEPNHESLSGNNQEK.D +	29	29
Pseudopodium-enriched		Deamidated (NQ)		
atypical kinase 1				
*S9Y3S9_CAMFR	493.7978	K.QTAAQLILK.A + Deamidated (NQ)	29	29
Core histone macro-				
H2A.1 isoform 2				
S9XXW2_CAMFR	490.7250	R.QDGSVDFGR.K	29	29
Fibrinogen beta chain				
*S9YGX6_CAMFR	746.3763	K.TTEILVANDKACR.L + Deamidated	29	29
PAS domain-containing		(NQ)		
serine/threonine-				
protein kinase				
*T0NMU1_CAMFR	487.7771	R.SKTEQLLR.D	28	28
SH2 domain-containing				
protein 7				
*TOMIT6_CAMFR	420.2258	R.AHGREIR.K + Deamidated (R)	28	28
Serine-tRNA ligase,				
mitochondrial				
*S9W449_CAMFR	424.2454	R.ASLEPGGGPR.G	28	28
Fc receptor-like protein				
5				

<sup>†</sup>Ions score is -10\*Log(P), where P is the probability that the observed match is a random event. Individual ions scores > 22 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 lons score 20.