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DISEASE IN WILDLIFE OR EXOTIC SPECIES

Hepatic Amyloidosis in a Chronically Entangled Grey Seal (*Halichoerus grypus*)

James E F Barnett[*], Janet A Gilbertson[†], Natalie Arrow[‡], Julian D Gillmore[†], Philip N Hawkins[†], Lizzi Larbalestier[‡], Dan Jarvis[‡], Sue Sayer[§] and Mark E Wessels[¶]

** Cornwall Marine Pathology Team, Falmouth, Cornwall, [†] National Amyloidosis Centre, UCL and Royal Free London NHS Foundation Trust, London, [‡] British Divers Marine Life Rescue, Uckfield, East Sussex, [§] Seal Research Trust, Hayle, Cornwall and [¶] Finn Pathologists, Weybread, Norfolk, UK*

Summary

Grey seal (*Halichoerus grypus*) entrapment in fishing gear is well documented, consisting of two forms: peracute underwater entrapment and chronic entanglement. We now highlight a previously undescribed sequela to chronic entanglement in a female grey seal estimated to be at least 2 years of age. The animal was first observed in September 2018 on the coast of north Cornwall, southwest England, with a large encircling neck wound consistent with monofilament net entanglement. In April 2021, it was admitted for attempted rehabilitation but had to be euthanized after 9 days due to clinical deterioration despite treatment. At post-mortem examination, the seal was in poor nutritional state, the nose to flipper length was low for its estimated age and the liver was markedly enlarged, pale and friable in texture with evidence of recent and previous hepatic haemorrhage. Histopathology revealed hepatic amyloidosis and evidence of amyloid in one kidney and one adrenal gland. Proteomic analysis of microdissected amyloid from the liver indicated type AA amyloid. Chronic entanglement is the most plausible cause of AA amyloidosis in this animal, indicating that amyloidosis should be considered as a pathological sequela and welfare concern associated with chronic entanglement of grey seals.

Keywords: AA amyloidosis; grey seal; marine mammal; net entanglement

Grey seal (*Halichoerus grypus*) entrapment in fishing gear in European waters is well documented (Baker *et al*, 1998; Vincent *et al*, 2005; Allen *et al*, 2012; Osinga *et al*, 2012; Cosgrove *et al*, 2016; Barnett *et al*, 2021). There are two recognized forms of entrapment in fishing gear: peracute underwater entrapment leading to near-immediate death, and chronic entanglement. The latter potentially includes seals caught during active fishing operations that are partially freed from fishing gear and animals that have interacted with, and become entangled in, lost fishing gear.

Amyloidosis is a general term used to describe a group of diseases caused by the pathological extracellular deposition of protein in a specific characteristic fibrillar conformation in various tissues throughout the body. Amyloid deposits disrupt the structure and function of affected organs and appear on light microscopy as an amorphous, eosinophilic, hyaline, extracellular substance. When stained with Congo red, they appear pink and produce diagnostic green birefringence when viewed under cross-polarized light. Some 20 or so proteins can form amyloid fibrils, but reactive systemic amyloidosis (type AA) is probably the most common form in animals (Miller and Zachary, 2017).

Correspondence to: J E F Barnett (e-mail: jamesbarnettvet@gmail.com).

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Serum amyloid A (SAA) is an apolipoprotein that is formed primarily in the liver following stimulation by proinflammatory cytokines, and one of its roles is as a chemoattractant in inflammatory processes (Woldemeskel, 2012). SAA is present in most species at trace levels of just a few mg/L, but production is up-regulated by up to several 1,000-fold as a response to a wide variety of acute phase stimuli, including infections, inflammation, neoplasia and most other types of significant tissue damage. AA amyloid deposition eventually occurs in a proportion of animals in which there is a sustained elevated concentration of SAA. It is the most common form of systemic amyloidosis in domestic animals and has also been reported in a wide range of non-domestic species (Cullen and Stalker, 2016).

Amyloidosis has been reported in grey seals. Reckendorf (2019) documented cases of systemic amyloidosis in grey seals and Bergman *et al* (2001) described renal amyloidosis in three grey seals. One of the authors has also detected one other case of systemic amyloidosis and one case of splenic amyloidosis in grey seals from southwest England (MEW, unpublished observations). In the reported cases, the presence of amyloid appeared to be an incidental finding. We now describe, to the best of our knowledge, the first case of confirmed systemic, primarily hepatic type AA amyloidosis, which had a significant clinicopathological effect on the seal and which is likely to have occurred secondary to chronic entanglement in this species.

A female grey seal was first identified in September 2018 on the north coast of Cornwall, southwest England, with a large encircling neck wound consistent with monofilament net entanglement. Using photographic identification methods (Sayer *et al*, 2019) this seal was subsequently recorded 25 times over a period of just over 2.5 years. In December 2018, when it was possible to identify the seal and compare its length with juveniles that were known to be going through their first long annual moult at 12–18 months of age, the animal was estimated to be at least 2 years of age. In April 2021, at a presumed age of over 4 years (from her photographic identification records and length comparison), the seal was found trapped between rocks on a beach and was admitted for attempted rehabilitation due to the entanglement wound and her weakened physical state. At the time of rescue the seal weighed 44 kg and was lethargic, dehydrated, in poor body condition and easily handleable. Monofilament netting was found within the encircling wound, embedded up to a depth of 4 cm over the dorsal neck, but the granulating wound appeared largely free of active infection. During rehabilitation, the seal's treatment included oral fluids progressing to fish, intramuscular long-acting amoxicillin (Clamoxyl LA; Zoetis, www.zoetis.com), intramuscular meloxicam (Loxicom; Norbrook, www.norbrook.com) and regular flushing and cleaning of the wound with saline and dilute chlorhexidine (Hibiscrub; Mölnlycke, www.molnlycke.co.uk). After 9 days, the animal's condition deteriorated. A blood sample taken at this time revealed a poorly regenerative anaemia, hypoalbuminaemia, elevated gamma glutamyl transferase and aspartate transaminase activities compared with published reference intervals (Barnett and Bexton, 2016) and glutamate dehydrogenase activity and non-esterified fatty acid concentrations that were elevated when compared with those in domestic species (Table 1). Due to the seal's deteriorating condition, the decision was taken to carry out euthanasia.

At post-mortem examination, the seal weighed 43.5 kg and was 146.5 cm long from the tip of the nose to the tip of the hind flippers. The nose to flipper length was notably less than the expected length of a female grey seal of 4 years of age (approx. 175 cm; Hewer, 1964). The animal was in a poor nutritional state, with a sternal blubber thickness of only 1 mm, and the net wound encircling the neck had evidence of healing with epithelial proliferation of the edges (Fig. 1). The lungs were congested, oedematous and emphysematous, with emphysema extending into the mediastinum, pericardium, parietal pleura and the fascial planes between muscles ventral to the cervical vertebrae and dorsal to the oesophagus and trachea. There was a moderate nasal mite (*Halarachne halichoeri*) burden and a heavy ascarid worm infestation in the stomach, with associated gastric ulceration. These ascarids were not speciated but the species of ascarid nematode previously found in grey seals in southwest England are *Contracaecum osculatum* and *Anisakis simplex* (Barnett *et al*, 2000). The liver was markedly enlarged, weighing 9 kg, pale and friable in texture (Fig. 2). A haematoma, up to 1.5 cm deep, was present beneath the liver capsule along the entire cranial surface of the right medial cranial lobe, radiating scars were present on the cranial and caudal surfaces of the right lateral cranial lobe and there was subcapsular haemorrhage at the hilus. Approximately 2 L of blood were free in the peritoneal cavity.

Samples were collected for histopathology in 10% neutral buffered formol saline, processed routinely and embedded in paraffin wax. Sections (4 μm) were stained with haematoxylin and eosin (HE) and Congo red. Histopathological examination of the liver revealed diffuse loss of normal hepatic architecture characterized by abundant deposition of pale staining hyaline eosinophilic extracellular material

Table 1
Haematological and serum biochemical findings in a chronically entangled grey seal

Parameter (units)	*Result*	*Reference interval**
Red blood cells ($\times 10^{12}$/L)	3.03	4.00–7.00
Haemoglobin (g/dl)	10.3	17.0–24.0
Packed cell volume (10%)	33.0	45.0–70.0
MCV (fl)	108.9	90.0–130.0
MCH (pg)	34.0	30.0–0.0
MCHC (g/dl)	31.2	30.0–40.0
Platelets ($\times 10^9$/L)	685	180–780
White blood cells ($\times 10^9$/L)	9.5	5.0–19.0
Neutrophils ($\times 10^9$/L)	7.7	2.0–12.0
Band neutrophils ($\times 10^9$/L)	0.0	Not available
Lymphocytes ($\times 10^9$/L)	1.4	0.0–6.0
Monocytes ($\times 10^9$/L)	0.2	0.0–3.0
Eosinophils ($\times 10^9$/L)	0.2	0.0–2.0
Basophils ($\times 10^9$/L)	0.0	0.0–1.0
Reticulocyte count ($\times 10^9$/L)	18	Not available
Total protein (g/L)	73.0	50.0–90.0
Albumin (g/L)	27.5	29.0–50.0
Urea (mmol/L)	15.8	7.0–22.0
Creatinine (μmol/L)	22	0–100
Aspartate transaminase (U/L @ 37°C)	293	0–200
Glutamate dehydrogenase (U/L @ 37°C)	209	Not available
Alkaline phosphatase (U/L @ 37°C)	155	0–600
Gamma glutamyl transferase (U/L @ 37°C)	797	0–100
Non-esterified fatty acids (μmol/L)	970	Not available

MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration.
*Barnett and Bexton (2016).

within sinusoids associated with disruption of hepatic cords and marked hepatocyte atrophy. The extracellular material stained with Congo red (Fig. 3) and had strong apple green birefringence under cross-polarized light, diagnostic of amyloid. Marked multifocal haemorrhage effaced the hepatic architecture, predominantly in subcapsular sites, with mild parenchymal haemorrhage. Within one section there was a focally extensive area of fibrosis with a multifocal mild lymphohistiocytic perivascular infiltrate.

Within the cortex of one adrenal gland there were multifocal areas characterized by disruption of normal sinusoids by deposition of similar eosinophilic hyaline extracellular material that stained with Congo red, resulting in separation of adrenal cortical cells. Pericapsular blood vessels had marked intramural amyloid deposition. A multifocal, minimal to mild, chronic, non-suppurative, lymphohistiocytic tubulointerstitial nephritis was also present and associated with positive staining with Congo red. Segmentally in glomeruli there were small red-stained deposits that expanded the mesangium and multifocal minimal to mild areas of similar staining in the medullary interstitium, all of which showed apple green birefringence.

Additional findings included: severe, chronic active, ulcerative and fibrosing dermatitis associated with the edges of the encircling net wound; marked pulmonary congestion with variable atelectasis; patchy alveolar oedema; multifocal interstitial oedema; and interstitial emphysema.

Amyloid investigation was performed at the UK National Amyloidosis Centre. Semi-serial formalin-fixed, paraffin-embedded (FFPE) liver tissue sections (2 μm or 6 μm) were used to determine the amyloid type.

Immunohistochemistry (IHC) labelling of the amyloid deposits was performed on 2 μm FFPE deparaffinized tissue sections using a panel of commercially available monospecific antibodies (Agilent, www.agilent.com), reactive with SAA protein, transthyretin and kappa and lambda immunoglobulin light chain. IHC was also performed using an in-house rabbit anti-human polyclonal antibody against human SAA protein (Hazenberg *et al*, 1990). IHC was performed on a manual platform using Impress detection kits with a Metal Enhanced 3,3'-diaminobenzidine Substrate Kit (Thermo Scientific; www.thermofisher.com) for visualizing the immunoreaction to determine the amyloid fibril type. Positive and negative controls were used in parallel. All sections from the case resulted in negative staining.

An alternative approach, proteomic analysis using laser dissection proteomic mass spectrometry, was undertaken. This method enables identification of amyloid as well as other proteins that are present within the sample (Canetti *et al*, 2020). For proteomic analysis, amyloid was laser dissected from 6 μm FFPE Congo red-stained liver tissue sections and captured into Eppendorf tubes using a Leica LM 7000 microscope (Leica, www.leica-microsystems.com). The captured amyloid was processed using trypsin as the digestion enzyme (Taylor *et al*, 2019), run on a mass spectrometer and data evaluated using MASCOT software to search the SWISS-PROT grey seal taxonomy (Mammalia) database. The amyloid protein identified was strongly suggestive of AA amyloid.

To the authors' knowledge, this is the first report of systemic type AA amyloidosis secondary to chronic entanglement in any pinniped species. The severe hepatic amyloidosis would account for the marked

Fig. 1. Monofilament net wound encircling neck of a chronically entangled grey seal.

hepatomegaly seen at necropsy and is also the likely cause of the hepatic rupture and haemorrhage, a sequela recorded in many species (Cullen and Stalker, 2016). Clinically, amyloid deposition in the liver would have had a profound effect on the seal's body condition through disruption of normal function (Pinney and Hawkins, 2012; Woldemeskel, 2012) including intermediary metabolism. A further key role of the liver is the production of insulin-like growth factors (IGFs) and, in mice, the lack of IGF-1 was found to lead to severe growth retardation, with the size of bones of the axial and appendicular skeletons being >25% less than in wild-type littermates (Yakar *et al*, 2018). If amyloid accumulation had occurred over a number of years, the effect on hepatic function could have had a significant impact on the animal's growth rate, as grey seal females continue to grow until they are at least 10 years of age (Hewer, 1964; Hauksson, 2007), and this is likely to have contributed to the seal's small size for its estimated age. Other likely factors contributing to the poor growth and body condition would include chronic protein loss from the cervical ulceration and difficulty in feeding, with possible social factors influencing access to feeding sites.

Why grey seals are the only species of phocid in which amyloidosis has been reported is unclear. Even in grey seals, a relatively small number of cases of amyloidosis have been reported, compared with the large number of inflammatory lesions reported in this species, at least on British coasts (Baker *et al*, 1998; Barnett *et al*, 2021). A similar situation has been found in humans, with less than 900 cases of AA amyloidosis in the whole of the UK over a period of more than 30 years despite the huge range of chronic inflammatory diseases that occur frequently in the general population (Ravichandran *et al*, 2020). The reason for this is again unknown, although an increased incidence of AA amyloidosis (approximately 10%) has been seen in people with rare genetic life-long underlying chronic inflammatory disorders (Lachmann *et al*, 2014). Similarly, in Shar Pei dogs, an unusually high incidence of AA amyloidosis has been attributed to the presence of an inherited underlying chronic inflammatory disorder causing a life-long elevation of SAA (Metzger *et al*, 2017).

The initial negative IHC results obtained in this study were anticipated. The use of monospecific antibodies towards human antigens to determine amyloid type in a seal is highly unlikely to be effective due to the specific binding of the antibody to a human antigen and not that of a seal.

Chronic entanglement was clearly a cause of chronic inflammation in this seal and therefore it is

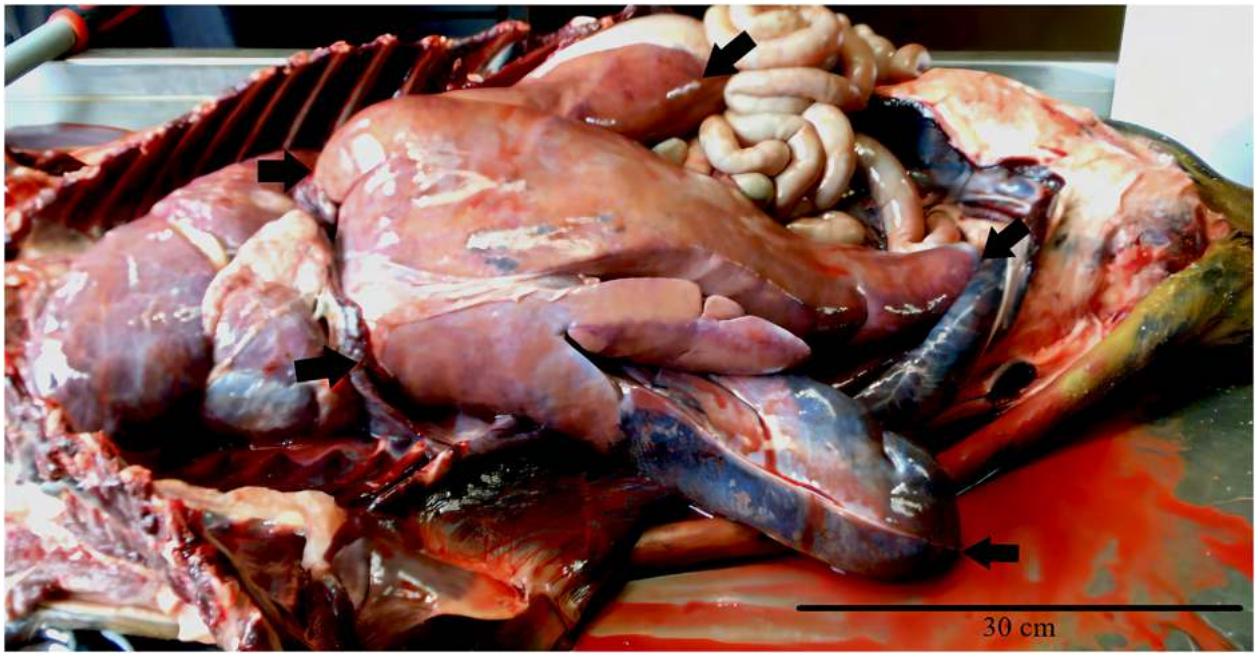

Fig. 2. Amyloidosis, liver, grey seal. Markedly enlarged, pale liver. Margins of liver (arrows). Extensive haematoma beneath capsule of right lobe.

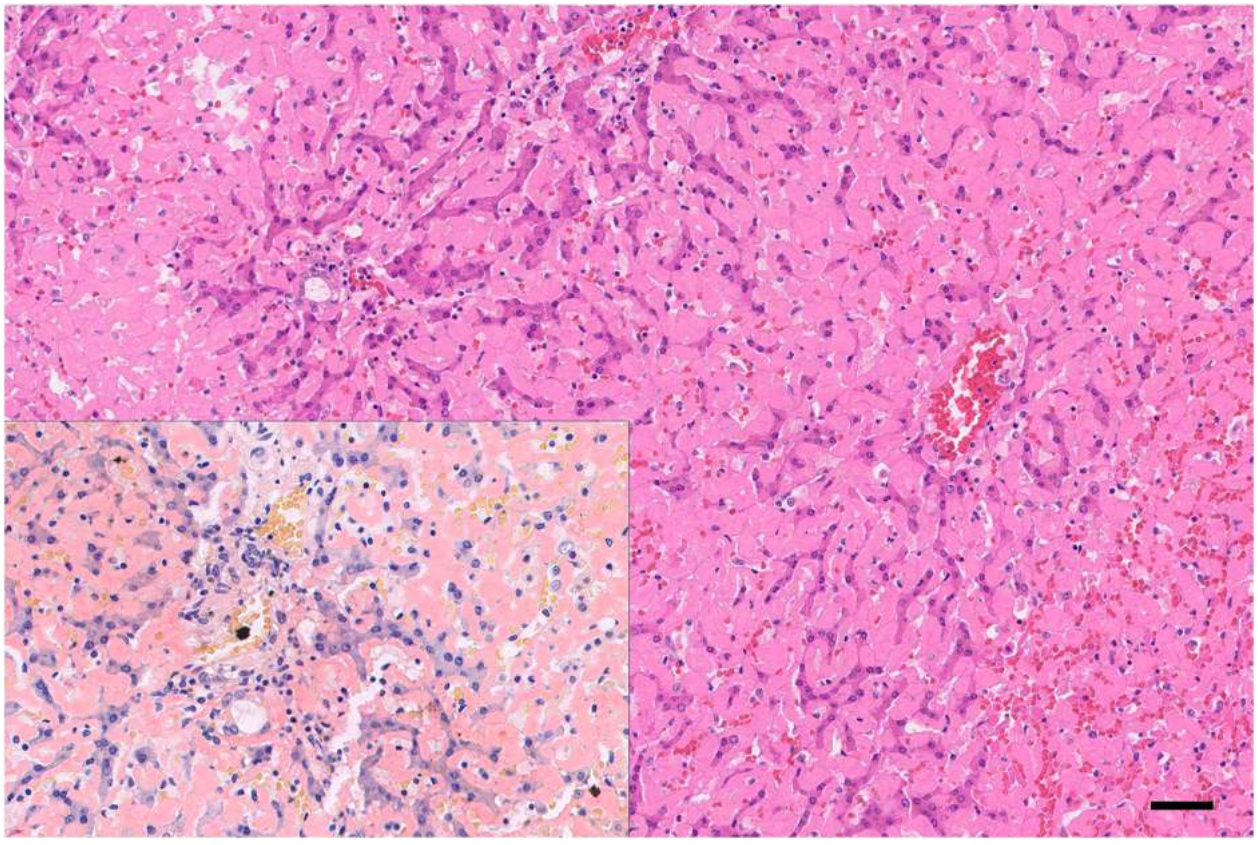

Fig. 3. Amyloidosis, liver, grey seal. Effacement of normal hepatic architecture due to extensive accumulation of extracellular amyloid within space of Disse, and severe hepatocyte loss and atrophy. HE. Bar, 50 μm. Insert: amyloid deposits stained with Congo red.

the most plausible explanation for the induction and progression of type AA amyloidosis and its subsequent effects on growth and nutritional state in this animal. Previously reported potential welfare implications of chronically entangled animals have included distress, pain, trauma, infection, soft tissue lesions and effects on mobility, feeding and behaviour (Butterworth, 2016). It clear that amyloidosis now needs to be considered as a further pathological sequela and welfare concern associated with chronic entanglement of grey seals.

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