PROJECT FINAL REPORT

Grant Agreement number: 222887

Project acronym: PRIORITY

Project title: Protecting the food chain from prions: shaping European priorities through basic and applied research

Funding Scheme: Large-scale integrating project

Period covered: from Oct. 1, 2009 to Sept. 30, 2014

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4.1 Final publishable summary report

Executive summary

Priority is an FP7-funded large scale integrating project. carried out from October 1, 2009 to September 30, 2014, whose objective was "**to protect Europe´s food chain from prions**". Prions are unique infectious agents composed solely of a misfolded protein that is able to propagate its aberrant, pathologic conformation. They were brought to the spotlight by the devastating Bovine Spongiform Encephalopathy (BSE) epizootic that affected Europe in the eighties and nineties of the last century, subsequently spreading to Humans and killing dozens of people in the form of variant Creutzfeldt-Jakob (vCJD) disease. In 2009 BSE was already under control and cases were declining. This was largely the result of policy directly derived from research, such as the ban on meat and bone meal (MBM), which research had identified as a source of prions, and active surveillance, possible with rapid tests developed in basic research projects. However, prions were still a threat. Atypical forms of prions with unpredictable transmission properties had been recently discovered; several cases of blood transfusion-associated infections had been reported, and results from a large number of tonsil analyses in the UK suggested an unexpected high number of asymptomatic infected individuals. The Priority consortium´s basic contention was that increasing our knowledge on basic aspects of the biology of prions, still obscure at the time, and developing new methods to study them was fundamentally necessary to development efficacious strategies to control and eradicate threes agents from our food chain. Therefore, we designed four blocks of research:

A. Prion structure, function, conversion and toxicity
B. Prion detection
C. Prion transmission and spreading
D. Prion epidemiology

The 21 members of the Priority consortium have worked in a coordinated manner, and made relevant advances in all four areas, including, but not limited to: 1) A low resolution structural model of PrP^{Sc}, that provides a basis for understanding prion propagation and transmission barriers. 2) Characterisation of key properties of recombinant prions. 3) Identification of the endosomal recyclying compartment and the cell membrane as the two key sites of conversion of PrP^{C} to PrP^{Sc}. 4) Identification of myelin maintenance as a key function of PrP^{C}, together with participation in other important routes related to neurodevelopment. 5) Identification of a key role of the flexible tail of PrP^{C} in prion associated toxicity, together with key signalling cascades involved in the conversion mechanism. 6) Development of an assay to discriminate prion strains based on luminescent conjugated polymers (LCPs). 7) A high throughput Förster resonance energy transfer (FRET)-based assay to detect prions. 8) Protein misfolding cyclic amplification (PMCA)-based methods to detect prions in milk, waste and blood. 9) Tools to detect atypical CJD cases based on imaging and biochemical approaches. 10) A real-time quaking-induced protein conversion (RT-QuIC)-based blood test capable of detecting prions in blood/CSF of preclinical sheep and humans. 11) Understanding the routes of prion propagation from the gut to the central nervous system. 12) Identifying an age-related decline of the immune system as the key cause of the decreased susceptibility of aged individuals to prion infection. 13) Identifying the key components of blood and milk that carry prion-related infectivity. 14) Concluding that atypical scrapie can transmit to Humans and that its strain properties change as it transmits between species. 15) Developing methods to assess the spread of prions through waste water. 16) Better decontamination methods for surgical instruments. 17) An improved method to decontaminate waste based on the use of microwaves. Together with conclusions of analysis of surveillance data through the period, all these advances have been combined and summarized into a position paper with policy recommendations. Among these, maintenance of a strict MBM feed ban, continuation of active surveillance and sustained efforts in basic prion research are key points.

Summary description of project context and objectives

Approximately twenty five years ago, the inception in the UK of Bovine Spongiform Encephalopathy (BSE), quickly brought the previously obscure "prion diseases" to the spotlight. The ensuing health and food crises that spread throughout Europe had devastating consequences. In United Kingdom (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 approximately 200 people to die from variant Creutzfeldt-Jakob disease (vCJD). Classical BSE appears to be now under control and will eventually disappear, as a result of the meat and bone meal (MBM) ban. Alternatively, a low incidence of BSE could remain endemic.

However, the danger of prions still threatens, and this was particularly evident when Priority was launched. The results from a large number of tonsil analyses in the UK suggested that there may be an alarmingly high number of asymptomatic PrP^{Sc} positive cases. Transmission through blood transfusion (confirmed in at least four cases) was another important concern (*Peden et al. 2004, Lancet 364:527-9*). In cattle, too, "atypical cases" of BSE (*Watts et al. 2006, PloS Pathog 2[3]:e26*) were worrysome, particularly since we did not understand the biochemical reason for the species barrier. Atypical ovine scrapie was another concern, as it could mask ovine BSE. Given the fact that scrapie is endemic and not likely to be eradicated soon, this was an important concern.

In this context, at the start of the **Priority** project, despite considerable progress in our understanding of prions and success in devising strategies to limit their spread, numerous holes still existed in our knowledge of their biology. Intense challenges still remained to definitively control and eradicate the threat posed by prions to our food and health. Thus, the overall objective of **Priority** was ***protecting Europe´s food chain from prions***. We designed four blocks to cover:

E. Prion structure, function, conversion and toxicity
F. Prion detection
G. Prion transmission and spreading
H. Prion epidemiology

A. Prion structure, conversion, function and toxicity. The overall objective of this block was **to increase our knowledge on basic aspects of the biology of prions**. It was, and is, our contention that an increased knowledge of such basic aspects is fundamentally necessary to development efficacious strategies to control and eradicate prions. We designed the following specific objectives:

- Understanding of the structure of prions, how it facilitates conversion, how it enciphers strain diversity and strain transmission barriers
- Understanding the function of PrP^{C}
- Identifying the location and key determinants of conversion at the cellular level
- Identifying toxic PrP species and downstream mechanisms mediating their action

Despite considerable efforts and some recent progress, the structure of PrP^{Sc} was largely unknown when **Priority** was launched. To understand how prions propagate, and therefore, to learn how to combat them efficiently, it is necessary to know the structure of PrP^{Sc}. Understanding conformationaly determined features of prions, such as the species barrier and strains, also depends on understanding the structure of PrP^{Sc}. The insoluble nature of PrP^{Sc} had precluded the use of NMR or X-ray diffraction techniques. FTIR had shown that PrP^{Sc} displays a considerably increased content in β-sheet. PrP^{Sc} was also known to form amyloid fibers. Electron crystallography of 2D crystals in PrP^{Sc} samples had led to the proposal of model based on a trimer of β-helical stackable PrP subunits (*Govaerts et al. 2004, PNAS 101:8342-7*). However, data were insufficient for a conclusion.

The cell biology of the PrP^{C} o PrP^{Sc} conversion was also poorly characterized. PrP^{C} visits a wide assortment of subcellular sites using many trafficking pathways, a complexity further

compounded by the part-time attachment of the PrPs to rafts (*Campana et al. 2005, Trends Cell Biol 15:102-11*). It was not known whether different routes can deliver infectious PrP to the intracellular site where conversion occurs and/or whether different sites for conversion exist inside the cell. Cell surface PrP^{C} internalizes by both clathrin and non-clathrin pathways, perhaps depending on the cell type and also on a poorly understood pathway dosage. By which pathway PrP^{Sc} gets into the cells was largely unknown. We considered that identification of the intracellular site(s) of prion conversion and the characterization of the factors involved in this mechanism at the cellular level would lead to the identification of new targets for therapy and to the design of new drugs.

The physiological function of PrP^{C} had remained unclear (*Aguzzi and Polymenidou 2004, Cell 116: 313-327). Prnp*$^{o/o}$ mice apparently seem to have a normal life expectancy (*Bueler et al. 1992 Nature 356:577-582*). In the past, Charles Weissmann's laboratory in collaboration with Aguzzi´s group had demonstrated that transgenic expression of certain amino-truncated variants of PrP^{C} causes early onset ataxia and death in PrP^{C} deficient mice *(Shmerling et al. 1998, Cell 93: 203-14)*. The fact that this phenotype is fully reverted by co-expression of a wild-type full-length PrP^{C} strongly indicates that neurodegeneration is related to interference with a pathway or a molecular mechanism related to PrP^{C}. Immediately prior to the launching of **Priority**, the Aguzzi lab had generated a new subset of transgenic mice expressing different truncated versions of PrP, that develop a neurodegenerative disorder which can be overcome by the co-expression of wild type PrP^{C} (*Baumann et al. 2007, EMBO J 26:538-47*). A specific white matter disease consisting in myelinated fiber degeneration was observed, suggesting a role for PrP^{C} in myelinated fiber maintenance. We therefore set out to work on elucidation of the function(s) of PrP armed with the hypothesis is that PrP could play a positive role in cell survival.

How prions cause neuronal death was another issue that remained enigmatic. It had been shown that PrP has to be present for PrP^{Sc} to exert its toxic effects (*Brandner et al. 1996, Nature 379: 339-43*). Further, in addition to fully translocated PrP, termed ^{SEC}PrP, two transmembrane forms with the C-termini pointing to the lumen or cytosol, ^{CTM}PrP or ^{NTM}PrP, respectively, had been described, and it had been suggested that regulation of ^{CTM}PrP in concert with the absence of ^{SEC}PrP might play a decisive role in PrP^{Sc} neurotoxicity. On the other hand, the mitogen activated kinase 1/2 (MEK) had been identified to modulate PrP conversion in permanently infected cell lines (*Nordstrom et al. 2005, J. Neurosci 25:8451-6*) providing a possible candidate for a signal transduction pathway important to regulate PrP conversion and toxicity; further, the mTOR pathway was shown to regulate PrP conversion by the Kristensson lab. These two pathways were thus candidates for cellular regulation of PrP conversion. E**xpected achievements in this block were:**

- A low resolution model of the structure of PrP^{Sc}
- Identification of the cellular site of conversion of PrP^{C} to PrP^{Sc}
- Identification of the key partners associated to PrP^{C} during its function
- Identification of key toxic PrP intermediates and pathogenic signalling routes.

<u>B. Prion detection:</u> The overall objective of this block was the **development of improved, sensitive tools for the study of prions, including a pre-clinical test**. Specific objectives were:

- Developing better tools and assays to study prions
- Developing better strain-specific reagents and tests
- Developing a pre-clinical test for detection of prions in blood
- Developing methods to detect prions in milk
- Developing methods to detect prions in soil and waste
- Developing pre-clinical tests to detect atypical CJD cases

At the time **Priority** was launched, one of the most urgent issues in the study of prion diseases was the insufficiency of highly sensitive prion detection tools. In particular, there was an urgent need for robust screening tools and sensitive assays that could be used for high throughput screening of inhibitors for the prion aggregate formation. Furthermore, better tools were needed that allowed new experiments on PrP^{Sc} formation, conversion, species barriers, and prion transmission.

Variant CJD (vCJD) is transmitted via the food chain, and iatrogenically, via contaminated surgical equipment; but since 2003, however, four cases of vCJD transmission via blood transfusion are documented (*Llewelyn et al. 2004, Lancet 363:417-421*). vCJD-infected individuals are asymptomatic for prolonged times despite infectivity in their blood. This raises the risk for donations of contaminated blood and inadvertent transmission of the disease. **Expected achievements were:**

- Neurospheres able to replicate different prion strains for the study of species/strain barriers
- Luminescent conjugated polymer (LCP)-based strain-specific reagents
- A preclinical blood prion test
- Biochemical/imaging tests capable of detecting atypical CJD pre-clinically

C. Prion transmission and spreading. The overall objective of this block was **understanding the spread and flow of prions within organisms**. We designed two areas of activity:

- Understanding the basic mechanisms of prion spread at the organism level
- Understanding the flow dynamics of prions in fluids (blood and milk)

When **Priority** was launched, data suggested that host age and inflammation might influence prion uptake from mucosal sites, or expand their tissue distribution *(Glaysher and Mabbott 2007, J Immunol 178:3757-3766*). The detection of abnormal PrP in mammary glands from lentivirus/TSE co-infected ewes warned about the potential exposure of humans to TSEs through the consumption of small ruminant milk. Acute mastitis, a common infection in dairy animals, was thought to be potentially responsible for a modification of the blood/milk barrier and milk composition, and promote infectivity passage in milk (*Ligios et al. 2005*). These elements called for an urgent understanding of potential prion infectivity levels associated with ruminant blood and milk.

Clinical vCJD has occurred predominantly in young adults, suggesting that age-related factors influence susceptibility. This phenomenon appears to be a widespread and includes BSE in cattle and sheep scrapie. Data from members of the **Priority** consortium had shown that prion susceptibility was impaired in aged mice. These data implied that host age produces a significant barrier to prion transmission. **Expected achievements for this block were:**

- Improved understanding of the spread of prions at the organism level
- Improved understanding of infectivity dynamics of blood and milk

D. Prion epidemiology. The objective of this block was to **better understand the way in which different strains of prions are spreading through animals and humans, and the environmental factors that modulate such spreading**. This objective was broken into:

- Assessing the risk posed by atypical scrapie, atypical BSE, and small ruminant BSE
- Assessing the infectivity of different tissues of small ruminants infected by atypical scrapie
- Assessing the risks associated to atypical CJD and its possible transmission through blood
- Assessing the risk associated to transmission of prions through milk
- Understanding the risks associated with prions in soil and waste water
- Developing more efficient decontamination methods.

At the time the **Priority** project started, several atypical TSE forms had been identified: 1) Atypical BSE in cattle, which had been discovered in old (over 4 years) asymptomatic animals (*Casalone et al. 2004, PNAS 101:3065-70*); 2) Nor98 scrapie in sheep from genotypes that had been previously considered to be poorly susceptible to TSE (*Benestad et al. 2003, Vet Rec 153:202-8;* 3) Atypical scrapie clinically silent but which can affect sheep with ARR/ARR genotype.

Despite progress in TSE diagnosis methods, our abilities to detect atypical TSE with a satisfying sensitivity could not be assumed. Immediately prior to the launching of **Priority**, studies in transgenic mice models had shown that the atypical strain BSE-L could evolve, after interspecies passage, to a classical BSE agent and be at the origin the BSE epidemics, and that it could be able to cross human species barrier with higher virulence than BSE. Further, the abilities of atypical BSE and scrapie to: 1) cross species barriers (including human and farm animals) and 2) transmit between individuals from the same species were still unassessed. Together, these elements questioned our capacity to: 1) monitor TSE in herds; 2) identify emergence or re-emergence of a TSE epidemic in animals, 3) identify zoonotic TSE emerging risks. Atypical scrapie was a critical issue in the epidemiology of prion diseases and their risk to humans (Fediaevsky et al, BMC Vet Res 2008 4: 19)

Further, there was at the time no consistent information concerning the infectivity distribution in tissues of sheep affected with atypical scrapie and the demonstration of the involvement of ARR/ARR animals that were believed to be resistant to TSE, represented a major concern with regards to the sheep industry. Lack of data on transmissibility, combined with both the unknown incidence of atypical scrapie and involvement of animals with genotypes believed to be resistant to TSE, wered a major concern with regards to the efficiency of the food protection policy against TSE.

As regards human prion disease, according to concepts current at the time, there were 6 distinct molecular subtypes that represent different human prion strains in sCJD, which differ from vCJD. Factors which determine human strains were not understood and a potential zoonotic origin at least of some subtypes could not completely be excluded. In order to understand factors which determine strain diversity in humans, such patients have to be identified early. While progress had been made in CSF tests and MRI, definitive classification could only be achieved at autopsy. To study the determinants of strain diversity in humans, cases had to be identified early and thus *ante-mortem* tests were needed.

Little was known about the dissemination of prions, in the environment. Scrapie was known to be transmitted through contaminated environments where it is able to remain for years. Remarkably, scrapie infectivity persisted after burial in soil for at least 3 years and for years in some environments. All this suggested the role of the environment as a reservoir of prions. Some risk assessment studies had proposed a potential environmental route of prion contamination through sewage sludge produced in slaughterhouse wastewater treatment facilities. TSE testing in the abattoirs is performed after the completion of all slaughtering processes, so that specified risk material (SRM) could be released during animal manipulation and enter the sewage net.

Inactivation of prion protein infectivity is a long-standing issue in prion research (*Taylor 2004, Contrib Microbiol 11:136-145*). This applies both to decontamination of contaminated animal tissue (e.g. offal of the slaughter process and animal by-products) and to contamination of medical devices or diagnostic material (e.g. formalin fixed tissue from human patients). The work of members of the **Priority** consortium and others (*Yan et al. Infect Control Hosp Epidemiol 25:280-284*) had shown that modifications of routinely used inactivation methods lead towards significantly increasing safety. **Expected achievements in this block were:**

- An improved method for disposal of animal waste
- A waste water prion assessment protocol
- An improved method for decontamination of lab and surgical materials
- A position document presenting an up-to-date risk analysis of exposure of humans and animals to different strains of prions and the evaluation of current intervention strategies.

S&T results/foregrounds

Block A: Prion structure, conversion and toxicity

Using structural constraints obtained using a variety of methods, we have been able to reveal the basic characteristics of the **architecture of PrP^{Sc}**. This is the result of coordinated work of several members of the consortium, with the valuable collaboration of Holger Wille and Howard Young, from the University of Alberta, Canada. Based on images of GPI-anchorless PrP^{Sc} of unprecedented quality obtained by cryo-electron microscopy, followed by application of complex mathematical averaging and reconstruction techniques, we have determined that PrP^{Sc} forms fibers composed of two intertwined protofilaments, with β-strands parallel to the fiber axis. The cross-section of each protofilament is bean-shaped, with dimensions of ~3.5 x 5.5 nm. Based on FFTs of raw and averaged cryoEM images, there are three repeating structural elements along the fiber axis, at ~0.48, ~2 and ~4 nm. The features repeating every ~0.48 nm correspond to β-strands stacked along the fibril axis (amyloid cross-beta architecture), while the ~2 nm-spaced features would correspond to the stacking of individual PrP^{Sc} subunits, which would therefore consist of 4-rung β-solenoids, which also agrees with geometric considerations considering the volume of protofilaments and the mass of each PrP^{Sc} subunit (Fig. 1). Volumes obtained by helical reconstruction show no evidence of any α-helices on the surface of the protofilaments.

The 2 nm-high PrP^{Sc} subunit stack along the axis (~2 nm axial repeat in the FFT). There is a pairing of subunits along the axis, as determined by ~4 nm signals in FFTs of averaged cryo-EM images. At this point it is not possible to discern whether such pairing is a consequence of a face-to-face stacking or, as in HET-s a feature such as retraction of a loop, repeating every other subunit in a head-to-tail stacking arrangement.

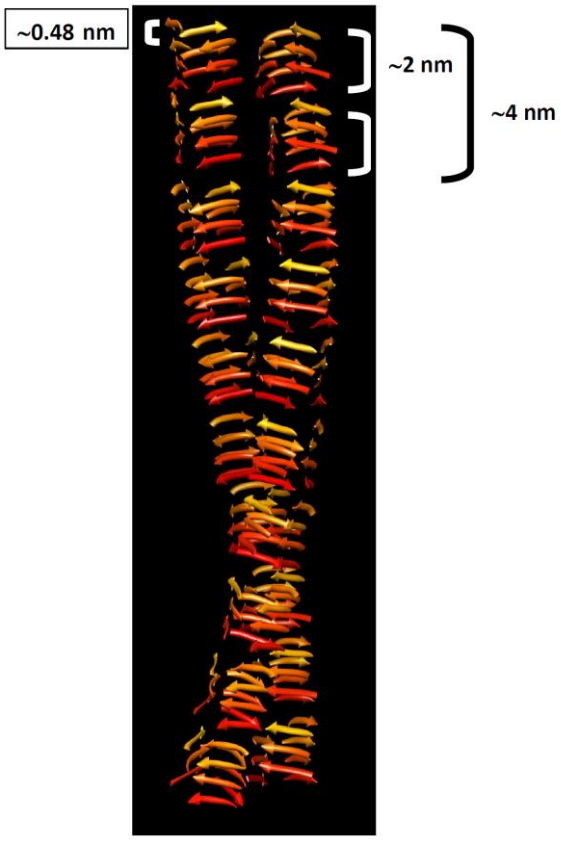

Fig. 1: The structure of PrP^{Sc}

A temptative threading of the PrP sequence into the PrP^{Sc} has been constructed based on: 1) even distribution of residues among the 4 rungs of a PrP^{Sc}; 2) localization of proline residues outside β-strands; 3) localization of PK susceptible sites (determined in GPI-anchorless PrP^{Sc} but also consistent with partial data obtained from wt PrP^{Sc}) also outside of β -strands, in connecting loops; localization of glycosylation sites also outside of β -strands; 4) localization of the two cysteine residues at bonding distance; 5) localization of as few charged residues facing inside β-solenoids.

Our model allows an understanding of prion propagation: PrP^{Sc} would act as a template; in particular, the uncoupled β-strands of the upper and lower rungs are "sticky" (Richardson & Richardson, PNAS 2002, 99: 2754–2759); PrP^{C} would unfold completely; in its unfolded state, it would refold on the upper/lower rungs of PrP^{Sc}, adopting β-sheet conformation in certain stretches to match the "sticky" uncoupled β-strands . Once a first rung is completed, the process would repeat by self-coiling and formation of successive (four) rungs.

Our model also allows an understanding of strains and transmission barriers: A prion strain would be a particular and specific structural variation of the general theme. The key structural feature defining transmission properties of a strain would be the specific topography of the upper and lower rungs. Thus, one particular strain will be characterised by specific topographic features in its upper and lower rungs, such as the length of β-strands, conformation of connecting loops etc. The position of specific residues may introduce steric hindrances, charges etc. that will determine the ease of difficulty of templating of an incoming unfolded PrP molecule. Templating difficulties may increase if there are differences in sequence (species barriers).

The cell biology of the **conversion PrP^{C} into PrPSc** was poorly understood, although it was clear that the intracellular trafficking of the protein has a major role in this process. It was not clear whether different routes can deliver infectious PrP to the intracellular site of conversion and/or whether different sites for conversion exist inside the cell. In addition where PrP is degraded and how it spread between cells, its of primarily importance for the development of the patogenesis.

We have used neuronal cell models of prion infection (e.g. N2a, CAD and GT1 cells) to analyse both the endocytic pathway of PrP and the intracellular site where PrP^{C}- PrP^{Sc} conversion occurs. We identified the perinuclear recycling compartment (ERC) as a major site for prion conversion in different cells (GT1, N2a, CAD) infected with two different prion strains (139A and RML. These data were confirmed in primary hippocampal neurons (Caputo *et al.*, in preparation).

Another important site for conversion is the plasma membrane, where a strong PrP^{Sc} signal can be found. Electron microscopy has provided key insights on plasma membrane-localized conversion. Furthermore, a new cell compartment containing PrP^{Sc} termed “strings” has been identified by means of fluorescence microscopy. In the meantime, it has been recently proposed that autophagy plays an essential role in neuronal homeostasis and its dysfunction facilitates neurodegeneration. We have confirmed upregulation of the autophagic pathway in different prion infected cells. Interestingly, the increased autophagic flux upon scrapie infection could neither eliminate PrP^{Sc} nor prevent its endogenous synthesis suggesting that autophagy is not involved in scrapie degradation. On the other hand we demonstrated that HO-tamoxifen leads to cholesterol accumulation in lysosomes and induces PrP^{Sc} degradation in these organelles. These data show a link between cholesterol and PrP trafficking and an autophagy independent role of lysosomes in prion degradation (Fig. 2).

To visualize PrP^{Sc} in living cells we successfully created fluorescently labeled PrP^{Sc} by coupling PrP^{Sc}-enriched brain homogenate with Alexa fluor (Gousset et al. Nat Cell Biol, 2009). This fluorescent agent is fully infectious in primary neuronal culture and has been successfully used to show the role of dendridic cells (DC) in the spreading of prions to primary neurons though Tunneling nanotubes (TNTs), an intercellular connecting structure. More recently we have found that the spreading of PrP^{Sc} thorugh TNTs occurs inside endolysosomal vesicles (Zhu, Victoria *et al*, submitted). Furthermore, following exposure via skin scarification, we could also demonstrate the

propagation of prions from the skin to the draining lymphatic nodes occurs via dermal classical DCs, independently of langerin(+) cells (Watne *et al*. J. Leukoc. Biol. 2012).

In order to characterize the axonal transport of PrP^{Sc}, we have established cortical and striatal primary neuronal circuits in microfluidic chambers. The uptake and the transport of labelled PrP^{Sc} were assessed by live imaging in time course experiments which indicated an efficient transport both retrogradly as anterogradly of Alexa-fluor PrP^{Sc}. We also found that transfer of PrP^{Sc} from one neuron to the connected one results in spreading of the infection. However this only occurs in mature networks (late infection). We postulated that early infection could impair synaptic establishment or could be associated with a neuronal cytotoxicity and so could impair the prion spreading (Tixador *et al*., submitted).

In summary, we now understand the basic elements of where prions replicate in the cell, inclusing how they transmit from cell to cell.

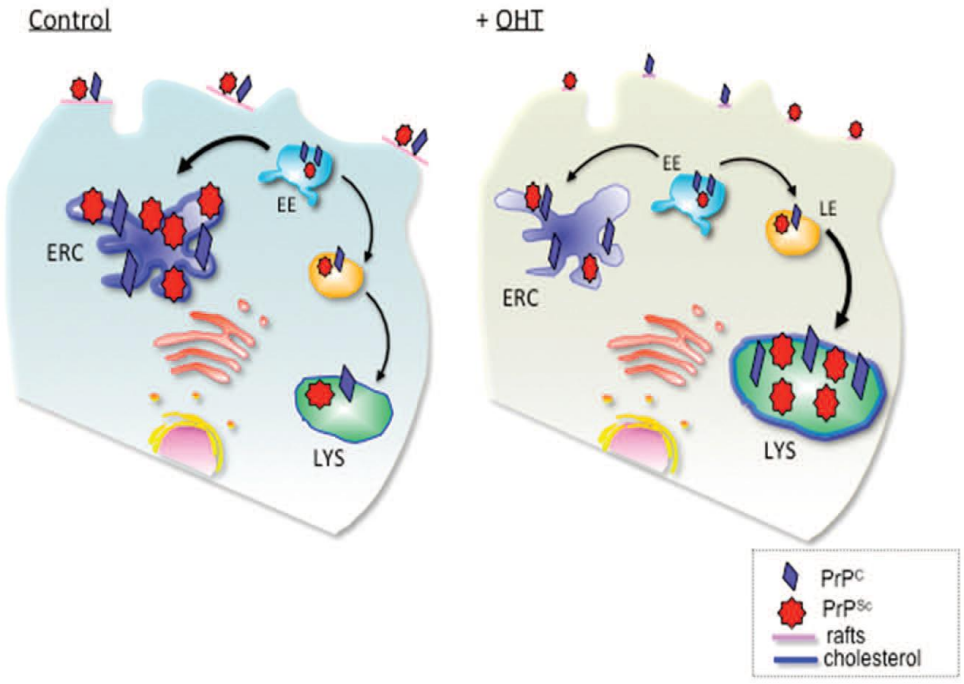

Figure 2: Schematic presentation of PrP trafficking in infected cells and upon OHT treatment*. In infected cells PrP^{C} and PrP^{Sc} interact at the plasma membrane in cholesterol-rich lipid domains called lipid rafts. Upon internalization, both PrP^{C} and PrP^{Sc} can recycle via the endosomal recycling compartment (ERC) or can be routed for degradation in lysosomes. Subcellular cholesteroldistribution influences PrP^{Sc} trafficking in the endocytic pathway. In untreated, infected cells, the majority of PrP recycles through the cholesterol-rich ERC, supporting conversion of PrP^{C} to PrP^{Sc}. Treatment with 4-hydroxytamoxifen (OHT) induces cholesterol accumulation in enlarged late endosomes (LE). PrP^{Sc} production and degradation defines the cellular load of infectious prions. We propose that 4-hydroxytamoxifen-induced changes in PrP^{Sc} trafficking favor PrP^{Sc} degradation. EE, early endosomes; LYS, lysosomes (Adapted from Marzo et al., J. Cell Sci, 2013).*

Our search for the **function(s) of PrP^{C}** started by studies aimed at localization of PrP in the central and peripheral nervous system. We reasoned that identifying the location sites might cast light on possible functions of PrP^{C}, which might help us understand prion-associated pathology.

By using cryo-immunoelectron microscopy and electron tomography we have studied the localization of PrP in the developing and adult central nervous system. PrP^{C} has been found at the synaptic cleft of some synapses in wt adult hippocampus stratum oriens and reduced numbers of synapses were observed in adult Prnp-/- mice. These results indicate that PrP^{C} may not be directly involved in synapse formation.

We have extensively investigated the localization and the functional consequences of PrP in the peripheral nervous system using mouse models. In particular, electron microscopy (including cryo-immuno electron microscopy), immunofluorescence, genetic, biochemical and functional analyses have been applied to investigate the chronic demyelinating polyneuropathy which develop in Prnp-/- mice with aging. These data pointed towards a crucial role for neuronal PrP^C for myelin maintenance in the peripheral nervous system (Bremer *et al*., Nat Neurosci 2010).

In this respect, we have found that transgenic overexpression of Shadoo was not able to rescue the chronic demyelinating polyneuropathy typically seen in Prnp-/- mice. Therefore we concluded that Shadoo does not bear a PrP^C-like myelinotrophic activity. We have established an *in vitro* system to study the molecular mechanisms underlying this physiological process and have identified the first 110 amino acids of PrP^C as the domain crucially involved in myelin maintenance (Küffer *et al*., manuscript in preparation).

In parallel studies, we have investigated a possible role of PrP in the development of the nervous system. We have determined, by using microarrays, the differential signature induced by different dosages of PRNP. These experiments were developed in living mice as well as in neuroblastoma cells (N2a). In addition, we determined the participation of PrP^C in neurotransmission, especially in gluitamatergic transmission. PrP^C participates modulating the natural functioning of the glutamate receptor GluR4/6. In neurons lacking PrP^C the receptor is always active and mediates neurotoxic effects. These neurotoxic effects are modulated by increased presence of reactive oxygen species in these cells. These results point to the presence of PrP^C in the postsynaptic level. In addition, we have determined the differential expression of miRNA in the neocortex of different PrP^C variants. Some of these miRNAs are implicated in cell control and proliferation. At this respect, we have determined that Cyclin D1 is a main target of the regulation mediated by PrP^C. Cyclin D1 controls cells cycle in a EGFR dependent manner. Thus our results indicate that PrP^C modulate EGFR functions in neurons. In parallel studies we have determined that PrP^C modulates the proliferation of stem cells in the adult hippocampus. In addition, PrP^C also regulates the proliferation and differentiation of oligodendrocyte precursors cells in the brain. Lastly in kinase assays we have seen that the activity of ERK1/2, MEK and JNK3 is modulated by PrP^C.

Finally, we have also studied the role of PrP in the immune system. Classical studies comparing non isogenic wildtype and knockout mice suffer from the fact that the two groups differ for an undetermined number of polymorphic genes flanking the targeted locus ("the flanking gene problem"). Therefore we have investigated whether this phenomenon might have implication in studies aimed at identifying the physiological role of PrP through comparison of Prnp-/-and Prnp+/+mice. We have selected an immune phenotype previously reported in Prnp-/- mice (increased phagocytosis of apoptotic cells) and have searched for polymorphic Prnp-flanking genes potentially responsible for this phenotype.By combining formal genetics, next-generation sequencing and functional studies, we have found that the polymorphic, Prnp-flanking gene Sirpa, rather than Prnp itself, controls phagocytosis of apoptotic cells (Nuvolone *et al*., J Exp Med 2013).

Furthermore, we used immortalized murine myeloid DCs in combination with a bacterial infection model to study the role of PrP^C in dendritic cell maturation and antigen presentation. We found significant alterations in the subcellular localization of PrP^C during functional maturation of DCs. Overexpression of PrP^C resulted in an increase in the turnover of surface MHC I involved in vacuolar cross-presentation. Based on these data we propose a model in which PrP^C regulates the uptake of particulate antigens as well as the turnover of surface recycled MHC I and thereby improves the cross-presentation efficiency of DCs (Fig. 3; Stitz *et al*., manuscript in preparation).

Regarding **mechanisms of toxicity**, two of our strategies were to identify cell targets of deletion mutant toxicity and PrP^C interaction partners. In this respect, we have established a protocol for the identification of a high-molecular weight complex containing PrP^C in brains under native conditions based on specific elution after precipitation with antibody POM2. We have performed extensive

biochemical studies including mass spectrometry and the results are compatible with the complex being a homo-oligomer of PrP^C. PrP^C-containing high molecular weight complex displays a

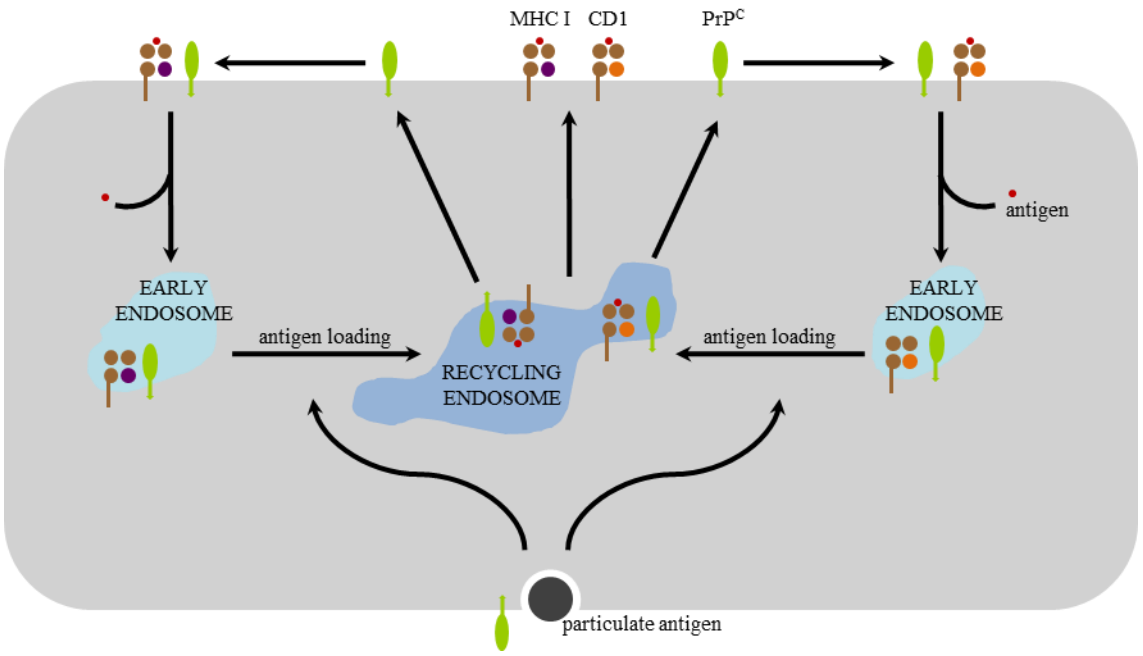

Fig. 3: Working model on the functional role of PrP^C in MHC I antigen presentation. *PrP^C regulates vacuolar cross-presentation of DCs by taking influence on antigen uptake and the turnover of surface recycled MHC I.*

different electrophoretic mobility in brains of mice carrying toxic deletion mutants of PrP^C (Calella *et al*., manuscript in preparation).

Also, we have applied biochemical approaches and found that the CC2 is a critical domain of PrP^C for the interaction with the α2δ subunit of voltage-gated calcium channels (VGCC) *in vivo* (Senatore, unpublished data).

By using different deletion mutants of PrP^C and conditional cell-type restricted ablation or expression of PrP^C *ex vivo* and *in vivo*, we uncovered a critical role for the flexible tail (FT) of PrP^C as the effector domain of PrP^C-mediated neuronal death (Fig. 4). Ligands targeting the α1 and α3 helices of the PrP^C globular domain induced rapid neurotoxicity in cerebellar organotypic cultured slices. Toxicity was prevented by deletions of the FT or by preincubation with antibodies against the FT, suggesting that the FT has neurotoxic properties and is required to transmit the toxic signals originating from the globular domain (Fig. 4 and Sonati *et al*., Nature 2013).

In light of these data, to investigate the role of FT of PrPC in triggering neuronal death in vivo, we generated a mouse line expressing a membrane-anchored version of the FT (FTgpi) of PrP^C. We found that FTgpi mice develop a progressive, fatal neurodegeneration morphologically and biochemically similar to that triggered by anti-GD antibodies. This process was associated with a conspicuous unfolded protein response through activation of the PERK pathway similar to what Mallucci and colleagues reported during prion infection (Dametto *et al*., manuscript in revision).

Cellular mechanisms play a role in conversion of the normal prion protein PrP^C to the disease-associated protein PrP^{Sc}. The cells provide not only PrP^C, but also still largely undefined factors required for efficient prion replication. Previously, we have observed that interference with ERK and p38-JNK MAP kinase pathways has opposing effects on formation of prions indicating that the process is regulated by a balance in intracellualar signaling pathways. In order to obtain a “flow-

chart" of such pathways, we have now studied activation of MEK/ERK and mTORC1 downstream targets in relation to PrP^{Sc} accumulation in GT1-1 cells infected with the RML or 22L prion strains.

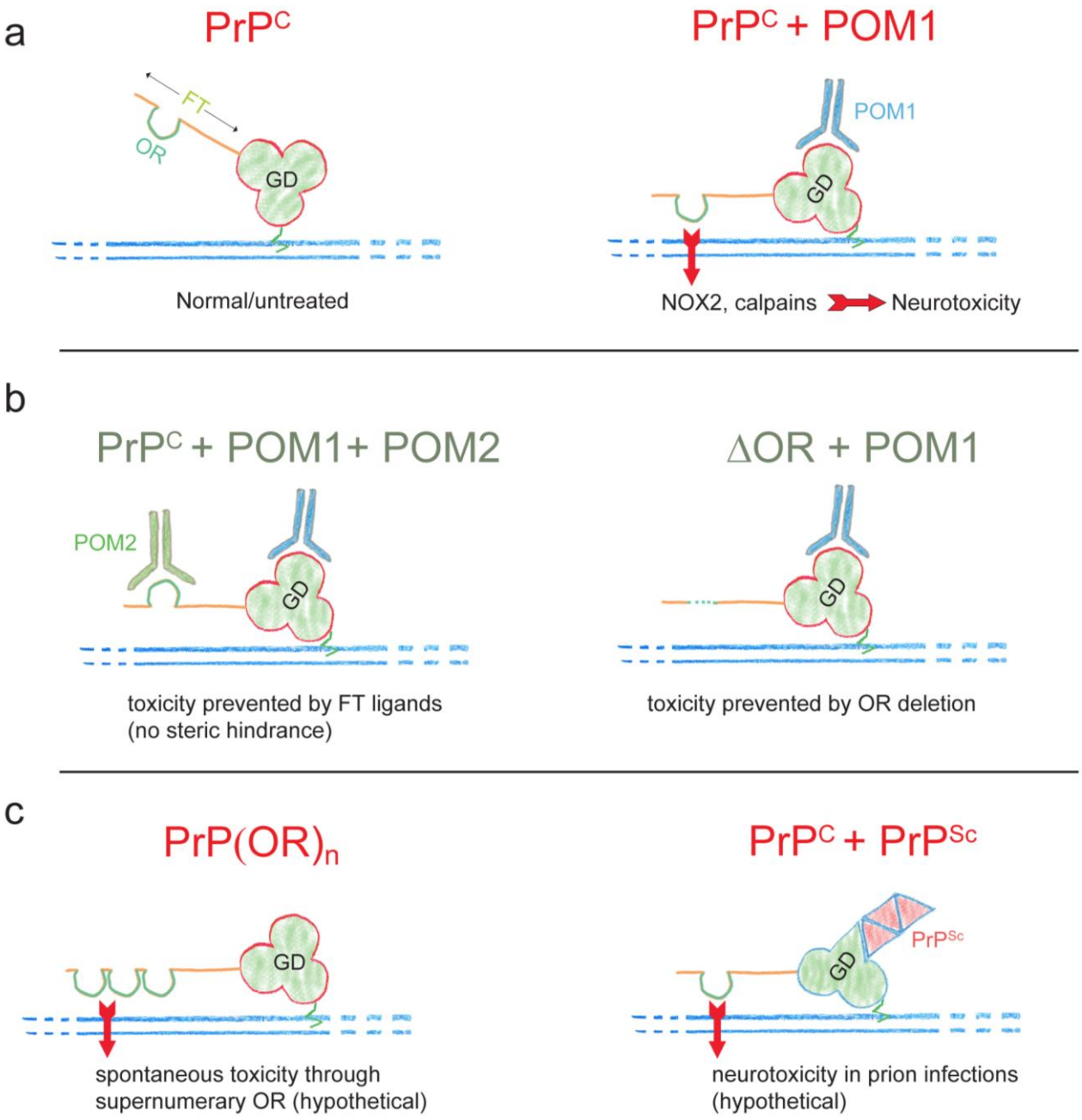

Fig. 4

Models of PrP-mediated toxicity. a, The cellular prion protein PrP c consists of a long flexible tail (FT) and a globular domain (GD) tethered to the membrane (blue) via a GPI anchor (green). GD ligands (holoantibodies, F(ab) fragments, or scFv minibodies) trigger neuronal loss, perhaps by altering the conformation of the FT relative to the membrane or to other molecules. **b**, Toxicity was abolished by pretreatment with FT ligands that engaged the octapeptide repeats (OR), or by deleting the OR. **c**, Supernumerary OR are associated with hereditary Creutzfeldt-Jakob disease and may plausibly also exert neurotoxicity through the FT. Bona fide prion infections may also conceivably utilize the same mechanism, as hypothesized in the right panel.
Reproduced from Sonati et al. Nature 2013

We have shown that inhibition of mTORC1 with rapamycin causes a reduction of PrP^{Sc} accumulation at similar low levels as seen when the interaction between the translation initiation factors eIF4E and eIF4G downstream mTORC1 is inhibited using 4EGI-1. No effect was seen following inhibition of molecules (S6K1 and Mnk1) that links MEK/ERK signalling to mTORC1-mediated control of translation. Instead, stimulation (high [KCl] or [serum]) or inhibition (MEK-inhibitor) of prion formation was associated with increased or decreased phosphorylation of the neuronal transcription factor Elk1, respectively. Our studies have shown that prion formation can be modulated by translational initiating factors, and suggests that MEK/ERK signaling plays a role in the conversion of PrP^{C} to PrP^{Sc} via an Elk1-mediated transcriptional control. Altogether, our studies indicate that prion protein conversion is under the control of intracellular signals, which hypothetically, under certain conditions may elicit irreversible responses leading to progressive neurodegenerative diseases (Fig. 5).

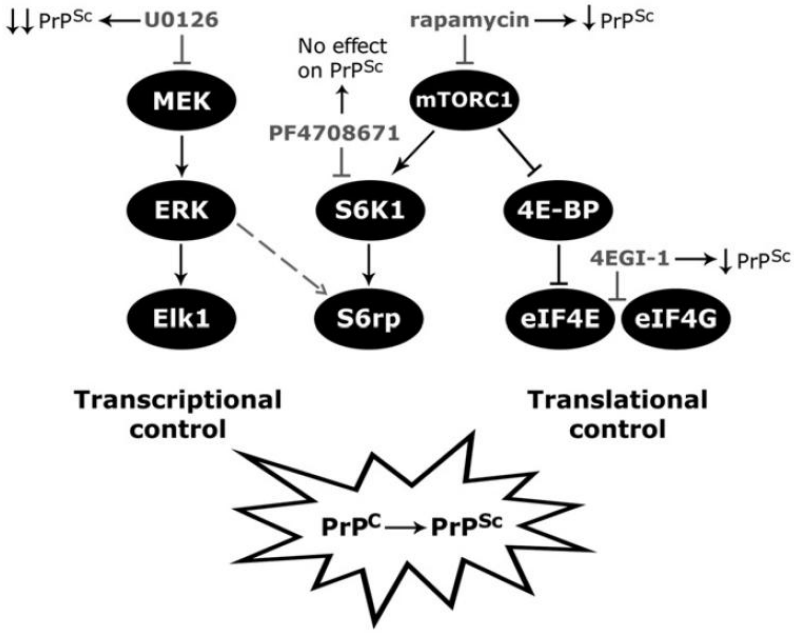

Fig. 5: Signalling cascades and prion conversion.

Finally, as part of this block, a glovebox was developed to handle electron microscopy samples into a Vitrobot without disturbing the environment. In the glovebox, electron microscopy samples can be prepared in a temperature and humidity controlled environment. More details are provided in section 4.2.

Block B: Prion detection

We have developed prion assays based on the use of conformationally sensitive luminescent conjugated polymers (LCPs) that provide a direct link between spectral signal and protein conformation. In particular, we have established optimized protocols to study different human prion strains in formalin-fixed paraffin-embedded tissues as well as in frozen tissues. These protocols can be applied to a large library of conformationally sensitive LCPs with different chemistries and optical properties. The consistency of spectral pattern of plaques from individual cases and differences among various cases – including cases falling within the same group according to current classification systems – could reflect peculiar structural features of prions in different cases (Leske, unpublished data).

We have also established a sensitive, homogeneous-phase fluorescence resonance energy transfer (FRET) assay for PrP^{C} and PrP^{Sc} based on the energy transfer between two fluorophores, Europium (Eu3+) and allophycocyanin (APC). This enabled us to establish a novel, fast, and convenient cell-based bioassay termed Digital Prion Infectivity Assay (dPIA) using an *ad hoc* developed mathematical approach to calculate the prion infectivity titer.We validated the dPIA with standard mouse prion inoculum RML6 (Rocky Mountain Laboratory). We further established the homogeneous-phase FRET assay for the hamster prion protein measurement using the FRET antibody pair 3F4-Eu2+ and POM1-APC. This FRET antibody pair is also suitable for the detection of human prion proteins. This system is being applied to a fully robotized automatic platform and protocols for high-throughput projects related to prion infectivity studies (Li *et al*., manuscript in preparation).

Also, based on recombinant antibody fragments, we generated peptides that specifically bind PrP^{Sc} and the normal prion protein (PrP^{C}), and furthermore, modified these peptides such that they would also be strain-specific, i.e. recognize only variant CHF but not sCJD, for example. We also generated ligands specifically binding to a neurotoxic but non-infectious form of PrP^{C}, CTMPrP, and charcterized this isoform in a cell cutlure model and its occurrence and function in brains of wild type animals.

We produced and characterized a collection of neurosphere cultures obtained from full brain from E14 foetuses of transgenic mice expressing prion protein (PrP) of bank vole, cow, sheep, pig and human, as well as from wild type mouse (129-OLA and FVB background), PrP null mouse and murine PrP over-expressing mouse (Tg20).

We also proposed to study the ability of these neurospheres to replicate homologous and heterologous prion strains. Infection experiments in neuropheres were iniciated using mouse cells and mouse adapted prions from different origins. We studied the ability of undifferentiated and differentiated neurospheres to replicate several prion strains. In these experiments we use neurosphere cultures isolated from129/Ola, FVB, Prnp0/0 and Tga20 mice, which over-express murine PrP. We were unable to detect PrPres accumulation in dividing neurosphere cultures after prion infection with two different mouse adapted scrapie inocula, RML and 22L. In contrast, with differentiated neurosphere cultures expressing PrP^{C} (129/Ola, FVB and Tga20) a successful PrPres amplification was observed in very short time experiments when infected with the RML and 22L innocula, implying that cell differentiation improve prion replication in these cultured cells. The mouse BSE adapted inoculum (301C) was not amplified in these neurosphere cultures neither before nor after differentiation, suggesting that these cell cultures show differential prion strain susceptibility (Fig. 6).

After the enormous effort done trying to found the adequate conditions for prion replication in neurospheres cultures, we concluded that neurosphere cultures are not suitable models for prion replication other than mouse adapted scrapie in murine neurosphere. However, when murine cells were inoculated with a panel of prions harbouring PrP sequence other than that of PrP expressed in the cells to be infected (heterologous prions), all results were negatives and none of the neurosphere cultures were able to replicate heterologous prions. Taken together all these results, we concluded that neurospheres cultures are not suitable models for prion replication other than mouse adapted scrapie in murine neurospheres, and are not able to replicate heterologous prions from other species.

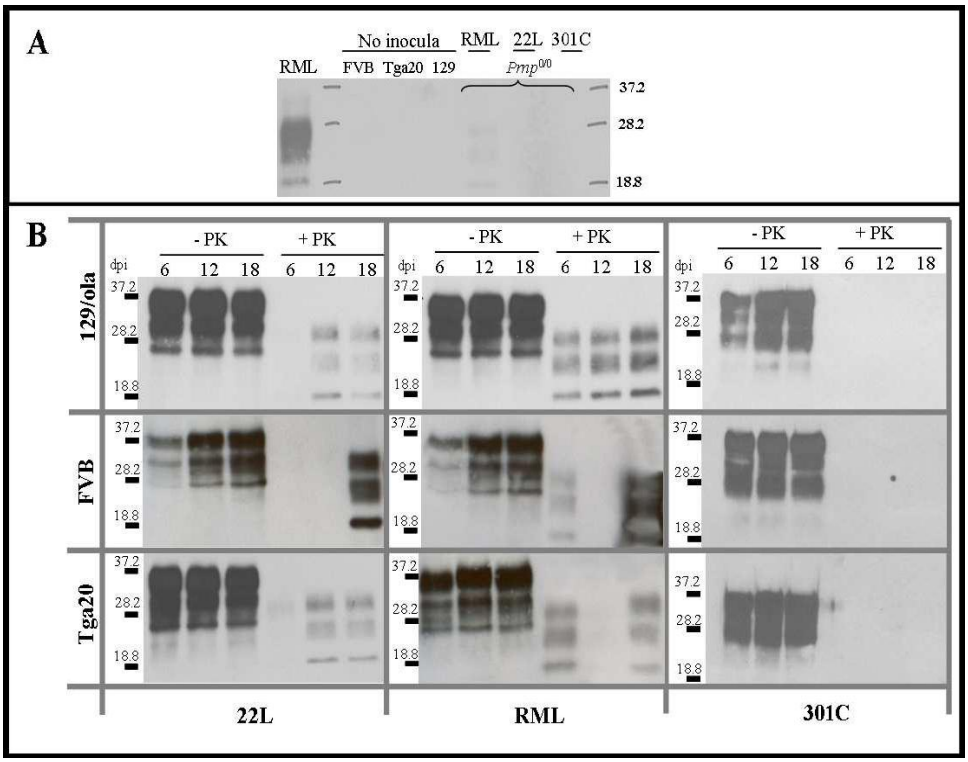

Figure 6: Infection of differentiated neurospheres. *(A) Western blot showing the controls of cells without inocula and Prnp0/0 cells with the three inocula of study from the experiment of neurosphere infection during the differentiation, all samples are digested with PK. The time point for the Prnp0/0 cells harvesting was 18 dpi. (B) Panel of western blots using the Sha31 mAb illustrating the result of the neurosphere infection during the differentiation with the three neurosphere lines versus the three inocula.*

A key objective of this block was to develop and validate a blood test for TSEs and use it as a diagnostic tool for the determination of disease pathogenesis in blood of TSE infected organisms.

A highly sensitive immunoassay, named Prionics®-Check vCJD V3.2, was developed with the following characteristics:

- It is reliable and robust sandwich ELISA, able to detect PrP^{Sc} in spiked human samples.
- The diagnostic specificity of Prionics®-Check vCJD V3.2: 99.97% (analysis of over 3000 human plasma samples in three independent evaluation studies conducted with the National Institute for Biological Standards and Control (NIBSC)).
- The analytical sensitivity of the Prionics®-Check vCJD V3.2: up to 10^{-5} based on a 10% vCJD positive brain homogenate spiked into human plasma.

The performance criteria for the development of a vCJD diagnostic test are outlined in the List A of Annex II of Reg (EC) 98/79 (IVD Regulation) and are defined in the Common Technical Specifications (CTS) to this regulation. Accordingly the analytical sensitivity and specificity as defined in the Common Technical Specifications (CTS) could be topped by the Prionics®-Check vCJD V3.2

Subsequently, we developed a test based on the amplification method real-time quaking induced conversion assay (eQuIC). In collaboration with Dr. B. Caughey, from the Laboratory of Persistent Viral Diseases, NIH/NIAID Rocky Mountain Laboratories the highly sensitive eQuIC was applied successfully to prion contaminated animal blood samples. Correspondingly, blood samples from mouse, sheep and hamster collected at the clinical phase of the disease could be discriminated

from non-infected samples. Moreover, this assay proved successful in the detecition of sCJD contaminated CSF samples (Cramm et al., Mol. Neurobiol. 2014).

Block C: Prion transmission and spreading.

The uptake of orally inoculated prions from the gut lumen and their transfer to the peripheral nervous system was investigated by histologically using immunofluorescence and ultrastructurally by cryo-immunogold. Data from these experiments is summarised in the cartoon below (Fig. 7): (1) Data suggested that prion uptake from gut lumen occurs predominantly via large LAMP1 positive endosomes of enterocytes in the follicle associated epithelium (FAE) with (2) much lower levels via M cells. (3) FAE enterocytes release prions to SED with Gpa33+ exosomes. (4) SED macrophages uptake and release Gpa33+ exosomes and prions to their environment. (5) Prions and Gpa33+ exosomes spread to the germinal centers by TBMs (6) Prions and FAE-derived Gpa33+ exosomes accumulate on surface of FDCs in the germinal centers. Prions start to replicate on the surface of FDCs. (7) Prions replicate and spread to CNS via enteric nerves close to Peyer's patches.

We have also investigated the ontology of follicular dendritic cells (FDCs), which play an important role for the microanatomical plasticity of lymphoid tissue and are crucially involved, as mentioned, in peripheral prion replication. By combining mouse genetics, kidney transplantations, marker-assisted FACS sorting and renal subcapsular transplantation of candidate precursor cells, we discovered that FDCs originate from ubiquitous perivascular precursors expressing platelet-derived growth-factor receptor β (PDGFRβ) (Krautler Cell 2012).

We have elucidated the reason why vCJD affects younger individuals: Ageing has a critical effect on splenic microarchitecture, and this in turn has a dramatic influence on susceptibility to BSE (Fig. 8).

Additionally:

- We have investigated the role of milk fat globule epidermal growth factor 8 (Mfge8) in prion pathogenesis. We found that Mfge8-dependent engulfment of cerebral apoptotic neurons plays an important role in prion disease (Kranich J Exp Med 2010).

- We have investigated the cellular and molecular requirements for prion colonization of lymph nodes. By combining mouse genetics and treatment with LTβR antagonist we discovered that TNFR1-independent prion accumulation in lymph nodes relies on LTbR signaling and the presence of high endothelial venules (HEV) (O'Connor PLoS Pathogens 2012).

- We have generated new BAC transgenic mice expressing lymphotoxin α and lymphotoxin β under the Mfge8 promoter (Mfge8-LTαβ) to investigate the role of lymphotoxin signaling for FDC development (Zhou Manuscript in preparation).

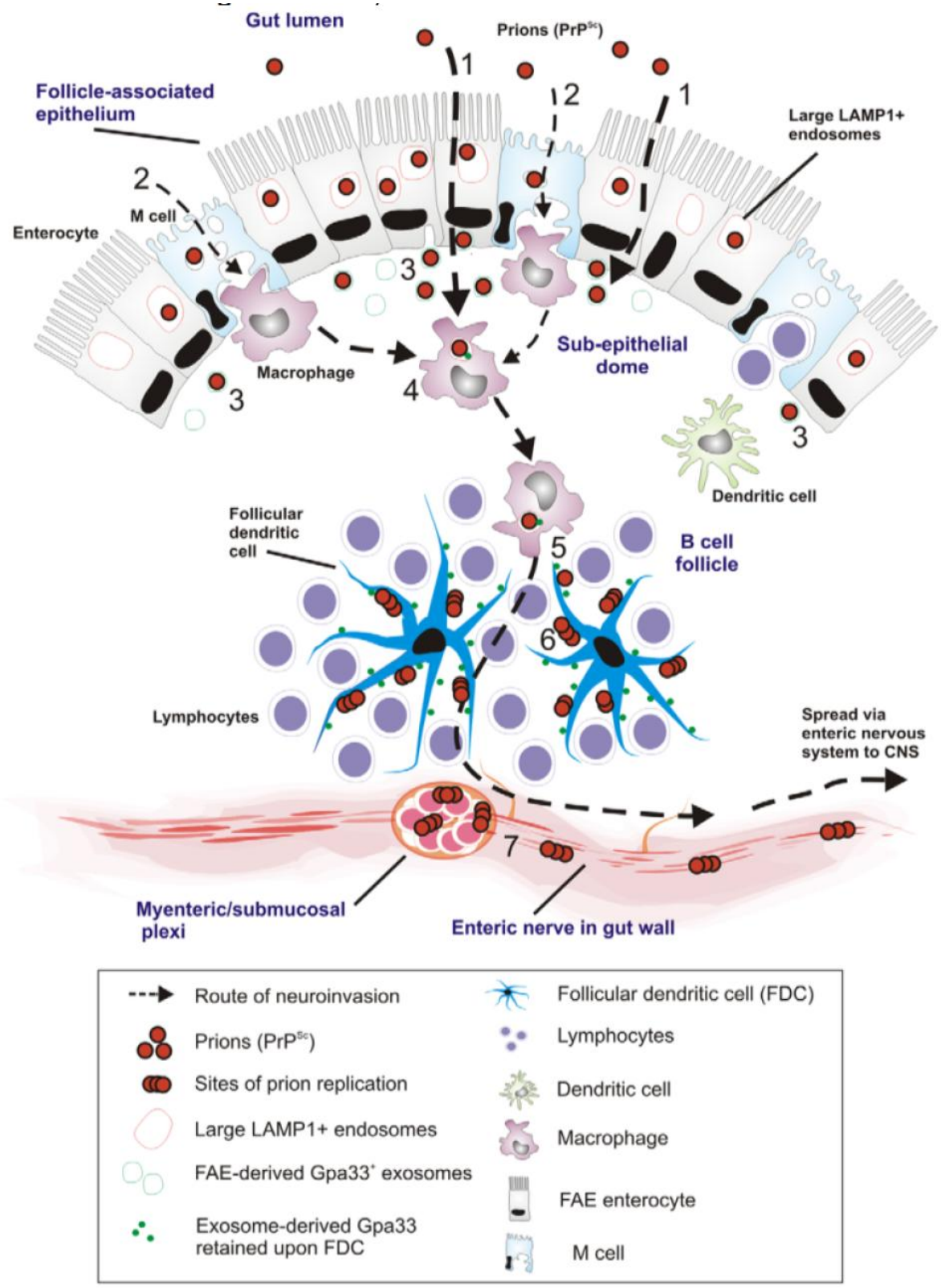

Fig. 7: Proposed model for prion neuroinvasion from the gut lumen via Peyer´s patches to the enteric nervous system. Adapted from Kujala et al., PLoS Pathogens 2011.

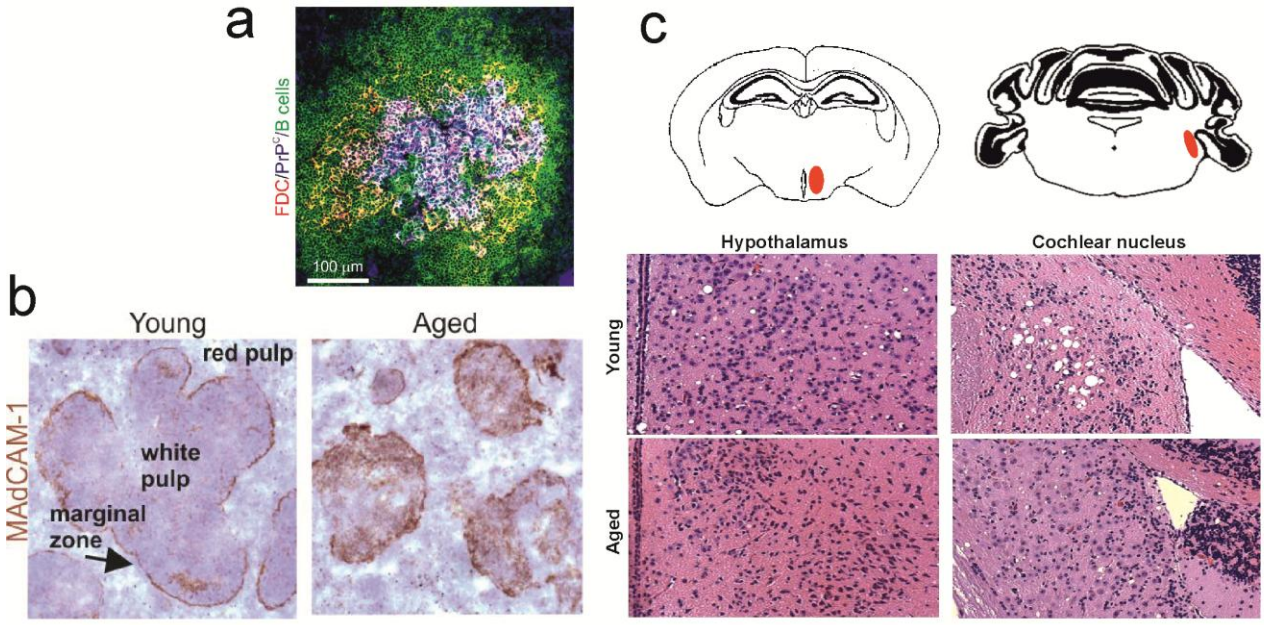

Fig. 8: The effects of aging on the splenic microarchitecture dramatically influence susceptibility to BSE infection. *a) Follicular dendritic cells (FDC) are important sites of prion replication in the spleen. b) Aging causes disturbances to the status of FDC and the splenic microarchitecture. c)As a consequence, FDC are unable to trap and retain prions such as scrapie of BSE reducing disease susceptibility. Adapted from Brown et al. 2012 J. Virol & Brown et al. 2014 J. Gen. Virol.*

- We have been unable to establish human hemato-lymphoid system mice to test whether sites of granulomatous inflammation are potential sites of prion accumulation in humans since immunodeficient Prnp knockout mice were found to be resistant to engraftment of human hematopoietic cells. Additional work has clarified that this unexpected phenotype was due to a genetic artifact of Prnp knockout line. Based on these observations, we have redirected our efforts to investigate artifactual phenotypes of Prnp knockout mice due to polymorphic Prnp-flanking genes. By combining formal genetics, next-generation sequencing and functional studies, we have found that Sirpa polymorphisms control phagocytosis of apoptotic cells (Nuvolone J Exp Med, 2013).

A key objective of this block was to elucidate infectivity dynamics in blood and milk. Our results allow us to conclude, with respect to blood:

- Presence of infectivity was demonstrated in the plasma, white blood cells and red blood cell concentrates of a vCJD affected patient
- In animal models, prionemia was demonstrated to be an early and persistent event (it appears within the 2-3 months that follow contamination)
- In animal models the transfusion of 200µL is sufficient to transmit the disease with a 100% efficacy
- The efficacy of the disease transmission by the transfusion route is more dependent on the viability of the transfused cell than on their level of infectivity
- In animal models, leuko-depletion of labile blood products display a high but not absolute efficacy against TSE transmission risk

- A PMCA method allowing the detection of minute amount of vCJD prion in the blood was developed In animals vCJD/BSE models, this method is efficient to detect prionemia in asymptomatic individuals
- The method is also efficient to identify vCJD agent in the blood of affected patients.

In the colostrum and the milk:

- Low but consistent levels of infectivity were evidenced in cream, skimmed milk and cellular fractions prepared from colostrum and milk collected in asymptomatic but scrapie infected small ruminants
- Chronic mastitis did not impact on the level of prions shed by colostrum and milk.

Block D: Prion epidemiology

Studies on atypical scrapie were identified as a key element of this block, given the potential risk associated to this agent. We studied the permeability of Human, bovine and porcine species barriers to atypical scrapie agent transmission. Experiments in transgenic mice expressing bovine, porcine or human PrP^C suggest that this TSE agent has the intrinsic ability to propagate across these species barriers including the Human one. Upon species barrier passage the biological properties and phenotype of atypical scrapie seem to be altered. Further experiments are currently ongoing (in the framework of this project but also in other projects) in order to: (i) characterize the properties of the prion that emerged from the propagation of atypical scrapie in tg Hu; (ii) to confirm that the phenomena we observed are also true for atypical scrapie isolates other than the ones we have studied.

In parallel, studies in shep have concluded that:

- Atypical scrapie can be transmitted by both oral and intracerebral route in sheep with various PRP genotypes
- Low but consistent amount of infectivity accumulates in peripheral tissue (mammary gland, lymph nodes, placenta, skeletal muscles, nerves) of sheep incubating atypical scrapie.

The combination of data from all our studies leads us to conclude that:

- Atypical scrapie passage through species barriers can lead to the emergence of various prions including classical BSE (following propagation in porcine PRP transgenic mice).
- Atypical scrapie can propagate, with a low efficacy, in human PrP expressing mice. **This suggests the existence of a zoonotic potential for this TSE agent.**

Pursuing another important objective of this block, we aimed at improving the early differential diagnosis of atypical molecular CJD subtypes and a further characterization of the Human prion strains. We took advantage of our surveillance systems, and asked neurologists and psychiatrists to report on all cases of progressive dementia in young people, and patients visited and examined using a standardised protocol for neuropsychological and neurological examination. The patients were classified according to established diagnostic criteria: 248 sCJD patients with known molecular subtype were investigated. Psychiatric symptoms were defined according to Möller and collaborators and the AMDP system (Study Group for Methods and Documentation in Psychiatry, German) (AMDP, Psychiatrie) and were collected by direct examination by study physicians or extracted from medical documentation. Our data were compared with published data on variant CJD (vCJD). Psychiatric symptoms were common in sCJD patients (90%) and mostly found already at the disease

onset (agitation in 64% of the patients, hallucinations in 45%, anxiety in 45%, depression in 37%). All psychiatric symptoms but illusions were found early in the disease course. Psychiatric symptoms in sCJD were less frequent than in vCJD. To find biochemical parameter for diagnosis of distinct molecular CJD subtypes we hypothesized that the PRNP codon 129 gene-polymorphism has not only a strong influence on the susceptibility, the progression of the disease but it might also change the physiological metabolism of PrP^{C}. Therefore, we analysed the cerebrospinal fluid (CSF) from different groups of patients by 2-dimensional Western blotting. Using the 2-D immunoblot technique with the detection antibody 3F4, we investigated PrP^{C} charge isoform profiles in the CSF derived from sCJD (MM, MV and VV) patients. The intensity of the 12 most abundantly expressed PrP^{C} charge isoforms was determined by densitometric analysis. The isoforms 5-10 showed the highest expression. Even though we failed to detect a specific isoform of PrP^{C} in one group, significant distinctions in the signal intensities of certain PrP^{C} charge isoforms could be observed. When we compared different sCJD codon 129 genotypes our data analysis revealed a significant increase of the isoforms 11 and 12 in sCJD MM and MV patients. Our findings indicated for the first time that the physiological metabolism is changed in dependence from the codon 129 MV sCJD genotypes. The up-regulation of certain PrP^{C} charge isoforms might be connected to the risk to develop sCJD and they might be helpful to discriminate among the groups.

Taking advantage of the RT-QuIC assay developed for the diagnosis of prion diseases (*vide supra*, section on block B), we developed assays adapted to CSF and obtained a high sensitivity of approximately 86 % and specificity off 99%. Moreover, we developed this assay further and analysed the prion seeding efficiency in humans with different forms of genetic and sporadic prion diseases. We propose that the application of the RT-QuIC method can be extended to show that PrP^{Sc} seeds from different prion diseases convert recombinant PrP with a different efficiency. To investigate the effect of type of prion disease on RT-QuIC response, we used CSF from sCJD, gCJD E200K and V210I, FFI and non-prion disease control cases. Lag phases and the intensity of ThT fluorescence signal over a time period of 80 h were measured for quantification of RT-QuIC response. Interestingly, type of prion disease (sporadic vs. genetic) and the *PRNP* mutation (E200K vs. V201I and FFI), codon 129 genotype and PrP^{Sc} type affected RT-QuIC response. In genetic forms, type of mutation showed the strongest effect on the observed outcome variables. In sporadic CJD, MM1 patients displayed a higher RT-QuIC signal maximum compared to MV1 and VV1. Age and gender were not associated with RT-QuIC signal but patients with a short disease course showed a higher seeding efficiency of the RT-QuIC response, suggesting that PrP^{Sc} characteristics in the CSF of human prion disease patients are associated with disease subtypes and the stage of the disease.

Further work on proteomics (Fig. 9) and peptidomics based application were applied for the biomarkers identification in different CJD subtype patient´s CSF and blood. Different protein separation techniques including 2D-Difference gel electrophoresis (DIGE), in-gel digestion and peptide extraction were applied to human biological fluids and identified/characterise novel candidates for diagnostic marker. The level of Desmoplakin, 14-3-3 and tau was measured in 58 sCJD patients and 81 control patients including 45 cases with an elevated 14-3-3 level. Our study revealed that desmoplakin showed a low positive rate accompanied by a very low false positive rate. Therefore, desmoplakin might be a promising CSF marker to rule out 14-3-3 false positive cases in sCJD differential diagnosis. Transcriptomic analysis of sCJD brain samples were performed to gain insights into the potential functional role of prion biomarkers in disease pathology. Interestingly, desmoplakin mRNA was found to be highly upregulated in sCJD and in the sCJD-MM1 mouse model, suggesting that altered Desmoplakin levels in the biological fluids of prion disease patients may be linked to its upregulated expression in the brain. Furthermore, we examined the proteome of the frontal cortex of patients with the two most common sCJD subtypes (MM1 and VV2) by 2D DIGE analysis and mass spectrometry. We found that 46 proteins were differentially expressed (7

common expressed, 4 opposite, rest displayed subtype-specific alterations). Most of differentially regulated proteins were involved in signaling transduction processes, neuronal activity, cell cycle and death, as well as in structure and motility. High-throughput transcriptomic analysis partially validated the findings at proteomic level. Finally, RNA-seq in the CJD mouse model revealed a set of genes which expression is regulated at pre-clinical stages and involved in synaptic transmission, while analysis performed at clinical stages indicates that Calcium related genes as well as protease and phosphatase families such as cathepsins and dual-specificity phosphatase respectively are highly diregulated in the CJD mouse model.

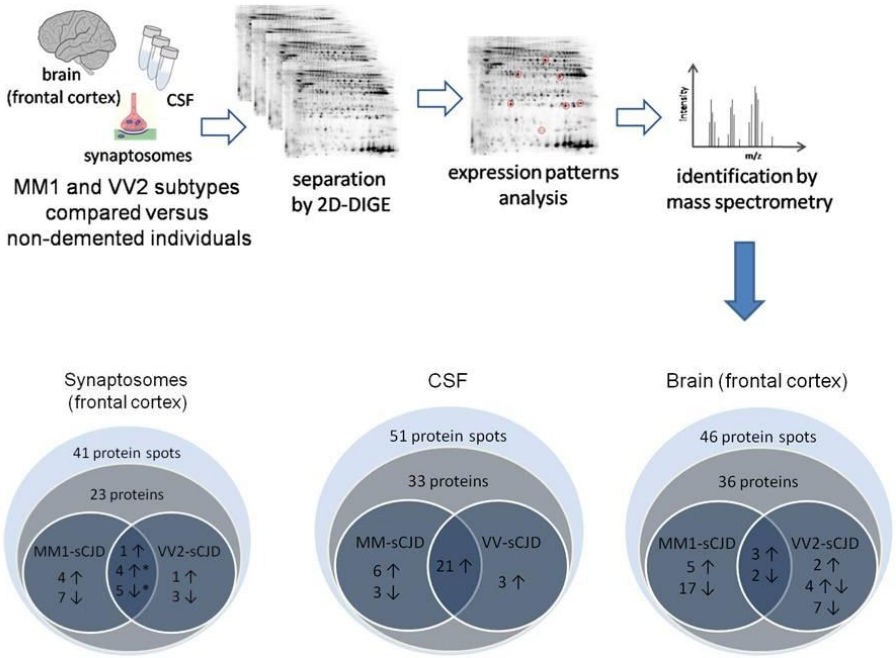

Figure 9: Differential expressional regulation of proteins in subtype-specific CJD patients.

We have also extensively investigated the occurrence of the most frequent CJD-specific mutation E200K in the general Slovakian population. We suspected that there might exist many asymptomatic carriers of the mutation, and this is indeed what we found, even in regions of Slovakia with the lowest incidence of this genetic form. Confirmed mutation in persons without any relation to $gCJD^{E200K}$ patients signals a possible iatrogenic CJD-risk in the general population and indicates as reasonable the preventive genetic testing in donors of high risk tissues or organs. In parallel studies in the same country, comparison of the mean age difference in patients of three generations in $gCJD^{E200K}$ affected families confirmed the existence of an "anticipation" phenomenon, i.e. decreasing mean age at clinical onset and death. Detailed analysis demonstrates an increasing anticipation with increasing number of affected generations as well as with significantly increased environmental Mn level, resulting in Mn/Cu disbalance in the brain. Anticipation is not influenced by gender, polymorphism M129V or by the 1st or 2nd degree relationship of affected family members.

Results provide evidence that anticipation is decisive for the repeatedly observed, significantly increased annual incidence of $gCJD^{E200K}$ and it participates in the gradual, slow increase of its annual incidence. The main clinico-epidemiological impact of the anticipation concerns the incidence of $gCJD^{E200K}$, the individual prognosis and prospectively also prophylactic treatment of asymptomatic carriers of the most spread mutation E200K.

We have also investigated the possible influence of the CJD-specific mutation E200K on the human-sheep species barrier. Comparison of humanized Tg mice with and without the E200K mutation, inoculated i.c. with classic sheep scrapie or gCJDE200K prions have shown no differences either in attack rate (100%), or incubation time, clinical signs and histopathological findings in gCJD infected groups, and significant differences in typical scrapie inoculated lines. While in Tg mice with the mutation E200K the transmission was successful in 15%, no infections occurred in the Tg line without mutation. Also, no infections occured after oral inoculation. Besides that, striking differences were observed in lesion profile and Western blot patterns.

These results provide the first evidence on the influence of the most frequent CJD- specific mutation E200K on the investigated species barrier, but the low attack rate (15%) observed, only after ic. infection, indicates extremely low risk of scrapie for humans, even in carriers of the E200K mutation.

With respect to **prion decontamination methods**, we have evaluated an alkaline detergent in combination with an oxidizing agent (Sekumatic FR and Sekumatic Oxivario), assessing its efficacy to eliminate prions adsorbed on a steel surface, as a model for decontamination of surgical instruments. According to the Standard Prion-Protocol of AFSSAPS (Agence Française de Sécurité Sanitaire des Produits de Santé), in order to understand the mechanism of an agent or a process against prions, for example, whether the prions are denatured or only washed away, it is required that an *in vitro* test (with/in standardised hard water) for the same brain homogenate contaminated steel wires to be performed additionally in the same way as the wires which are to be implanted. *In vitro* qualitative study of possible detachment, destabilization and degradation effects of Sekumatic reprocessing agents against pathological prion proteins bound to steel surfaces was therefore performed by Western blot. The tests were carried out without any mechanical force. With a concentration of 0.5%, the agent Sekumatic FR is found to lead to detachment of prion protein from the steel wires and renders the infectious prion proteins sensitive to proteinase K digestion (destabilization) after incubation at 55°C for 5 min. When the same batch of wires further treated with 0,8% of Sekumatic FR plus 0,7% of Sekumatic Oxivario at 55°C for 10 min, there were no prion proteins detectable in the supernatant (degradation). However, there are still residue prion proteins left on the steel wire surface, although it was barely detectable in the eluate from the wires on the Western blot.

We also valuated an alkaline detergent agent with different pH values against prions adsorbed on steel surface by Western blot. The detachment, destabilization and degradation effects of alkaline agents with different pH (pH 12.3, pH 11.7, pH 11.13 and pH 10.9) were investigated against pathological prion proteins bound to steel surfaces by Western blot using the same protocol described in the first study. Western blot assays have shown that treatment of steel wires, which were contaminated with 10% Scrapie hamster brain homogenate, with 1.0% of four alkaline agents at 55°C for 10 min led to prion protein degradation in the supernatant and on the wires and rendered the pathological prion proteins no longer resistant to Protease K digestion. Alkaline agent with a pH value of 12.3 has the strongest effect among the four agents. Compared to the water treatment control group, the alkaline groups had stronger prion protein signals in the supernatant, indicating detachment of prion protein away from the wire surface.

We also tested inactivation of pathological prion proteins bound to steel wires by a combination of heat drying and steam sterilization. In this case, prion inactivation was assessed by bioassay. Our study showed, to our knowledge for the first time, that dry heat (<120°C) combined with steam sterilization could lead to prion inactivation. Although the mechanism of the prion inactivation in this case is unknown at present since prions are resistant to both dry heat and autoclaving, we speculate that during dry heating, prions on steel surface might be modified through Maillard reactions. Maillard reaction may lead to structural changes of prion protein, which may facilitate further protein structural damages by steam sterilization. This procedure merits further study. Dry heating and steam autoclaving should be studied separately and consecutively, that may provide the information whether the mechanism of prion inactivation shown in this report is a synergic effect.

Using an alternative approach, we studied the combined effect of $CuSO_4$ and H_20_2 on the infectivity of formaline fixed and native brain in gCJD with the PRNP mutation E200K (gCJDE200K), characterized by experimentally confirmed high transmissibility. The efficiency of decontamination evaluated by immunohistochemical detection (Abs 3F4 and 6H4) of PrP^{Sc} and by SAF revealed significant reduction of PK resistant prion, in both, fixed and embedded slides, as well as in the native tissue, but residual PrPres or SAFs were present. Increased concentration and exposition of tested chemical decontamination provided no evidence of increased effect on this residual infectivity. Decontamination has no influence on the quality of histological / immunohistological slides.

Correlation of the immunohistochemical and biochemical findings with biological control of the infectivity (bioassay, comparing contaminated versus contaminated and decontaminated needles used for i.c. inoculation of gCJD brain suspension) confirmed residual (15%) infectivity after treatment by $CuSO_4$ and H_20_2.

Significantly reduced infectivity caused by $CuSO_4$ and H_20_2 in fixed and embedded brain tissue, may considerably increase the safety of histological / immunohistopathological examination, necessary for definite diagnosis in suspected human prion disease.

Another objective was **to study the inactivation of prions in animal-by-products** (category 1 and 2 materials). We assessed the suitability of microwave treatment (thermo-pressure-hydrolysis) to inactivate prion proteins including the treatment of category 1 and 2 material (REGULATION (EC) No 1069/2009). To improve the sensitivity of our detection system (Western blotting assay) for remaining prion proteins after inactivation we established a PMCA protocol including 5 rounds of amplification. For the studies the hamster adapted scrapie strain 263K: Syrian hamster brain homogenates, BSE strain 301V, mouse-passaged BSE prions, and mouse brain homogenates were used. We evaluated three different temperatures and incubation times at a constant pressure of 1.5 bar (90° C, 120 ° C, 150° C, 5 min, 10 min, and 15 min).

No 263K and no 301V prions were detectable by Western blot after at least 10 min and 120 °C microwave treatment. 263K prions still detectable after treatment at 90°C and 120°C, 5 min. No significant differences between category 1 and category 2 substrates were observed.

By integrating PMCA (5 rounds) in our detection assay it was possible to achieve identical sensitivities of detection as with the classical bioassay, i.e. we could detect up to log7 infectivity reduction after microwave treatment.

In summary, the results showed that the most efficient microwave treatment of prions 263K and 301V is using 120° C for at least 15 min or 150° C for at least 10 min. Quantitative analysis of prion inactivation efficacy was possible after the integration PMCA into the detection procedure.

Another key objective in this block was the study of **dynamics of prions in solids and wastewater**. Experimental protocols for the detection of prions in environmental matrices, including slaughterhouse and urban wastewater, river water and seawater have been developed and their

sensitivity using WB assays has been described. In particular, we have developed the following standard operation procedures (SOPs):

- Concentration of PrP^{Sc} from slaughterhouse and urban wastewater
- Concentration of PrP^{Sc} from river water
- Concentration of PrP^{Sc} from seawater
- Release of PrP^{Sc} from soils
- Release of PrP^{Sc} from MBM

Armed with these SOPs, we studied the stability of BSE in urban wastewater using WB assays and mouse bioassays using the Tg110 mouse line. In this study, the persistence of BSE infectious agent in sewage has been assessed by both PrP^{Res} immunoblotting and mouse bioassay in a long-term incubation study (Note: in the following discussion the operational term PrP^{Res},rather than PrP^{Sc}, will be used, to signify that data refer to PK-resistant PrP as detected in Western blots. Results indicated that no PrP^{Res} was detected after 150 days of incubation and consistent with this, a statistical regression model estimated 2-logs decay in 151day. Surprisingly, in contrast, no reduction in infectivity was observed during this period. Similarly, BSE infectivity remained unaltered after incubation in PBS for 265 day, whereas PrP^{Res} levels dropped progressively over the length of the study. These results indicate that in sewage and PBS, prion infectivity persists longer and with different dynamics than its commonly used marker PrP^{Res}. Thus, mathematical models computed on the basis of PrP^{Res} detection were unable to predict inactivation of prion infectivity. It is also reasonable to assume that conventional wastewater treatments with low retention times could have a very limited impact on prion infectivity. This data is essential for the development of accurate risk assessment analysis for BSE and other prion diseases in the environment.

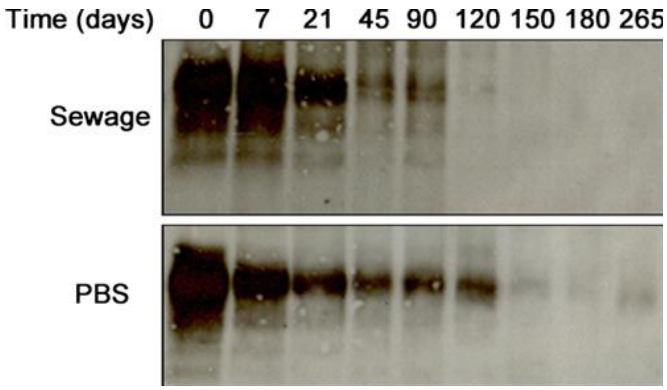

Fig. 10: Detection of PrP^{Res} in sewage and PBS contaminated with BSE*. Aliquots of each sample were collected and concentrated on the indicated day. After proteinase K treatment, PrPRes was detected by IB.*

Considering the poor performance of detection of PrP^{Res} by WB, we turned to PMCA. PMCA proved to be a useful tool to detect PrPSc in wastewater samples. While negative results for PrP^{Res} were obtained for samples spiked from dilutions -2 (1:100; 25 µg of brain infect tissue).by WB, when PMCA was applied, positive results were obtained up to -5 dilutions (1:100000; 0,02 µg of infected brain tissue). MBM seemed to be a matrix with less inhibitory compounds than wastewater, and the results were consistent and reproducible, showing that PMCA represented a very useful tool to detect PrP^{Sc} in this matrix. Negative results for PrP^{Res} (WB) were obtained in samples spiked from dilutions -2 (1:100; 22 µg of brain infect tissue). Analyzing the samples by PMCA, all dilutions (-1 to -5; 220-0,02 µg of brain infect tissue) were positive and inhibition problems were not detected. Due to the relevance of wastewater as a source of contamination and the economic interest of MBM,

the methods for wastewater and MBM were selected for standardization and validation studies (inter and intra–laboratory assays). The statistical analysis concluded that there were no significant differences between samples in both, MBM and wastewater.

We also analysed the dissemination and stability of prions in the environment and contaminated superficial water, and evaluated critical factors affecting the survival of prions in the environment. BSE results were published (Maluquer de Motes, Environmental Res. 117:1-7, 2012). A quadratic regression model with higher confidence (R2=0.95), estimated its decay as 1 log (t90) after 111 days and 2 logs (t99) after 151 days. However, inoculation of transgenic mice with a sample that had been incubated in sewage for 150 days demonstrated that no decay in infectivity had occurred. For Scrapie Dawson strain a quadratic regression model was predicted. The point estimates of t90 for the diverse matrices showed t90s of 148.93 days in slautherhouse wastewater, 70.64 days in river water and 82,87 days in seawater, showing significantly higher valuesif the water matrices were previously autoclaved, proving the important role of the microbiota in the reduction of PrP^{Res} in water matrices.

We also analyzed the role of viruses as microbial source tracking tools to identify fecal/urine contamination of human or animal sources in water. A new ovine polyomavirus (OPyV) was identified by our team in Barcelona and a protocol for the detection and quantification of ovine polyomavirus in water was defined with a specific qPCR **assay**. Besides, the use of bovine polyomavirus (BPyV) has been also proposed for tracing bovine sources of environmental contamination. The results obtained in our laboratory using the specific quantitative PCR assay developed by Hundesa et al., 2010can be used to determine the excretion level and concentration of bovine polyomaviruses in slaughterhouse wastewater, including raw water, flocculated water, biosolids and effluent water. Tracing bovine polyomavirus can thus be utilized as a very useful method to assess potential bovine contamination in waste and in the environment, as an exceptionally sensitive method, representing alsoa complementaryprotocol for the potential presence of prion contaminationalong with tracing of PrP^{Sc}.

Finally, the stability of the viral markers, using HAdV and JCPyV, as viral models at 20°C±2 over 166 days was analyzed in slaughterhouse wastewater samples (raw and flocculated) containing ovine and bovine contamination. The statistical analysis of estimated t90, t99, t99,9, t99,99 showed values of t90 of few days and t99.99 of 175.75 and 141 days for adenovirus and polyomavirus respectively.

Potential impact (including the socio-economic impact and the wider societal implications of the project so far) and the main dissemination activities and exploitation of results (10 pages max)

(This section is based on the Position Paper written by the Priority consortium and disseminated among policymakers and stakeholders).

Bovine spongiform encephalopathy (BSE), "mad cow disease" as it was named by the media, created a global European crisis in the eighties and nineties, with very serious health and economic implications. BSE is caused by prions, infectious proteins that constitute a novel and still incompletely understood class of infectious and pathogenic agents. Classical BSE now appears to be under control, with 18 EU Member States having achieved the World Organisation for Animal Health (Office International Epizooties) 'negligible risk' status. This is to a great extent the result of a research effort that identified the sources of prions in meat and bone meal (MBM), developed analytical tools to test animals and thus provided the tools to guide policy. **Priority** (www.prionpriority.eu) is an FP7-funded project through which 21 European research institutions and SMEs joined efforts throughout September 2010 to September 2014 to conduct coordinated basic and applied research on prions. In this section we summarize the main conclusions of the Priority consortium and the potential impat and societal implications of the project. We advance our main conclusions and recommendations, in particular as they might affect public policy, including a detailed elaboration of the evidence that led to them. Our main recommendations are:

a. The issue of re-introducing ruminant protein into the food-chain

The opinion of the members of **Priority** is that sustaining an absolute feed ban for ruminant protein to ruminants is the essential requirement, especially since the impact of non-classical forms of scrapie in sheep and goats is not fully understood and cannot be fully estimated. Therefore, the consortium strongly recommends prohibiting re-introduction of processed ruminant protein into the food-chain. Arguments in support of this opinion are:

- the large (and still uncharacterized) diversity of prion agents that circulate in animal populations;
- the uncertainties related to prion epidemiology in animal populations;
- the unknown efficacy of industrial processes applied to reduce microbiological risk during processed animal protein (PAP) production on most prion agents;
- the intrinsic capacity of prions to cross interspecies transmission barriers;
- the lack of sensitive methodology for identifying cross contamination in food.
- the evolution of natural food chains in nature (i.e. who eats whom or what) has generated an efficient barrier preventing, to some extent, novel prion epidemies and that this naturally evolved ecology should be respected.

The consortium is also hesitant to introduce processed ruminant proteins into fish food considering the paucity of data on prion infections in fishes and sea animals including those of mammalian origin, and the risk of establishing an environmental contamination of the oceans that cannot be controlled.

b. Atypical prion agents and surveillance

Atypical prion agents (see below) will probably continue to represent the dominant form of prion diseases in the near future, particularly in Europe. Atypical L-type BSE has clear zoonotic potential, as demonstrated in experimental models. Similarly, there are now some data that seem to indicate that the atypical scrapie agent can cross various species barriers. Moreover, the current EU policy for

eradicating scrapie (genetic selection in affected flocks) is ineffective for preventing atypical scrapie. The recent identification of cell-to-cell propagation and the protein-encoded strain properties of human neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease, suggest that they bear the potential to be transmissible even if not with the same efficiency as CJD. More epidemiological data from large cohorts are necessary to reach any conclusion on the impact of their transmissibility on public health. Re-evaluations of safety precautions may become necessary depending on the outcome of these studies.

In that context it would appear valuable

- to develop knowledge related to the pathogenesis and inter-individual transmission of atypical prion agents in ruminants (both intra- and inter-species)
- to improve the sensitivity of detection assays that are applied in the field towards this type of agent
- to maintain a robust surveillance of both animal and human populations

c. The need for extended research on prions

Intensified searching for a molecular determinants of the species barrier is recommended, since this barrier is a key for many important policy areas - risk assessment, proportional policies, the need for screening of human products and food. In this respect, prion strain structural language also remains an important issue for public health for the foreseeable future. Understanding the structural basis for strains and the basis for adaptation of a strain to a new host will require continued fundamental research. Prions maintain a complex two-way relationship with the host cell and fundamental research is needed on mechanisms for their transmission, replication and cause of nervous system dysfunction and death.

Early detection of prion infection, ideally at preclinical stage, also remains crucial for development of effective treatment strategies in humans affected by the disease.

<u>Position of the Priority consortium</u>

Nearly 30 years ago, the appearance in the UK of Bovine Spongiform Encephalopathy (BSE) quickly brought the previously obscure “prion diseases” to the 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 in Europe to die from variant Creutzfeldt-Jakob disease (vCJD) (http://www.cjd.ed.ac.uk/).

<u>Origins of prion epidemies</u>

Classical BSE now appears to be under control, with 18 EU Member States having achieved the World Organisation for Animal Health (Office International Epizooties) ‘negligible risk’ status (May 2014; http://www.oie.int/en/animal-health-in-the-world/official-disease-status/bse/list-of-bse-risk-status/), and the remaining MS assessed as ‘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 principle driver of the epidemic was identified as prions in Meat and Bone Meal (MBM). Tests based on prion protein-specific antibodies were developed, allowing detection of infected animals, and a better understanding of disease pathogenesis and the distribution of infectivity in edible tissues; experimental investigation of transmission barriers between different species allowed a rational estimation of risks, etc. 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.

In spite of this progress, prions are still a threat. Epidemiological re-assessment indicates that the ~10 year incubation period separating the peaks of the BSE and the vCJD epidemics is probably

too short. 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 conformer prion protein PrPSc. 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 cases with other prion diseases.

The human prion problem

The clinical cases of vCJD identified to date have all shared a common 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, raising the possibility that genotype does not confer resistance but instead modulates incubation period. Apart from the two UK studies, the lymphoid tissues of non-CJD patients have not been examined for the presence of PrPSc, so, these cases may not solely represent pre-clinical vCJD, but also other forms of prion disease.

Recent experiments in highly susceptible mouse models indicate the presence of infectivity in blood or blood components at late disease stages in sporadic CJD. The significance of this experimental finding for humans has to be explored in more detail and, 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 those with signs of infection or abnormal PrP accumulation in peripheral tissue could have infective blood, posing the risk for transmission via blood products, which has been clearly demonstrated in experimental models, and confirmed in several cases of vCJD in man. Altogether, these data clearly demonstrate the potential risk of a second wave of vCJD, particularly when the number people identified with lymphoid accumulation of PrPSc (16/32,411) 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 on cases of “atypical” BSE in cattle throughout the EU and beyond may lead to a new epidemic, particularly since we still do not understand all factors determining the species barrier. Ovine scrapie is another concern, because it could mask ovine BSE, presumably transmissible to humans. 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 these forms of disease will persist in the global animal population. The low prevalence of these disease forms makes effective surveillance very challenging. However, there is a clear risk attendant on ignoring these cases without an understanding of their possible zoonotic potential, particularly when most forms of human disease have no established aetiology. In summary, atypical cases of BSE and scrapie presently clearly outnumber classical cases in cattle and sheep in all member states.

We will highlight the state-of-the-art knowledge and point out scientific challenges and the major questions for research. Strategic objectives and priorities in Europe in the future for research that aims to control, eliminate or eradicate the threat posed by prions to our food and health are also indicated.

The **Priority** project has focused on 4 themes, namely the structure, function, conversion and toxicity of prions; detection of prions; mechanisms of prion transmission and spreading and epidemiology of prion diseases. This paper summarizes the opinions/positions reached within these themes at the end of the project.

1. Prion structure, function, conversion and toxicity

State of the art

The mechanisms for conversion of the normal cellular PrPC to PrPSc, as well as strain diversity and transmission barriers, are structurally enciphered. Thus, it is essential to understand the structure of PrPSc in order to design methods to interfere with prion propagation and spread, but it can also be of diagnostic significance if alternative and/or earlier markers could be identified - especially in vivo.

The PrPSc forms double amyloid fibers made up of two intertwined fibrils, each 3-4 nm wide, with no regular pitch. Fiber X-ray diffraction data and geometric considerations strongly suggest that each PrPSc monomer stacked in each protofilament is a 4-rung β-solenoid, with a high structural resemblance to the HET-s fungal prion. Limited proteolysis studies corroborate that PrPSc contains stretches of high resistance to PK (presumably □-strands) interspersed with short stretches with a higher proteolytic susceptibility, presumably loops and turns. The C-terminal stretch (180-231) is the most PK resistant region, arguing that no residual □ -helical structure remains in PrPSc.

Unraveling the early events in structural prion formation in sporadic forms of prion disease is of major importance, since the conversion of PrPC to PrPSc is the central event in prion diseases. Hereditary prion diseases are associated with about forty point mutations of the gene (PRNP) that codes for PrP, Most of the variants associated with these mutations are located in the globular domain of the protein.

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. In sharp contrast with 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 solved yet.

Major questions and scientific challenges

Many aspects of prion replication can be demonstrated in vitro in systems containing only PrPC and PrPSc. At first approximation, prion propagation can thus be reduced to a biophysical problem dealing with alternative conformations, amyloid structures, and conformational coercion. However, like other pathogens, prions maintain a complex, two- way relationship with the host cell. It is clear that prions propagate in their natural hosts much more efficiently that they do in vitro. The host cell provides both the molecular species (such as PrPC) and the molecular mechanisms required for the prion propagation. Questions related to (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, remain largely unsolved.

Regarding the role of intracellular trafficking in prion diseases it is clear that the endosomal compartment has an important role in prion conversion, while prion structures called “string” containing both PrPC and PrPSc are found on the cell surface of infected cells. Furthermore, novel structures called tunneling nanotubes (TNTs) appear to have a major role in spreading PrPSc between cells in cultures, however better tools are needed to evidence their presence and role in vivo. One major scientific challenge is to better understand the structural basis of the different prion strains and 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 PrPSc monomers in a propagative PrPSc stack (either oligomer or protofilament in a fiber); in turn, transmission barriers consist of steric hindrances hampering continuation of the cross-β stack by an

incoming PrP strand being molded onto the pre-existing PrPSc assembly. Related to this is the question of why some prions are more dangerous than others for humans. In addition, since sporadic prion diseases affect mainly aging people (average age of onset being around 65 years old) a challenge will be to investigate age-related factors that promote these sporadic diseases to develop. 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 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 PrPSc.
- Structural analysis of the various point mutations present in the globular domain of PrPSc can unveil common folding traits that may allow to a better understanding of the early conformational changes leading to the formation of monomeric PrPSc.
- Analyses 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" can provide novel strategies aiming at blocking prion propagation and toxicity.
- Development of new treatment strategies for individuals affected by CJD.

2. Prion detection

State of the art

Post mortem detection systems of PrPSc in the central nervous system and lymphoreticular tissue are nowadays widely used for surveillance of BSE and Scrapie in sheep and goat. These rapid tests have greatly improved the detection of infected animals 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 PrPSc in body fluids like blood or cerebrospinal fluid. Even though these tests still need to be improved in sense of time to result and robustness they open up new ways for live tests to detect prions.

Opinions – Positions:

The emergence of in vitro amplification technologies such as protein misfolding cyclic amplification (PMCA) and real time quaking induced conversion (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) collected from affected individuals, and their ability to do this has been demonstrated in both sheep and human samples. 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 80's . Despite those initial difficulties PCR is now a basic lab technology.

Prions may be considered also as potential environmental contaminants and their stability in the environment, wastewater and soils must be evaluated as valuable parameter for developing risk assessment studies. Prions are extremely resistant to inactivation and it has been demonstrated that prions can survive in soil for years. In recent years, deposition of scrapie and chronic wasting disease (CWD) infectivity in the environment through biological fluids and/or faeces has been proved, and, BSE and scrapie can also be introduced anthropogenically by transporting infectious prions via landfill leach or slaughterhouse wastewater. Furthermore, there is the possibility of discharged

contaminated urine, feces and blood from CJD patients. All of this suggests strongly 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 evaluate the fate of infectious prions in the environment and the potential sources of contamination.

Major questions and scientific challenges

A major scientific challenge is to develop new, and refine existing, prion detection methods that could have applications in pharma 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 waste water treatments for the removal of prions need to be assessed.

Strategic objectives and priorities in the future

- Improving the performances and robustness of in vitro prion amplification technology
- Establishing a relationship between the presence of PrPSc, 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 matrix.
- Redefining the techniques available to optimize the detection of prions in diverse environmental matrices, with validated protocols.
- Water samples impacted by infected animal excreta and waste water must be analyzed for any potential role in the transmission of prion diseases, producing data on the potential dissemination of prions in these areas.

3. Prion transmission and spreading

State of the art

Insights into the mechanisms by which prions enter the brain to induce neurodegenerative diseases, and on how they may exit an organism through body fluids, as well as on and how host factors (such as age and inflammation) affect these processes, are essential for a better understanding of the factors affecting prion transmission and pathogenesis.

Opinions-Positions:

Strategic objectives and priorities in the future

We still do not know the precise cellular mechanisms by which prion infections get in or out of an animal, or how to detect/control either of these processes. Knowledge of these processes would assist both diagnostic and therapeutic intervention strategies.

Major questions and scientific challenges

By which cellular mechanisms do prions propagate from the periphery to the central nervous system to cause disease, and how are they released into body fluids for spread into the environment?

Are species barriers to prions “rigid/absolute” and related to the prion strain, or can they, at the individual host level, be affected by host variables such as other infections and inflammatory disorders, and age?

Another important question is whether animal milk presents a risk for spread of prions to humans.

Strategic objectives and priorities in the future

- Identifying the organelle(s) and molecules involved in cell to cell prion spreading and release in the body fluids

- Better understanding of when to target diagnostic/therapeutic strategies based on age/species.
- Development of host cell-directed interventions to prevent propagation and spread of prions.
- Which decontamination procedures should be implemented in practice?
- Identifying the organelle(s) and molecules involved in cell to cell prion spreading and release in the body fluids
- Better understanding of when to target diagnostic/therapeutic strategies based on age/species.
- Development of host cell-directed interventions to prevent propagation and spread of prions.
- Which decontamination procedures should be implemented in clinical practice?

4. Prion epidemiology

State of the art

Considerable efforts have been made by the EU in recent years through the implementation of rigorous regimes to control prion infections in cattle, sheep and goats as well as leucodepletion of human blood. An array of regulations such as the introduction of the feed ban, an effective surveillance and monitoring system, the destruction of SRM and the establishment of culling strategies for small ruminants by member state authorities had a significant impact on the incidence and spread of the 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.

Chronic Wasting Disease (CWD) is a naturally-occurring prion disease in cervids, which has reached epidemic proportions in the US and Canada. 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. 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. 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 processing 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, faeces and blood from CJD or vCJD patients. In humans, several molecularly-defined disease subtypes have been described. However, neither the molecular basis nor the epidemiological significance of these so called sporadic disease subtypes are understood.

Opinions -Positions:

A better understanding of the way in which different strains of prions are spreading between animals and human beings, and the environmental factors that modulate such spreading is essential to design methods to prevent the spread of prions within the communities. Crucial to prevention of the spread of prions are improved methods for decontamination and disposal of animal waste as well as assessment of prions in waste water and soils.

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. 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. Most recently, two new cases of non-classical BSE were diagnosed in Germany 2013/14, a country where BSE had last been seen in 2009. The overall picture of atypical/sporadic BSE is complicated by the fact that these two new cases of BSE appear to be different from known atypical cases in cattle. 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 (Sept. 2014) atypical scrapie has become the most common form of disease detected in sheep causes quite some concern. None of the measures implemented for disease control in small ruminants has affected the prevalence of atypical disease, despite being shown to be effective for the control of classical scrapie.

A major point of concern is therefore the occurrence of atypical cases of BSE, which in light of the new types of atypical BSE in Germany (2013/14) and earlier (2011) in Switzerland, would remain undetected with any reduction in surveillance. The occurrence of atypical BSE in elderly 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 an Opinion indicating 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.

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

Major questions and scientific challenges

Although the epidemiology of atypical cases supports the hypothesis of a spontaneous origin, they can be experimentally transmitted and therefore present a risk. Also 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 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 PrPSc levels. Improved PrP markers must be defined to be used as target parameter when considering infectivity.

Strategic objectives and priorities in the future

- The existence of atypical prions in cattle and small ruminants, which were until now unknown, and the new concept of "prionopathies" in humans clearly show that appropriate prion agent surveillance should be maintained in both the animal and human populations, and that surveillance tools should be reviewed, expanded and developed according to the state of current scientific knowledge.
- Definition of suitable wastewater treatments that would reduce the possibility of prion dissemination in the environment.
- Implementation of a study of the potential presence of CWD in Europe, including surveillance programs for the detection of CWD prions and studies of their behavior in the environment.
- Development of programmes for education and awareness within farming communities and vets/medics, in particular, as a frontline surveillance

• Establishing more comprehensive continuous- molecular strains defined- surveillance of all forms of human prion diseases for early identification of atypical cases and potential outbreaks in humans.
• Development and definitions of strain-specific therapeutic options in classical and atypical CJD in humans.
• Identification of cellular factors that control prion uptake, replication and release for host-directed drug targeting

Project website: www.prionpriority.eu

List of beneficiaries:

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B7	Lothar Stitz	Friedrich Loeffler Institut - Bundesforschungsinstitut fuer Tiergesundheit, Tuebingen	FLI	Germany
B8	Albert Taraboulos	The Hebrew University, Jerusalem	HUJI	Israel
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B12	Rosina Gironés	Universitat de Barcelona	UB	Spain
B13	Olivier Andreoletti	Institut National de la Recherche Agronomique, Toulouse	INRA	France
B14	Klaus Roth	SMP GmbH - Pruefen Validieren Forschen, (SME) Tuebingen	SMP	Germany

B15	Marion Simmons	The Secretary of State for Environment, Food and Rural Affairs, Weybridge	VLA	United Kingdom
B16	Sylvie Benestad	Veterinaerinstituttet - National Veterinary Institute, Oslo	NVI	Norway
B17	Eva Mitrova	Slovenska Zdravotnicka V Bratislave, Bratislava	SZU	Slovakia
B18	Giuseppe Legname	Scuola Internazionale Superiore di Studi Avanzati, Trieste	SISSA	Italy
B19	Bas Lemmens	Universiteit Maastricht, Maastricht	UM	The Netherlands
B20	Alex Raeber	Prionics AG (SME), Zurich	PRIONICS	Switzerland
B21	Reinhard Boehm/Ludwig Hölzle	Universitaet Hohenheim	UHOH	Germany

Contact: Jesús R. Requena (Project oordinator), CIMUS Biomedical Research Institute, University of Santiago de Compostela, Avda. de Barcelona s/n 15782, Santiago de Compostela, Spain,

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4.2 Use and dissemination of foreground

Priority was conceived as a project focused mainly on the acquisition of knowledge. It was our contention, from the outset, that only by better understanding the basic biology of prions, how they propagate, where, what are their mechanisms of toxicity, how the spread among tissues and animals, what is their persistence in the soil and wastewater, how they can be better detected and eliminated...we would set the foundations to better fight them and thus protect our food. In consequence, the most important outputs of **Priority** consist of a list of more than 100 scientific publications, with some more still in the print, under review or in preparation, to be published within the next months. These constitute the most valuable foreground generated by **Priority**, and have been made public and shared with the scientific community.

Section A (public)

TEMPLATE A1: LIST OF SCIENTIFIC (PEER REVIEWED) PUBLICATIONS, STARTING WITH THE MOST IMPORTANT ONES										
NO.	Title	Main author	Title of the periodical or the series	Number, date or frequency	Publisher	Place of publication	Year of publication	Relevant pages	Permanent identifiers[2] (if available)	Is/Will open access[3] provided to this publication?
1	Regulation of $GABA_A$ and Glutamate Receptor Expression, Synaptic Facilitation and Long-Term Potentiation in the Hippocampus of Prion Mutant Mice.	Rangel et al.	PLoS One	4(10)	PLOS	San Francisco	2009	4(10): e7592.		Yes
2	NMR structure of the human prion protein with the pathological Q212P mutation reveals unique structural features.	Ilc et al.	PLoS One	5(7)	PLOS	San Francisco	2010	5(7):e11715		Yes
3	Characterization of the role of dendritic cells in prion transfer to primary neurons	Langevin et al.	Biochem. J	431	Portlad Press	London	2010	189-98.		No
4	Structural facets of disease-linked human prion protein mutants: a molecular dynamic study	Rossetti et al.	Proteins	78	John Wiley	Hoboken, NJ	2010	3270-80		No
5	Combining independent drug classes into superior, synergistically acting hybrid molecules.	Müller-Schiffmann et al	Angew. Chem. Int. Ed.	49	John Wiley	Hoboken, NJ	2010	8743-46.		Yes
6	Prion infection of differentiated neurospheres	Hervá et al.	J. Neurosci. Methods	188	Elsevier	Amsterdam	2010	270-275		No
7	Toward the Molecular Basis of Inherited Prion Diseases: NMR Structure of the Human Prion Protein with V210I Mutation	Biljan et al.	J. Mol. Biol	412	Elsevier	Amsterdam	2011	660–673		Yes

[2] A permanent identifier should be a persistent link to the published version full text if open access or abstract if article is pay per view) or to the final manuscript accepted for publication (link to article in repository).

[3] Open Access is defined as free of charge access for anyone via Internet. Please answer "yes" if the open access to the publication is already established and also if the embargo period for open access is not yet over but you intend to establish open access afterwards.

8	Common Structural Traits across Pathogenic Mutants of the Human Prion Protein and Their Implications for Familial Prion Diseases	Rosetti et al.	J. Mol Biol	411	Elsevier	Amsterdam	2011	700-712		Yes
9	Probing structural differences between PrPC and PrPSc by surface nitration and acetylation: evidence of conformational change in the C-terminus	Gong et al.	Biochemistry	50	American Chemical Society	Washington DC	2011	4963-72		No
10	Aerosols transmit prions to immunocompetent and immunodeficient mice	Haybaeck et al.	PLoS Pathog	Jan 13 2011	PLOS	San Francisco	2011	7(1):e1001257		Yes
11	Prion disease blood test using immunoprecipitation and improved quaking-induced conversion. 2011,	Orrú et al.	MBio.	2(3)	American Soc. for Microbiology	Washington DC	2011	2(3). pii: e00078-11.		No
12	Biological Effects and Use of PrPSc- and PrP-Specific Antibodies Generated by Immunization with Purified Full-Length Native Mouse Prions.	Petsch et al.	J. Virol.	85	American Soc. for Microbiology	Washington DC	2011,	4538–4546.		No
13	Quinpramine ameliorates rat experimental autoimmune neuritis and redistributes MHC class II molecules..	Meyer zu Hörste et al.	PLoS One,	6	PLOS	San Francisco	2011	6:e21223		Yes
14	Prion uptake in the gut: identification of the first uptake and replication sites...	Kujala et al.	Plos Pathog	7	PLOS	San Francisco	2011	7(12): e1002449		Yes
15	Biological Effects and Use of PrPSc- and PrP-Specific Antibodies Generated by Immunization with Purified Full-Length Native Mouse Prions. 85:	Petsch et al.	J. Virol.	85	American Soc. for Microbiology	Washington DC	2011	4538–4546.		No
16	Neuroprotective role of PrPC against kainate-induced epileptic seizures and cell death depends on the modulation of JNK3 activation by GluR6/7–PSD-95 binding.	Carulla et al.	Mol. Biol. Cell	22	Am. Soc. for Cell Biol	Bethesda	2011	3041–3054		No
18	Atypical/Nor98 scrapie infectivity in sheep peripheral tissues.	Andreoletti et al.	PLoS Pathog	7	PLOS	San Francisco	2011	7: e1001285.		Yes
19	Structural Organization of Mammalian Prions as Probed by Limited Proteolysis.	Vázquez-Fernández, E. et al.	PLoS One,	7	PLOS	San Francisco	2012	7(11): e50111.		Yes
20	PK-sensitive PrPSc Is Infectious and Shares Basic Structural Features with PK-resistant PrPSc.	Sajnani et al.	PLoS Pathog.	8	PLOS	San Francisco	2012	8(3): e1002547.		Yes

21	Multifaceted roles of tunneling nanotubes in intercellular communication.	Marzo et al.	Front Physiol.	3	Frontiers	Lausanne	2012	72		Yes
22	Prionemia and leukocyte-platelet-associated infectivity in sheep transmissible spongiform encephalopathy models. 2012,	Lacroux et al.	J. Virol.	86	American Soc. for Microbiology	Washington DC	2012	2056–2066.		No
23	Role of the cellular prion protein in oligodendrocyte precursor cell proliferation and differentiation in the developing and adult mouse CNS.	Bribián et al.	PLoS One	7	PLOS	San Francisco	2012	,7(4): e33872.		Yes
24	Persistence of the bovine spongiform encephalopathy infectious agent in sewage.	Maluquer de Motes et al.	Environ. Res	117	Elsevier	Amsterdam	2012	1-7		No
25	Prion Pathogenesis Is Faithfully Reproduced in Cerebellar Organotypic Slice Cultures.	Falsig et al.	PLoS Pathog	8	PLOS	San Francisco	2012	8(11): e1002985		Yes
26	Prion propagation, toxicity and degradation.	Aguzzi & Falsig	Nat. Neurosci.	15	Nature Publishing Group	London	2012	936-939.		No
27	Five Questions on Prion Diseases..	Aguzzi & Zhu.	PLoS Pathog	8	PLOS	San Francisco	2012	8(5): e1002651		Yes
28	Determining the role of mononuclear phagocytes in prion neuroinvasion from the skin.	Wathne et al.	J. Leukoc. Biol.	91	Society for Leukocyte Biology	Bethesda	2012	817-28.		No
29	The Effects of Host Age on the Transport of Complement-Bound Complexes to the Spleen and the Pathogenesis of Intravenous Scrapie Infection.	Brown et al.	J. Virol.	86	American Soc. for Microbiology	Washington DC	2012	25–35		No
30	PK-sensitive PrPSc Is Infectious and Shares Basic Structural Features with PK-resistant PrPSc.	Sajnani et al.	PLoS Pathog.	8	PLOS	San Francisco	2012	8(3): e1002547		Yes
31	Sporadic Creutzfeldt–Jakob disease subtype-specific alterations of the brain proteome: Impact on Rab3a recycling.	Gawinecka et al.	Proteomics.	12	John Wiley	Hoboken, NJ	2012	3610-20.		No
32	Impact of Leucocyte Depletion and Prion Reduction Filters on TSE Blood Borne Transmission.	Lacroux et al.	PLoS One	7	PLOS	San Francisco	2012	7(7): e42019.		Yes
33	Highly Efficient Prion Transmission by Blood Transfusion.	Andreoletti et al.	PLoS Pathog.	June; 8	PLOS	San Francisco	2012	8(6): e1002782.		Yes
34	Structural Rearrangements at Physiological pH: Nuclear Magnetic Resonance Insights from the V210I	Biljan et al.	Biochemistry	51	American Chemical Society	Washington DC	2012	7465−7474.		No

	Human Prion Protein Mutant.									
35	Structural basis for the protective effect of the human prion protein carrying the dominant-negative E219K polymorphism.	Biljan et al.	Biochem. J.	446	Portlad Press	London	2012	243–251.		No
36	Report about four novel mutations in the prion protein gene. 2013,	Schelzke et al.	Dement Geriatr Cogn Disord.	35(3-4):	Karger	Basel	2013	229-37.		No
	Subtype-specific synaptic proteome alterations in sporadic Creutzfeldt-Jakob disease.	Gawinecka et al	J Alzheimers Dis.	37	IOS Press	Amsterdam	2013	51-61		Yes
38	Association of prion protein genotype and scrapie prion protein type with cellular prion protein charge isoform profiles in cerebrospinal fluid of humans with sporadic or familial prion diseases.	Schmitz et al.	Neurobiol Aging	35	Elsevier	Amsterdam	2014	1177-88		No
39	Impact of the cellular prion protein on amyloid-β and 3PO-tau processing.	Schmitz et al.	J Alzheimers Dis.	38	IOS Press	Amsterdam	2014	551-65		Yes
40	Characteristic CSF prion seeding efficiency in humans with prion diseases. 2014,	Cramm et al.	Mol Neurobiol.	May 9 **epub ahead of print**	Springer	Berlin	2014			No
41	Anchorless 23-230 PrPC Interactomics for Elucidation of PrPC Protective Role.	Zafar et al..	Mol Neurobiol.	Jan 5 **e-pub ahead of print.**	Springer	Berlin	2014			No
42	Detection of infectivity in blood of persons with variant and sporadic Creutzfeldt-Jakob disease.	Douet et al.	Emerg Infect Dis.	20			2014	114-7		No
43	Glycogen synthase kinase 3 beta (GSK3beta) at the tip of neuronal development and regeneration.	Seira and Del Rio.	Mol Neurobiol.	49	Springer	Berlin	2014	931-44		No
44	Gene expression resulting from PrP(C) ablation and PrP (C) overexpression in murine and cellular models..	Llorens et al	Mol Neurobiol.	49	Springer	Berlin	2014	413-23		No
45	Neurotoxicity of prion peptides mimicking the central domain of the cellular prion protein.	Vilches, S., et al.	PLoS One,	8(8)	PLOS	San Francisco	2013	8(8): e70881.		Yes
46	Cellular prion protein modulates beta-amyloid deposition in aged APP/PS1 transgenic mice.	Ordonez-Gutierrez et al.	Neurobiol Aging,	34	Elsevier	Amsterdam	2013	2793-804.		No
47	Microarray and deep sequencing cross-platform analysis of the mirRNome and isomiR variation in response to epidermal growth factor.	Llorens et al.	BMC Genomics,	14	BioMed Central	Berlin	2013	371-5		Yes

48	PrP(C) regulates epidermal growth factor receptor function and cell shape dynamics in Neuro2a cells..	Llorens et al	J Neurochem,	127	John Wiley	Hoboken, NJ	2013	124-138		No
49	A highly expressed miR-101 isomiR is a functional silencing small RNA. .	Llorens et al.	BMC Genomics,	14	Biomed Central	Berlin	2013	104-107		Yes
50	Prion formation correlates with activation of translation-regulating protein 4E-BP and neuronal transcription factor Elk1.	Allard et al.	Neurobiol. Dis.	58C	Elsevier	Amsterdam	2013	116-122		No
51	Prion strings and networks on the surface of infected cells.	Rouvinski et al.	J Cell Biol.	204	Rockefeller Univ. Press	New York	2013	423-41		No
52	Description of a novel viral tool to identify and quantify ovine faecal pollution in the environment.	Rusiñol et al.	Sci Total Environ.	1	Elsevier	Amsterdam	2013	458-460		No
53	De novo prions.	Benetti et al	Biol Rep.	2	Springer	Berlin	2010	46-50		No
54	Neurodevelopmental expression and localization of the cellular prion protein in the central nervous system of the mouse.	Benvegnù et al.	J Comp Neurol	518	John Wiley	Hoboken NJ	2010	1879-91.		No
55	NMR structure of the human prion protein with the pathological Q212P mutation reveals unique structural features.	Ilc et al.	PLoS One.	5(7)	PLOS	San Francisco	2010	5(7):e11715		Yes
56	56. A system-level approach for deciphering the transcriptional response to prion infection.	Zampieri et al.	Bioinformatics.	27	Oxfor Journals	Oxford	2011	3407-14.		No
57	Early structural features in mammalian prion conformation conversion.	Legname	Prion	6	Landes	Austin	2012	37-39		Yes
58	Dominant-negative effects in prion diseases: insights from molecular dynamics simulations on mouse prion protein chimeras. 2012,	Cong et al.	J Biomol Struct Dyn.	31(8)	Adenine Press	Shenectady NJ	2013	829-40		No
59	In vitro aggregation assays for the characterization of α-synuclein prion-like properties. Prion. 2014, 8(1).	Narkiewicz et al.	Prion	8(1)	Landes	Austin	2014	34-56		Yes
60	Probing early misfolding events in prion protein mutants by NMR spectroscopy.	Giachin et al.	Molecules	18	MDPI	Basel	2013	9451-76.		Yes
61	Prion protein accumulation in lipid rafts of mouse aging brain.	Agostini et al.	PLoS One.	8	PLOS	San Francisco	2013	8:e74244.		Yes
62	SAXS structural study of PrPSc	Amenitsch	Prion	7(6)	Landes	Austin	2013	496-500		Yes

	reveals ~11 nm diameter of basic double intertwined fibers. Prion.	et al.								
63	Accelerated clinical course of prion disease in mice compromised in repair of oxidative DNA damage.	Jalland et al.	Free Radic Biol Med.	68	Elsevier	Amsterdam	2014	1-7		No
64	Golgi sorting regulates organization and activity of GPI proteins at apical membranes.	Paladino et al.	Nature Chemical Biology,	10	Nature Publishing Group	London	2014	350-7		No
65	Exploring the role of lipids in intercellular conduits: breakthroughs in the pipeline.	Delage & Zurzolo	Frontiers in Plant Science	Dec 10, 4	Frontiers Media	Lausanne	2013	504		No
66	The Fate of PrP GPI-Anchor Signal Peptide is Modulated by P238S Pathogenic Mutation.	Guizzunti & Zurzolo.	Traffic	15	John Wiley	Hoboken NJ	2013	78-93		No
67	Not on the menu: autophagy-independent clearance of prions..	Browman & Zurzolo	Prion	7	Landes	Austin	2013	286-90		Yes
68	Myo10 is a key regulator of TNT formation in neuronal cells.	Gousset et al.	Journal of Cell Science,	126(Pt 19):	Cambridge Univ. Press	Cambridge	2013	4424-35.		No
69	Pathogenic prions deviate PrP(C) signaling in neuronal cells and impair A-beta clearance.	Pradines et al.	Cell Death and Disease.	4	Nature Publishing Group	London	2013	4:e456 (2013).		No
70	Spontaneous Generation of Infectious Prion Disease in Transgenic Mice.	Torres et al	Emerging Infectious Diseases,	19	CDC	Atlanta	2013	1938-47.		Yes
71	Bovine spongiform encephalopathy induces misfolding of alleged prion-resistant species cellular prion protein without altering its pathobiological features.	Vidal et al.	J Neurosci.	33	Stanford University Highwire Press	Stanford	2013	7778-86.		Yes
72	Cellular prion protein modulates β-amyloid deposition in aged APP/PS1 transgenic mice	Ordóñez-Gutiérrez et al.	Neurobiology of Aging	34	Elsevier	Amsterdam	2013	2793-804.		No
73	PrP(C) regulates epidermal growth factor receptor function and cell shape dynamics in Neuro2a cells..	Llorens et al.	J Neurochem	127	John Wiley	Hoboken NJ	2013	124-38		No
74	Detection of infectivity in the blood of vCJD and sCJD affected patients..	Douet et al	Emerging Infectious Diseases,	20	CDC	Atlanta	2014	114-7		Yes
75	A template for new drugs against Alzheimer's Disease.	Aguzzi et al	Cell	154	Cell Press/Elsevier	Amsterdam	2013	1182-1184		No
76	The immunobiology of prion diseases.	Aguzzi et al.	Nature Reviews. Immunology,	13	Nature Publishing Group	London	2013	888-902.		No
77	Mutations in the gene encoding PDGF-B cause brain calcifications in humans and mice.	Keller et al.	Nature Genetics	45	Nature Publishing Group	London	2013	1077-1082		No

78	SIRPα polymorphisms, but not the prion protein, control phagocytosis of apoptotic cells.	Nuvolone et al.	Journal of Experimental Medicine	210			2013	2539-2552.		No
79	The toxicity of antiprion antibodies is mediated by the flexible tail of the prion protein.	Sonati et al.	Nature	501	Nature Publishing Group	London	2013	102-106.		No
80	Evidence of sub-clinical prion disease in aged mice following exposure to bovine spongiform encephalopathy.	Brown & Mabbott.	Journal of General Virology	95	Society for General Microbiology		2014	231-243		No
81	Aging and the mucosal immune system in the intestine	. Mabbott et al.	Biogerontology,	April 5 (epub ahead of print)	Springer	Berlin	2014			No
82	Aging-induced proteostatic changes in the rat hippocampus identify ARP3, NEB2 and BRAG2 as a molecular circuitry for cognitive impairment	Ottis et al.	. PLoS ONE	8	PLOS	San Francisco	2013	8: e75112.		Yes
83	Characterization of a single-chain variable fragment recognizing a linear epitope of Abeta: a biotechnological tool for studies on Alzheimer's disease?	Dornieden et al.	PLoS ONE,	8(3)	PLOS	San Francisco	2013	8(3):e59820.		Yes
84	Hybrid molecules synergistically acting against protein aggregation diseases...	Korth et al	Curr Top Med Chem	13	Bentham Science	Sharjah	2013	2484-90.		No
85	Aggregated proteins in chronic mental diseases: DISC1opathies.	Korth	Prion	6	Landes	Austin	2012	1-8		Yes
86	Human and rat brain lipofuscin proteome.	Ottis et al.	Proteomics	15-16	John Wiley	Hoboken, NJ	2012	2445-54.		No
87	C-terminal fragment of N-cadherin accelerates synapse destabilization by β-amyloid.	Andreyeva et al.	Brain	135	Oxford Journals	Oxford	2012	2140-54.		No
88	Cellular prion protein participates in amyloid-beta transcytosis across the blood-brain barrier.	Pflanzner et al.	Blood Flow and Metabolism	32	Nature Publishing Group	London	2012	628-33.		No
89	Biological effects and use of antibodies generated by immunizing with full length native prions.	Petsch et al.	Journal of Virology	85	Am. Soc. Microbiology	Washington DC	2011	4538-46		No
90	Quinpramine ameliorates rat experimental autoimmune neuritis and redistributes MHC class II molecules.	Meyer zu Hörste et al.	PLoS ONE,	6(6)	PLOS	San Francisco	2011	6(6):e21223.		Yes
91	Genetic polymorphism at codon 129 of the prion protein gene is not associated with primary	Stüve et al.	Archives of Neurology,	68	American medical Assoc		2011	264-265.		No

	progressive multiple sclerosis.									
92	Presenilin-1 (PSEN1) but not amyloid precursor protein (APP) mutations present in mouse modles of Alzheimer's disease attenuate the response to -secretase modulators (GSMs) regardless of their potency and structure	. Hahn et al.	Journal of Neurochemistry	116	John Wiley	Hoboken NJ	2011	385-95.		No
93	Combining independent drug classes into superior, synergistically acting hybrid molecules.	Müller-Schiffmann et al.	Angewandte Chemie International Edition,	49	John Wiley	Hoboken NJ	2010	8743-46		No
94	Pharmacological PrP-Silencing Accelerates CNS Autoimmune Disease via TCR Signaling.	Hu et al	Brain	133	Oxford Journals	Oxford	2010	375-388.		No
95	Oral treatment with the amyloid-b oligomer precipitating substance D3 improves pathology of Alzheimer's transgenic mice.	Funke et al.	ACS Chemical Neuroscience	1	American Chemical Society	Washington DC	2010	639-48.		No
96	Structure-activity relationship of tocopherol derivatives suggesting a novel non-antioxidant mechanism in antiprion potency.	Muyrers et al	Neuroscience Letters,	469	Elsevier	Amsterdam	2010	122-6		No
97	Complementarity determining regions of a funtional anti-PrP scFv orchestrate conformation-specificity and antiprion activity.	Muller-Schiffmann et	Molecular Immunology	46	Elsevier	Amsterdam	2009	532-40.		No
98	Hybrid compounds - from simple combinations to nanomachines.	Müller-Schiffmann et al.	BioDrugs	26	Springer	Berlin	2012	21-31		No
99	Rapidly progressive Alzheimer's disease.	Schmidt et al	Archives of Neurology	68	American Medical Assoc.		2011	1124-30.		No
100	Quinpramine - a promising compound for treating immune-mediated demyelination of the nervous system	Meyer zur Hörste et al.	Drug News & Perspectives	23	Thomson Reuters	web	2010	287-94		Yes
101	Prions, proteinase K and infectivity.	Sajnani & Requena	Prion	6	Landes	Austin	2012	430-2		Yes
102	Oxidation of methionine 216 in sheep and elk prion protein is highly dependent upon the amino acid at position 218 but is not important for prion propagation.	Silva et al.	Biochemistry	52	American Chemical Society	Washington DC	2013	2139-47.		No
103	The structure of the infectious prion protein: Experimental data and molecular models	Requena & Wille.	Prion	Jan 1 (8)	Landes	Austin	2014	60-6		Yes

TEMPLATE A2: LIST OF DISSEMINATION ACTIVITIES

NO.	Type of activities[4]	Main leader	Title	Date/Period	Place	Type of audience[5]	Size of audience	Countries addressed
1	Scientific meeting	*All*	NeuroPrion 2010	8-10 Sept 2010	*Salzburg*	*Scientists, stakeholders*	*750*	worldwide
2	Scientific meeting	*All*	NeuroPrion 2011	May 16-19	*Montreal*	*Scientists, stakeholders*	*500*	worldwide
3	Scientific meeting	*All*	NeuroPrion 2012	May 9-12 2012	*Amsterdam*	*Scientists, stakeholders*	*500*	worldwide
4	Scientific meeting	*All*	NeuroPrion 2013	May 26-29 2013	*Banff*	*Scientists, stakeholders*	*500*	worldwide
5	Scientific meeting	*All*	NeuroPrion 2014	May 27-30 2014	*Trieste*	*Scientists, stakeholders*	*500*	worldwide
5	*Radio program (* Swedish Radio P3)	*KI*	”Prions - the evil proteins”	Nov 3-5, 2012, and May 4, 2013	*Stockholm*	*Young listeners*		Sweden
7	Daily Newspaper piece. http://www.dailymedi.com	KI	reports of the Conference CJD Surveillance System in Asia	Feb 13, 2012	Seoul	General public		South Korea
8	Conferences within the Universities of Braga, Coimbra and Lisbon joint PhD program. PhDESC, Retreat	KI	Microbes and Brain Dysfunctions	June 1-2, 2012	Vila de Ancora	Graduate students		Portugal

[4] A drop down list allows choosing the dissemination activity: publications, conferences, workshops, web, press releases, flyers, articles published in the popular press, videos, media briefings, presentations, exhibitions, thesis, interviews, films, TV clips, posters, Other.

[5] A drop down list allows choosing the type of public: Scientific Community (higher education, Research), Industry, Civil Society, Policy makers, Medias, Other ('multiple choices' is possible).

9	Lecture	KI	Prions and Neurodegenerative Diseases	May 29, 2014	Izmir	undergraduate, graduate and postgraduate students in Biomedicine and Biotechnology.	350	Turkey
10	Written report to the the Science and Technology Committee of the UK Parliament (Enquiry on blood and tissue safety following the sCJD crisis)	PRIONICS	Efforts in developing a vCJD blood test	Feb 2014	London	UK Parliament Members		UK
11	Oral report to the Science and Technology Committee of the House of Commons (Enquiry on blood and tissue safety following the sCJD crisis)	PRIONICS	Efforts in developing a vCJD blood test	5 March 2014	London	UK Parliament Members		UK
12	Lecture	UMG-GOE	Differentialdiagnose dementieller Erkrankungen: Stellenwert der Liquoranalytik. 6. Dresdner Liquorsymposium Dorint Hotel Dresden.	17 May 2014	Dresden	Scientists and clinicians		Germany
13	Lecture	UMG-GOE	Prion and Prion-like Diseases. Indo-German Workshop in Trivandrum, 30.11-1.12.2013	30, Nov. 2013	Trivandrum	Scientists, students		India
14	Lectures: International conference, Center of Extrapyramidal Disorders. Russian Society of Neurologists and Russian Medical Academy of Postgraduate Education	UMG-GOE	Creutzfeldt-Jakob Krankheit. Extrapyramidal disorders- Yesterday Today Tomorrow.	24-26 October 2013	Moscow	Graduate students, clinicians, scientists		Russia
15	Lecture, The Australian and New Zealand Neuropsychiatry and Behavioural Neurology Conference 2012, Melbourne Brain Centre, The University of Melbourne, Melbourne, Australia	UMG-GOE	The differential diagnosis in rapid progressive dementia.	19 October 2012	Melbourne	Scientists, clinicians		Australia
16	Lecture: Workshop Klinische Immunologie, Neurologische Klinik, Universitätsklinikum Freiburg, 13.-15. Oktober 2011	UMG-GOE	Prionerkrankungen-Wege zur Diagnose und Therapie	October 14, 2011	Freiburg	Clinicians		Germany
17	Nespeaper piece "La Voz de Galicia"	USC	Reunión de investigadores sobre los priones en Santiago	Dec 4, 2012	Santiago de Compostela	General public	500.000	Spain
18	Media coverage: http://www.bbsrc.ac.uk/news/health/2013/131204-n-prions-vcjd-and-immune-relay.aspx	UEDIN	Media interest in the Brown et al study (BBSRC)	2013	London	General public/scientists		UK
19	Radio programme "Efervesciencia"	USC	"Prions"	8/04/13	Santiago de Compostela	General public	2 million	Spain
20	Lecture: MJFF/Kinetics Workshop: Is Parkinson’s Disease a Prion-like Disorder? Palo Alto, CA	NKI	Prion Transfer within	September	Palo Alto	Scientists,		USA

	Presenter: Susan Godsave		the Hippocampus	12, 2012		clinicians		
21	Poster:, 10th Dutch EndoNeuroPsycho Meeting, Lunteren	NKI	Insights into prion replication and pathology from cryo-immunogold EM studies of mouse hippocampus	May 29-31, 2012	Lunteren	Scientists, clinicians, students		The Netherlands
22	Poster:, 8th FENS forum of neuroscience, Barcelona	NKI	Insights into prion replication and pathology from cryo-immunogold EM studies of mouse hippocampus	July 10-14, 2012	Barcelona	scientists		Spain
23	Meeting with patients´ families, activity during the II Spanish Prion Meeting	USC	Meeting with families of patients with genetic CJD	Dec 4, 2011	Madrid	Patients´ families		Spain
24	Lecture by Juan Maria Torres.. JORNADAS CISA-INIA/MAGRAMA/LCV. . Place: Salón de Actos CISA-INIA:	CISA	Cepas de priones en especies ganaderas: detección, transmisión, potencial zoonótico y factores determinantes de susceptibilidad/resistencia	03-03-2014-	Valdeolmos , Madrid.	Animal health technicians, veterinarians, surveillance workers, students		Spain
25	Several lectures, posters, participation in the organization, Ist Spanish Prion Meeting	CISA, USC, UB	Different aspects of prion biology	25-26/11/2010	Bilbao	Scientists, students, Animal health technicians, veterinarians, surveillance workers, clinicians, families	80	Spain
26	Several lectures, posters, participation in the organization, 2nd Spanish Prion Meeting	CISA, USC, UB	Different aspects of prion biology	01-02/12/2011	Madrid	Scientists, students, Animal health technicians, veterinarians, surveillance workers, clinicians, families	80	Spain
28	Organization, Ist Iberian Prion Meeting	CISA, USC, UB	Different aspects of prion biology	3-4 Dec 2012	Santiago de Compostela	Scientists, students, Animal health technicians, veterinarians, surveillance workers, clinicians, families	60	Spain/Portugal with participation of other European countries

29	Several lectures, posters, assistance in the organization, 2nd Iberian Prion Meeting	CISA, USC, UB	Different aspects of prion biology	2-3 Dec 2013	Faro	Scientists, students, Animal health technicians, veterinarians, surveillance workers, clinicians, families		Portugal/Spain with participation of other countries
30	Article: Investigación y Ciencia (Spanish edition of Scientific American) http://www.investigacionyciencia.es/noticias/el-lado-bueno-del-prion-10944	SISSA	EL LADO BUENO DEL PRION	Tuesday, 5 March, 2013	Barcelona	General public		Spain, Spanish speaking countries
31	Article: Il Piccolo	SISSA	LA FACCIA BUONA DEL PRIONE, PER FAR CRESCERE IL NOSTRO CERVELLO	Tuesday, 19 February, 2013	Trieste	General public		Italy
32	TV interview GIUSEPPE LEGNAME Telequattro https://www.youtube.com/watch?v=b8EuLTB7iqY	SISSA	Intrivista a Giuseppe Legname	Wednesday, 5 February, 2014	Trieste	General public		Italy
33	Article: Real Clear Science http://www.realclearscience.com/2014/01/28/prions_origin_of_039evil039_conformation_pdf_257254.html	SISSA	PRIONS: ORIGIN OF 'EVIL' CONFORMATION	Tuesday, 28 January, 2014	Web-based	General public		world
34	The Roslin Institute has an active programme of public engagement with research, supported by a dedicated Public Engagement Officer. This includes an annual public Open Day attracting 400-500 visitors and offering direct engagement between researchers and members of the public, as well work with local schools and participation in science festivals and other external events. we designed and delivered activities at the Institute's Open Day based on data generated from PRIORITY	UEDIN		September 2013 and October 2014	Edinburgh	General public	400-500	UK
35	Seminars: Byron Calgua (2011 y 2012) and Rosina Gironés (2010, 2013) Seminar: "Prions as infectious agents and as environmental contaminants" in the Master Program: Advanced Microbiology, in the course: Sanitary Virology, Department of Microbiology, University of Barcelona (2010-2013)	UB	"Prions as infectious agents and as environmental contaminants"	2010, 2011, 2012, 2013	Barcelona	Graduate students		Spain
36	Lecture: Adriano Aguzzi at ZURICH.MINDS 2013: Fatal Attraction Between Proteins Youtube, channel ZURICH.MINDS https://www.youtube.com/watch?v=bFe3L48WgQQ	UZH	Fatal Attraction Between Proteins	2013	Zurich	Students/general public		Switzerland/ web
37	Lecture: Adriano Aguzzi: Youtube, channel cellvideoabstracts https://www.youtube.com/watch?v=ahBQqRHdI70	UZH	Origin of Follicular Dendritic Cells		web	Students, scientists		web
38	Lecture: Adriano Aguzzi: Youtube, channel uzhch https://www.youtube.com/watch?v=ujqrFt2qbFw	UZH	The flexible tail of the prion protein poisons brain cells		web	Students, general public		web
39	Lecture: Associazione Alzheimer onlus website	UZH	Coda flessibile dei prioni avvelena le		web	General public		Web (Italian speaking

	http://www.alzheimer-riese.it/index.php?option=com_content&view=article&id=3037:coda-flessibile-dei-prioni-avvelena-le-cellule-cerebrali&catid=36:ricerche&Itemid=233		cellule cerebrali					countries)
40	Newspaper article, Neue Zürcher Zeitung http://www.nzz.ch/wissen/wissenschaft/wie-der-protein-schwanz-die-bildung-von-zellgiften-ausloest-1.18126161	UZH	Wie der Protein-Schwanz die Bildung von Zellgiften auslöst (by Stephanie Lahrtz)	31/07/2013	Zurich	General public		Switzerland
41	Press article: Le Temps http://www.letemps.ch/Page/Uuid/c80cb752-fac4-11e2-94ac-7e2bb1c97fd0/Maladies_%C3%A0_prions_la_queue_flexible_est_coupable	UZH	Maladies à prions: la queue flexible est coupable	August 1, 2013	Geneva	General public		Switzerland
42	TV/radio interviews: SRF http://www.srf.ch/player/tv/news-clip/video/adriano-aguzzi-so-haben-wir-die-maeuse-infiziert-?id=a405e94d-170f-4692-92a9-48971ea637a7	UZH	Adriano Aguzzi: So haben wir die Mäuse infiziert.	13/01/2011	Zurich	General public		Switzerland
43	Radio interview: SRF 4 News aktuell http://www.srf.ch/player/radio/srf-4-news-aktuell/audio/professor-adriano-aguzzi-im-gespraech?id=9651e2a2-b76d-4486-9698-a5c08b3a9dcd	UZH	Professor Adriano Aguzzi im Gespräch	26.03.2014	Zurizh	General public		Switzerland

Section B (Confidential[6] or public: confidential information to be marked clearly)
Part B1

TEMPLATE B1: LIST OF APPLICATIONS FOR PATENTS, TRADEMARKS, REGISTERED DESIGNS, ETC.					
Type of IP Rights[7]:	**Confidential** Click on YES/NO	Foreseen embargo date dd/mm/yyyy	Application reference(s) (e.g. EP123456)	Subject or title of application	Applicant (s) (as on the application)
Patent	**Yes**	n.a.	WO2012/099884A1	Methods for Amplification and Detection of Prions	Orru, C., Caughey, B., Kuhn, F., Schroeder, B. and Raeber, A.

[6] Note to be confused with the "EU CONFIDENTIAL" classification for some security research projects.

[7] A drop down list allows choosing the type of IP rights: Patents, Trademarks, Registered designs, Utility models, Others.

Part B2
Please complete the table hereafter:

Type of Exploitable Foreground[8]	Description of exploitable foreground	Confidential Click on YES/NO	Foreseen embargo date dd/mm/yyyy	Exploitable product(s) or measure(s)	Sector(s) of application[9]	Timetable, commercial or any other use	Patents or other IPR exploitation (licences)	Owner & Other Beneficiary(s) involved
Device	*Glovebox + usability*	***YES***	*n.a.*	*Glovebox*	*1. Medical 2. Industrial inspection*	*n.a.*	*no*	*Maastricht University*
TEST	**TEST BASED ON AMPLIFICATION AND DETECTION OF PRIONS**	**YES**	N.A.	BLOOD PRION TEST	MEDICAL, AGRICULTURAL	N.A.	APPLICATION: WO2012/099884A1	PRIONICS, NIH.

[19] A drop down list allows choosing the type of foreground: General advancement of knowledge, Commercial exploitation of R&D results, Exploitation of R&D results via standards, exploitation of results through EU policies, exploitation of results through (social) innovation.
[9] A drop down list allows choosing the type sector (NACE nomenclature) : http://ec.europa.eu/competition/mergers/cases/index/nace_all.html

Foreground obtained throughout WP7 of the Priority grant will be used towards the development and marketing of a blood test for vCJD. In collaboration with blood transfusion centers in the UK and France, prototype blood tests based on the eQUiC technology will be validated and submitted to regulatory authorities for review in order to obtain marketing authorization. Manufacturing and distribution of kits and technology for blood testing for vCJD will be either done in-house or out-licensed to third parties.

To handle electron microscopy samples into a Vitrobot without disturbing the environment, a **Glovebox** (Fig. 11) was developed. In the **Glovebox**, electron microscopy samples can be prepared in a temperature and humidity controlled environment. After the sample is ready for vitrification, the **Glovebox** can be positioned under the Vitrobot and vitrification can take place. The sample will be transported under the necessary right conditions into the Vitrobot.

Especially the new M4I devision of Maastricht University (Maastricht Multimodal Molecular Imaging Institute) will use the **Glovebox** for nanoscopic research and mass spectrometry.

The **Glovebox** has been tested by several users. During the tests future developments concerning the **Glovebox** were born. One of the improvements is a special application whereby cells are cultured in a unique gas environment. The gas will be supplied and controlled in the **Glovebox**. Also gas mixtures will be possible and quantities of gas in time will be programmed. For this application the **Glovebox** has to be applied with mass flow controllers of several gases. Also the lab should be prepared for the use of gases.

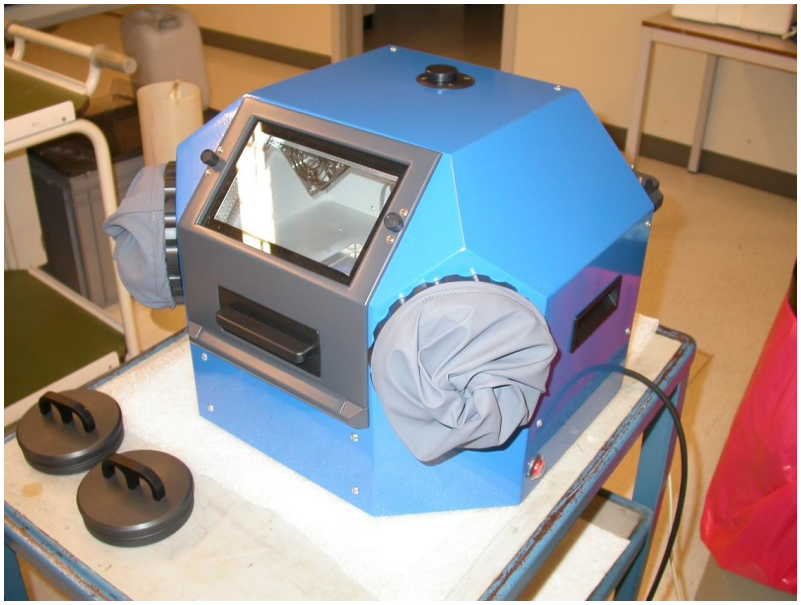

Fig. 11: Glovebox

4.3 Report on societal implications

Replies to the following questions will assist the Commission to obtain statistics and indicators on societal and socio-economic issues addressed by projects. The questions are arranged in a number of key themes. As well as producing certain statistics, the replies will also help identify those projects that have shown a real engagement with wider societal issues, and thereby identify interesting approaches to these issues and best practices. The replies for individual projects will not be made public.

A General Information *(completed automatically when **Grant Agreement number** is entered.*	
Grant Agreement Number:	222887
Title of Project:	Priority
Name and Title of Coordinator:	Jesús R. Requena, Ph.D.

B Ethics	
1. Did your project undergo an Ethics Review (and/or Screening)? • If Yes: have you described the progress of compliance with the relevant Ethics Review/Screening Requirements in the frame of the periodic/final project reports? Special Reminder: the progress of compliance with the Ethics Review/Screening Requirements should be described in the Period/Final Project Reports under the Section 3.2.2 *'Work Progress and Achievements'*	***Yes***
2. Please indicate whether your project involved any of the following issues (tick box) :	***YES***
RESEARCH ON HUMANS	
• Did the project involve children?	
• Did the project involve patients?	X
• Did the project involve persons not able to give consent?	
• Did the project involve adult healthy volunteers?	
• Did the project involve Human genetic material?	
• Did the project involve Human biological samples?	X
• Did the project involve Human data collection?	X
RESEARCH ON HUMAN EMBRYO/FOETUS	
• Did the project involve Human Embryos?	
• Did the project involve Human Foetal Tissue / Cells?	
• Did the project involve Human Embryonic Stem Cells (hESCs)?	
• Did the project on human Embryonic Stem Cells involve cells in culture?	
• Did the project on human Embryonic Stem Cells involve the derivation of cells from Embryos?	
PRIVACY	
• Did the project involve processing of genetic information or personal data (eg. health, sexual lifestyle, ethnicity, political opinion, religious or philosophical conviction)?	
• Did the project involve tracking the location or observation of people?	
RESEARCH ON ANIMALS	
• Did the project involve research on animals?	X
• Were those animals transgenic small laboratory animals?	X
• Were those animals transgenic farm animals?	
• Were those animals cloned farm animals?	

• Were those animals non-human primates?	
RESEARCH INVOLVING DEVELOPING COUNTRIES	
• Did the project involve the use of local resources (genetic, animal, plant etc)?	
• Was the project of benefit to local community (capacity building, access to healthcare, education etc)?	
DUAL USE	
• Research having direct military use	
• Research having the potential for terrorist abuse	

C Workforce Statistics

3. Workforce statistics for the project: Please indicate in the table below the number of people who worked on the project (on a headcount basis).

Type of Position	Number of Women	Number of Men
Scientific Coordinator	0	1
Work package leaders	3	10
Experienced researchers (i.e. PhD holders)	12	25
PhD Students	15	11
Other		

4. How many additional researchers (in companies and universities) were recruited specifically for this project?	**15**
Of which, indicate the number of men:	6

D Gender Aspects

5.	**Did you carry out specific Gender Equality Actions under the project**?	X ○	Yes No

6. Which of the following actions did you carry out and how effective were they?

		Not at all effective				Very effective
x	Design and implement an equal opportunity policy	○	○	○	○	X
x	Set targets to achieve a gender balance in the workforce	○	○	○	X	○
❑	Organise conferences and workshops on gender	○	○	○	○	○
❑	Actions to improve work-life balance	○	○	○	○	○
x	Other: Gender equality committee					

7. Was there a gender dimension associated with the research content – i.e. wherever people were the focus of the research as, for example, consumers, users, patients or in trials, was the issue of gender considered and addressed?

○ Yes- please specify

X No

E Synergies with Science Education

8. Did your project involve working with students and/or school pupils (e.g. open days, participation in science festivals and events, prizes/competitions or joint projects)?

○ Yes- please specify

x No

9. Did the project generate any science education material (e.g. kits, websites, explanatory booklets, DVDs)?

○ Yes- please specify

x No

F Interdisciplinarity

10. Which disciplines (see list below) are involved in your project?

X Main discipline[10]: **1.5**

X Associated discipline[10]: **3.3** ○ Associated discipline[10]: **4.2**

G Engaging with Civil society and policy makers

11a	**Did your project engage with societal actors beyond the research community?** *(if 'No', go to Question 14)*	x ○	Yes No

11b If yes, did you engage with citizens (citizens' panels / juries) or organised civil society (NGOs, patients' groups etc.)?

○ No

○ Yes- in determining what research should be performed

○ Yes - in implementing the research

X Yes, in communicating /disseminating / using the results of the project

[10] Insert number from list below (Frascati Manual).

11c In doing so, did your project involve actors whose role is mainly to organise the dialogue with citizens and organised civil society (e.g. professional mediator; communication company, science museums)?	○ X	Yes No

12. Did you engage with government / public bodies or policy makers (including international organisations)

○	No
○	Yes- in framing the research agenda
X	Yes - in implementing the research agenda
x	Yes, in communicating /disseminating / using the results of the project

13a Will the project generate outputs (expertise or scientific advice) which could be used by policy makers?

○	Yes – as a **primary** objective (please indicate areas below- multiple answers possible)
x	Yes – as a **secondary** objective (please indicate areas below - multiple answer possible)
○	No

13b If Yes, in which fields?

Agriculture	X	Energy		Human rights	
Audiovisual and Media		Enlargement		Information Society	
Budget		Enterprise		Institutional affairs	
Competition		**Environment**	x	Internal Market	
Consumers		External Relations		Justice, freedom and security	
Culture		External Trade		Public Health	x
Customs		Fisheries and Maritime Affairs	x	Regional Policy	
Development Economic and Monetary Affairs		**Food Safety**		Research and Innovation	
		Foreign and Security Policy		Space	
Education, Training, Youth		Fraud		Taxation	
Employment and Social Affairs		Humanitarian aid		Transport	

13c If Yes, at which level?

- ○ Local / regional levels
- ○ National level
- x European level
- ○ International level

H Use and dissemination

14. How many Articles were published/accepted for publication in peer-reviewed journals?	**103 (more pending)**
To how many of these is open access[11] provided?	**36**
How many of these are published in open access journals?	**36**
How many of these are published in open repositories?	
To how many of these is open access not provided?	
Please check all applicable reasons for not providing open access:	
❑ publisher's licensing agreement would not permit publishing in a repository ❑ no suitable repository available x no suitable open access journal available ❑ no funds available to publish in an open access journal ❑ lack of time and resources ❑ lack of information on open access ❑ other[12]: ……	

15. How many new patent applications ('priority filings') have been made? *("Technologically unique": multiple applications for the same invention in different jurisdictions should be counted as just one application of grant).*		**1**
16. Indicate how many of the following Intellectual Property Rights were applied for (give number in each box).	Trademark	
	Registered design	
	Other	
17. How many spin-off companies were created / are planned as a direct result of the project?		**0**
Indicate the approximate number of additional jobs in these companies:		

18. Please indicate whether your project has a potential impact on employment, in comparison with the situation before your project:

❑	Increase in employment, or	❑	In small & medium-sized enterprises
❑	Safeguard employment, or	❑	In large companies
❑	Decrease in employment,	x	None of the above / not relevant to the project
❑	Difficult to estimate / not possible to quantify		

19. For your project partnership please estimate the employment effect resulting directly from your participation in Full Time Equivalent (*FTE = one person working fulltime for a year*) jobs:	*Indicate figure:*

[11] Open Access is defined as free of charge access for anyone via Internet.
[12] For instance: classification for security project.

Difficult to estimate / not possible to quantify	X

I Media and Communication to the general public

20. As part of the project, were any of the beneficiaries professionals in communication or media relations?

○ Yes x No

21. As part of the project, have any beneficiaries received professional media / communication training / advice to improve communication with the general public?

○ Yes X No

22 Which of the following have been used to communicate information about your project to the general public, or have resulted from your project?

❑	Press Release	X	Coverage in specialist press
X	Media briefing	X	Coverage in general (non-specialist) press
❑	TV coverage / report	X	Coverage in national press
X	Radio coverage / report	❑	Coverage in international press
❑	Brochures /posters / flyers	x	Website for the general public / internet
❑	DVD /Film /Multimedia	X	Event targeting general public (festival, conference, exhibition, science café)

23 In which languages are the information products for the general public produced?

x	Language of the coordinator	x	English
x	Other language(s)		

***Question F-10*:** Classification of Scientific Disciplines according to the Frascati Manual 2002 (Proposed Standard Practice for Surveys on Research and Experimental Development, OECD 2002):

FIELDS OF SCIENCE AND TECHNOLOGY

1. NATURAL SCIENCES

1.1 Mathematics and computer sciences [mathematics and other allied fields: computer sciences and other allied subjects (software development only; hardware development should be classified in the engineering fields)]

1.2 Physical sciences (astronomy and space sciences, physics and other allied subjects)

1.3 Chemical sciences (chemistry, other allied subjects)

1.4 Earth and related environmental sciences (geology, geophysics, mineralogy, physical geography and other geosciences, meteorology and other atmospheric sciences including climatic research, oceanography, vulcanology, palaeoecology, other allied sciences)

1.5 Biological sciences (biology, botany, bacteriology, microbiology, zoology, entomology, genetics, biochemistry, biophysics, other allied sciences, excluding clinical and veterinary sciences)

2 ENGINEERING AND TECHNOLOGY

2.1 Civil engineering (architecture engineering, building science and engineering, construction engineering, municipal and structural engineering and other allied subjects)

2.2 Electrical engineering, electronics [electrical engineering, electronics, communication engineering and systems, computer engineering (hardware only) and other allied subjects]

2.3. Other engineering sciences (such as chemical, aeronautical and space, mechanical, metallurgical and materials engineering, and their specialised subdivisions; forest products; applied sciences such as

geodesy, industrial chemistry, etc.; the science and technology of food production; specialised technologies of interdisciplinary fields, e.g. systems analysis, metallurgy, mining, textile technology and other applied subjects)

3. MEDICAL SCIENCES

3.1 Basic medicine (anatomy, cytology, physiology, genetics, pharmacy, pharmacology, toxicology, immunology and immunohaematology, clinical chemistry, clinical microbiology, pathology)

3.2 Clinical medicine (anaesthesiology, paediatrics, obstetrics and gynaecology, internal medicine, surgery, dentistry, neurology, psychiatry, radiology, therapeutics, otorhinolaryngology, ophthalmology)

3.3 Health sciences (public health services, social medicine, hygiene, nursing, epidemiology)

4. AGRICULTURAL SCIENCES

4.1 Agriculture, forestry, fisheries and allied sciences (agronomy, animal husbandry, fisheries, forestry, horticulture, other allied subjects)

4.2 Veterinary medicine

5. SOCIAL SCIENCES

5.1 Psychology

5.2 Economics

5.3 Educational sciences (education and training and other allied subjects)

5.4 Other social sciences [anthropology (social and cultural) and ethnology, demography, geography (human, economic and social), town and country planning, management, law, linguistics, political sciences, sociology, organisation and methods, miscellaneous social sciences and interdisciplinary , methodological and historical S1T activities relating to subjects in this group. Physical anthropology, physical geography and psychophysiology should normally be classified with the natural sciences].

6. HUMANITIES

6.1 History (history, prehistory and history, together with auxiliary historical disciplines such as archaeology, numismatics, palaeography, genealogy, etc.)

6.2 Languages and literature (ancient and modern)

6.3 Other humanities [philosophy (including the history of science and technology) arts, history of art, art criticism, painting, sculpture, musicology, dramatic art excluding artistic "research" of any kind, religion, theology, other fields and subjects pertaining to the humanities, methodological, historical and other S1T activities relating to the subjects in this group]

2. FINAL REPORT ON THE DISTRIBUTION OF THE EUROPEAN UNION FINANCIAL CONTRIBUTION

This report shall be submitted to the Commission within 30 days after receipt of the final payment of the European Union financial contribution.

Report on the distribution of the European Union financial contribution between beneficiaries

Name of beneficiary	Final amount of EU contribution per beneficiary in Euros
1.	
2.	
n	
Total	