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Keynote Lectures

Pathogenesis and therapy of genetic prion disease: insights from transgenic mice

Roberto Chiesa, PhD

Head of Laboratory of Prion Neurobiology at the Neuroscience Department at the Mario Negri Institute.

Doctor Chiesa graduated in Biology, majoring in genetics, at the University of Pavia, Italy, and became interested in neurodegeneration during his PhD, where he investigated amyloid neurotoxicity in vitro. He then focused on mechanisms of neurodegeneration in vivo and how abnormally folded proteins are neurotoxic. At Washington University, St. Louis, USA, Dr. Chiesa worked on genetic prion diseases. He generated Tg(PG14) mice, which express an insertional PrP mutation and show marked progressive neurodegeneration. Only another model existed, the Tg(P101L) made in Prusiner lab, and together these strengthened the case for pathogenicity of PrP mutations. Dr. Chiesa pursued this line of research when he returned to Italy in 2001. Since then he has led an independent group in the Neuroscience Department at the Mario Negri Institute, focusing on two central questions: 1) What causes neuronal dysfunction in inherited prion diseases? 2) How do different PrP mutations direct the disease phenotype? He developed a program whose main objectives were to explore the mechanisms of neurodegeneration in transgenic mice and primary neuronal cultures, with the ultimate goal of devising therapeutic approaches. Recently, he also became interested in studying the role of protein misfolding in cerebellar degeneration in Marinesco-Sjögren syndrome, a rare genetic disease of early infancy, and of prion-like propagation of tau in chronic neurodegeneration following traumatic brain injury.

Genetic prion diseases are dominantly inherited degenerative brain disorders caused by mutations in the gene encoding the prion protein (PrP). Different PrP mutations cause different diseases, including genetic Creutzfeldt-Jakob disease (gCJD), fatal familial insomnia (FFI) and Gerstmann-Sträussler-Scheinker (GSS) syndrome. We generated transgenic mice expressing gCJD, FFI- and GSS-associated mutations, and found that they recapitulate key phenotypic features of the corresponding human disorders. The mutant PrPs in these mice form conformationally distinct aggregates that accumulate in different intracellular compartments, causing different morpho-functional abnormalities of the secretory pathway. How this may contribute to the disease phenotype and the implication for therapy will be discussed.

Early cytopathogenesis in mutant huntingtin mouse and human carriers

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Ana Cristina Rego is Associate Professor with Aggregation and CNC/CIBB Group Leader at University of Coimbra. Main research interests focus on neuronal mechanisms involving mitochondrial deregulation in neurodegenerative disorders and the role of targeted neuroprotective strategies. She has identified early mitochondrial and redox changes linked to glutamatergic synapses in Alzheimer's and Huntington's diseases. She published over 120 papers (Web of Science h-index=45; >8000 citations) in journals such as J.Neurosci, FRBM, ARS, NBA, ARR. She mentored 19 PhD and 46 MSc students. She coordinated 25 competitive grants and participated in 18 other, funded by FCT, HighQ Foundation, EHDN or private foundations, including SCML and FLAD prizes. She is ad hoc reviewer for Parkinson's United Kingdom, Alzheimer's Res. United Kingdom, FWF (Austria), ANR (France), AMRF (NZ), Telethon Fond. (IT), ISF (Israel), Wellcome Trust (UK), COST, JPND, among others, and for several scientific journals. She was member of the ERA-Chair and ERAatUC team; currently she is Vice-President of the Portuguese Brain Council and President of the Portuguese Society for Neuroscience (SPN).

Huntington's disease (HD) is a genetic neurodegenerative movement disease that largely affects the striatum. HD has a progressive course with increasing motor disability, linked with neuropsychiatric and cognitive impairment, ultimately leading to dementia and death. As in other neurodegenerative diseases, spreading of the aggregate-forming protein, mutant huntingtin (mHTT), has been described, progressively affecting the cortex and other brain areas. Deficits in mitochondrial function and mitochondrial-related redox deregulation have been attributed to HD. However, whether these changes occur in in vivo in early stages of the disease has been unclear. In PET studies performed in mouse and human brain we analyzed tissue overreduced states in premanifest/presymptomatic and prodromal mHTT carriers. This was correlated with increased levels of reactive oxygen species, altered mitochondrial function and morphology at early stages when assessing *ex vivo* HD human skin fibroblasts and striatal mitochondria isolated from YAC128 transgenic mouse. HD human induced pluripotent stem cells and derived neural stem cells also showed deregulated mitochondria, concomitantly with increased release of extracellular vesicles. Data demonstrate mHTT-imprinted mitochondrial modifications and redox unbalance at early HD stages, which may facilitate the progression of this debilitating brain disorder.

CWD strain characterization in North America

Debbie McKenzie, PhD

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Dr. McKenzie received her PhD in Medical Biochemistry from the University of Calgary, Alberta. From 1988 to 2008, she was a research scientist at the University of Wisconsin at Madison, working with Drs. Richard Marsh and Judd Aiken. Her studies combined biochemical analyses of infectious prions with prion infectivity studies in rodents and deer. When Chronic Wasting Disease (CWD) was first identified in Wisconsin in 2002, CWD became her main research focus, with an emphasis on CWD genetics and strains. In 2008, she moved to the Centre for Prions and Protein Folding Diseases at the University of Alberta where she continues to focus on emergence of CWD strains and host range. Her research team has identified several CWD strains with variable host ranges. She was the lead on a recently completed Genome Canada project, "Systems Biology and Molecular Ecology of Chronic Wasting Disease", a project involving 12 research teams.

Chronic wasting disease (CWD) is a prion disease affecting both captive and wild cervids and is a serious ongoing management issue for maintaining iconic cervid species. The geographical range of CWD continues to expand with 4 Canadian provinces and 30 US states identifying CWD in their cervid populations. Within endemic areas, the disease prevalence continues to increase, with some areas having disease prevalence above 60%. Our research team focuses on two aspects of CWD: strain adaptation and environmental detection/persistence.

As CWD agents are transmitted between different cervid species and between the same species having different prion protein gene (Prnp) polymorphisms, novel CWD strains are generated. We are currently characterizing the biological and biochemical properties of these strains, including host range. Changes in host range will increase the potential for transmission to economically important species.

Cervids infected with CWD prions shed infectious agent via saliva, urine, and feces throughout the course of infection. These infectious biofluids remain bioavailable in the environment via binding to soil and vegetation. Our current work involves the detection of CWD in soil samples from endemic areas as a prelude to determining environmental persistence.

Chronic wasting disease in European cervids

Romolo Nonno, PhD

Head of the Italian Reference Laboratory for genetics and strain typing of animal TSEs; Responsible for strain typing activities within the European Reference Laboratory for TSEs.

Doctor Romolo Nonno graduated in Veterinary Medicine and obtained his PhD in Pharmacology in 1998 at the University of Milano, where he continued his research as Postdoctoral Fellow until 2000. Since 2000, he is working at the Istituto Superiore di Sanità in Rome (Italy), where he focussed his research activities on prion diseases of animals and humans, mainly working on the characterization and discrimination of prion strains and on their interspecies transmissibility. Romolo Nonno is the head of the Italian Reference Laboratory for genetics and strain typing of animal TSEs (as a part of the Italian NRL on TSEs) and is responsible for strain typing activities within the European Reference Laboratory for TSEs.

Romolo Nonno, Sylvie L. Benestad

Chronic wasting disease (CWD) is a relentless epidemic disorder caused by infectious prions that threatens the survival of cervid populations and raises increasing public health concerns in North America. In Europe, CWD was detected for the first time in wild Norwegian reindeer (*Rangifer tarandus*) and moose (*Alces alces*) in 2016. Since then, Norway implemented an intensified testing programme in wild and captive cervids and the EU adopted a surveillance programme in member states that have a reindeer and/or a moose population (Estonia, Finland, Latvia, Lithuania, Poland and Sweden). Overall, 40 cases of CWD were detected in wild cervids, involving 3 countries (34 cases in Norway, 4 in Sweden and 2 in Finland) and 3 cervid species: 20 cases in reindeer, 17 in moose and 3 in red deer (*Cervus elaphus*). So far, CWD has not been detected in captive cervids in Europe.

CWD in reindeer was detected only in Norway, in two neighbouring wild reindeer populations. The pathological features were similar to CWD in North American (NA) cervids, with involvement of lymphoid tissues in the pre-clinical phase and supposedly contagious spread. The semi-isolated reindeer population in which the first case was identified was culled and tested for CWD, showing an estimated prevalence in adults of 1,2% (19 cases). A new case was diagnosed in a neighbouring reindeer population in 2020. In contrast, CWD in moose and red deer showed epidemiological and pathological features different from CWD in Norwegian reindeer and in NA cervids. Positive cases were characterised by advanced age, lack of lymphoid involvement and distinctive patterns of protease-resistant PrPSc.

Strain typing by bioassay in rodent models showed that the CWD prion strain affecting Norwegian reindeer is different from NA CWD prions, as well as from the multiple prion strains detected in European moose and red deer.

Emerging evidences will be discussed in light of their implications for understanding the origin, the nature and the ecology of CWD in Europe, for implementing CWD control strategies and for the safety of humans will be presented.

Recent high-resolution insights into prion strain diversity: can cryo-EM explain prion glycoform signatures?

Szymon Manka, PhD

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Doctor Romolo Nonno graduated in Veterinary Medicine and obtained his PhD in Pharmacology in 1998 at the University of Milano, where he continued his research as Postdoctoral Fellow until 2000. Since 2000, he is

working at the Istituto Superiore di Sanità in Rome (Italy), where he focussed his research activities on prion diseases of animals and humans, mainly working on the characterization and discrimination of prion strains and on their interspecies transmissibility. Romolo Nonno is the head of the Italian Reference Laboratory for genetics and strain typing of animal TSEs (as a part of the Italian NRL on TSEs) and is responsible for strain typing activities within the European Reference Laboratory for TSEs.

Recent cryo-EM studies of infectious, *ex vivo,* prion fibrils from hamster 263K and mouse RML prion strains revealed a broadly similar, parallel in-register intermolecular β-sheet (PIRIBS) amyloid architecture in both strains. Rungs of the fibrils are composed of single prion protein (PrP) monomers that fold to create distinct N- and C-terminal lobes. However, observed structural variations between these fibrils are conflated by differences in hamster/mouse PrP sequence, which precludes resolving how divergent prion strains can emerge from an identical PrP substrate. It is, therefore, now important to obtain more prion fibril structures from the same species and/or of strains that can cross the mouse/hamster species barrier to gain further insight into the mechanism of prion strain diversity. Another puzzle pertains to the presence of paired fibril assemblies in *ex vivo* prion preparations. Are they biologically relevant? It remains to be seen if the future collection of near-atomic resolution structures of both single and double-protofilament assemblies can uncover the structural basis for unique glycoform ratios characterising various prion strains.

Chronic wasting disease risk assessment in Portugal: results and future work

Maria dos Anjos Pires, PhD

Principal Investigator (PI) of the project "Chronic wasting disease risk assessment in Portugal" funded by the Portuguese Foundation for Science and Technology.

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The project intitles "Chronic Wasting Disease Risk Assessment in Portugal" reference PTDC/CVT-CVT/29947/2017, was financed by FCT with 239.897,23€, started in October 2018, and it was extended until September 2022 in consequence of the COVID pandemic situation.

Three institutions are associated with the best success of the proposed aims: UTAD, the lead institution, INIAV with the skills for the TSE diagnosis, and the IPCB, the major institution for samples harvester.

This project has 7 tasks, developed during these years.

Task 1, Training and sample collection is complete. Five hundred samples from 3 different species of cervids that live in Portugal (*Cervus elaphus, Dama dama* and *Capreolus capreolus*) were harvested in hunting in Castelo Branco and the north of Portugal, at UTAD.

In task 2, the lymph nodes and brainstem of these animals were screened for PrPres at INIAV, which became all negative. The central nervous system histopathology is ongoing, and no severe pathologies have been diagnosed yet. Most cases of *Capreolus capreolus* were from necropsies and other lesions were diagnosed as parasites and traumatic lesions, and one case of renal carcinoma.

Task 3 is the determination of *prnp* genotypes in the Portuguese cervid population and will be exposed in oral communication: "*Chronic wasting disease (CWD) risk assessment in Portugal: - The genetic approach to study prion protein gene (PRNP) variability in Portuguese populations of three cervid species: Red deer, Fallow deer and Roe deer*".

In task 4 we intend to determine the identification of other risk factors as related to the positive cases of BSE and scrapie near the wild hunted cervid population, imports of cervids and hunting tourism.

We also proposed to make a transmission assay of atypical scrapie to cervid Tg-mice. In this 5th task, the transmission ability of atypical scrapie to cervids is being studied by bioassay in transgenic mouse models expressing either Deer PrP (226Q-DePrPTg146) or Elk PrP (226E-ElkPrPTg152). To do this, these two newly generated mouse lines have been inoculated with a collection of Portuguese Atypical Scrapie isolates. Currently, these experiments are underway but we will have to wait to have conclusive results.

The last 2 tasks are in progress, statistical analysis and modelling, defining the risk of occurrence of prion diseases in cervids in Portugal, and the dissemination of the results in international meetings, international papers, and book chapters contributions. This Iberian Congress is one example of this project dissemination.

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Oral Communications:
Prion diseases and prion-like diseases in humans

O1 | Regional differences in neuroinflammation-associated gene expression in the brain of sporadic Creutzfeldt-Jakob disease patients

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Neuroinflammation is an essential part of neurodegeneration. Yet, current understanding of neuroinflammation associated molecular events in distinct brain regions of prion disease patients is insufficient to lay the ground for effective treatment strategies targeting this complex neuropathological process. To address this problem, we analyzed expression of 800 neuroinflammation associated genes to create a profile of biological processes taking place in frontal cortex and cerebellum of patients, who suffered from sporadic Creutzfeldt-Jakob disease. The analysis was performed using NanoString nCounter technology, human neuroinflammation panel+. The observed gene expression patterns were regionally and sub-regionally distinct, suggesting variable neuroinflammatory response. Interestingly, the observed differences could not be explained by the molecular subtypes of sporadic Creutzfeldt-Jakob disease. Furthermore, analyses of canonical pathways and upstream regulators based on differently expressed genes indicated an overlap between biological processes taking place in different brain regions. This suggests that even smaller scale spatial data reflecting subtle changes in brain cells' functional heterogeneity and their immediate pathologic microenvironments are needed to explain the observed differential gene expression in a greater detail.

O2 | Clearance of variant CJD prions in vivo by the Hsp70 disaggregase system

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The metazoan Hsp70 disaggregase protects neurons from proteotoxicity that arises from the accumulation of misfolded protein aggregates. Hsp70 and its co-chaperones disassemble and extract polypeptides from protein aggregates for refolding or degradation. The effectiveness of the chaperone system decreases with age and leads to accumulation rather than removal of neurotoxic protein aggregates. Therapeutic enhancement of the Hsp70 protein disassembly machinery may counter late-onset protein misfolding neurodegenerative diseases. In the context of prion disease, it is not known if stimulation of protein aggregate disassembly paradoxically leads to enhanced formation of seeding competent species of disease-specific proteins and acceleration of neurodegenerative disease. We tested the hypothesis that modulation of Hsp70 disaggregase activity perturbs mammalian prion-induced neurotoxicity and prion seeding activity. To do so we used PrP transgenic *Drosophila* that authentically replicate mammalian prions. RNASeq identified that Hsp70, DnaJ-1 and Hsp110 gene expression was down-regulated in prion-exposed PrP *Drosophila*. We showed that RNAi knockdown of Hsp110 or DnaJ-1 gene expression in variant CJD prion-exposed human PrP *Drosophila* enhanced neurotoxicity, whereas over-expression mitigated toxicity. Strikingly, prion seeding activity in variant CJD prion-exposed human PrP *Drosophila* was ablated or reduced by Hsp110 or DnaJ-1 over-expression, respectively. Our data show the metazoan Hsp70 disaggregase facilitates clearance of mammalian prions and that its enhanced activity is a potential therapeutic strategy for human prion disease.

O4 | Generation and characterization of a new model for $G_{114}V$ Gerstmann-Sträussler-Scheinker disease

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$G_{114}V$ mutation leads to an early-onset form of Gerstmann-Straüssler-Scheinker syndrome (GSS). It is located within the palindrome sequence of PrP and immediately upstream of the glycine-rich region. $G_{114}V$-GSS patients show low PrPSc levels mainly detected by immunohistochemistry as fine deposits. The study of the onset, progression, molecular mechanisms involved and for testing possible treatments, will benefit for the generation of transgenic mouse models of GSS although almost all attempts to produce transgenic mouse models for human genetic TSEs in the human PrP sequence had been unsuccessful.

Two different hemizygous transgenic mouse lines expressing the human PrP protein harboring the mutation $G_{114}V$ were generated and characterized, lines Hu-Tg749-V_{114} and Hu-Tg741-V_{114}. In addition, Hu-Tg741-V_{114} mice were crossed with Hu-Tg340-G_{114} to generate and characterize the heterozygous line Hu-Tg740-GV_{114}. Spontaneous prion generation was detected in all lines at the end of mouse lifespan. The biochemical features of PrP^{res} were characterized by the presence of an "atypical" pattern showing a band with approximately 7-8 kDa. Brain PrP^{res} in heterozygous Hu-Tg740-GV_{114} mice was probed to be formed exclusively by V_{114}-PrP and not by the wild-type allele G_{114}-PrP. A second passage in Hu-Tg741-V_{114} mouse line using the spontaneously generated Hu-Tg741-V_{114} prions reduced survival times and increased attack rates.

Although more research is needed to further characterize human V_{114} prions, these transgenic lines offer an excellent tool to model GSS in the proper human PrP sequence.

O5 | Role of cellular prion protein in synucleinopathies

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Aims: Synucleinopathies, such as Parkinson's disease are a class of neurodegenerative diseases and characterized by deposits of aggregated alpha-synuclein (aSyn) in neurons and glia. It is supposed, that misfolded oligomeric aSyn converts natively folded monomeric aSyn in a templated-induced conversion process (prion-like pathway) into toxic oligomers, which can advance to the formation of pathologic fibrils. In this work, we focused on cellular prion protein (PrPC) as a potential receptor protein of aSyn, supporting internalization and interaction of both proteins.

Methods: To investigate a conceivable direct interaction, we subjected recombinant human PrPC as well as recombinant human monomeric and oligomeric aSyn to surface plasmon resonance spectrometry (SPR). SH-SY5Y (SHWT) and stable PRNP transfected SH-SY5Y PrP (SHPrP) cells were treated with monomeric and oligomeric aSyn under same conditions.

Results: We demonstrate the effect of PrPC on the internalization of aSyn and the interaction between both proteins. SPR results presented monomeric and oligomeric aSyn as direct interaction partners of PrPC. Oligomeric aSyn showed higher binding affinity to PrPC than monomeric aSyn. SHPrP cells showed a significantly higher amount of internalized oligomeric aSyn compared to SHWT cells.

Conclusions: Altogether, our experiments indicate PrPC as a receptor for aSyn, promoting the internalization and interaction of each other that could contribute to a better understanding of the pathological mechanism in synucleinopathies, which is important for future therapies or diagnostics.

O6 | AMYSEEDS: targeting amyloid beta seeds at the initial stage of Alzheimer's disease

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The recent failure of several amyloid beta (Aβ)-based Alzheimer's disease (AD) clinical trials despite their promising laboratory results has been attributed, among other reasons, to a wrong starting time (too late in the staging of AD), when the Aβ pathology has started and the progress of the disease may be irreversible. Consequently, developing disease modifying therapies directed to interfere with the initial steps of the disease is the next rational research avenue. However, the lack of a clear target at AD early stage hinders the development of new therapies. This project builds on recent findings that the pathogenic Aβ seeds might be present in AD patients before the start of Aβ-associated pathology (early seeds). Those seeds conformation and/or biochemical nature might be different from Aβ seeds isolated from brains of late-stage AD patients. Thus, this project intends to isolate and characterize these early Aβ seeds from human brain tissue and, based on their structural features, find new therapeutic agents with potential to delay AD when applied early enough. To this end, we will take advantage of a recently developed in vitro cell assay to select brain samples containing Aβ seeds, and a new phase-transition based method to isolate such seeds. Biochemical and structural characterization of the seeds will allow the identification of distinct features which will be used for the screening of compounds with capacity to block the seeds. The efficacy of such compounds will be both *in vitro* and *in vivo* tested.

Oral Communications: Prion diseases in animals

O7 | Defining the strain properties of emergent forms of chronic wasting disease

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Cervid PrP (CerPrP) coding sequences are generally invariant except at codon 226. Whereas deer,

reindeer and moose PrP encode glutamine at residue 226 (Q226), elk or red deer may encode glutamate (E226) at this position. In order to precisely assess the effects of this primary structural difference we created Gt mice in which the murine PrP coding sequence was replaced with that of CerPrP-Q226 or CerPrP-E226, referred to as GtQ226 and GtE226 mice. The differential responses of GtQ226 and GtE226 mice to CWD prions underscores the importance of this key primary structural difference on CWD pathogenesis at the level of strain selection and, by extension, a rigorous and definitive means to identify and characterize the properties of novel CWD prion strainas. Accordingly, our recently published studies showed that Norwegian and North American CWD are caused by different prion strains. Here we report the properties of prions causing newly emergent forms of CWD in additional European and Asian locations. We find that while prions causing CWD in South Korean cervids share properties with those of CWD prions causing epidemic CWD in North America, newly emergent forms of CWD in Scandinavian countries show high levels of strain diversity and instability.

O8 | Generation of human chronic wasting disease in transgenic mice

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Chronic wasting disease (CWD) results from the accumulation of an infectious misfolded conformer (PrP^{Sc}) of cellular prion protein (PrP^{C}) in the brains of deer and elk. It has been spreading rapidly throughout many regions of North America, exported inadvertently to South Korea, and more recently identified in Europe. Mad cow disease has caused variant Creutzfeldt-Jakob disease (vCJD) in humans and is currently the only known zoonotic prion disease. Whether CWD is transmissible to humans remains uncertain. We report here the generation of the first CWD-derived infectious human PrP^{Sc} using elk CWD PrP^{Sc} to initiate the conversion of human PrP^{C} from normal human brain homogenates with in vitro protein misfolding cyclic amplification (PMCA). Western blotting with a human PrP selective antibody confirmed that the PMCA-generated protease-resistant PrPSc was derived from the human PrP^{C} substrate. Two lines of humanized transgenic mice expressing human PrP^{C} with either Val or Met at the polymorphic codon 129 developed clinical prion disease following intracerebral inoculation with the PMCA-generated CWD-derived human PrP^{Sc}. Diseased mice exhibited distinct PrP^{Sc} patterns and neuropathological changes in the brain. Our study, using PMCA and animal bioassays, provides the first evidence that CWD PrP^{Sc} has the potential to overcome the species barrier and directly convert human PrP^{C} into infectious PrP^{Sc} that can produce bona fide prion disease when inoculated into humanized transgenic mice.

Supported in part by CJD Foundation, CDC, NIH, Michael J. Fox Foundation for Parkinson's Research, Alzheimer's Association, Alzheimer's Research United Kingdom (ARUK), and Western Brain Institute.

O9 | High transmissibility of splenic prions in cervidized transgenic mice as a promising diagnostic marker for acquired human CWD cases

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Whether CWD prion can infect humans in real life remains controversial. Multiple in vitro CWD-seeded human PrP conversion experiments and some animal model studies indicate that the species barrier for CWD to human transmission can be overcome. One of the challenges of CWD zoonosis studies is the lack of a reliable marker for identification of acquired human CWD cases, should they occur.

We have detected a couple of prion-positive spleens

in a group of humanized transgenic mice inoculated with certain CWD isolates. Such experimentally generated splenic humanized CWD prions (termed eHuCWD^{sp}) appear indistinguishable from prions in the brain sCJDMM1 patients on Western blot. Significantly, we found that eHuCWD^{sp} can efficiently infect not only humanized transgenic mice but also cervidized transgenic mice (Tg12). Tg12 mice infected by eHuCWD^{sp} produced prions and brain pathology that are practically identical to those of CWD-infected Tg12 mice. In contrast, prions from the spleen of humanized transgenic mice inoculated with sCJDMM1 (termed sCJD^{sp}), similar to prions from sCJDMM1 patient brains, is poorly transmissible in the Tg12 mice. These data demonstrate that high transmissibility of splenic prions in cervidized transgenic mice is a unique feature of acquired human CWD prions, and it may serve as a reliable marker to identify the first acquired human CWD cases.

O10 | Chronic wasting disease (CWD) risk assessment in Portugal - the genetic approach to study prion protein gene (PRNP) variability in Portuguese populations of three cervid species: Red deer, Fallow deer and Roe deer

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Among the transmissible spongiform encephalopathies (TSEs), chronic wasting disease (CWD) in cervids is now the rising concern in wildlife within Europe, after the first case was detected in Norway in 2016, in a wild reindeer and until October 2021, a total of 34 cases were described in Norway, Sweden and Finland. The establishment of risk assessment projects, even in countries with no cases of CWD is very important to forecast possible contaminations.

The study of the genetics of the prion protein gene, *PRNP*, has been proved to be a valuable tool for determining the relative susceptibility to TSEs. In the present study we analyzed the exon 3 of *PRNP* gene in 235 samples from three species: red deer (*Cervus elaphus*), fallow deer (*Dama dama*) and Roe deer (*Capreolus capreolus*). Three single nucleotide polymorphisms (SNPs) were found in red deer – codon A136A, codon T98A, codon Q226E – and no sequence variation was found in fallow deer and roe deer. The low genetic diversity found in our samples are compatible with the ones found in previous studies in Europe. The comparison of our population with North American populations, suggest that the free-ranging deer from our study may present susceptibility to CWD, although lack of experimental data and the necessity of large survey are necessary to evaluate these populations.

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O11 | Identification of biomarkers associated with endoplasmic reticulum stress in natural scrapie

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The accumulation of misfolded proteins such as PrP^{Sc} can alter endoplasmic reticulum homeostasis triggering the unfolded protein response (UPR). In this pathogenic event, the molecular chaperones play an important role. Several reports in humans and animals have suggested that neurodegeneration is related to endoplasmic reticulum stress in diseases caused by the accumulation of misfolded proteins. In this study,

we investigated the expression of three endoplasmic reticulum stress markers: PERK (endoplasmic reticulum kinase), BiP (binding immunoglobulin protein) and PDI (Protein Disulfide Isomerase) in sheep affected by natural scrapie in clinical and preclinical stages of the disease by immunohistochemical and western blot analyses. We observed a significant overexpression of BiP, PERK and PDI in clinical sheep compared to healthy controls in various areas of the brain. Our results suggest that the neuropathological and neuroinflammatory phenomena that develop in prion diseases cause endoplasmic reticulum stress in brain cells, leading to the accumulation of these proteins in different areas of the brain and triggering an UPR, which at the beginning could be a neuroprotective event, however, prolonged UPR activation could initiate neurodegeneration.

O12 | Ovine atypical scrapie failed to transmit to cattle

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Classical bovine spongiform encephalopathy (BSE) in cattle was caused by the recycling and feeding of meat and bone meal contaminated with a transmissible spongiform encephalopathy (TSE) agent but it still remains unknown whether the agent originated from sheep or cattle. This study aimed to determine whether atypical scrapie could cause a BSE-like disease when transmitted to cattle.

Two groups of calves (five and two) were intracerebrally inoculated with atypical scrapie brain homogenate from two sheep with atypical scrapie. Controls were five calves intracerebrally inoculated with saline solution (group 1, culled as age-matched control for test group cull) and one non-inoculated animal (group 2). Cattle were clinically monitored until clinical end-stage or at least 96 months post inoculation (mpi). After euthanasia, the brain was collected for potential transgenic mouse bioassay and examined for TSE by Western immunoblot and immunohistochemistry.

Three of the five animals from test group 1 were lost due to intercurrent diseases at 23, 46 and 91 mpi, one animal was culled with BSE-like clinical signs at 48 mpi and one clinically unremarkable animal was culled at 106 mpi. Both test group 2 animals were culled with no obvious TSE signs at 106 mpi. One of the control animals in each group was also culled because of intercurrent diseases. None of the animals tested positive for TSEs. Bioassay in tg338 and tg110 mice using the brain of the clinical suspect was negative. The results do not provide any evidence that cattle are susceptible to atypical scrapie.

Oral Communications: Prion structure and biology

O13 | Cryo-EM Structure of the Amyloid Exon of a Human Prion-like Protein

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The Heterogeneous ribonucleoprotein D-like (hnRNPDL) is a ubiquitously expressed prion-like RNA-binding protein required for RNA-processing. hnRNPDL has three alternative splicing (AS) isoforms differing in the number of low complexity domains (LCD): hnRNPDL1, hnRNPDL2 and hnRNPDL3. Mutations of the conserved Asp259 at the C-terminal Tyr/Gly-rich LCD of hnRNPDL cause autosomal dominant limb-girdle muscular dystrophy-3 (LGMDD3). Thus, elucidation of the molecular mechanisms underlying amyloid transitions in this protein is of biomedical relevance. We report the cryo-electron microscopy (cryoEM) maps at 2.5 Å resolution and the corresponding atomic model of hnRNPDL amyloid filaments reconstituted in vitro. The high-resolution structure revealed that fibril core is built up by the cross-β packing of a highly hydrophilic LCD region into a single filament. Importantly, this region includes Asp259, and precisely matches the sequence

of the exon absent in hnRNPDL3, a soluble isoform that differs in subnuclear localization and function from the rest. All in all, our results highlight the differential role played by this domain and provides the first report of a functional amyloid exon.

O14 | Mass spectrometry-based quantitation of methionine oxidation to assess prion structures

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Prions (PrP^{Sc}) induce a natively expressed prion protein (PrP^{C}) to adopt the prion conformation. PrP^{C} and PrP^{Sc} possess identical primary structures but differ in their 2', 3', and quaternary structures. PrP^{C}'s secondary structure is composed mostly of disordered motifs, α-helical and β-sheet motifs. PrP^{Sc}'s secondary structure contains β-sheet or disordered motifs, with no α-helix. Two structures accommodate these constraints, the parallel in-register intermolecular β-sheet (PIRIBS) structure and the four-rung β-solenoid (4RβS) model.

The chemical environment and consequent chemical reactivity of a protein's amino acids is determined by the protein's conformation. If the same protein can be refolded into more than one conformation, then the same amino acid may react differently with the same added reagent in a conformation-dependent way. Unlike conformation, covalent changes remain after a protein is denatured. In this way conformation-dependent differences can be captured for subsequent analysis. The methionines in the prion conformation are in a different chemical environment and may react differently than the same methionines in the natively expressed prion protein (PrP^{C}) conformation.

We reacted the Sc237 strain of hamster-adapted scrapie and recombinant hamster prion protein with hydrogen peroxide. The samples were analyzed by mass spectrometry to quantitate the extent of methionine oxidation. As expected, the exposed surface methionines of rPrP were more readily oxidized than those non-surface exposed methionines in the protein's interior. In the prion conformation, the same methionines reacted differently. We compared our empirical results with those predicted by the PIRIBS structure and 4RβS model. Neither exactly matched our results.

O16 | Atypical scrapie evolution through the species barrier in bovine PrP during PMCA propagation

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During intra- or cross-species transmission the prion conformational characteristics might be changed altering its phenotypical characteristics, thus emerging new prion strains. There are two main theories, non-mutually exclusive, that could explain prion strain emergence during species barrier propagation: the 'deformed templating' and the 'conformational selection model'. According to the 'deformed templating' or mutation model, the new host forces a shift in PrP^{Sc} conformation when the prion is unable to replicate, as a result emerging prion strains *de novo*. On the other hand, the 'conformational selection' theory postulates that prion isolates are a conglomerate of PrP^{Sc} conformations with a majoritarian structure within it (the most stable energetically favourable conformation). During cross-species transmission the species barrier acts as a filter where other PrP^{Sc} conformers might be selected.

In previous studies, we showed the emergence of the bovine spongiform encephalopathy agent (BSE-C) due to the transmission of atypical scrapie (AS) onto bovine PrP. In this work, we analysed the evolutionary dichotomy in the AS transmission based on the differential strain characteristics of thermostability and *in vitro* prion amplification by PMCA. Mutation seems to be the main mechanism responsible for BSE-C emergence.

O17 | A non-PrP^{Sc} PrP prion

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While infectivity is the *sine qua non* condition to define a *bona fide* prion, some recombinant full-length amyloids also propagate and accumulate in the brain but cannot cause clinical signs, at least in the first passage.

Recently some of us have shown that full length PrP amyloids are infectious to transgenic (Tg) Tg7 mice. Here Tg mice expressing 1x of the bank vole I109 PrP sequence were intracerebrally inoculated with a bank vole 23-231 PrP amyloid. All the mice developed clinical signs consisting of ataxia, kyphosis, lethargy and body weigh lost with an average incubation time of 198±8 days. Brains of clinically sick mice accumulate PK-resistant PrP fragments of ~20, ~16 and ~13 kDa. These fragments, where recognised by the C-terminal antibody SAF84 (160-170), but not by SAF83 (126-164). Moreover, when brain homogenates were treated with PNGaseF, all the mentioned bands collapsed in a ~13 kDa band, corresponding to the ~9.5 kDa PK-resistant amyloid core plus the GPI anchor.

These results demonstrate the existence of an infectious recombinant full-length PrP amyloid that has an attack rate of 100% in its first passage in a 1x expression model and presents period of incubation even shorter than some recombinant PrP^{Sc} strains.

In view of the recent results of cryo-EM, showing that PrP^{Sc} and PrP amyloids share a PIRIBS architecture, it is no wonder that both conformers can be infectious, despite their different size. Thus, PrP amyloids must be also considered *bona fide* prions.

O18 | Insight into the key features of a new method allowing the spontaneous formation of *bona fide* recombinant prions *in vitro* within minutes

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Spontaneous misfolding of recombinant prion protein in vitro, one of the most useful tools to study the main event underlying Transmissible Spongiform Encephalopathies of sporadic and genetic origin, has always rendered highly variable results. The different methodologies developed to achieve the spontaneous generation of infectious prions in vitro have given rise to a large spectrum of misfolded proteins and amyloid aggregates with distinct properties. From those unable to propagate and cause disease in vivo or those requiring overexpressing animal models and multiple serial inoculations, to infectious prions with high titers, able to cause disease in wild-type animals. Despite the success of some procedures, such as the PMCA using recombinant mouse PrP and polyanionic cofactors, generation of synthetic bona fide prions was inconsistent, obtaining on other occasions non-infectious products with the same method. Herein, we break down a novel and easily scalable methodology that consistently leads to the spontaneous misfolding of recombinant PrP into infectious, high titer, *bona fide* prion preparations, allowing the understanding of the minimal requirements for such an event. Using recombinant PrP from bank vole complemented with dextran as substrate, we have optimized the Protein Misfolding Shaking Amplification

(PMSA) to obtain infectious synthetic prions within minutes. Additionally, it allows the formation of a variety of strains with specific and differential features. Thus, through fine-tuning of PMSA operational conditions, we can consistently produce highly infectious recombinant prions in a spontaneous manner, offering an invaluable tool to study every aspect contributing to spontaneous prion formation.

Scientific Posters:
Prion diseases and prion-like diseases in humans

P1 | Brain Tissue-Based Diagnostics of Rare and RT-QuIC-negative Prion Disease Subtypes

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Prion diseases are rapidly progressing, neurodegenerative protein misfolding diseases. The commonest prion disease caused by misfolding cellular prion protein is sporadic Creutzfeldt-Jakob disease (sCJD). Current sCJD classification consists of 14 molecular subtypes defined by misfolded prion protein isoform, patient's genotype at polymorphic codon 129 in the prion protein gene and distinct neuropathological features such as spongiosis severity and misfolded prion protein accumulation pattern in different brain regions.

Real time quaking-induced conversion (RT-QuIC) method is widely applied for ante-mortem diagnostics of prion disease, and it is an excellent tool providing quick and highly accurate (>90%) positive/negative-type of answers based on cerebrospinal fluid sample analysis of sCJD suspected patients. However, the RT-QuIC method cannot distinguish between different disease molecular subtypes, and, most importantly, does not detect some of the rarer subtypes.

We present 3 recent, unique sCJD cases that would have been impossible to diagnose without neuropathological and molecular examination of brain samples.

The 3 cases include the first in the world sCJD molecular subtype VV1 with 1-Octapeptide Repeat Deletion polymorphism, the first in Denmark Variably Protease Sensitive Prionopathy, and a case of sporadic Fatal Insomnia presenting with Parkinsonism. RT-QuIC test was negative in 2 of the cases and was not performed in the last case.

It is strongly encouraged to still consider autopsy-based diagnostics in patients with suspected sCJD or an atypical fast progressing dementia.

P2 | GSS A117V in transgenic mice expressing vole PrPC: a fast and versatile model of human prion disease with early biomarkers

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Gerstmann-Sträussler-Scheinker (GSS) syndrome is a rare genetic form of prion pathology characterized by a longer disease course and clinical and neuropathological manifestations differing from those of Creutzfeldt-Jakob disease (CJD). It can be caused by a number of different mutations in the PRNP gene, the most common of which is P102L. GSS was long thought

to be a non-transmissible proteinopathy given the low capacity of these prions to infect wild-type and human PrP^{C} -expressing transgenic animal models. However, it was later proven that the infectiousness of a particular prion isolate depends also on the model chosen. For example, GSS was found to be highly transmissible to bank voles, causing a neurological disease and the accumulation of PrP^{Sc} in the brain. In this study, we present a new model of prion disease consisting of bank vole PrP^{C} - expressing mice (TgVole) at approximately physiological levels (1x) experimentally infected with one isolate of GSS linked to the mutation A117V. GSS A117V transmits by intracerebral route to TgVole (1x) with high efficiency and extremely short incubation periods of 67 ± 1 dpi. Additionally, GSS A117V transmits by intraperitoneal route to TgVole (1x) with a period of 72 ± 3 dpi, being nearly the same as the intracerebral route. To further characterize the prionopathy triggered by GSS A117V prions in the aforementioned TgVole (1x) murine model and to identify biomarkers in easily accessible body fluids, we performed a kinetic study by collecting blood samples along the incubation period of the animals and analyzed two biomarkers: neurofilament light chain (NfL) and β-synuclein (β-syn). The combination of the brief incubation period by intracerebral and, importantly, intraperitoneal route with the possibility to track the disease progression by means of serum biomarkers postulates this new TgVole model as an ideal *in vivo* approach for preclinical studies on future treatments for human transmissible spongiform encephalopathies.

Scientific Posters: Prion diseases in animals

P3 | Identification of the presence of a scrapie resistance gene in sheep on the territory of the Republic of Serbia

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Monitoring of scrapie in sheep and goats has been carried out for many years in Serbia. Despite many years of monitoring, only one case of this disease has been reported in sheep, but given that, there are numerous cases of the disease in some neighboring countries, it is very important to determine the presence of resistant genes in the sheep population in Serbia. Genetic susceptibility to scrapie is influenced mainly by the prion protein polymorphisms of codons136, 154, and 171. The ARR allele is considered to provide very strong resistance against classical scrapie and the VRQ genotype is the most susceptible. In order to examine the genetic makeup of sheep in Serbia related to scrapie, we optimized TaqMan probes of real-time polymerase chain reaction (qPCR) techniques for three codons. We analyzed blood samples from 100 sheep using qPCR and the results showed that AA homozygous for the 136 codon were the most common. For codon 154 the most frequent genotype was RR and for codon 171 the most frequent genotype was QQ.

P4 | Mineral licks: a chronic wasting disease monitoring proposal

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Described in the 60s in the United States, Chronic Wasting Disease (CWD) is now widespread in North America (NA) and, recently, some unrelated cases have been reported in Europe. This prion disease, which affects both free-ranging and captive cervids, is characterized

by a notable spread, being a sign of it the expansion of CWD in NA to 30 states of the US, 4 provinces of Canada and, through the importation of diseased animals, to Korea. That remarkable spread of CWD is due to its high horizontal transmission capacity, which is mainly caused by the presence of prions in the fluids of infected cervids and promoted by characteristic clinical signs such as polyuria and sialorrhea, contributing to their release into the environment. Thus, places where cervids congregate act as foci of CWD transmission, such as those surrounding mineral licks where they gather. Mineral licks are locations where animals can take essential mineral nutrients from deposits of salts and other minerals and can be naturally occurring or artificial. Here, we explore the possibility of taking advantage of mineral licks as CWD transmission fomites and use them for monitoring the potential expansion or emergence of new cases of the disease. For that, laboratory-prepared CWD-infected saliva has been added to a salt lick to emulate a mineral lick used by a CWD-infected cervid. The resulting CWD-spiked mineral lick has been processed and submitted to RT-QuIC in order to assess if naturally CWD-infected mineral licks could be used for CWD monitoring.

P5 | Monitoring the presence of CWD in the population of Slovenian red deer

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Chronic Wasting Disease (CWD) was first discovered in European cervids in 1996. To date, it has been confirmed in reindeer (*Rangifer tarandus tarandus*), moose (*Alces alces*), and red deer (*Cervus elaphus*). Although official monitoring for CWD was conducted in Slovenia only in 2006 and 2007, we collected and examined 190 brains from red deer and 2 brains from Canadian deer - wapiti (*Cervus canadensis*) between 2002 and 2021. Most brains were from healthy animals (161 samples or 83.9%), 23 (12.0%) from dead animals, and 8 (4.2%) from animals with clinical neurological signs. All samples were examined with rapid post-mortem tests, histopathology, and, in most cases, immunohistochemistry and were all negative for CWD. To determine the genotypes of the prion protein gene (*PRNP*) in the Slovenian red deer population, DNA was isolated from the muscles of 15 animals sent for CWD testing, and the *PRNP* genotype was determined by sequencing. Although only a small number of animals were genotyped, nucleotide variants at 11 *PRNP* codons of 12 currently described polymorphisms were identified in these animals from the Slovenian red deer population (approximately 8000 red deer hunted yearly). Previous studies have shown that cervides with all currently reported genotypes of *PRNP* could be affected by CWD, but some polymorphisms are associated with lower attack rate of CWD. Our results indicate that the Slovenian red deer population is heterogeneous with respect to CWD genotypes and may also be susceptible to CWD.

P6 | Lack of evidence of transmission on 1st passage of idiopathic human prion disease to small ruminant mouse models Tg338 and Tg501

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About 90% of Creutzfeldt-Jakob disease cases are classified as sporadic (sCJD), that is, occur infrequently, randomly and without a known cause. It is a fatal

neurodegenerative disease with an incidence of 1-1.5 cases *per* million, without treatment or prophylaxis at present. Epidemiological studies have been so far unable to establish a causal relationship between sCJD and prion diseases in animals.

The zoonotic potential of sheep scrapie was demonstrated in 2014 (Cassard et al., Nature Communications) through inoculation of transgenic mice overexpressing the human prion protein with scrapie isolates. The resulting prion disease was indistinguishable from that occurring after sCJD inoculation in the same model and, while these results do not demonstrate that sCJD is caused by scrapie prions, they do show that the transmission barrier between ovine and human prions is not absolute.

To further assess this zoonotic risk, we have prepared inocula from 3 sCJD cases (MM1, MV2 and VV2) and 2 VPSPr cases (MM and MV) to verify if it is possible to recover the scrapie phenotype. No evidence of transmission has been found on a first passage in Tg338 nor Tg501 ovinized mouse bioassays (second passages ongoing).

In vitro amplification of the same isolates in Tg338 and Tg501 brain homogenates rendered identical results, with the exception of CJD-MM1 prions, for which PrP^{res} could be detected. However, the similarity of the detected prion with that arising spontaneously in unseeded Tg338 amplification reactions, suggests it may not derive from the propagation of the human seed, and thus, that the transmission barrier between human prions and sheep is greater than in the opposite direction.

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Scientific Posters:
Prion Structure and Biology

P7 | Effect of the octapeptide repeat region on prion strain properties and spontaneous misfolding propensity of PrP

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Transmissible spongiform encephalopathies (TSE) or prion diseases are fatal neurodegenerative disorders affecting both humans and other mammals. These diseases are caused by the aberrant isoform (PrP^{Sc}) of the cellular prion protein (PrP^{C}), which in ways yet to be deciphered causes amyloid aggregation in the brain, consequently leading to neuropathological changes. From a structural point of view, the PrP presents a highly ordered C-terminal globular domain with three α-helices and two β-sheets, and an unstructured N-terminal domain. The N-terminal domain contains the octapeptide repeat region (OR), a tandem repetition of up to five octapeptide sequences. It is worth noting that alterations in the number of OR have been related to an early or delayed disease onset, due to a potential variation in conversion ability from PrP^{C} to PrP^{Sc}. It is currently known that the C-terminal domain is sufficient for prion conversion and infectivity, however, that does not exclude the OR region from having an effect in disease development and act as a potential modulator. Thus, to assess the effect of the OR in misfolding we tested the ability of recombinant PrP (recPrP) variants presenting a range from 0OR to 14OR to spontaneously convert into PrP^{sc} *in vitro*. We furthermore tested the propagation capacity of some of these recPrP in the presence of distinct infectious seeds to determine if the OR region could have any role on the conservation of strain characteristics. Using Protein Misfolding Shaking Amplification (PMSA), we could assess the effect of this region on prion misfolding.

P8 | Strain-specific morphology of reactive astrocytes in prion diseases

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Background: Reactive astrogliosis is a common marker of human and animal prion diseases. Recently, an increasing number of studies argued the neuroprotective or neurotoxic role of astrocytes during prion pathogenesis. Emerging evidence highlights that several factors modulate astrocytic phenotype, such as the involved brain region, host genotype and prion strain. The influence of strain is still debated. Here, we investigated the relationship between strain and astrocytic phenotype in bank voles.

Methods: Seven human- and animal-derived prion isolates representative of six vole-adapted strains were selected. One vole-adapted strain was isolated from both a human case of Gerstmann-Sträussler-Scheinker (GSS) and a spontaneous disease in transgenic mouse over-expressing bank vole prion protein (TgL1). Astrocytic phenotype was analysed by morphological and morphometric approaches using immunohistochemistry and Fiji software. The relationship between astrocytes and patterns of PrP^{Sc} deposition was also investigated.

Results: All prion strains produced astrogliosis in brain regions affected by spongiosis and PrP^{Sc} deposition. Mediodorsal thalamic nucleus (MDTN) was affected in all strains, therefore it was the region selected to compare astrocytes involvement. The morphological analysis of resident astrocytes in MDTN showed that astrocytes displayed phenotypic heterogeneity and distinctive strain-specific morphology. Interestingly, the similar morphology of reactive astrocytes confirmed the resemblance of the strain isolated from both GSS and TgL1.

Conclusion: This study confirmed the heterogeneous response of astrocytes to prions and highlighted the pivotal influence of prion strain on astrocyte phenotype.