Acquired equine polyneuropathy of Nordic horses - a conspicuous inclusion body Schwannopathy

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Abstract

Acquired equine polyneuropathy (AEP), formerly also known as Scandinavian knuckling syndrome, is one of the most prevalent polyneuropathies in equids in Norway and Sweden, with more than 400 cases registered since first observations in 1995. Despite geographical clustering and an association to forage feeding, its aetiology remains unknown. Clinically AEP is characterized by knuckling due to dysfunction of metatarsophalangeal extensor muscles. This neuropathological study aimed to gain further insights in the pathobiology of AEP and its underlying aetiopathogenesis. We thereby confirmed that all affected horses suffered from similar large fiber neuropathy, exhibiting conspicuous Schwann cell inclusions in most samples, suggestive of a primary disruption of Schwann cell metabolism leading to inclusion body schwannopathy with secondary inflammatory changes. The degree of nerve pathology was not predictive of clinical outcome.

Keywords: Knuckling; schwannopathy; demyelination; inclusion body; inflammatory; nerve fiber teasing.

Abbreviation: Acquired equine polyneuropathy: AEP

1. Introduction

The first case clusters of a unique neuromuscular syndrome in horses characterized by knuckling in the metatarsophalangeal joints were observed in Norway in 1995 (1) and Sweden in 1998 (2). Since then, more than 400 cases have been identified throughout Norway, Sweden and Finland, making this disease the most prevalent polyneuropathy in equids in this part of the world (3-7). The syndrome was associated with~~to~~ peripheral nerve lesions, but the cause has not yet been identified. The disease was initially~~sometimes~~ referred to as "Scandinavian knuckling syndrome" ~~in the beginning~~, but is now~~lately~~ known as acquired equine polyneuropathy (AEP).

AEP affected~~s~~ horses and ponies are of a wide spectrum of breeds, uses and comprise~~,~~ all sexes and ages, except for foals. Clinically, the disease is characterized by digital extensor dysfunction, primarily affecting the pelvic limbs resulting in knuckling in the metatarsophalangeal joints (1, 3, 4) (Fig. 1). In mild cases, knuckling occurs~~is presented~~ only rarely unless provoked by e.g. tight circling or sudden stop from trot. Apart from these manipulations of movement, digital extensor dysfunction may be exacerbated with sudden distress, which requires careful handling during clinical examination of more severe cases (4). Horses with AEP do not appear ataxic. The horses are otherwise alert, responsive with normal appetite and clinical variables are within normal limits. There have been no significant abnormalities on laboratory analysis of blood or cerebrospinal fluid when examined (1, 2).

The clinical disease course is highly variable. In the most severe and acute cases, horses suddenly knuckle and stand~~rest~~ on the dorsal metatarsophalangeal region without being able to correct the abnormal limb position for seconds to minute(s). Such cases are often unable to get up from recumbency, even with assistance. In less severe and more prolonged disease courses, horses knuckle intermittently for months before they either improve slowly, or suddenly deteriorate and become recumbent. Horses that remain able to rise and stand with or

without support mostly recover completely with long convalescence. Intermittent knuckling has,~~ve~~ however, been observed for up to 17 months after onset, with a median duration of clinical signs of 4.4 months (4). Case fatality rates vary ~~in~~between outbreaks and range from 29% to 53% (1, 4, 8).

Typically, AEP affects more than one, but not all horses in a stable and has a seasonal pattern with most cases appearing during winter and springtime, indicating an environmental trigger (1, 4, 5). A specific~~certain~~ aetiology has not been associated to AEP despite extensive studies, but almost all cases have been fed wrapped forage, indicating an alimentary risk factor of unclear nature (1, 4). However, analysis of the hygienic, botanical, chemical and microbiological composition of wrapped forage have so far failed to identify a disease causing agent (unpublished data) (4).

Despite ~~the high disease prevalence~~the relative large number of cases, the sparse availability of fresh material for peripheral nerve studies has hitherto limited the possibilities to clarify the pathobiology of AEP from the tissue perspective. Post-mortem examination of the nervous system of 22 horses diagnosed with AEP in Norway (1) and a number of horses in Sweden (unpublished) indicated a polyneuropathy, but obtained tissues did not allow for further classification. The only in depth investigation reported was from ~~in~~one single horse from Finland and it revealed schwannopathic features and nerve~~-~~-fibre ~~-~~invasive inflammation (3). Whether these lesions are characteristic of AEP remains yet unknown, in particular because~~as~~ this horse also was ataxic (3), which is unusual for the majority of AEP cases (1, 4). Hence, it was the aim of this study to clarify peripheral nerve and muscle changes of an extended series of AEP horses presenting with classical clinical signs, in order to approach the underlying pathological mechanisms and aetiological triggers.

2. Material & Methods

2.1 Included horses

Horses were recruited from outbreaks of AEP reported to the Equine Clinic, Norwegian University of Life Sciences (NMBU) or National Veterinary Institute, Sweden, between 2005 and 2014. In accordance with former published diagnostic algorithms (1, 4), inclusion criteria were a clinical history of repeated bilateral pelvic limb knuckling without ~~overt~~ signs of ~~a central nervous system disease~~involvement of the nervous system of the head or other abnormal clinical signs. Exclusion criteria included: 1) primary musculoskeletal disorders affecting the metatarsophalangeal joint, 2) neuromuscular junction disorders, 3) spinal ataxia ~~or indication of any other central nervous system (CNS) involvement~~ or 4) primary muscle disease and other causes of non-neurologic pelvic limb weakness~~paresis~~. All horse owners consented for the results to be included in this study.

Based on neurological examination by authors (SHO, GG,~~)~~12 cases) , and ~~videos and/or~~ veterinary records from the neurological evaluation performed by local veterinarians with or without videos (four cases), the clinical severity of each case was graded at least two times; at onset and at time of sampling, some cases also in between. Severity were graded I-IV according to a semiquantitative grading system established earlier (1) (Table 1, video 1 and 2). Biopsies and autopsies were performed for diagnostic reasons.

2.2 Sampling

In the cases that were euthanized on humane grounds due to deterioration or an uncertain prognosis, samples were taken at autopsy. Fascicular nerve specimens were taken from one or more of the following sites: recurrent laryngeal nerve, median nerve, lateral digital palmar nerve, femoral nerve, sciatic nerve, tibial nerve, common and superficial peroneal nerve and lateral digital plantar nerve (supplementary item). If possible, nerves were collected from

both sides of the body, particularly in the case of the recurrent laryngeal nerves-. Specimens from spinal nerve roots were resected after extensive laminectomy.

Biopsies from appendicular muscles including triceps, extensor carpi radialis, quadriceps (vastus lateralis), tibialis cranialis and/or extensor digitalis longus and gluteal muscles were harvested, and specimens were immediately shipped overnight to the laboratories for processing. Cases that were recovering had at least one skeletal muscle biopsy taken.

2.3 Histological processing

2.3.1 Nerve processing

All nerve samples underwent the routine biopsy protocol established at the Neuropathology Laboratory, Ludwig-Maximilians University of Munich (LMU), Germany. It includes: 1) paraffin embedding for assessment of epineurial, interstitial and vascular abnormalities, 2) semithin sections, 3) nerve fibre teasing (NFT) for assessment of myelinated nerve fibre characteristics and 4) transmission electron microscopy (TEM) for identification of subcellular changes and unmyelinated fibre pathologies.

Paraffin embedding was preceded by whole-trunk immersion in 10% neutral buffered formalin for at least 24 hours, after which the fascicles were trimmed and underwent an ascending ethanol series and immersion in liquid paraffin using an automatic tissue processor (Hypercenter®, Shandon Inc.). Sections were cut at 3 µm and subsequently stained by haematoxylin-eosin (HE), Goldner's trichrome stain (GTS), and picrosirius red-alcian blue staining (PICRAB) (9). Upon paraffin embedding, transverse sections of the spinal cord samples were performed and stained with HE and trichrome to evaluate neural versus interstitial and vascular changes.

For semithin histology, NFT and TEM, large fascicles were gently separated and immersed in 2.5% glutaraldehyde in 0.1M Soerensen's phosphate buffer for 1-2 hours. Thereafter they

were incubated in washing buffer. A series of fascicular full trunk samples of 2 mm length were obtained using a razor blade on the proximal and distal edges of the specimens. These pieces were subjected for transverse and longitudinal sectioning. They were post-fixed in 2% osmium tetroxide, dehydrated by an ascending ethanol series and embedded in epoxy resin. Semithin sections were processed at 0.5 µm thickness and stained by p-phenylene diamine and modified Richardson's stain, using azure II methylene blue and safranin-O (10). On semithin scout sections, candidate areas were identified for TEM, trimmed, sectioned at 50 nm, mounted on copper grids and contrasted with lead citrate and uranyl-acetate. Ultrathin sections were stored in an exsiccator until ultrastructural examination. Trimmed fascicles also were impregnated in 2% osmium tetroxide, washed in phosphate buffer before undergoing NFT after immersion in glycerol with and without haematoxylin counterstaining (10).

The neuromorphological investigation employed standard algorithms for peripheral nerve diagnostics (11) and analyzed samples from neurological horses in comparison to age- and breed-group matched non-neurologic controls (74 horses; 46 female/28 male, 6 weeks to 28 years) available through the archive of the Neuropathology Laboratory, LMU, Germany. Checklist for myelinated nerve fibre evaluation included abnormalities of Schwann cell nucleus and perikaryon, the presence of Schwann cell inclusions, the thickness and integrity of compacted and uncompacted myelin, the width and content of the nodal gap, the axonal diameters, the density, distribution and morphology of the axonal cytoskeleton and axoplasmic organellae, the frequency and spatial distribution of the axon-Schwann cell network (ASN), the appearance of the inner and outer endoneurial sheath and the presence of nodal gap cells, fibre-adhesive and fibre-invasive immune cells. The appearance of unmyelinated nerve fibres, including C-fibre axons and their ensheathing Remak cells and basilar laminae as well as the presence of collagen pockets and empty Schwann cell subunits were evaluated at ultrastructural level. All histological investigations were carried out at a

Zeiss Axiophot® equipped with a CCD camera with magnifications ranging from x125 to x1000. TEM was performed at a Zeiss EM10®, at 80kV, with a magnification of x1500 to x100.000.

2.3.2 Immunohistology of nerves

Upon histological evaluation, immunohistochemical labelling techniques were employed for assessment of endoneurial immune cell infiltrates and endoplasmic reticulum stress.

The following markers were applied for immune cell phenotyping: T-cell marker CD3 (monoclonal mouse, clone F7.2.38, 1:200, Dakocytomation, Glostrup, Denmark), B-cell marker CD79a (monoclonal mouse, clone HM57, 1:500, Dakocytomation, Glostrup, Denmark), lysozyme (polyclonal rabbit, 1:200, Linaris, Freiburg, Germany) and MAC387 (polyclonal rabbit antibody, 1:1000, Linaris, Freiburg, Germany) labelling histiocytes and macrophages. Detection of humoral factors was performed using antibodies directed at horse IgG (polyclonal rabbit, Linaris, 1:100, Freiburg, Germany). These markers were applied on deparaffinised sections and on selected teased fibres after fixation with 4% paraformaldehyde and treatment with 20M sucrose. Endoplasmic reticulum (ER) dysfunction was evaluated via the ER chaperone and signaling regulator GRP78/BiP.

Immunohistochemical procedures on sections employed antigen retrieval with microwave treatment in citrate buffer (20 min, pH 6.0, 800 W), overnight incubation with the primary antibodies at 4°C, avidin-biotin enhancer (ABC kit, Linaris, Freiburg, Germany) and a diaminobenzidine hydrochloride detection kit. Whole mount immunohistochemistry of teased fibres was conducted as single and double labelling study. Following microwave treatment, incubation with each primary antibody was carried out for 5 days at 37°C in a humid chamber. Immersion with the second primary antibody was preceded by LinBloc® (Linaris,

Freiburg, Germany) treatment. Histogreen® (Linaris, Freiburg, Germany) was used as second chromagen.

2.3.3 Muscle processing

Between one and four biopsies from different muscles were examined for individual horse (supplementary item 1). Samples were immersed in liquid nitrogen and processed to frozen and formalin fixed slides stained with HE, periodic acid Schiff (PAS) with and without diastase pretreatment and Masson`s trichrome techniques. In two cases modified Gomori trichrome and fibre typing with adenosine triphosphatase (ATPase) and nicotinamide adenine dinucleotide (NADH) tetrazolium reductase staining was also performed.

2.4 Data analysis

Nerve lesion scores (0-3) were obtained for myelinated fibre loss, actual demyelinating and axonal pathologies, Schwann cell changes and inflammatory features (12). Lesion occurrence and scores were compared in between acute (≤4 weeks disease history) and chronic (≥8 weeks disease history) cases using chi square /Fisher´s exact test and Mann Whitney test. The interdependence between nerve lesions and clinical grades was evaluated via Kendall-Tau test. P values ≤ 0.05 were accepted indicating significance.

3. Results

3.1 Demographics and management

Sixteen horses from Norway and Sweden were included in the study. Case horses were aged between 1 and 25 years (mean 10), represented all sexes and 9 different breeds (Table 2). Stabling included both small units with less than 10 horses and large stables with up to 80 horses. Prevalence of AEP at farm level varied and ranged from 1 affected out of 50 horses to 10 out of 14. All cases had been fed wrapped forage preceding the disease. Two cases (No. 1 and 7) had been stabled in farms together with AEP cases earlier, but at that point without any

clinical signs of AEP. Horse No. 1 was stabled together with ~~exposed to~~ a single AEP horse a year before and was then, in the present study, part of~~affected by~~ a large outbreak involving 10 out of 14 stablemates. Horse No. 7 was stabled at a farm with several AEP affected horses four years prior to inclusion in the study, and at that time found to be neurologically normal~~unaffected~~ by one of the authors (SHO). The horse thereafter changed stables and had no history of neurological deficits during the four years that followed~~to come~~. She was then the only horse diagnosed with AEP at the farm.

3.2 Clinical course

The included horses represented all four severity grades at onset of disease, see Table 2 and Fig. 2 for details.~~The severity of clinical signs at onset of the disease was grade I in four horses, grade II in six horses, grade III in four horses and grade IV in two horses (Table 2, Fig. 2).~~ Owners had elected euthanasia in 14 out of 16 case horses. The duration of the disease before sampling (observation time) was four weeks or less and classified as acute in six horses, ~~six and seven weeks respectively in two horses,~~ and more than eight weeks, classified as chronic. in eight horses, ~~four of which more than a year~~ (Table 2). ~~Based on this, six horses (No. 1-6) with clinical disease history of ≤4 weeks were classified as acute and eight horses (No. 7-14) with ≥8 weeks duration, as chronic.~~ The two surviving horses with six and seven weeks observation time (No. 15, 16) had only muscle biopsies taken and were not included in the statistical analysis.

In five horses neurological deterioration (n=3 grade III to grade IV, n=2 grade I to grade IV) occurred over 10 days to 4 months (Fig. 2). In three horses with grade I (n=2) or grade III (n=I) neurological deficits remained constant whereas in the remaining eight horses remission in clinical signs was observed. In six of the eight horses, recovery was incomplete (initial grade II, grade I at time of euthanasia). ~~A neurological deterioration was seen in five horses during an observational time of ten days to four months. Three of these horses progressed~~

~~from grade III to IV, and two deteriorated from grade I on first examination, to grade IV at time of euthanasia (Fig. 2). Neurological deficits remained constant during the observation period of seven days to two months in three horses (3/16), presenting with grade I (n=2) or grade III (n=1). Remission of clinical signs was seen in 8/16 horses. Recovery was incomplete in six horses which had showed grade II compromise at initial presentation, and grade I at time of euthanasia (observational time of four months to two years).~~ Two surviving ponies improved from grade IV to grade I and grade III, respectively, within the 6-7 weeks that passed from clinical onset to biopsy. They made a complete recovery from clinical signs of AEP within six months following sampling, and remained free during the following years.

3.3 Pathology

3.3.1 Tissue availability

In total, 105 nerve samples were collected from 14 horses that were subject to euthanasia. The samples originated from of up to 15 different nerve-sites from both sides of the body. From five horses, spinal nerve roots were resected. The samples contained one isolated dorsal root ganglion (DRG) (1/5), DRG plus postganglionic dorsal root and subganglionic ventral root (3/5) or not further specified fragments of non-ganglionic nerve roots (1/5). In depth evaluation of muscles and/or nerves was performed in all 14 euthanized horses (Table 2), in the two surviving case horses tissue diagnostics was limited to muscle biopsies. In addition, full autopsy was performed in four horses, according to respective consent of the owners.

3.3.2. Nerve pathology

All 14 AEP cases showed significant and rather uniform peripheral nerve changes extending throughout all sampling sites, with minor random variations (Table 3). At stage of sampling, all nerves exhibited mild to moderate loss of myelinated nerve fibres (MF), with or without

large-fiber predominance (7 of each) (Fig. 3). Total MF drop-out appeared mildly more advanced in acute versus chronic cases (p=0.03, Table 3).

In all but one horse (Table 2 No. 7, a chronic case), the nerves showed axonopathic MF features. Axonal atrophy with subsequent internodal myelin sheath crenation, inner and out-folded myelin loops and concentric myelin sheath adjustment was most prevalent, affecting 13/14 horses. More conspicuously, axonal swelling was noted due to abnormal axoplasmic aggregation of mitochondrial, multivesicular and dense bodies plus proliferation of axon-Schwann cell network in three acute (No. 2, 3, 6) and one chronic case (No. 9). Finally, four acute (all but No. 1 and 4) and five chronic cases (No. 8, 10-12, 14) presented with various stages of Wallerian degeneration. Amongst axonal changes, acute cases showed higher degrees of axonal atrophy (P<0.02; Table 3) if compared to chronic presentations. No significant differences were seen regarding occurrence and stage of Wallerian degeneration. There was however an ~~weak~~interdependence between Wallerian degeneration and severity degree of clinical signs in the acute cases (P=0,02, r=0.80~~22~~), with more pronounced Wallerian degeneration seen in the most severe cases.

Myelin sheath changes were evident throughout acute and chronic cases (Table 3, Fig. 3), including the single case lacking axonal pathologies (No. 7). With exception of horse No. 12, demyelinating features affected large fibre types only. These comprised interspersed or clustered demyelinated and hypomyelinated segments in all horses as well as paranodal demyelination with stepped remyelination and formation of pseudo- or hemi-nodes in two acute (No. 2, 6) and three chronic cases (No. 7, 9, 14) and dysmorphic paranodes in four acute (No. 1, 2, 5, 6) and two chronic cases (No. 12, 14).

Myelin sheath destruction was associated with fibre-adherent (12/14) (No. 1-11, 14) and even fibre-invasive (9/14) (No. 1-3, 5-7, 10, 11, 14) mononuclear round cells in a majority of cases (Table 3, Fig. 4). The degree of fibre-directed infiltrates in acute cases was statistically linked

to the severity degree of clinical signs (P=0.04; r=0.83), with more infiltrates seen in the most severe cases. All but one horse (13/14, exception No. 5) further presented with a diffuse lymphohistiocytic infiltration of the endoneurium that mainly expressed T-cell marker CD3 and lysozyme followed by a few CD79a-positive B-lymphocytes. Investigation of teased whole mount fibres was consistent with very mild immunopositivity for IgG within the myelin spiral.

Dyscompaction of myelin was noted in one acute (No. 3) and four chronic AEP cases (No. 7, 8, 12, 13) in terms of tomacula (No. 8, 12, 13), adaxonal and interlamellar ballooning (No. 3, 7).

Severe hypertrophy of nearly all Schwann cell perikarya was observed in 12/14 AEP horses (all but No. 13 and 14), with higher scores for the acute cases (P=0.04, Table 3). All six acute and five chronic cases presented with highly conspicuous amorphic perinuclear Schwann cell inclusions (Fig. 5). The inclusions stained osmiophobic, pale-azurophilic and GRP78/BiP-positive, on immunohistochemistry. On electron microscopy, they resembled flocculent electron dense material suggestive of non-filamentous protein accumulation. The content appeared not to be bound by a membrane, but was rather indistinctively separated from the cytosol. Apparently independent of the clinical stage, all but two cases (No. 1 and 13) showed hyperplastic Schwann cells and supernumerary Schwann cell processes ("onion bulbs") centered on demyelinated incompletely remyelinated fibres.

Insights from 2-15 sampling sites in 7/14 horses (No. 1, 4, 9, 10, 12, 13, 14) ruled out significant proximodistal gradients and asymmetric nerve affection with regards to axonal changes. This contrasts to proximal predominance of inflammatory features in one acute (No.3) and two chronic cases (No. 7, 11). Furthermore, in two horses with bilateral peroneal nerve sampling, Schwann cell inclusions were seen in one side only. Another single acute

case (No. 5) with four sites investigated, presented with the ~~-~~peroneal nerve being most severely affected by all type of changes.

All three DRG (3/5) showed patchy increase of satellite cells and some Nageotte bodies in DRG. Lymphoplasmocytic aggregates were occasionally seen in all five animals. In three samples out of four containing distinctive dorsal and ventral roots, the inflammatory changes were more prominent in the dorsal roots.

3.3.3. Muscle pathology

A total of 24 muscle biopsies were sampled from 7 cases (supplementary item 1, Table 2, No. 2, 3, 6, 7, 11, 15, 16). All samples showed similar changes, namely~~had~~ occasional fibres with euchromatic peripheral nuclei, and degrees of very mild to moderate multifocal myofibre atrophy with mild small group atrophy and occasional anguloid fibres. Intramuscular axons were rarely observed and appeared normal. Overtly angular fibres were rare, however in four of the cases the changes may be significant enough to be due to mild denervation. Two cases showed marked atrophy of both fibre types. One of these had been recumbent for a significant amount of time and additionally showed occasional single fibre necrosis of hypertrophic fibres. In no samples was there evidence of arteritis, cellular infiltrate or apparent replacement of fibres with adipose or fibrous tissue.

4. Discussion

Although the total number of AEP affected horses is not very high, it is the most prevalent polyneuropathy in horses in a geographically restricted area. ~~AEP is a highly prevalent emerging, but geographically restricted, polyneuropathy in horses that~~ It presents uniformly ~~presents~~ with knuckling in the metatarsophalangeal joints due to extensor weakness~~paresis~~ or flexor-extensor incoordination. This study identified a high level of pathomorphological homogeneity amongst the multiple investigated nerves, biopsy sites and individuals

throughout the affected farms in Scandinavia. Very much like our first observation in nerves from a Finnish AEP affected horse, the cases from Norway and Sweden presented predominantly with a hitherto undetermined inclusion body schwannopathy and recurrent inflammatory demyelination. According to the lack of respective neurological and veterinary reportsliterature and our own laboratory files, comprising several thousand clinical cases since the 1980's, a similar bimodal neuropathy has not been recognized previously. In equids, the closest reported equivalent to AEP is a knuckling neuropathy described in three young horses from Japan (13, 14). Most of the resemblance herein refers to the clinical presentation, demyelinating-remyelinating features and some Wallerian degeneration seen predominantly in large myelinated fibers (13, 14). However, there is no reference in the Japanese case studies to Schwann cell changes and inflammatory features similar to what we see in AEP. In contrast to AEP, moreover, Japanese cases also demonstrated denervation of limb muscles. In Nordic horses affected by AEP, evidence of denervation was only subtle and inconsistent. Even the surviving horse that presented with disability grade III on sampling, showed disuse atrophy and paresis of the muscle due to demyelination rather than denervation. Hence, the dropout of large myelinated fibres in AEP nerves is supposed to result from a decay of Ia/Ib afferents rather than motor axons. Credence to this hypothesis is lent by the relative preservation of spinal ventral roots if compared to dorsal roots in a smaller series of cases (not shown).

Similar to the fibre dropout, schwannopathic features mainly were restricted to those cells enveloping large myelinated fibres. In contrast to the earlier case with aggregates seen in the rough endoplasmic reticulum (rER) (3), large cytoplasmic Schwann cell inclusions were not membrane bound at time of sampling. On the other hand, they consistently stained immunopositive for BiP/GRP78, indicating that the inclusions indeed may result from defective posttranslational protein processing, irrespective of rER membrane preservation. BiP/GRP78 belongs to the group of peptide-binding molecular chaperones that interact with

protein-folding intermediates to prevent protein aggregation by keeping it in a folding-competent state (15). Chaperones guarantee that only properly assembled and folded proteins are able to leave the rER, while unfolded or misfolded proteins will accumulate, awaiting proteosomal degradation. Several circumstances such as macromolecular crowding, oxidative stress, exposure to toxins, and aging may impair protein folding and/or affect rescue mechanisms such as ubiquitination/proteosomal activity and autophagy (16). Consequently, the triggers of AEP appears either to directly interfere with protein folding and rescue mechanisms or incite one of the named prerequisite disturbances. Even though a toxic principle is very likely, there is no poison known to us that is likely to reproduce exactly these changes. Misfolding of proteins and pathological aggregation in experimental settings also are known to enhance the immunogenicity of proteins explaining the autoimmune side effects of certain drugs and nanoparticles (17). Sporadic inclusion body myositis (sIBM) is a natural example of how misfolding and dysfunctional proteosomal pathways may lead to cellular autoimmune responses (16). Sporadic inclusion body myositis is the most common human myopathy presenting over the age of 40 years. Respective muscle fibre inclusions also stain positive for peptide-binding chaperones, disulfide isomerases and lectin chaperones, all of which individually document unfolding and/or misfolding of peptide chains and glycoproteins. Sporadic inclusion body myositis is an acquired immune-mediated myopathy, but the susceptibility to sIBM and progression of disease appear to segregate with certain HLA haplotypes (18, 19). The employed immune effector cascades recruit cytotoxic T cells and autoantibodies. That autoimmunity does not tell the whole story has been nicely demonstrated by the general failure of immunosuppressive treatment in sIBM (20). Even though the comparison is tempting, AEP epidemiology does not indicate an MHC haplotype association (4). It also is not restricted to a certain age segment but affects all breeds, ages and sexes non-selectively. Moreover, in contrast to sIBM, there is no exact match between the extent of histopathological damage and clinical disability. This renders an unseen factor

likely, one that interferes with nerve fibre~~sensory~~ function at the level of impulse conduction or neurotransmission. Hence, even if myelination is maintained, affected Schwann cells may be partially dysfunctional. Factors may e.g. interfere with transmembranous transport and detoxification at paranodes and Schmidt-Lanterman clefts (21). Alternatively, nerve conduction may be impaired at the level of nodal axolemma or within the dorsal root ganglia (DRG). Preliminary DRG investigations indeed revealed occasional degeneration of sensory neurons in single AEP cases. Moreover, in the context of autoimmunity, humoral factors that not necessarily lead to cell-mediated myelinotoxicity require consideration. Such soluble factors are involved in cases of Guillain-Barré syndrome (GBS) in people (22). Axonal conduction block can be caused by antibodies neutralizing transient voltage-gated Na^+ channels clustered at the node of Ranvier (23). Immunmodulatory treatment may remove antibodies or other factors inferring with Na^+ channel function improving nerve function ahead of possible structural restoration (22). Weak immunopositivity for intralesional immunoglobulins and the lack of correlation between the nerve fiber damage and the clinical impairment, render humoral immune mechanisms in AEP possible. Clarification as to whether these comprise anti-ganglioside antibodies as in GBS (24) awaits the availability of species specific serological tests for neural autoantibodies.

Peripheral nerve lesions in AEP cases are far more widespread than the clinical picture would suggest. This for example is evident in the recurrent laryngeal nerve, the longest ~~peripheral~~ nerve in equids, where the observed pathological lesions would be expected to compromise laryngeal function causing stridor. However, this has neither been observed clinically by roaring nor has endoscopy performed in some AEP affected horses shown any laryngeal paresis. A slap-test (25) has been performed in most cases examined by the authors, but only rarely have a decreased leftsided reflex been noted (4). Mild symmetrical laryngeal hemiplegia could however go unnoticed if the horse is not exercised.

Investigation of nerve samples~~biopsies~~ allows for a specific AEP diagnosis and exclusion of relevant differential diagnoses, but it does not reflect the extent of dysfunction nor the clinical outcome. A more stringent evaluation of the neurophysiological impact of AEP pathology would require electrodiagnostics. In humans and small animals, electrophysiological investigations, rather than nerve biopsy, provide important determinants for peripheral nerve diagnosis as in the clinical work-up of GBS (22, 26). In horses, nerve conduction studies require~~implicate~~ general anesthesia or deep sedation, both of which relaxes the horse to a point where knuckling is easily induced ~~may worsen the clinical signs in AEP~~ and thus were declined by the owners. Diagnosis of mild and early AEP can therefore be challenging since it purely depends on observation of knuckling, which may happen intermittently and easily be missed by the owner and veterinarian. Thereby, estimation of disease duration can be underestimated, unless the animals were in daily use at disease onset, as in the present study.

Neuropathies in humans are generally classified as acute if the time from onset to peak of signs is less than four weeks (22, 26, 27), while a clinical course over more than eight weeks is considered chronic (28, 29). Although not fully comparable since euthanasia ended the clinical course, we concluded that the six horses that were euthanized within four weeks were clinically in the acute phase of the disease. At odds with the short clinical disease-history however, histopathology featured chronic changes mainly such as onion bulbs. This corresponds to the acute onset seen in up to 16% of human patients diagnosed with chronic inflammatory demyelinating polyneuropathy (30, 31) and sporadically described in animals (32). As lesions progress and maybe converge, secondary features such as loss of fibres, secondary type of Wallerian degeneration, may mask the primary mode of lesion. The lag between induction and clinical manifestation of AEP further compromises the retrospective analysis of exposure to environmental factors such as feed, toxins and infectious pathogens.

The acute cases comprised the clinically most severely affected horses; five out of six were grade III or IV at the time of euthanasia. In the chronic group, only one horse was grade IV, the remaining seven were all grade I at the point of sampling. Interestingly, the significant difference between the two groups, with more extensive myelinated fiber loss and compound axonal pathologies in the acute group remained true also for the only grade I horse in the acute group and the grade IV case in the chronic group. The intercorrelation between fibre-directed infiltrates as well as Wallerian degeneration and clinical impairment in the acute group may very well be biased because of few horses included and only one mildly affected horse in this group. There was no correlation when comparing severity grades and infiltrates in all cases, disregarding disease duration. As much as the clinical examination focuses on disability, the grade of clinical compromise does not necessarily predict the disease course or outcome, as demonstrated by the various severity degrees and disease duration in the included cases. Indeed, with dedicated owners and cooperative patients many horses will overcome the disease, independent of the grade on admission or the maximal score of disability during the observational period (4, 33). As long as the animals rise and are able to stand with or without assistance every 24 hours (see supportive online material), full recovery may be possible. As nicely demonstrated in case No. 16, a show jumper pony, even horses with grade IV clinical signs may return to full performance levels. A transient and/or low exposure to AEP triggers may result in transient and mild clinical signs. The timeline of prodromal disease development however is unclear and peripheral nerves in horses that have fully recovered from AEP have yet to be investigated.

In conclusion, histopathological findings in AEP affected horses are strikingly similar despite variation in clinical severity and duration of disease at sampling, and comprise a re- and demyelinating, predominantly large fibre, neuropathy with conspicuous Schwann cell

inclusions. In contrast, muscle biopsies present with surprisingly mild changes. The aetiology remains unclear but an environmental toxin resembles the most likely pathogen.

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Reference List

1. Hanche-Olsen S, Teige J, Skaar I, Ihler CF. Polyneuropathy associated with forage sources in Norwegian horses. J Vet Intern Med. 2008;22(1):178-84.

2. Gustafsson K, Ronéus M. (Outbreaks of neurologic disorders in horses). Sven Vet Tidning. 2000;52(5):253-9.

3. Hahn CN, Matiasek K, Syrja P, Jokinen TS, MacIntyre N, Tulamo RM. Polyneuropathy of Finnish horses characterised by inflammatory demyelination and intracisternal Schwann cell inclusions. Equine Vet J. 2008;40(3):231-6.

4. Gröndahl G, Hanche-Olsen S, Bröjer J, Ihler CF, Jäderlund KH, Egenvall A. Acquired equine polyneuropathy in Norway and Sweden: a clinical and epidemiological study. Equine Vet J Suppl. 2012(43):36-44.

5. Wolff C, Egenvall A, Hanche-Olsen S, Gröndahl G. Spatial and temporal distribution of incidence of acquired equine polyneuropathy in Norway and Sweden, 1995-2012. BMC Vet Res. 2014;10:265.

6. Telama H, Alho J, Virtala A-M, Tulamo R-M. Acquired equine polyneuropathy- a review and an account of Finnish outbreaks. Suomen Eläinlääkärilehti. 2011;117(5):301-8.

7. Hanche-Olsen S. Acquired equine polyneuropathy - clinical, pathological and epidemiological aspects [Philosophiae Doctor]. Oslo, Norway: Norwegian University of Life Sciences; 2017.

8. Fjordbakk CT, Strand E, Hanche-Olsen S. Surgical and conservative management of bilateral dynamic laryngeal collapse associated with poll flexion in harness race horses. Vet Surg. 2008;37(6):501-7.

9. Kaemmer D, Bozkurt A, Otto J, Junge K, Klink C, Weis J, et al. Evaluation of tissue components in the peripheral nervous system using Sirius red staining and immunohistochemistry: a comparative study (human, pig, rat). J Neurosci Methods. 2010;190(1):112-6.

10. Wieczorek LA. Nerve teasing as a diagnostic aid in detection of peripheral neuropathies: methodology and interpretation [Dissertation]. Munich: Ludwig-Maximilians-University; 2002.

11. Gross S, Fischer A, Rosati M, Matiasek L, Corlazzoli D, Cappello R, et al. Nodo-paranodopathy, internodopathy and cleftopathy: Target-based reclassification of Guillain-Barre-like immune-mediated polyradiculoneuropathies in dogs and cats. Neuromuscul Disord. 2016;26(12):825-36.

12. Pamphlett R, Sjarif A. Is quantitation necessary for assessment of sural nerve biopsies? Muscle Nerve. 2003;27(5):562-9.

13. Furuoka H, Mizushima M, Miyazawa K, Matsui T. Idiopathic peripheral neuropathy in a horse with knuckling. Acta Neuropathol. 1994;88(4):389-93.

14. Furuoka H, Okamoto R, Kitayama S, Asou S, Matsui T, Miyahara K. Idiopathic peripheral neuropathy in the horse with knuckling: muscle and nerve lesions in additional cases. Acta Neuropathol. 1998;96(4):431-7.

15. Chambers JE, Marciniak SJ. Cellular mechanisms of endoplasmic reticulum stress signaling in health and disease. 2. Protein misfolding and ER stress. Am J Physiol Cell Physiol. 2014;307(8):C657-70.

16. Vattemi G, Engel WK, McFerrin J, Askanas V. Endoplasmic reticulum stress and unfolded protein response in inclusion body myositis muscle. Am J Pathol. 2004;164(1):1-7.

17. Ratanji KD, Derrick JP, Dearman RJ, Kimber I. Immunogenicity of therapeutic proteins: influence of aggregation. J Immunotoxicol. 2014;11(2):99-109.

18. Mastaglia FL. Sporadic inclusion body myositis: variability in prevalence and phenotype and influence of the MHC. Acta Myol. 2009;28(2):66-71.

19. Needham M, James I, Corbett A, Day T, Christiansen F, Phillips B, et al. Sporadic inclusion body myositis: phenotypic variability and influence of HLA-DR3 in a cohort of 57 Australian cases. J Neurol Neurosurg Psychiatry. 2008;79(9):1056-60.

20. Gang Q, Bettencourt C, Machado P, Hanna MG, Houlden H. Sporadic inclusion body myositis: the genetic contributions to the pathogenesis. Orphanet J Rare Dis. 2014;9:88.

21. Scherer SS, Deschênes SM, Xu YT, Grinspan JB, Fischbeck KH, Paul DL. Connexin32 is a myelin-related protein in the PNS and CNS. J Neurosci. 1995;15(12):8281-94.

22. Vucic S, Kiernan MC, Cornblath DR. Guillain-Barre syndrome: an update. J Clin Neurosci. 2009;16(6):733-41.

23. Weber F, Rüdel R, Aulkemeyer P, Brinkmeier H. Anti-GM1 antibodies can block neuronal voltage-gated sodium channels. Muscle Nerve. 2000;23(9):1414-20.

24. Dilley A, Gregson NA, Hadden RD, Smith KJ. Effects on axonal conduction of anti-ganglioside sera and sera from patients with Guillain-Barre syndrome. J Neuroimmunol. 2003;139(1-2):133-40.

25. Greet TR, Jeffcott LB, Whitwell KE, Cook WR. The slap test for laryngeal adductory function in horses with suspected cervical spinal cord damage. Equine Vet J. 1980;12(3):127-31.

26. Asbury AK, Cornblath DR. Assessment of current diagnostic criteria for Guillain-Barre syndrome. Ann Neurol. 1990;27 Suppl:S21-4.

27. Van der Meché FG, Van Doorn PA, Meulstee J, Jennekens FG. Diagnostic and classification criteria for the Guillain-Barre syndrome. Eur Neurol. 2001;45(3):133-9.

28. Research criteria for diagnosis of chronic inflammatory demyelinating polyneuropathy (CIDP). Report from an Ad Hoc Subcommittee of the American Academy of Neurology AIDS Task Force. Neurology. 1991;41(5):617-8.

29. Barohn RJ, Kissel JT, Warmolts JR, Mendell JR. Chronic inflammatory demyelinating polyradiculoneuropathy. Clinical characteristics, course, and recommendations for diagnostic criteria. Arch Neurol. 1989;46(8):878-84.

30. McCombe PA, Pollard JD, McLeod JG. Chronic inflammatory demyelinating polyradiculoneuropathy. A clinical and electrophysiological study of 92 cases. Brain. 1987;110 (Pt 6):1617-30.

31. Trojaborg W. Acute and chronic neuropathies: new aspects of Guillain-Barre syndrome and chronic inflammatory demyelinating polyneuropathy, an overview and an update. Electroencephalogr Clin Neurophysiol. 1998;107(5):303-16.

32. Molin J, Márquez M, Raurell X, Matiasek K, Ferrer I, Pumarola M. Acute clinical onset chronic inflammatory demyelinating polyneuropathy in a dog. Muscle Nerve. 2011;44(3):441-4.

33. Hanche-Olsen S, Kielland C, Ihler CF, Hultin Jaderlund K. Long-term follow-up of Norwegian horses affected with acquired equine polyneuropathy. Equine Vet J. 2017.