Prion, 10:165–181, 2016
Published with license by Taylor & Francis
ISSN: 1933-68961933-690X online
DOI: 10.1080/19336896.2016.1175801

PERSPECTIVE

The Priority position paper: Protecting Europe's food chain from prions

Jesús R. Requena [a], Krister Kristensson[b], Carsten Korth[c], Chiara Zurzolo[d], Marion Simmons[e], Patricia Aguilar-Calvo[f], Adriano Aguzzi[g], Olivier Andreoletti[h], Sylvie L. Benestad[i], Reinhard Böhm[j], Karen Brown[k], Byron Calgua[l], José Antonio del Río[m], Juan Carlos Espinosa[f], Rosina Girones[l], Sue Godsave[n], Ludwig E. Hoelzle[j], Michael R. Knittler[o], Franziska Kuhn[p], Giuseppe Legname[q], Paul Laeven[r], Neil Mabbott [k], Eva Mitrova[s], Andreas Müller-Schiffmann[c], Mario Nuvolone[g], Peter J. Peters[t], Alex Raeber[p], Klaus Roth[u], Matthias Schmitz[v], Björn Schroeder[p], Tiziana Sonati[g], Lothar Stitz[o], Albert Taraboulos[w], Juan María Torres[f], Zheng-Xin Yan[u], and Inga Zerr[v,x]

[a]CIMUS Biomedical Research Institute, University of Santiago de Compostela, Santiago de Compostela, Spain;
[b]Karolinska Institute, Stockholm, Sweden;
[c]University of Düsseldorf, Düsseldorf, Germany;
[d]Institut Pasteur, Paris, France;
[e]APHA, Weybridge, UK;
[f]CISA, INIA, Madrid, Spain;
[g]University of Zürich, Zürich, Switzerland;
[h]ENVT-INRA, Toulouse, France;
[i]Norwegian Veterinary Institute, Oslo, Norway;
[j]University of Hohenheim, Stuttgart, Germany;
[k]University of Edinburgh, Edinburgh, UK;
[l]University of Barcelona, Barcelona, Spain;
[m]IBEC, Barcelona, Spain;
[n]Netherlands Cancer Institute, Amsterdam, The Netherlands;
[o]Friedrich Löffler Institut, Insel Reims, Germany;
[p]Prionics, Zürich, Switzerland;
[q]SISSA, Trieste, Italy;
[r]University of Maastricht, Maastricht, The Netherlands;
[s]Medical University of Slovakia, Bratislava, Slovakia;

[t]The Maastricht Multimodal Molecular Imaging Institute, University of Maastricht, Maastricht, The Netherlands;
[u]SMP GmbH, Tübingen, Germany;
[v]Universitätmedizin Göttingen, Göttingen, Germany;
[w]The Hebrew University, Jerusalem;
[x]German Center for Neurodegenerative Diseases (DZNE), Göttingen, Germany

ABSTRACT. Bovine spongiform encephalopathy (BSE) created a global European crisis in the 1980s and 90s, with very serious health and economic implications. Classical BSE now appears to be under control, to a great extent as a result of a global research effort that identified the sources of prions in meat and bone meal (MBM) and developed new animal-testing tools that guided policy. Priority (www.prionpriority.eu) was a European Union (EU) Framework Program 7 (FP7)-funded project through which 21 European research institutions and small and medium enterprises (SMEs) joined efforts between 2009 and 2014, to conduct coordinated basic and applied research on prions and prion diseases. At the end of the project, the Priority consortium drafted a position paper (www.prionpriority.eu/Priority position paper) with its main conclusions. In the present opinion paper, we summarize these conclusions.

With respect to the issue of re-introducing ruminant protein into the feed-chain, our opinion is that sus taining an absolute ban on feeding ruminant protein to ruminants is essential. In particular, the spread and impact of non-classical forms of scrapie and BSE in ruminants is not fully understood and the risks cannot be estimated. Atypical prion agents will probably continue to represent the dominant form of prion diseases in the near future in Europe. Atypical L-type BSE has clear zoonotic potential, as demonstrated in experimental models. Similarly, there are now data indicating that the atypical scrapie agent can cross various species barriers. More epidemiological data from large cohorts are nec essary to reach any conclusion on the impact of its transmissibility on public health. Re-evaluations of safety precautions may become necessary depending on the outcome of these studies.

Intensified searching for molecular determinants of the species barrier is recommended, since this bar rier is key for important policy areas and risk assessment. Understanding the structural basis for strains and the basis for adaptation of a strain to a new host will require continued fundamental research, also needed to understand mechanisms of prion transmission, replication and how they cause nervous system dysfunction and death. Early detection of prion infection, ideally at a preclini cal stage, also remains crucial for development of effective treatment strategies.

KEYWORDS. atypical scrapie, atypical BSE, BSE, CJD, prion, scrapie

The Priority Position

About 30 y ago, the appearance of BSE in the United Kingdom (UK) quickly brought the previously obscure "prion diseases" into the public spotlight. The ensuing health and food crises that spread throughout Europe had devastating consequences. In the UK alone, there were more than 36,000 farms directly affected by BSE and the transmission of BSE prions to humans via the food chain has caused over 200 people, most of

Correspondence to: Jesús R. Requena; CIMUS Biomedical Research Institute, University of Santiago de Compostela-IDIS, Avda. de Barcelona s/n, 15782 Santiago de Compostela, Spain; Email: jesus.requena@usc.es

Received February 5, 2016; Revised March 28, 2016; Accepted April 1, 2016.

them in Europe, to die from variant Creutzfeldt-Jakob disease (vCJD).[1]

Classical BSE now appears to be under control, with 18 EU member states having achieved the World Organization for Animal Health (*Office International des Epizooties*) 'negligible risk' status[2] and the remaining member states assessed as presenting 'controlled' risk. Of note, research, including EU-funded research, has played a key role in this success: while the origin of the infection was never defined, the principal driver of the epidemic was identified as prions in MBM.[3] Tests based on the use of prion protein-specific antibodies were developed, allowing detection of infected carcasses, and a better understanding of disease pathogenesis and the distribution of infectivity in edible tissues, and experimental investigation of transmission barriers between different species allowed an estimation of risks.[4] All of this led to the implementation of rational and effective policies, such as the MBM ban to protect the animal feed chain, and the Specified Risk Material (SRM) regulations to protect the human food chain (see reference 5 for an update on relevant EU legislation[5]).

In spite of this progress, prions are still a threat. Epidemiological re-assessment, and data on late-onset kuru in 129M/V heterozygous *PRNP* carriers,[6] indicate that the period separating the peaks of the BSE and the vCJD epidemic ($\sim$10 years) is probably too short to include potential late-onset vCJD.[7-8] In addition, results from a large number of human tonsil and appendix analyses in the UK suggest that there may be a high number of asymptomatic individuals who are positive for the disease-associated prion protein conformer, PrP^{Sc}.[10] While vCJD is the only form of human prion disease that has been consistently demonstrated to have lymphoreticular involvement, there has been no systematic investigation of lymphoid tissue in humans with other prion diseases.

The Human Prion Problem

The clinical cases of vCJD identified to date have all shared a common prion protein (PrP) genotype, M129M, although one pre-clinical case was confirmed as an M129V heterozygote, and it has been mooted that perhaps only the M129M proportion of the population is susceptible. However, in the UK appendix study, PrP accumulation was described in samples representing every codon 129 genotype. This raises the possibility that *PRNP* genotype does not confer resistance but instead modulates incubation period.[10]

Recent experiments in highly susceptible mouse models indicate the presence of infectivity in blood or blood components at late disease stages in sporadic CJD.[11] The significance of this experimental finding for humans has to be explored in more detail, although, at the present time, there is no evidence for the transmission of prions via blood in sporadic CJD. However, a likely scenario is that all cases with signs of infection or abnormal PrP accumulation in peripheral tissue could have infective blood, posing the risk for transmission via blood products, and this has been clearly demonstrated in experimental models,[12] and confirmed in several cases of vCJD in humans.[9,13] Altogether, these data clearly demonstrate the potential risk of a second, late-onset wave of vCJD, particularly when the number of people identified with lymphoid accumulation of PrP^{Sc} (16/32411) gives a prevalence estimate in the UK of 493 per million, much higher than the number of clinical cases seen to date.

The Animal Prion Problem

An increasing number of reports of cases of "atypical" BSE in cattle throughout the EU and beyond, in part a reflection of increased testing and awareness, may be a forewarning of a new epidemic, particularly since we still do not understand all the factors determining the species barrier. Ovine scrapie is another concern, because it could mask ovine BSE, presumably transmissible to humans. While ovine scrapie and BSE can be analytically told apart *post-mortem*, differentiation is not easy in live animals– the clinical sign differences being very subtle.[14] Scrapie is endemic and not likely to be eradicated soon, although current control measures are effective at greatly reducing disease incidence. Atypical forms, which may be spontaneous, are not affected by these control measures and they will persist in the global

animal population. These disease forms now predominate, and effective surveillance is very challenging, in part as a consequence of the absence of clusters, which makes their identification rather demanding in terms of labor and costs. Atypical cases of BSE and scrapie presently clearly outnumber classical cases in cattle and sheep in many member states. There is a clear risk attendant on ignoring these cases without an understanding of their possible zoonotic potential, or their potential to become an animal health problem should current feed controls be relaxed, particularly when most forms of human disease have no established etiology.[15]

The Priority Project

The Priority project was launched in September 2009. A Large Integrating Project within the EU FP7, its immediate objective was to "protect Europe's food chain from prions," while its wider scope was to understand prions by using basic and applied research. Priority focused on 4 themes, namely: a) the structure, function, conversion and toxicity of prions; b) detection of prions; c) mechanisms of prion transmission; and d) spreading and epidemiology of prion diseases. The project ended in September 2014. Research associated with the project has resulted in publication of more than 100 scientific papers (www.prionpriority.eu), and the elaboration of a Position paper with the main conclusions of the consortium at the end of the project. The current paper summarizes the opinions/positions reached within these themes at the end of the project. The full Priority position paper can be found in: www.prion priority.eu/Priority position paper.

PRION STRUCTURE, FUNCTION, CONVERSION AND TOXICITY

The mechanisms for conversion of the normal cellular prion protein, PrP^{C} to PrP^{Sc}, as well as strain diversity and transmission barriers, are structurally enciphered.[16,17] Therefore, it is essential to understand the structure of PrP^{Sc} in order to design methods to interfere with prion propagation and spread.

PrP^{Sc} forms double amyloid fibers made up of 2 intertwined fibrils, each ~3–5 nm wide, with no regular pitch. Fiber X-ray diffraction data, cryo-EM analysis and geometric considerations strongly suggest that each PrP^{Sc} monomer stacked in each protofilament is a 4-rung β-solenoid, with a high structural resemblance to the HET-s fungal prion.[18,19] Limited proteolysis studies corroborate that PrP^{Sc} contains stretches of high resistance to proteinase K (PK), presumably β-strands, interspersed with short stretches with a higher proteolytic susceptibility, presumably loops and turns.[20] The C-terminal stretch (~180–231) is the most PK resistant region, arguing that no residual α-helical structure remains in PrP^{Sc}.[20] While this knowledge of the structure of PrP^{Sc} is still far from complete, it allows us to hypothesize how propagation of PrP^{Sc} might occur: templating must involve the upper- and lowermost rungs of the β-solenoid. These edge strands are "sticky;" they will propagate their hydrogen-bonding pattern into any amyloidogenic peptide they encounter.[21] In fact, the β-strands of native proteins based on a β-solenoid motif have evolved to contain capping loops and other structures to block unregulated stacking. Furthermore, the elimination of the capping structures results in edge-to-edge driven oligomerization.[22] Thus, the upper and lower β-solenoid rungs could template an incoming unfolded PrP molecule, creating additional β-solenoid rungs. Once the initial additional β-rung has formed, it creates a fresh "sticky" edge ready to continue templating until the whole incoming unfolded PrP molecule is fully converted into another copy of the infectious conformer, *i.e.*, a complete 4-rung solenoid stacked on its template.

It is noteworthy that the molecular forces responsible for the templating, *i.e.*, hydrogen-bonding, charge interactions, aromatic stacking, and steric constraints, are fundamentally similar to those operating during DNA replication. Obviously, the exquisite specificity of the A:T and G:C pairings is lacking and instead, a much more complex array of forces controls the pairing of the pre-existing and nascent β-rungs.

Unraveling the early events in structural prion formation in sporadic forms of prion disease is of major importance, since the

conversion of PrP^{C} to PrP^{Sc} is the central event in prion diseases. Hereditary prion diseases are associated with about 40 point mutations of the *PRNP* gene that codes for PrP. Most of the variants associated with these mutations are located in the globular domain of the protein.[23]

Opinions-Positions

The basic tenet of the prion theory, *i.e.* that protein misfolding can be faithfully propagated, is now widely accepted. Moreover, novel data increasingly implicate similar "prionoid" principles in the pathogenesis of "proteinopathies" such as Alzheimer's, Huntington's and Parkinson's disease.[24,25] In sharp contrast to this fundamental understanding, and despite the development of many new tools for prion research, the most basic mechanistic details of how prions function and how they cause disease have not been completely solved yet.

Major Questions and Scientific Challenges

Many aspects of prion replication can be demonstrated *in vitro* in cell-free systems containing PrP^{C} and PrP^{Sc}.[26] At first approximation, prion propagation can thus be reduced to a biophysical problem dealing with alternative conformations, amyloid structures, and conformational coercion.[19] However, like other pathogens, prions maintain a complex, 2-way relationship with the host cell. It is clear that prions propagate in their natural hosts much more efficiently than they do *in vitro*.[27] The host cell provides both the molecular species (such as PrP^{C}) and the molecular mechanisms required for prion propagation. Although now we are beginning to understand the molecular underpinnings of (i) the uptake of prions by the host cell and relevance of intracellular pathways for prion conversion, (ii) the influence of host cell signals and factors on prion replication, (iii) the normal function of the prion protein and pathogenesis, *i.e.*, mechanisms by which prions cause dysfunctions or damage to the neurons, and (iv) the transfer of prions to neighboring cells, there is still much to cover in these areas.

Regarding the role of intracellular trafficking in prion diseases, amyloidic prion structures called "strings" containing PrP^{Sc} are found on the surface of infected cells,[28,29] while the endosomal compartment is also strongly implicated in prion conversion.[30] Furthermore, novel structures called tunneling nanotubes (TNTs) appear to have a major role in spreading PrP^{Sc} between cells in cultures,[31] however better tools are needed to demonstrate their presence and role *in vivo*.[32]

One major scientific challenge is to better understand the structural basis of the different prion strains and the mechanisms behind the transmission barriers between animal species. However, we are now in a position to state that strains are specific molecular topologies of the upper and lower surfaces of PrP^{Sc} monomers in a propagative PrP^{Sc} stack (either oligomer or protofilament in a fiber); in turn, transmission barriers consist of steric hindrances hampering continuation of the cross-β stack by preventing an incoming PrP strand from being molded onto the pre-existing PrP^{Sc} assembly.[19] Related to this is the question of why some prions are more pathogenic than others for humans. An additional challenge will be to investigate age-related factors that promote development of sporadic prion diseases, since these mainly affect aging people (average age of onset is around 65 y old). Another major effort to understand basic mechanisms of the disease would be required to develop adequate and early therapies for humans affected by the diseases. This input can only come from the scientific community because there is a clear lack of industrial investment to study and develop compounds for the treatment of CJD affected individuals.

Strategic Objectives and Priorities in the Future

- More data of a higher resolution are needed to understand the structural basis of prion strain transmission barriers, e.g. by NMR-based, deuterium exchange analyses of recombinant PrP^{Sc}.
- Structural analysis of the various point mutations present in the globular domain of PrP^{Sc} should unveil common folding traits that may allow a better understanding of the early

conformational changes leading to the formation of monomeric PrP^{Sc}.
- Analyses should be carried out combining high resolution imaging tools and neurophysiology that leads to a better understanding of the function of the prion protein and the intercellular transmission of its pathological isoforms.
- Analyses of the cause of prion toxicity and identification of host cell-derived factors that are "partners in crime" should provide novel strategies aimed at blocking prion propagation and toxicity.
- Treatment strategies for individuals affected by CJD and of prophylactic approaches for carriers of pathogenic PRNP mutations should be developed.

PRION DETECTION

Post mortem detection systems for PrP^{Sc} in the central nervous system (CNS) and lymphoreticular tissue are nowadays widely used for surveillance of BSE and scrapie in animals.[33] These rapid tests have greatly improved the detection of infected carcasses before their entry into the human food chain. Lately the development of highly sensitive methods like protein misfolding cyclic amplification (PMCA) and real time quaking induced conversion (RT-QuIC) made it possible to detect even minor amounts of PrP^{Sc} in body fluids like blood or cerebrospinal fluid.[34] Even though these tests still need to be improved to allow widespread use in routine laboratories, their robustness has been demonstrated. They open up new ways for live tests to detect prions.[35]

Opinions-Positions

The emergence of *in vitro* amplification technologies such as PMCA and RT-QuIC represents a real revolution for prion detection. These techniques display sufficient sensitivity to allow prion detection in the body fluids (such as blood and cerebrospinal fluid) collected from affected individuals, and their ability to do this has been demonstrated in both sheep and human samples.[34] However, at the moment they remain of limited robustness and the mechanisms and analytical conditions which allow amplification of misfolded PrP remain largely unknown. Such issues are similar to those encountered when PCR was developed in the 1980s. Despite those initial difficulties PCR is now a basic research laboratory technique.

Prions may also be considered as potential environmental contaminants and their stability in the environment, wastewater and soils must be evaluated as a valuable parameter for developing risk assessment studies. Prions are extremely resistant to inactivation and it has been demonstrated that they can survive in soil for years.[36,37] In recent years, deposition of scrapie and chronic wasting disease (CWD) infectivity in the environment through biological fluids and/or faeces has been proved,[38] and BSE and scrapie can also be introduced anthropogenically by transporting infectious prions via landfill leach or slaughterhouse wastewater.[39,40] All of this strongly suggests that infectious prions can enter the environment, and could be transported via water, resulting in exposure of both humans and animals to infectious prion diseases. Therefore, it is critical to adapt and develop analytical methods and strategies to evaluate the fate of infectious prions in the environment and the potential sources of contamination.[40,41]

Major Questions and Scientific Challenges

A major scientific challenge is to develop new, and refine existing, prion detection methods that could have applications in pharmaceutical screening, consumables testing, environmental monitoring (e.g., allowing re-population of previously affected farms), and *in vivo* diagnostics.

The behavior and stability of prions in the environment and wastewater have to be better defined, and the efficiency of wastewater treatments for the removal of prions needs to be assessed.

Strategic Objectives and Priorities in the Future

- Improving the performance and robustness of *in vitro* prion amplification techniques.

- Establishing a relationship between the presence of PrP^{Sc}, as demonstrated in an environmental matrix by *in vitro* amplification methodology, and the risk of prion transmission for an individual that would be exposed to such a matrix.
- Redefining the techniques available to optimize the detection of prions in diverse environmental matrices, with validated protocols.

PRION TRANSMISSION AND SPREADING

Following peripheral exposure, some acquired prion strains accumulate and replicate in the secondary lymphoid organs (SLO) such as the gut-associated lymphoid tissues (GALT) in the gastrointestinal tract. This amplification stage in the SLO is critical for the efficient spread of these prion strains to the central nervous system through a process termed neuroinvasion. In the past 15 y there has been intensive research into this early phase of the disease process.[42,43] Identification of the important cellular and molecular processes required for the establishment of prion infection in the GALT and subsequent neuroinvasion has implications beyond academic interest, as experiments have shown that treatments that block this process can dramatically reduce susceptibility to peripherally-acquired prions.[44,45] Indeed, the demonstration that vCJD in humans most likely arose due to consumption of BSE-contaminated food has focused attention on whether immunotherapeutic approaches, such as anti-prion vaccines, may represent a valid therapeutic strategy against prion infection.[46]

Opinions-Positions

Although control measures have helped to limit further spread of BSE in Europe, prions continue to pose important health, welfare and economic problems to the livestock industry worldwide and have direct consequences for human health. Countries outside Europe are reporting BSE cases, and novel BSE variants with uncertain transmission potential have been described. BSE has also been identified in goats, raising concern that it may have infected sheep.[47,48] Little is known of the mode of transmission of CWD in North America and the consequences to livestock and human health. The transmission potential of atypical forms of sheep scrapie is also uncertain. Many important questions remain concerning the pathogenesis and modes of transmission of prion diseases in animals and humans. The lack of prion-specific preclinical diagnostics compounds the problems for disease identification, treatment and eradication. Since future outbreaks of prion disease are likely to be orally-acquired, a thorough analysis of the factors that influence susceptibility to orally-acquired prions will enhance our understanding of the factors which increase the risk of disease transmission, improve preclinical diagnosis and help identify novel targets for prophylactic and therapeutic intervention.

A unique subset of stromal cells within the B-cell follicles of secondary lymphoid tissues, termed follicular dendritic cells (FDC), are considered to be the essential early sites of prion accumulation and replication.[44,45,49] Data from experimental scrapie prion transmissions to mice show that initial replication in the GALT of the small intestine such as the Peyer's patches is critical for efficient neuroinvasion.[50,51] As well as expressing high levels of cellular prion protein, these cells trap and retain the prions on their surfaces, where they are amplified above the threshold level required to achieve infection of local peripheral nerves.[49,52-54] However, the requirement for initial accumulation and replication on FDC is not absolute. Under certain circumstances some prion strains or isolates may accumulate in lymph nodes in association with high endothelial venules independently of FDC.[55] Also, some prion strains such as BSE appear to achieve neuroinvasion from the gastrointestinal tract without any apparent amplification step in the GALT.

Following their amplification within the SLO, prions subsequently infect the peripheral nerves within these tissues and spread along them to the CNS.[56,57] How prions initially

spread from the FDC and infect the peripheral nervous system is uncertain. A role for classical dendritic cells in this process has been proposed in which these cells may shuttle prions between FDC and nearby peripheral nerves.[58] Alternatively, tunnelling nanotubes which extend and connect cells may act as interconnecting conduits through which prions may be transferred between cells.[31]

The demonstrations that immune cells played an important role in the establishment of prion infections, and that prions were present in significant burdens in the SLO for the duration of the preclinical phase,[59] suggested that vCJD had the potential to be accidentally transmitted horizontally between humans via the transfusion of blood or blood products from an infected individual.[9] As a consequence of these findings, procedures such as leukodepletion were introduced in an attempt to reduce the potential for vCJD to be transmitted via the transfusion of blood or blood products.

Sites of chronic inflammation, by inducing the formation of ectopic FDC-containing B-cell follicles or granulomatous lesions, might influence the organ tropism of prions and induce or enhance local prion infectivity of tissues that otherwise would not be considered at risk.[60,61] The detection of disease-specific PrP in mammary glands from lentivirus/prion co-infected ewes warned about the potential exposure of humans to prions through the consumption of small ruminant milk. Sheep with scrapie and lentiviral mastitis accumulate prions within the ectopic follicles in the inflamed mammary glands. Importantly, these sheep also secreted levels of prions in their milk which were sufficient to transmit disease to suckling lambs.[62] Subsequent studies in non-mastitic goats and sheep have since suggested that milk and colostrum may transmit classical scrapie from an infected ewe to their offspring.[63-65] Together, these elements called for an urgent understanding of potential prion infectivity levels associated with ruminant blood and milk.

The consumption of BSE-contaminated meat products is the most likely source of vCJD in humans.[66] During the BSE epidemic in the UK it is estimated that up to approximately 500,000 infected cattle were slaughtered for human consumption.[67] Surprisingly, despite the likely widespread exposure of the UK human population to BSE prions, most clinical cases of vCJD have occurred almost exclusively in young adults (median age at onset of disease, 26 years; median age at death, 28 years). The factors responsible for the age-related incidence of vCJD are uncertain as the possibility that young adults were exposed to greater levels of BSE by dietary preference is unproven.[68] Data from experimental prion transmissions to mice suggest that the effects of aging on the microarchitecture of SLO dramatically reduce susceptibility to peripherally-acquired prion disease.[69-72]

Major Questions and Scientific Challenges

After ingestion, BSE prions in cattle appear to be able to infect the nervous system independently of consistently detectable replication within the GALT. The rules which govern this for BSE and other prion isolates are currently uncertain, since oral transmission of BSE prions to other species appears dependent on agent replication in the GALT prior to neuroinvasion. An ability to predict the transmission characteristics following the identification of a novel prion isolate will be important for assessing human and animal health risks.

Much progress has been made in our understanding of how prions are initially conveyed from the site of exposure to cells within the SLO upon which they replicate.[42,43,73] For example, after oral exposure prions may cross the gut epithelium via M cells to infect the Peyer's patches.[74,75] How prions subsequently infect the FDC within the B-cell follicles is uncertain, but a role for classical dendritic cells in this process has been considered.[56,76]

Once the prions have been amplified on the surfaces of FDC above the threshold required for neuroinvasion, they infect the enteric nerves within the intestine,[56,77] and spread via the peripheral nervous system (both sympathetic and parasympathetic) to the CNS.[78,79] Although the relative positioning of the FDC and sympathetic nerves within the SLO appears to influence the rate of neuroinvasion, the

precise mechanism by which this occurs is uncertain.

The transmission of prion strains within the same host species is typically efficient and causes disease in the recipients with highly reproducible disease characteristics. However, inter-species prion transmissions upon first passage are typically characterized by their low efficiency and extended disease incubation periods. This effect on prion transmission is termed the 'species barrier' effect. Many factors are known to have an important influence on the inter-species transmission of prions such as polymorphisms and mutations in the PRNP gene (which encodes PrP^C), and biophysical aspects of templating events are clearly important (see above). Unfortunately the precise molecular mechanism/s responsible for the species barrier effect is uncertain. An ability to predict the potential for a novel prion isolate to have the potential to transmit to other species, especially humans, is crucial to restrict and control future prion disease outbreaks.[80]

Pioneering studies have shown that both passive and active immunization against PrP are feasible approaches to attempt to block experimental prion diseases.[81,82] Many natural prion infections are orally acquired, and mucosal vaccination against prions appears to be effective and the most appropriate method for protection against orally acquired prion infections.[83] However, a mucosal vaccine may offer little protection against accidental iatrogenic CJD transmissions via contaminated blood or blood products, tissues or contaminated surgical instruments. Therefore, an ideal anti-prion vaccine should be able to induce both strong mucosal and systemic anti-PrP antibody responses. However, the cellular prion protein is almost ubiquitously expressed in the mammalian host, and so the potential for any anti-prion vaccines to bind to host PrP^C and cause autoimmunity or cell toxicity must be carefully considered. A recent study has provided valuable information on the factors which influence the ability of anti-prion antibodies to cause rapid neurotoxicity.[84] These data suggest it may be possible to identify those vaccines which induce the development of potentially neurotoxic anti-PrP antibodies.

Strategic Objectives and Priorities in the Future

- Thorough characterization of the cellular relays by which prions are propagated from peripheral sites of exposure to the central nervous system should be carried out.
- Determination of the factors which control prion replication in SLO, and why this is obligatory for the efficient neuroinvasion of some, but not all, prion strains.
- Determination of the molecular characteristics, other than PrP^C expression, which render cells such as FDC susceptible to prions.
- Identification of the mechanisms involved in cell-to-cell prion spreading.
- Assessment of how prions are released into body fluids and how this may contribute to their contamination of the environment, and their transmission between hosts.
- Development of effective immunotherapeutic strategies to protect the host against peripherally acquired prions, and a better understanding of when to target these strategies based on host age/species.
- Understanding of the underlying factors which determine the species barrier effect, to enable the prediction of the risks to human and animal health posed by novel prion strains.
- Development of a highly sensitive and specific diagnostic test to identify prion-infected individuals from the early preclinical stages of disease, and gaining of a more accurate understanding of prevalence of these diseases in populations.
- To enable the above to be achieved efficiently, it will be important to establish consistent protocols and experimental models to enable meaningful comparisons to be made between studies generated in different laboratories.

PRION EPIDEMIOLOGY

In recent years considerable disease control efforts have been made by the EU to control

prion infections in cattle, sheep and goats through the implementation of rigorous regimes. An array of regulations such as the introduction of the feed ban, a compulsory surveillance and monitoring system, the removal and destruction of SRM and the establishment of culling strategies for small ruminants by member state authorities have all had a significant impact on the incidence and spread of disease.

Undoubtedly, these measures have been responsible for reducing the number of BSE cases detected in the EU from 2,167 in 2001 (15 member states) to 68 cases in 2009, 44 cases in 2010 and down to 7 cases in 2013/14 (September 2014) in 27 member states.

CWD is a naturally-occurring prion disease in domestic and wild cervids (moose deer and elk), which has reached epidemic proportions in the US and Canada.[85] However, it has not been detected in Europe so far, even though there is significant trafficking of potentially contaminated materials between continents. The potential presence of CWD in Europe is not continuously monitored, although there was a single EU-wide cervid surveillance exercise undertaken some years ago in the context of the EU Regulations for BSE and scrapie, which detected no cases. The deposition of scrapie and CWD prions in the environment occurs through biological fluids and/or faeces.[39,86] Data depict a scenario where prions may accumulate in the environment due to direct shedding from pre-clinical animals, and remain infectious in soil and water for periods of time long enough to permit transmission to susceptible individuals.[87,88] Thus CWD, unlike BSE is horizontally and vertically transmitted among captive and wild animals, so it is extremely difficult to eradicate. Given that both red deer and reindeer are native to Europe, there is no reason to assume that CWD could not become a problem in the European continent.

Although the scenario for BSE may not be exactly the same (BSE prions are present at much lower levels in extraneural tissues than in the CNS), deposition of BSE prions in the environment may occur due to burial of carcasses and mortalities, and to a lesser extent, through biosolids generated in water treatment plants by the processing of infected animals which had not been identified and removed. Presumably this scenario occurred frequently during the BSE epidemic. Furthermore, there is the possibility of discharged contaminated urine, feces and blood from CJD or vCJD patients.[89-91]

In humans, several molecularly-defined disease subtypes have been described. [92-95] However, neither the molecular basis nor the epidemiological significance of these so called sporadic disease subtypes are understood. Analysis of the genetic CJD-risk in the general population, focused on the PRNP mutation E200K, confirmed the occurrence of asymptomatic ("healthy") carriers of this mutation without their known relation to CJD patients. The first data on the overall most common CJD-specific mutation in the general population signal possibly an iatrogenic CJD risk, as well as a hidden iatrogenic explanation in some sporadic CJD cases.[96]

Opinions-Positions

A better understanding of the way in which different strains of prions may spread between animals and human beings, and the environmental factors that modulate this, is essential to help design methods for the prevention of the spread of prions within communities. Improved methods for decontamination and disposal of animal waste as well as assessment of prions in wastewater and soils are crucial to prevention of the spread of prions.

Although still declining, BSE has not been eradicated so far, and one might question if eradication is generally achievable, particularly when the potential for sporadic cases of BSE is considered.[97] The occurrence of these atypical cases of BSE, in light of alleged new types of atypical BSE in Germany (2013/14) and earlier (2011) in Switzerland, would remain undetected with any reduction in surveillance, and is therefore a major point of concern.[98] Furthermore, sporadic cases of BSE appear to be significantly different from orally acquired BSE in many aspects. The most obvious differences in such atypical/sporadic BSE cases are the

tendency for the diseased animals to be in the last third of the life span for cattle, and to present a different molecular phenotype of the prion protein.[99] Most recently (2013/2014), 2 new cases of non-classical BSE were diagnosed in Germany, a country where BSE had last been seen in 2009. The overall picture of atypical/sporadic BSE is complicated by the fact that these 2 new cases of BSE appear to be different from known atypical cases in cattle.

The occurrence of atypical BSE in older cows with an extended pre-clinical phase poses a particular challenge. Even in classical BSE, depending on different testing scenarios, the European Food Safety Authority (EFSA) has published a report[97] in which the surveillance data indicate the possibility of missing BSE cases in healthy or at risk animals. In the consortium's opinion the chance of spread of BSE within the cattle population can be regarded as negligible as long as the feed ban is still operative. Likewise, under the present regulatory regimens the exposure risk for humans is very low.

Cases of atypical scrapie in sheep and goats as well as BSE in sheep and goats further complicate the picture. The fact that in the years 2010 - 2014 (September 2014) atypical scrapie has become the most common form of disease detected in sheep in many countries causes quite some concern. None of the breeding measures implemented for disease control in small ruminants have affected the prevalence of atypical disease, despite being shown to be effective for the control of classical scrapie, because both the epidemiology of the disease and the genetics of host susceptibility are different for classical and atypical scrapie.[97]

Prion diseases cannot be eradicated, especially the spontaneous diseases, and it is the opinion of the consortium that a continuous robust targeted surveillance of both animal and human populations is still required.

Major Questions and Scientific Challenges

Although the epidemiology of atypical TSE cases supports the hypothesis of a spontaneous origin, they can be experimentally transmitted and therefore present a risk. Also the stability of these prions upon passage is not yet known: they may become more infectious following either inter- or intra-species passage. A major scientific challenge is therefore to understand the basic biology and key components determining susceptibility and transmissibility.

More information about the susceptibility of prions to inactivation treatments in wastewater treatment plants and their stability relative to environmental factors is necessary. Further analysis would, thus, be necessary to understand if improvements to increase biological inactivation are a real solution for prion inactivation in wastewater treatment plants.

Data have indicated that the inactivation of infectious BSE in the environment cannot be estimated only by the measurement of protease resistant PrP^{Sc} levels. Improved PrP markers must be defined to be used as target parameters when considering infectivity.

Strategic Objectives and Priorities in the Future

- Appropriate prion agent surveillance should be maintained in both the animal and human populations, and surveillance tools should be reviewed, expanded and developed according to the state of current scientific knowledge, given the existence of atypical prions in cattle and small ruminants, which were unknown until recently, and the new concept of "prionopathies" in humans.
- Definition of suitable wastewater treatments that would reduce the possibility of prion dissemination in the environment should be accomplished.
- Water samples impacted by infected animal excreta and wastewater should be analyzed for any potential role in the transmission of prion diseases, producing data on the potential dissemination of prions in these areas.
- A study of the potential presence of CWD in Europe, including a targeted surveillance program for the detection of CWD

prions and studies of their behavior in the environment, should be implemented
- Development of programs for education and awareness within farming communities and veterinary and medical personnel, in particular, as a frontline surveillance, should be carried out.
- Establishment of more comprehensive continuous, molecular strain-defined surveillance of all forms of human prion diseases for early identification of atypical cases and potential outbreaks in humans.
- Determination of the epidemiological risk and the significance of the relatively large number of asymptomatic carriers of human PrP^{Sc} (tonsil, appendix studies)
- Strain-specific therapeutic options in classical and atypical CJD in humans should be developed and defined.

CONCLUDING REMARKS

After the initial shock caused by the BSE crisis, the scientific community reacted vigorously and in a short period of time provided essential knowledge and tools to policymakers and society at large that were critical to curb, reverse and eventually control the epidemic in cattle. The decisive support and funding provided by the EC to researchers in the prion field played a key role in this endeavor. From our current vantage point, it is critical that policies based on scientific knowledge that have proven to be highly efficacious are not relaxed based on short-sighted economic considerations. Furthermore, research efforts need to be continued (and funded) as important basic aspects of prion biology still remain unsolved, despite impressive advancements in recent years. Beyond the obvious benefit of protecting the food chain, prion biology is being increasingly recognized as relevant to the understanding of a subset of neurodegenerative ailments, such as Alzheimer's and Parkinson's diseases that kill millions every year.[25] Knowledge obtained from the study of prions and prion diseases might prove to be of enormous interest in the near future not just to keep our food safe.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

ACKNOWLEDGMENTS

We are indebted to the many colleagues, postdoctoral researchers, Ph.D. students and technicians who have contributed to the studies of the Priority project, and whose names cannot be listed for lack of space, and to Martín Cacheiro (University of Santiago de Compostela), for superb management of the project.

FUNDING

Funding was provided by the EC through FP7 222887 "Priority."

ORCID

Jesús R. Requena http://orcid.org/0000-0003-1954-4975

Neil Mabbott http://orcid.org/0000-0001-7395-1796

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