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LETTER TO THE EDITOR

PRION SEARCH AND CELLULAR PRION PROTEIN EXPRESSION IN STRANDED DOLPHINS

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The recent description of a prion disease (PD) case in a free-ranging bottlenose dolphin (*Tursiops truncatus*) prompted us to carry out an extensive search for the "disease-associated" isoform (PrP^{Sc}) of the cellular prion protein (PrP^{C}) in the brain and in a range of lymphoid tissues from 23 striped dolphins (*Stenella coeruleoalba*), 5 bottlenose dolphins and 2 Risso's dolphins (*Grampus griseus*) found stranded between 2007 and 2012 along the Italian coastline. Three striped dolphins and one bottlenose dolphin showed microscopic lesions of encephalitis, with no evidence of spongiform brain lesions being detected in any of the 30 free-ranging cetaceans investigated herein. Nevertheless, we could still observe a prominent PrP^{C} immunoreactivity in the brain as well as in lymphoid tissues from these dolphins. Although immunohistochemical and Western blot investigations yielded negative results for PrP^{Sc} deposition in all tissues from the dolphins under study, the reported occurrence of a spontaneous PD case in a wild dolphin is an intriguing issue and a matter of concern for both prion biology and intra/inter-species transmissibility, as well as for cetacean conservation medicine.

The recent description of a naturally occurring prion disease (PD), or transmissible spongiform encephalopathy (TSE), in a free-ranging bottlenose dolphin (*Tursiops truncatus*) (1) is an intriguing

finding and a matter of concern for a number of reasons. First of all, apart from this being the first report of a spontaneous PD condition in any cetacean and, more in general, in any aquatic mammal, it should be also emphasized that the prion protein (PrP) gene (*PRNP*) sequence from eight cetacean species has previously been shown to be very close to that of cattle, sheep and goat (2), thus providing additional support to the existence of a common ancestor for ruminants and cetaceans (3).

Secondly, on the basis of the high sequence homology between cetacean *PRNP* gene and that of the aforementioned ungulate species, which may naturally develop bovine spongiform encephalopathy (BSE) and scrapie (4), susceptibility to and occurrence of TSEs in cetaceans have been deemed plausible (2). Additionally, one of the most, if not even the most intriguing feature of a TSE-like condition in a free-living cetacean refers to the route(s) through which such prion infection may have been acquired by the animal under study (1).

Following the description of this really

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impressive PD case in a wild dolphin (1), we decided to extensively investigate our collection of tissue specimens from stranded cetaceans, in order to properly assess whether any evidence of PrP^{Sc} deposition occurred within these tissues.

To accomplish this, we selected a total of 30 free-ranging odontocete cetaceans found stranded between 2007 and 2012 along the Italian coastline, 23 of which were striped dolphins (*Stenella coeruleoalba*), 5 of them being bottlenose dolphins and the remaining 2 being Risso's dolphins (*Grampus griseus*).

Three striped dolphins and one bottlenose dolphin showed microscopic lesions of encephalitis, with no evidence of spongiform brain lesions being detected in any of the 30 wild cetaceans investigated herein. More in detail, these three striped dolphins exhibited a multifocal, subacute to chronic, non-suppurative morbilliviral encephalitis, while the bottlenose dolphin showed a multifocal, non-suppurative encephalitis (secondary to concurrent *Morbillivirus* and *Toxoplasma gondii* infection), associated with a severe, *Staphylococcus aureus*-induced purulent encephalitis.

Paraffin-embedded sections, along with homogenates of the brain and a range of lymphoid tissues (spleen, mesenteric, pulmonary and prescapular lymph nodes) from the above cetaceans, were submitted to an in-depth search for the "disease-associated" isoform (PrP^{Sc}) of the cellular prion protein (PrP^{C}) by means of *ad hoc* immunohistochemistry (IHC) and Western blot (WB) investigations. More in detail, F89 (F89/160.1.5) and F99 (F99/97.6.1), two commercially available (*VMRD,* Inc., Pullman, WA, USA) anti-PrP mouse monoclonal antibodies (mAbs), were utilized in parallel for both IHC and WB analyses, with the 6H4 murine mAb (Prionics AG, Switzerland) being additionally employed for WB investigations.

Furthermore, on paraffin-embedded sections obtained from well-preserved tissue samples (brain, spleen, mesenteric, pulmonary and prescapular lymph nodes) of 10 striped dolphins and 3 bottlenose dolphins, we also tried to evaluate, by means of IHC, the expression pattern(s) of PrP^{C} within such tissues. Indeed, the preservation status represents a matter of crucial concern in stranded cetaceans, the corpses of which are frequently found in advanced *post-mortem* autolysis, a condition which may dramatically

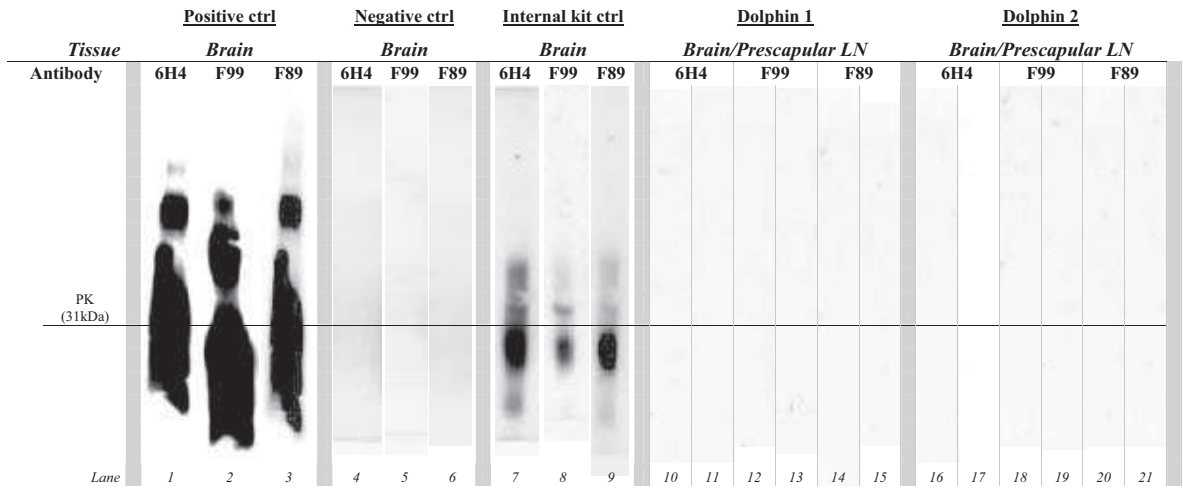

Fig. 1. *Results of Western blot (WB) investigations for PrP^{Sc} deposition in tissues (brain and lymphoid tissues) from stranded cetaceans, with WB analyses having been carried out by means of 6H4, F99 (F99/97.6.1) and F89 (F89/160.1.5) anti-PrP mouse monoclonal antibodies (mAbs). Molecular weight (MW) marker: Proteinase K (PK).* ***Lanes 1-3****: Positive control (ctrl) brain from a scrapie-affected sheep.* ***Lanes 4-6****: Negative ctrl brain from a scrapie-uninfected sheep.* ***Lanes 7-9****: Internal kit positive ctrl brain sample (Prionics AG, Switzerland).* ***Lanes 10, 12, 14****: Striped dolphin (Stenella coeruleoalba). Brain tissue samples with no evidence of PrP^{Sc} deposition.* ***Lanes 11, 13, 15****: Striped dolphin (S. coeruleoalba); same animal as in lanes 10, 12, 14. Prescapular lymph node (LN) tissue samples with no evidence of PrP^{Sc} deposition.* ***Lanes 16, 18, 20****: Striped dolphin (S. coeruleoalba). Brain tissue samples from another animal showing no evidence of PrP^{Sc} deposition.* ***Lanes 17, 19, 21****: Striped dolphin (S. coeruleoalba); same animal as in lanes 16, 18, 20. Prescapular LN tissue samples with no evidence of PrP^{Sc} deposition.*

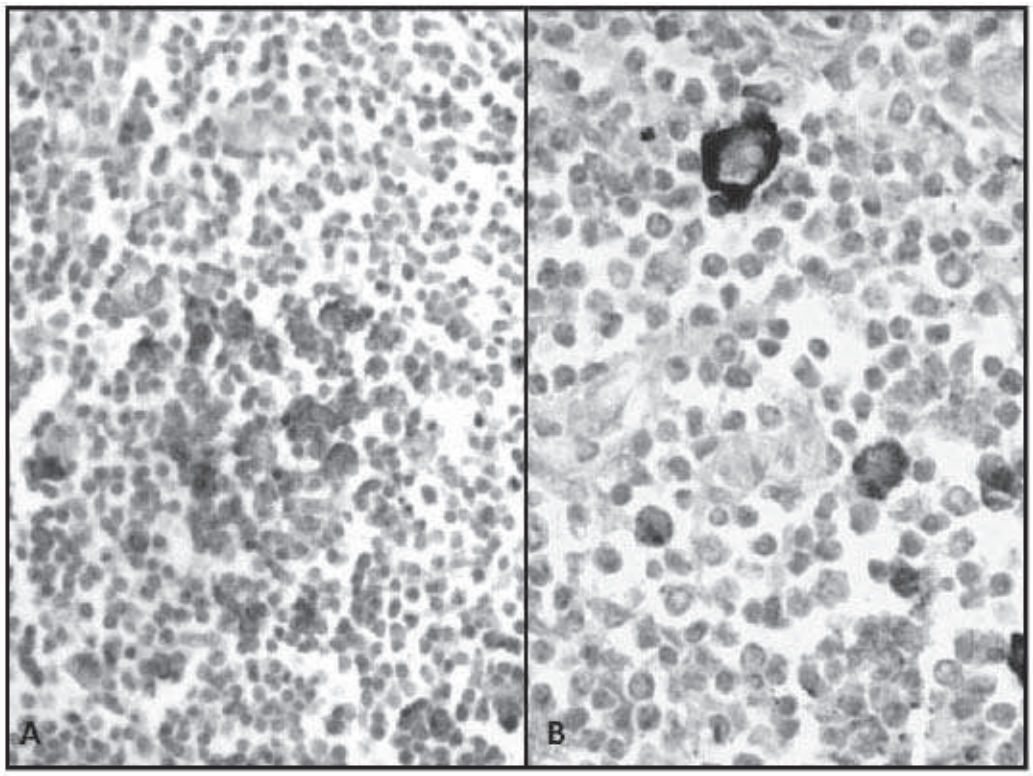

Fig. 2. *Mesenteric LN from a striped dolphin (S. coeruleoalba). A prominent PrP^C immunoreactivity (IR) is apparent in cellular elements which are topographically and morphologically consistent with follicular dendritic cells (FDCs) residing in secondary lymphoid organs and tissues (panel **A**). Prescapular LN from another striped dolphin (S. coeruleoalba). A strong PrP^C IR pattern is shown on the membrane and, to a lesser extent, within the cytoplasmic compartment of FDC-like cells (panel **B**). Final magnification: x250 (x20 objective, panel A); x500 (x40 objective, panel B). PrP immunohistochemistry (IHC) with the F99 mAb. Mayer's haematoxylin counterstain.*

affect, in its turn, the results of the evaluation of PrP^C expression within their tissues, provided that PrP^C - differently from PrP^{Sc} - is very sensitive to proteolytic digestion (4).

All IHC and WB analyses which were performed, albeit extensive, showed no evidence of PrP^{Sc} deposition in all tissues from the dolphins under study (Fig. 1). Interestingly enough, however, we were able to document a prominent PrP^C immunoreactivity (IR) at the level of the cerebral parenchyma and of the lymphoid tissues investigated herein, with such PrP^C IR pattern being particularly evident along neuronal axons and dendrites from many brain regions, as well as on the membrane and - to a lesser degree - inside the cytoplasm of cells which appeared to be topographically and morphologically consistent with follicular dendritic cells (FDCs) residing in secondary lymphoid tissues (Fig. 2, panels A and B).

On the basis of the results obtained herein, no evidence of PrP^{Sc} deposition was found in any of the stranded cetaceans under study. Differently from the PD case reported in a wild dolphin (1), however, none of the 30 animals included in our survey showed any evidence of spongiform degeneration inside their brain. In this respect, while we feel it would be important to add prions and TSE-like diseases to the list of neurotropic pathogens and neurological conditions affecting free-ranging cetaceans, we also believe that the prominent PrP^C IR detected within the brain and in

lymphoid tissues from the dolphins investigated herein provides a solid ground of biological plausibility for prion colonization of the above cetacean tissues. This is additionally underscored by

the finding of a PrP^C expression pattern which was topographically and morphologically consistent with that of FDCs. As a matter of fact, new insights have been recently gained into FDC role and biological significance (5), with FDCs residing inside host's lymphoid tissues being also of crucial relevance for prion replication and for the early pathogenesis of natural and experimental scrapie (6-8), which is unanimously considered to be the PD, or TSE "prototype" (9).

As previously mentioned, one of the most, if not even the most challenging feature of a PD condition in free-living cetaceans refers to the route(s) through which infection may be acquired. In this respect, whenever a "sporadic" form of PD developed in a dolphin, similarly to what has been known for over 90 years in humans (4), this would probably lead to a different "scenario" from that which we would face in the case of wild dolphins being found to be susceptible to an "infectious" PD condition, as in the well-documented example of sheep scrapie (4). In fact, this would represent an additional threat to cetaceans, the health and conservation status of which are dramatically impacted nowadays by many other biological and anthropogenic *noxae* (10). With reference to the above, further issues of concern regard the animal source(s) of infection and its putative "land-to-sea" flow, as already hypothesized for *Toxoplasma gondii* infection in sea otters (*Enhydra lutris*) and bottlenose dolphins (10), or the alternative existence of an "exclusively marine" cycle of infection. Finally, a particularly relevant issue is that addressing the "strategies" adopted by dolphin prions for their persistence within the marine environment, considering their progressive dilution and dispersal in sea water.

In conclusion, despite the negative results of our study, we still believe that the documented occurrence of a spontaneous TSE-like condition in a wild dolphin (1) is an intriguing issue and a matter of concern for both prion biology and intra/inter-species transmissibility, as well as for cetacean conservation medicine.

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