Gene expression profiling of prion-infected brains: a novel disease signature for neurodegeneration in non-human primates and in humans

Academic Year 2014/2015

CANDIDATE
Silvia Vanni

SUPERVISOR
Prof. Giuseppe Legname

CO-SUPERVISOR
Dr. Maura Barbisin
“Each one of us here today will, at one time in our lives, look upon a loved one who is in need and ask the same question: we are willing to help, Lord, but what, if anything, is needed? For it is true [...] it is those we live with and should know who elude us. But we can still love them – we can love completely, without complete understanding.”
INDEX

List of abbreviations................................................................. 1

Abstract........................................................................................ 5

Introduction.................................................................................... 10

Prion protein structure................................................................. 12
Prion protein expression and function........................................... 14
PrP<sup>Sc</sup> formation...................................................................... 15
Prion diseases.................................................................................. 16
Animal TSEs.................................................................................. 17
Human TSEs.................................................................................... 19
Genetic prion diseases................................................................. 20

Sporadic prion diseases................................................................. 23
Sporadic Creutzfeldt-Jakob disease (sCJD).................................... 23
Variably Protease-Sensitive Prionopathy (VPSPr)........................... 25

Acquired prion diseases................................................................. 26
Variant Creutzfeldt-Jakob disease (vCJD)...................................... 26
Kuru.............................................................................................. 28
Iatrogenic Creutzfeldt-Jacob disease (iCJD)................................... 29

Prion-like diseases.......................................................................... 30
Alzheimer’s Disease (AD).............................................................. 33
Parkinson Disease (PD)............................................................... 33
Amyotrophic lateral sclerosis (ALS)............................................. 35
Tauopathies................................................................................... 36
Non-human primate models .................................................. 37
Diagnosis .................................................................................. 39
Therapy .................................................................................... 41
Functional genomic approach in prion disorders ................. 42

Aim of the work ........................................................................... 42

Materials and methods .............................................................. 43

Ethics statement ........................................................................ 43
Animals ..................................................................................... 44
Patient samples .......................................................................... 45
Tissues and RNA extraction ....................................................... 45
Immunoblot analysis .................................................................... 46
Microarray analysis using the GeneChip® Rhesus Macaque genome array ................................................................. 47
Microarray data analysis ............................................................... 47
Reverse transcription and qPCR ................................................... 49
Immunohistochemistry on human brain slices ....................... 52
Availability of supporting data .................................................... 52

Results ....................................................................................... 53

PrP\textsuperscript{Sc} content in brain tissue of macaques .............. 53
Microarray analysis of brain gene expression in cynomolgus macaques ................................................................. 55
Functional classification of differentially expressed genes (DEGs) in macaques ................................................................. 57
Identification of biologically relevant networks in macaques ...... 59
Validation of differentially expressed genes in macaques by RT-qPCR .............................................................................. 62
Validation of the gene signature in humans by RT-qPCR .......... 74
Immunohistochemistry ............................................................... 84
Discussion ................................................................................. 85
Conclusions ............................................................................. 100
Appendix .................................................................................. 103
Bibliography ............................................................................. 108
LIST OF ABBREVIATIONS

TSE = transmissible spongiform encephalopathy
PrP<sup>c</sup> = cellular prion protein
PrP<sup>Sc</sup> = scrapie prion protein
PRNP = prion protein gene
BSE = bovine spongiform encephalopathy
CJD = Creutzfeldt-Jakob disease
vCJD = variant Creutzfeldt-Jakob disease
sCJD = sporadic Creutzfeldt-Jakob disease
iCJD = iatrogenic Creutzfeldt-Jakob disease
fCJD = familial Creutzfeldt-Jakob disease
AD = Alzheimer’s disease
PD = Parkinson’s disease
CBD = corticobasal degeneration
RIN = RNA integrity number
RT-qPCR = quantitative reverse transcription polymerase chain reaction
FC = fold change
ORF = open reading frame
GPI = glycophaspatidylinositol
CDS = coding DNA sequence
RBV = red-backed vole
GAPDH = glyceraldehyde-3-phosphate dehydrogenase
ACTB = actin beta
RPL19 = ribosomal protein L19
HBB = hemoglobin beta
HBA2 = hemoglobin alpha2
TTR = transthyretin
APOC1 = apolipoprotein C1
SERPINA3 = serpin peptidase inhibitor clade A
USP16 = ubiquitin specific peptidase 16
NR4A2 = nuclear receptor subfamily 4 group A member 2
ALAS2 = 5'-aminolevulinate synthase 2
RHAG = Rh-associated glycoprotein
PMCA = protein misfolding cyclic amplification
SDS-PAGE = sodium dodecyl sulphate - polyacrylamide gel electrophoresis
CWD = chronic wasting disease
FSE = feline spongiform encephalopathy
EUE = exotic ungulate encephalopathy
GSS = Gerstmann-Sträussler-Scheinker syndrome
FFI = fatal familial insomnia
CAA = cerebral amyloid angiopathy
VPSPr = variably protease-sensitive prionopathy
PK = proteinase K
ALS = amyotrophic lateral sclerosis
SAA = serum amyloid A
HD = Huntington’s disease
mHTT = mutant huntingtin
Aβ = amyloid beta
APP = amyloid precursor protein
DLB = dementia with Lewy bodies
MSA = multiple system atrophy
SOD1 = superoxide dismutase 1
TDP-43 = transactive response DNA binding protein 43
FTD = frontotemporal dementia
PSP = supranuclear palsy
AGD = argyrophilic grain disease
MAPT = microtubule-associated protein tau
BASE = atypical BSE
CSF = cerebrospinal fluid
EEG = electroencephalogram
RT-QuIC = real-time quaking induced conversion
DEG = differentially expressed gene
HRP = horseradish peroxidase
cDNA = complementary DNA
FDR = false discovery rate
RT = room temperature
IV = intravenous
IC = intracranially
LXR/RXR = liver X receptor / retinoid X receptor
FAM = fluorescein amidite
GFAP = glial fibrillary acidic protein
TNFSF10 = tumor necrosis factor ligand superfamily member 10
MT-CYTB = mitochondrial cytochrome b
MT-ND4 = mitochondrial NADH dehydrogenase subunit 4
IHC = immunohistochemistry
CNS = central nervous system
VLDL = very low density lipoprotein
EOAD = early onset Alzheimer’s disease
WT = wild type
Hb = hemoglobin
HDAC = histone deacetylase
TSA = thricostatin
SN = substantia nigra
ER = endoplasmic reticulum
ABSTRACT

Background: Prion diseases or transmissible spongiform encephalopathies (TSE) are a class of fatal infectious neurodegenerative disorders whose pathogenesis mechanisms are not fully understood. The diseases manifest as sporadic, genetic or acquired. So far, neither specific biomarkers for early diagnosis nor effective therapeutic targets have been identified. The pathological molecular component of the diseases is a misfolded isoform of the prion protein (PrP) denoted as prion. Mounting evidence suggests that in addition to gene coding for the PrP (PRNP) other genes may contribute to the genetic susceptibility of TSE. In this context, microarray-based gene expression analyses offer unique tools to approach neurodegenerative disorders. In particular, transcriptome profiling can be used to identify altered transcripts in response to pathogens, and select potential targets for novel therapeutic approaches. Up to date, a number of studies have been carried out in order to investigate the gene expression alterations occurring in prion-infected organisms, but most of them involved animal models such as mice, sheep and cattle, which are not closely related to humans. Several studies have been performed on non-human primates but none of them have investigated the genomic outcome of prion infection.

In this study, we performed the first large-scale transcriptome gene expression analysis on BSE-infected cynomolgus macaques (Macaca fascicularis), which are an excellent model for studying human acquired prion disease. Indeed, cynomolgus macaques are evolutionary very close to humans, have a high degree of amino acid homology in PrP sequence. In addition, like the human sequence, macaque PrP possesses the same polymorphism at codon 129. Furthermore, BSE can be transmitted either intracranially or orally to these animals leading to a disease that is
very similar to the human disorder, as regards preclinical incubation time, clinical symptoms and pathophysiology.

**Aim of the work:** The initial objective of the present work was to identify the main genes that are differentially expressed in the frontal cortex of intracranially infected monkeys compared to non-infected ones. This approach could shed some light on the biological processes underlying the pathogenesis of human prion diseases, which may therefore become potential targets for both diagnostic and therapeutic strategies.

Following the encouraging results obtained in monkeys, we decided to further confirm the dysregulation pattern highlighted in macaques in human prion disorders. At this step of the study, the final aim was to investigate the specificity of the identified gene signature for CJD in comparison to both healthy subjects and to other neurodegenerative diseases. This further analysis aimed at highlighting not only prion disease specific molecular mechanisms, but also potential common neurodegeneration processes.

**Methods:** Total RNA from the **gyrus frontalis superior** of 12 animals – 6 intracranially BSE-challenged (A1-A6), 1 orally BSE-infected (B6) and 5 non-infected age- and sex-matched control macaques (CovA, CovB, CovC, CovD1, CovD2) – was isolated homogenizing the material with micro pestles in TRIzol (Invitrogen). DNase I digestion was then performed and RNA was checked for quantity and purity on a NanoDrop 2000 spectrophotometer (Thermo Scientific™) and integrity on a 2100 Bioanalyzer (Agilent Technologies). Samples were labeled using the GeneChip 3’IVT Express Kit (Affymetrix) and hybridized to a GeneChip Rhesus Macaque Genome Array (Affymetrix). The bioinformatics analysis identified 300 probe sets that were up- or down- regulated about twofold (≥|1.95|). Because among them no candidate
appeared using FDR 0.05, we chose as criteria an unadjusted \( p \)-value of \( \leq 0.005 \) together with a fold change \( \geq 2.0 \). We then used the Ingenuity Pathways Analysis (IPA) to annotate genes according to their functional relationships and determine potential regulatory networks and pathways. In order to confirm the array results using an independent and more sensitive technique, we performed RT-qPCR for a subset of differentially expressed genes, with \textit{GAPDH} and \textit{ACTB} as reference genes. −RT controls were included in the plates for each primer pair and sample. The relative expression ratio was calculated using the \( 2^{-\Delta \Delta CT} \) method. Statistical significance was calculated with the unpaired student t-test (\( p<0.05 \)). Regarding human samples, we collected about 120 samples from frontal cortex of frozen postmortem brain tissue, including: prion-infected patients (vCJD, sCJD, iCJD), neurodegeneration affected patients (AD, PD, CBD, tauopathies) and controls (healthy subjects). RNA was extracted using TRIzol with PureLink® RNA Mini Kit (Life Technologies) and on-column DNase I digestion. Quantity and integrity were checked as above, and only samples with a RIN around 4.5 or higher were included in the study. Reverse transcription was then carried out using Superscript III and RT-qPCR was performed for the previously selected gene transcripts, with \textit{ACTB} and \textit{RPL19} as reference genes. In addition, for all macaque and human samples, erythrocyte markers expression analysis was performed in order to exclude any relevant blood contamination. \textbf{Results:} The microarray-based transcriptome analysis of brains from BSE-infected macaques revealed 300 transcripts with expression changes greater than twofold. Among these, the bioinformatics analysis identified 86 genes with known functions, most of which are involved in cellular development, cell death and survival, lipid metabolism and transport and acute phase response signaling. RT-qPCR was performed on selected gene transcripts in order to validate the differential expression in infected animals versus controls. The results obtained
with the microarray technology were confirmed and a five-gene signature was identified. In brief, HBB (hemoglobin, beta chain) and HBA2 (hemoglobin, alpha chain 2) were down-regulated in intracranially infected macaques, whereas TTR (transthyretin), APOC1 (apolipoprotein C1) and SERPINA3 (serpin peptidase inhibitor 3) were up-regulated. Interestingly, we found a completely different expression pattern for B6, the only orally-infected sample available, in comparison to the intracranially infected animals, for three genes (USP16, NR4A2, HBB), suggesting that the route of infection might play a substantial role in determining the gene expression regulation. Given that the autopsy procedure could have led to the presence of some blood in the brain material, we analyzed all the samples also for expression of two specific erythrocyte markers, ALAS2 (5'-aminolevulinate synthase 2) and RHAG (Rh-associated glycoprotein), in order to assess the reliability of the results related to the regulation of both chains of hemoglobin and exclude any major influence of potential blood contamination. RT-qPCR analysis for both markers revealed negligible blood contamination (C_T ≥ 35) within some samples.

Given the encouraging results found in macaques, we decided to investigate if this BSE-infection gene signature was reliable also in discriminating CJD patients from healthy ones. In humans, the disease that corresponds to BSE infection in macaques would be vCJD, which arose in human population in late 90s after consumption of BSE contaminated bovine meat. However, given the limited numbers of definitive diagnosed vCJD patients (slightly more than 200 worldwide, two of which are in Italy) and considered their reduced accessibility, we decided to extend our analysis also to sCJD patients. This would also allow us to shed some light on the possible differences in gene regulation mechanisms between acquired and sporadic human prion disorders. In addition, to better investigate the influence of different etiologies,
we also included some patients with iatrogenic CJD (iCJD), an acquired prion disease -as vCJD- but with a different origin, in this case patients that followed treatment with growth hormone derived from prion contaminated cadavers. Regarding control samples, we had to face the very limited availability of brain samples from healthy subjects, either age-matched with vCJD (around 30 years) or with sCJD (around 65 years). Therefore, we decided to introduce in our study some samples from patients with non-CJD neurodegenerative disorders as an additional “control” group; this would also enable the identification of possible prion-specific gene expression alterations. In general, the gene expression trend observed in macaques was confirmed in humans, with similar FC values, for four out of five genes: $HBA1/2$ is down-regulated in both sCJD cases and also in patients affected by other non-CJD neurodegenerative diseases, while $APOC1$, $TTR$ and $SERPINA3$ are up-regulated in CJD patients, but not in patients affected by other neurodegenerative diseases, as they show levels of expression not different from that of the healthy controls ($FC < |2|$).

**Conclusions:** In our work we used both microarray and RT-qPCR technologies that allowed us to identify a gene signature able to distinguish BSE-infected macaques from control animals. The identified genes are involved in oxygen transport and iron homeostasis ($HBB$, $HBA2$), cholesterol metabolism and lipid transport ($APOC1$, $SERPINA3$) as well as acute phase response ($SERPINA3$, $TTR$). Therefore, these results suggest that, in order to identify potential biomarkers and drug targets for prion diseases and other neurodegenerative disorders, a combination of various pathways has to be targeted, including oxygen homeostasis, cholesterol metabolism and inflammation response. Importantly, the dysregulation of four of these genes ($HBA2$, $APOC1$, $TTR$, $SERPINA3$) has been validated with similar FC values also in
CJD affected human samples, confirming the reliability of our previous analysis on BSE-infected monkeys and providing important hints on some prion-specific alterations in CJD disease. These results could be extremely helpful in understanding the mechanism underlying the progression of the disease, allowing for the identification of some key players which, if not being the cause of the onset, could however be some of the target genes affected by the disease. In addition, some of our findings support the hypothesis of a potential shared mechanism underlying the onset and the development of all neurodegenerative disorders. This is in agreement with recent data supporting the idea of a unifying role of prions in these diseases in general and maybe a prion-like behavior for most neurodegenerative disorder.

INTRODUCTION

In the last decades, the striking advancements of our knowledge in biomedicine have led to a significant improvement of health conditions. This fact has ultimately led to the lengthening of life expectancy: by 2050, almost 400 million people all over the world will be ≥ 80 years old. As a consequence, the prevalence of neurodegenerative disorders has rapidly increased. The fact of this augment is likely to represent a major social and economic issue in the near future, not only for high-income countries but also in low- and middle-income ones [1].

Even though great efforts have been dedicated to research for many years, we are still far from understanding the precise molecular mechanisms that lead to neurodegeneration in pathologies such as Alzheimer’s, Parkinson’s, Huntington’s or
Creutzfeldt-Jakob’s disease. In addition, for all these disorders no therapy is still available [2].

In general, these disorders share a common mechanism of aberrant folding of distinct proteins that, upon misfolding, become more prone to aggregation, and, in turn, may become highly neurotoxic. This mechanism is nowadays referred to as “prion paradigm”. The latter can be explained in a canonical model for the seeded aggregation of misfolded proteins: (i) recruitment of monomers by aggregates of misfolded proteins that impose their aberrant structure on the native polypeptides; (ii) growth and fragmentation of oligomers; (iii) spreading of the pathology [2]. Since the 1930s, when a spongiform encephalopathy of a sheep was shown to exhibit atypical infectivity; such as very long incubation period, absence of inflammation, no demonstrable microbial or viral agent, several other species, including humans, have been found to be affected by a similar pathology. Only half a century later, Prusiner and colleagues were able to show that the infectious agent of these transmissible spongiform encephalopathies (TSEs) consists solely of an abnormally folded protein, hereafter called “prion” – an acronym to indicate a proteinaceous infectious particle. The physiological cellular prion-related protein (PrP<sup>C</sup>) is a membrane-bound protein predominantly expressed in the nervous tissue, where it probably plays a role in neuronal development and function [3]. When in its misshapen state, the molecules aggregate with one another and impose its anomalous structure on benign PrP molecules. Prions thus act as corruptive templates (seeds) that initiate a chain-reaction of PrP misfolding and aggregation. As prions grow, fragment and spread, they cause neuronal loss perturbing the function of the nervous system and ultimately cause the death of the affected individual [4].
**Prion protein structure**

The human *PRNP* gene is located on the short arm of chromosome 20 and, in all mammals, contains three exons. The open reading frame (ORF) lies entirely within exon 3 and transcribes an mRNA of 2.1–2.5 kb in length, with approximately 50 copies/cell in neurons [5]. PrPC is the related encoded glycoprotein that resides in lipid rafts and is attached to the outer leaflet of the plasma membrane via a C-terminal glycosylphosphatidylinositol (GPI) anchor. Following the cleavage of the N-terminal signal peptide, PrPC is exported to the cell surface. Then, after ~5 h at the cell surface (its average half-life) it is internalized through a caveolae-dependent mechanism and is degraded in the endolysosome compartment [6]. Human PrPC is a 253 amino acid protein, which has a molecular weight of 35-36 kDa [7]. Its highly conserved structure is composed of a flexible unfolded N-terminal domain and an α-helical enriched globular domain; in the N-terminus of the protein there is a cleavable signal peptide and a glycine-rich octapeptides region – made up of four octarepeats - that binds copper and other divalent cations. In addition, a non-octarepeat copper binding site can be found immediately after this region. The C-terminal moiety is composed of a cleaved signal peptide, 3 α-helices and a short 2-stranded antiparallel β-sheet, and also contains a disulfide bridge linking α2-α3 helices and 2 N-linked glycosylation sites (Figure 1) [8]. PrPSc is present in un-, mono- or di-glycosylated forms, which correspond to the variable N-glycan attachment to two highly conserved residues, asparagine 181 and 197 in the human protein. While the ratios of the various glycosylated forms of PrPSc remain reasonably constant in uninfected individuals, the ratios of the glycosylated bands of PrPSc are highly variable in brains infected with different TSE agents. Moreover, it has been recently shown that the glycosylation status of PrPSc affects the inter-species transmissibility of some prion diseases [9].
As regards non-human primates, the *Macaca* genus is closely related to humans, thanks to a common ancestor ≈25 million years ago. Nevertheless, some studies have shown that in general macaques harbor much higher genetic diversity than humans [10]; however, Mauritian macaques used in this study have been known to show extremely low genetic diversity, due to their recent colonization [11]. It is known that amino acid substitutions in PrP gene sequence can alter susceptibility to TSE agents [12]. The macaque *PRNP* shares a 96% identity with the human sequence, with 32 residues differing within the CDS region (Figure 2). Similarly, also the two proteins show a 96% identity, with 9 residues (97, 100, 108, 138, 143, 155, 166, 168 and 220) that differ between macaque and human primary sequence.
Figure 2. Sequence identity between human and macaque PrP sequences.
Numbering is based on the human sequence. Dots indicate residues different from human residues. Alignment of the sequences was obtained from Ensembl database. Macaca mulatta and Macaca fascicularis protein sequences are identical.

Notably, both species display serine homozygosity at codon 170, whose polymorphism has been recently linked with transmission of infectivity in red-backed vole (RBV). Indeed, amplification of human vCJD PrP$^\text{Sc}$ in PMCA occurred only with RBV 170S/S and not in RBV 170N/N or 170S/N [13, 14]. Considering all these notions, we can assume that the macaques involved in this study represent an accurate model for a human species barrier.

**Prion protein expression and function**

Prion protein is highly conserved in mammals, where it is almost ubiquitously expressed, suggesting some relevant physiological roles particularly in the central nervous system and in the immune system. Indeed, together with neurons, lymphocytes and antigen presenting cells (such as dendritic cells and monocytes) show the highest levels of PrP$^\text{C}$ expression [15]. However, the biological functions of PrP$^\text{C}$ still remain elusive. Numerous experiments using mice and murine cell lines in
which the expression of PrP is abolished (Prnp<sup>0/0</sup>) suggest that PrP<sup>C</sup> is involved in several physiological functions such as myelin maintenance, metal homeostasis, apoptosis prevention and also hematopoiesis and erythroid differentiation [16]. In particular, in different cell culture models PrP<sup>C</sup> showed to promote neurite outgrowth and to prevent neuronal cell death, particularly through inhibition of the mitochondrial proapoptotic pathway [17-19]. PrP<sup>C</sup> is also involved in early synaptic transmission, in regulation of circadian activity and in memory formation [15]. In addition, Prnp<sup>0/0</sup> are resistant to prion infection and that neuron-restricted PrP<sup>C</sup> expression is sufficient to allow and sustain scrapie infection in transgenic mice [20]. Even if some evidences suggest an implication of PrP<sup>C</sup> in the activation of T lymphocytes and phagocytosis, the precise role of PrP<sup>C</sup> in both innate and adaptive immune system still remains unclear [15].

**PrP<sup>Sc</sup> formation**

The stochastic, genetically- or infectivity- linked misfolding of the essentially α-helical PrP<sup>C</sup> entails a 40% β-sheet enrichment of the aberrant PrP<sup>Sc</sup> isoform, whose different biophysical characteristics favor attraction and conformational conversion of other PrP<sup>C</sup> molecules. This process leads to the formation of soluble oligomers of increasing length (Figure 3). In this chain reaction of elongation, PrP<sup>Sc</sup> functions as a template that imposes its aberrant structure on native PrP<sup>C</sup> molecules, resulting in drastic modifications of PrP<sup>C</sup> biochemical properties [2]. Indeed, PrP<sup>Sc</sup> is insoluble in non-denaturant detergents, it is only partially hydrolyzed by proteases to form a fragment designated PrP 27-30, denoted after its electrophoretic mobility in reducing and denaturing SDS-PAGE, and it accumulates in the brain with a pattern distinct from the distribution of PrP<sup>C</sup> [21]. It has been speculated that the PrP<sup>C</sup> conversion to PrP<sup>Sc</sup> may occur in caveolae-like domains [7]. Oligomers and larger aggregates can
fragment, amplifying in this way the seeding capacity, and may ultimately deposit outside neurons as insoluble amyloid fibrils, characterized by ordered β-sheet repeats perpendicularly oriented to the fiber axis [2]. In particular, the oligomeric states of pathogenic mammalian prions are thought to be the toxic forms, and assembly into larger polymers, such as amyloid fibrils, seems to be a mechanism for minimizing toxicity [22].

**Figure 3. The prion paradigm of template-assisted, seeded protein aggregation.**
A schematic diagram shows the hypothetical series of events leading from the misfolding and self-aggregation of protein molecules to the formation of characteristic lesions. The assemblies that act as seeds for the templated misfolding of other molecules can vary in size from small oligomers to large polymers. These seeds initiate and sustain the disease process and may be the agents by which the aggregates proliferate and spread within the nervous system. In addition, small, oligomeric assemblies have been identified as cytotoxic agents in several instances. The lesions that result from the seeding cascade can occur as intracellular inclusions (such as neurofibrillary tangles or Lewy bodies) or extracellular masses (such as amyloid plaques). (from Walker 2015)

**Prion diseases**
Pathologically, prion diseases are marked by spongiform change (vacuolation) in the brain, neuronal loss, astrocytosis, and the accumulation of PrPSc [3]. TSEs are mostly idiopathic in origin and typically late-onset, while the genetic forms (about 10%) have a slightly earlier age at onset and a slower course. Iatrogenic cases are due to contaminated neurosurgical instruments, brains derivatives such as pituitary
hormones, corneal transplantation, dura mater graft. In addition, more recently, consumption of prion-tainted beef products and transfusion of prion-infected blood has occurred [2].

Prion diseases are infectious provided that the amino acid sequences between PrP\textsubscript{Sc} in the inoculum and PrP\textsubscript{C} expressed in the recipient are identical; therefore, PrP\textsubscript{Sc} from one species fail to or inefficiently transmit the disease to an organism expressing heterologous PrP\textsubscript{C}, a feature known as transmission barrier [23]. Furthermore, differences in incubation time can occur also when the invading PrP\textsubscript{Sc} and the host PrP\textsubscript{C} have identical sequences. This phenomenon is caused by the existence of prion strains that have the ability to generate distinct incubation times and neuropathological lesions in homologous hosts. Given that structural information for PrP\textsubscript{Sc} is not available at high resolution, this theory is currently based on distinct biochemical and biophysical properties of different strains. One tempting hypothesis would be that the transmission barrier is determined as well by distinct conformations of PrP\textsubscript{Sc} in different species [24].

**Animal TSEs**

Animal prion diseases include scrapie in sheep and goats, the aforementioned bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease (CWD) in cervids, transmissible mink encephalopathy in ranch-raised mink, feline spongiform encephalopathy (FSE) in cats and exotic ungulate spongiform encephalopathy (EUE) of captive wild ruminants. In contrast to humans, animal TSEs are generally acquired (Table 1).

Also, as already mentioned above, it is known that there are transmission barriers that operate at the molecular level, whose efficacy depends on compatibility between the PrP\textsubscript{Sc} from the infecting species and the PrP\textsubscript{C} from the host. Similarities in the
species-specific primary PRNP sequences may account for part of this effect, but prion strains and host PRNP polymorphic genotype may affect susceptibility in ways not yet fully understood [25].

Animals affected by TSEs increase the risk of transmission to humans, as in the case of BSE that captured worldwide attention in the 1990s because of food-borne transmission to humans, causing a fatal variant form of Creutzfeldt-Jakob disease (CJD). The frequency of classical BSE cases has declined dramatically following the implementation of appropriate measures to control animal feed. However, atypical forms of BSE have recently been described in Europe, Japan and North America, posing major concerns for public health [26-28]. These atypical forms are bovine amyloidotic spongiform encephalopathy (L-type BSE) and H-type BSE, both with a pathology and epidemiology distinct from classical BSE, thus representing distinct prion strains [25]. Some studies suggest that L-type BSE, but not H-type, is able to infect cynomolgus macaques [29, 30] and humanized transgenic mice, indicating that a zoonotic risk may exist [31].

Concerning cervids, in North America both the native and farmed deer and elk populations are currently suffering the rapid spread of CWD; whether it has the potential to be infectious to humans is uncertain and represents a relevant concern [32]. Indeed, while CWD transmission failed to occur in humanized mice overexpressing human PrP bearing the129M polymorphism [33-35], squirrel monkeys, but not cynomolgus macaques, are susceptible to CWD infection [36, 37]. Furthermore, brain homogenate from CWD infected cervids, used as a seed in PMCA reaction, was able to convert normal human PrP<sup>C</sup>, indicating that there is not an absolute barrier between human PrP and cervids PrP<sup>Sc</sup> [25].
Regarding sheep, both classical and atypical scrapie are considered to represent a negligible risk to the human population as they appear not to be infectious to humans [32].

<table>
<thead>
<tr>
<th>Animal transmissible spongiform encephalopathies</th>
<th>Probable aetiology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scrapie (in sheep and goats)</td>
<td>Acquired</td>
</tr>
<tr>
<td>Transmissible mink encephalopathy (TME, in mink)</td>
<td>Acquired</td>
</tr>
<tr>
<td>Chronic wasting disease (CWD, in deer and elk)</td>
<td>Acquired</td>
</tr>
<tr>
<td>Bovine spongiform encephalopathy (C-type BSE)</td>
<td>Acquired</td>
</tr>
<tr>
<td>Feline spongiform encephalopathy (FSE, in cats)</td>
<td>Acquired</td>
</tr>
<tr>
<td>H-type and L-type BSE</td>
<td>Idiopathic</td>
</tr>
<tr>
<td>Atypical scrapie (for example, Nor98 in sheep)</td>
<td>Idiopathic</td>
</tr>
</tbody>
</table>

Table 1. Animal transmissible spongiform encephalopathies (from Head 2012)

**Human TSEs**

Human prion diseases include of Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS), kuru, fatal familial insomnia (FFI), PrP cerebral amyloid angiopathy (PrP-CAA) and variably protease-sensitive prionopathy (VPSPr). CJD is by far the most common prion disorder in humans, with a worldwide incidence of 1-2 cases per million of population per annum; among these, the vast majority of cases are idiopathic and then termed sporadic CJD. With lower incidence are familial or genetic (fCJD or gCJD) cases and are found in association with a growing list of mutations in the ORF of the PrP gene, PRNP. The remaining are acquired (iCJD) through contaminated surgical instruments or cadaveric derivatives (growth hormone, dura mater and corneal transplantation) or, as mentioned above, following the consumption of infected bovine meat (vCJD) [38]. Besides CJD, there are other even rarer prion diseases, either inherited such as FFI and GSS, sporadic such as fatal insomnia or acquired due to mortuary feast rituals such as kuru (Table
2). Regardless of the origin, it seems as though PRNP gene has an influence on prion susceptibility [39]. Indeed, codon 129 is the site of a common methionine (M)/valine (V) polymorphism: in the Caucasian population around 51% of individuals are heterozygous (M/V), 37% M homozygous (M/M) and 12% are V homozygous (V/V) [38]. Interestingly, the phenotype of the prion diseases, whether sporadic, familial or acquired, often differs depending on the 129 genotype of the affected subject. Furthermore, more than 70% of sporadic and 100% of variant CJD are M homozygous, providing evidences of a genetic predisposition for human prion diseases [40]. Another well-known non-pathogenic polymorphism in the PRNP gene is glutamic acid (E) or lysine (K) at codon 219, which is mainly found in Asian and Pacific population where 219E/K heterozygosity seems to confer resistance against sCJD but not entirely against the acquired or genetic form of prion disease [41].

<table>
<thead>
<tr>
<th>Human prion disease</th>
<th>Probable aetiology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sporadic CJD (sCJD) and its subtypes</td>
<td>Idiopathic</td>
</tr>
<tr>
<td>Sporadic fatal insomnia (sFI)</td>
<td>Idiopathic</td>
</tr>
<tr>
<td>Variably protease sensitive prionopathy (VPSPr)</td>
<td>Idiopathic</td>
</tr>
<tr>
<td>Kuru</td>
<td>Acquired (sCJD)</td>
</tr>
<tr>
<td>Iatrogenic CJD (iCJD)</td>
<td>Acquired (sCJD)</td>
</tr>
<tr>
<td>Variant CJD (vCJD)</td>
<td>Acquired (BSE)</td>
</tr>
<tr>
<td>Familial or genetic CJD (fCJD or gCJD)</td>
<td>Genetic (PRNP mutations)</td>
</tr>
<tr>
<td>Gerstmann–Straussler–Scheinker disease (GSS)</td>
<td>Genetic (PRNP mutations)</td>
</tr>
<tr>
<td>Fatal familial insomnia (FFI)</td>
<td>Genetic (PRNP mutations)</td>
</tr>
<tr>
<td>Prion protein cerebral amyloid angiopathy (PrP-CAA)</td>
<td>Genetic (PRNP mutations)</td>
</tr>
</tbody>
</table>

Table 2. Human prion diseases
The probable source of infectivity in the acquired forms shown in parenthesis. (from Head 2012)

**Genetic prion diseases**

In particular, regarding familial prion diseases, there are a number of polymorphisms and more than 40 different pathogenic mutations in the human PRNP gene that are
linked to familial CJD, FFI or GSS; they include 35 point mutations and expansions or deletions within an unstable region of \( PRNP \) rich in proline, glycine and glutamine (octapeptide repeat region) (Figure 4). Interestingly, one to four additional octarepeats cause late-onset fCJD with a mean age of 62 years, while 5 to eight additional octarepeats cause early-onset fCJD or GSS with a mean age of 32 [22]. Curiously, one particular mutation, Asp178Asn, is related with fCJD – if valine is present at codon 129 – or FFI -if methionine is instead encoded at codon 129. In general, codon 129 determines earlier onset and a shorter course of genetic prion diseases for M/M homozygous individuals [39, 42].

Generally, these diseases manifest with cognitive difficulties, ataxia and myoclonus and show different neuropathological features according to different causative mutations. While fCJD is characterized by spongiform degeneration with diffuse astrogliosis throughout the brain cortex and deep nuclei, GSS shows multiple PrP-positive amyloid plaques and FFI presents a relative lack of spongiform degeneration together with neuronal dropout and gliosis in the thalamus and brain stem [43, 44]. The age at onset ranges from the third to the ninth decade of life, while the course ranges from a few months to several years with death occurring often from infection (pneumonia or urosepsis).

With regards to fCJD, progressive confusion and memory impairment are the first symptoms, and the typical age of manifestation is from 30 to 50 years. Clinical symptoms of GSS usually develop between 40 to 60 years of age, with cerebellar dysfunction (unsteady gait, mild dysarthria) together with spasticity and bradykinesia, and a slow progression from a few to seven years. A typical sign of FFI is insomnia, with disruptions in autonomic functions followed by ataxia. The age of onset for FFI spans from 40 to 50 years. Disease duration is generally 12 months in individuals homozygous for the disease causing mutation and becomes significantly longer in
heterozygous individuals (around 21 months. This suggests a gene dosage effect [32, 43].

Interestingly, mutations that generate a premature stop codon in the PRNP gene are known to cause a particular prion disease characterized by neurofibrillary tangles and PrP-positive amyloid fibril deposits in arteries and arterioles walls, a condition known as cerebral amyloid angiopathy (CAA). As for GSS, the truncated C-termini resulting from these mutations determine the loss of the GPI anchor, required to attach the protein to the outer leaflet of the plasma membrane; this indicates that the GPI moiety might interfere with the ability of PrP to form amyloid fibrils and that its absence could trigger the cerebrovascular amyloid deposition of these readily formed fibrils [45, 46].

Figure 4. Schematic representation of human PrP mutations and polymorphisms. The N-terminal octapeptide repeat motif is comprised of eight residues: P(H/Q)GGG(-/G)WGQ. Normal PrP contains five copies of this motif; a single OR deletion is considered a non-pathogenic polymorphism. However, insertional mutations consisting of one to nine additional OR are pathogenic. Polymorphisms and pathogenic mutations of the PRNP gene are represented above and below the schematic, respectively. Letters preceding the numbers indicate the normal amino acid residue for the position and letters following the numbers designate the new residue. (from Acevedo-Morantes 2014)
Sporadic prion diseases

Sporadic Creutzfeldt-Jakob disease (sCJD)

One other major disease modifier, together with the polymorphism at codon 129, is the type of PrP$^{\text{Sc}}$ present in the affected individual: indeed, type 1 PrP$^{\text{Sc}}$ is mainly associated with the MM genotype (60-70% of all sCJD cases) while type 2 PrP$^{\text{Sc}}$ is more often present with MV or VV genotype [39]. These two types of prions differ by the molecular weight of the unglycosylated band, which migrates at 21kDa in type 1 and at 19kDa in type 2, on Western blot [47]. Phenotypic heterogeneity indeed is one of the most intriguing features of prions, influencing the progression rate of the disease, the pattern of PK-resistant fragments of PrP$^{\text{Sc}}$, the neuropathological characteristics of brain lesions and transmissibility properties in mice. For instance, it has been recently shown that human MM1 sCJD prion replication rate in vitro is markedly higher than that of MM2 sCJD: this diversity is probably the consequence of specific structural features of each PrP$^{\text{Sc}}$ type that control the intrinsic growth rate of the aggregates [48]. The issue is further complicated by the fact that around 25% of sCJD cases present both type 1 and type 2 PrP$^{\text{Sc}}$, with their ratio varying among different brain regions, suggesting regional or cellular tropism of PrP$^{\text{Sc}}$ [47].

Based on the polymorphism at codon 129 and the type of PrP$^{\text{Sc}}$ present, sCJD has been historically divided into six phenotypically different subtypes:

**Subtype 1: MM1 and MV1** – this is the most common subtype of sCJD, with a large prevalence of MM homozygosity. Symptoms, mainly cognitive impairment, arise around a mean of 65 years of age, with a mean clinical duration of 4 months. Histopathologically, this subtype presents with diffuse fine spongiform degeneration particularly severe in the cortex, astrogliosis and neuronal loss. PrP immunostaining is synaptic-like.
Subtype 2: VV2 – this is the next common subtype, accounting for 16% of all sCJD cases, and is also known as “cerebellar” or “ataxic” variant. Indeed, the main difference with the subtype 1 relies on the fact that ataxia is the most recurrent symptom at diagnosis. One other distinctive characteristic is the presence of focal PrP plaque-like aggregates, especially in the cerebellum.

Subtype 3: MV2 – this subtype comprises around 9% of all sCJD cases. Its main characteristics are a longer clinical duration (average of 17 months) and the presence of kuru plaques in the cerebellum. Ataxia and cognitive impairment are almost equally the earliest signs of disease at the onset.

Subtype 4: MM2 – this is a quite rare subtype (2-8% of all cases) with a clinical duration similar to that of MV2 subtype including cognitive impairment as the first most common symptom. The hallmark here is the spongiform degeneration with large vacuoles, referred as “coarse spongiosis”, diffused in the cortex and thalamus together with astrogliosis.

Subtype 5: VV1 – it represents about 1% of all sCJD cases, with an early onset of, on average, 39 years and a clinical duration of about 15 months. The main symptom is a fronto-temporal dementia, with severe fine spongiform degeneration and a faint synaptic-like PrP immunostaining pattern.

Subtype 6: sporadic fatal insomnia (sFI) – this subtype, also referred as “thalamic” variant, accounts for about 2% of the sporadic prion diseases and it’s phenotypically identical to FFI. sFI is linked to the 129MM genotype and PrP^{Sc} type 2, but it differs from MM2 subtypes as it has a significantly longer clinical duration (average of 24 months) and the eventual presence of insomnia at diagnosis. The main histopathological feature is severe astrogliosis and neuronal loss in the thalamus without spongiosis. The PrP^{Sc} type 2 of sFI shows however a different glycoform ratio compared to that of FFI. Indeed, while in the genetic form of the disease there is a
marked under-representation of the unglycosylated form, in sFI the ratio of the three glycoforms is similar to that of the other sCJD subtypes. As mentioned above, about one fourth of all the sCJD cases present both type 1 and type 2 PrP$^\text{Sc}$, either in the same anatomical region or not, with predominance of type 1 in MM cases and predominance of type 2 in VV cases, which also dictates the related phenotype [39].

**Variably Protease-Sensitive Prionopathy (VPSPr)**

In 2008, a new human sporadic disease involving PrP was reported in the USA, with some other cases later identified also in the UK, Netherlands and Spain. This disease has a distinctive neuropathology characterized by the presence of microplaques, but it is defined by the observation that PrP$^\text{Sc}$ in the brain is less resistant to protease digestion than the PrP$^\text{Sc}$ in other human prion diseases and by the presence of N- and C-terminally cleaved ≈ 8kDa PK-resistant PrP [49]. The mean age of onset is around 67 years with an average duration of disease of about 30 months. However, in the presence of 129VV genotype there is a slight prevalence for an earlier onset and shorter disease duration. As for sCJD, this prion disease also displays heterogeneity, indeed according to the genotype at codon 129 VPSPr can be classified into three subtypes. In 129VV cases, which represent around 65% of total VPSPr cases, the first clinical symptoms are psychiatric signs such as mood and behaviour changes, speech deficit and cognitive impairment, while 129MV cases (≈23%) and 129MM subjects (≈12%) often present with parkinsonism followed by ataxia/myoclonus and progressive cognitive impairment. The common hallmark among all 129 genotypes is the presence of moderate spongiform degeneration with vacuoles in the major cerebral regions, together with occasional cerebellar microplaques in 129VV e 129MV cases; these genotypes generally have more severe lesions. Also, all three genotypes are characterized by a ladder-like
electrophoretic profile of PK-resistant fragments, consisting of 5 major bands migrating approximately at 26kDa, 23kDa, 20kDa, 17kDa and 7kDa, the latter being highly PK resistant in all subtypes. 129MM cases display relatively high PK resistance, the genotype 129MV display intermediate PK resistance while cases with the 129VV genotype show low or are even lacking band profile [50]. To date, no published studies on VPSPr transmissibility are available; however preliminary data indicate that transmission, if it occurs at all, is not efficient, specifically in comparison to CJD [49].

**Acquired prion diseases**

**Variant Creutzfeldt-Jakob disease (vCJD)**

In 1996, the National CJD Surveillance Unit reported a new form of human prion disease in the UK, now known as variant CJD (vCJD). This form of the disease has a clinical and pathological phenotype that is distinct from sporadic CJD. Interestingly, all confirmed cases of vCJD to date are MM homozygous at codon 129, a very strong association, but it is not yet clear whether the polymorphism is the major determinant of susceptibility or only of incubation time [32, 51]. Indeed, in 2008 in the UK a new case of vCJD has been diagnosed in a patient who was MV heterozygous at codon 129. However no autopsy was performed to confirm the diagnosis [52].

Experimental transmission studies of BSE and vCJD to inbred and transgenic mice found that the transmissible agent was the same, confirming the epidemiological evidence that vCJD is acquired from another species [40, 53]. The main distinguishing features of vCJD as opposed to sCJD are the clinical presentation, a younger age at clinical onset (mean around 28 years) and the speed at which the disease progresses to death (average of 13 months) [32]. Given that the most likely
route of infection from contaminated BSE-affected bovine meat is the oral one, it was predictable that both PrP\textsuperscript{Sc} and infectivity are detectable within lymphoid tissues. Indeed, unlike sCJD and iCJD, in vCJD the infectious agent can be detected in tonsils, lymph nodes, spleen and appendix even in the preclinical phase of the disease [53]. More importantly, this evidence suggests that lymphocytes may carry infectivity in blood during the incubation period of vCJD, which poses a major health issue considering that the estimated prevalence of asymptomatic individuals in the UK as determined by epidemiological studies and from the retrospective screening of appendix and tonsil tissues is around 1 per 2000-10000 [40, 54-56]. Furthermore, one recent model predicts more than 300 further cases in the next 150 years, most of which are predicted to be the result of blood-derived infection [57].

Regarding clinical features, the most common sign is dementia together with ataxia, involuntary movements and psychiatric features [58]. Currently, 226 vCJD cases have been reported worldwide, 175 had been UK residents during the high-risk period 1980-1996. To date, cases were registered in several other countries in addition to the UK, including France, Ireland, Italy, USA, Canada, Saudi Arabia, Japan, Netherlands, Portugal, Spain and Taiwan (data as of 29-04-2015 from National Creutzfeldt-Jakob Disease Research & Surveillance Unit). Successful prion disease transmission is thought to be dependent on the strain of the prion agent and its genetic compatibility with the host. Experimental transmission studies in mice have shown that the strain of agent causing vCJD has identical properties to that of BSE, which differs from the scrapie or sCJD strains [59].

Interestingly, although the UK population at different ages has been exposed to BSE in the food chain, the majority of vCJD cases are younger than 40-years-old [51]. This suggests a higher rate of dietary exposure, increased susceptibility to infection or a reduced incubation period in this age group [60]. Another possible explanation is
that as part of the development of the human immune system, there is a greater volume of gut-associated lymphoid tissues when younger, with a consequent higher volume of available tissue to absorb the infected material and therefore a more efficient infection [51].

**Kuru**

Kuru is an epidemic disease that in the 1950s had a dramatic impact on a restricted geographic area of the Papua New Guinea highlands, leading to more than 1800 deaths and therefore providing evidence for direct human-to-human prion transmission [61]. Indeed, the current hypothesis is that a sporadic case of CJD was propagated by mortuary feast practice of deceased tribe members, leading to an epidemic that affected predominantly women and children, the main participants of these mortuary feasts. In 1959 cannibalism was banned and since then no new cases have been reported.

As with other prion disorders, codon 129 homozygosity has been associated with an earlier onset and a shorter clinical duration. In general, the mean incubation period for kuru has been estimated to be around 12 years, however in some cases it may exceed five decades [62]. Some years ago a polymorphism was identified in codon 127 (G127V) whose heterozygosity seemed to provide strong resistance to kuru. Given the highly restricted geographic distribution of the protective V127-associated haplotype, it is believed that this genetic resistance developed as a consequence of the selective pressure imposed by kuru in the 20th century [61]. Very recently, the heterozygosity at codon 127 polymorphism confers complete protection against kuru and sCJD, but not vCJD in transgenic mice. Also, given the observation that V127 was seen exclusively on a M129 PRNP allele, its interaction with the common M129V PrP polymorphism was investigated. Notably, homozygosity for V127
polymorphism completely prevented the infection of 18 different human prion disease isolates –comprised of kuru, vCJD, iCJD and sCJD–, regardless of the 129 codon genotype of the inoculum. Therefore, this polymorphism seems to act in a different manner than that in codon 129. Indeed, while 129 polymorphism is partially protective against sCJD only in the heterozygous state, probably through inhibition of protein-protein interactions during the process of prion propagation, V127 seems to act as a dominant-negative inhibitor of prion conversion and propagation [63].

**Iatrogenic Creutzfeldt-Jakob disease (iCJD)**

iCJD is caused by the accidental transmission of prions from person to person during medical or surgical procedures. Several routes of transmission have been implicated such as a corneal transplant from a donor with sCJD [64], or human dura mater grafts from a contaminated commercial source that have resulted in over 200 iatrogenic infections worldwide [32, 65]. A similar number of cases arose from the use of growth hormone and gonadotropin derived from pituitary glands of CJD affected cadavers. One other minor route of transmission is represented by the re-use of neurosurgical instruments and intracerebral electrodes used on CJD affected patients. Regarding vCJD, but not sCJD, transmission via blood transfusion or infected blood-derived products in human patients has been reported in four clinical cases and one asymptomatic case, all in the UK [32, 55]. Interestingly, while the four symptomatic cases showed typical MM homozygosity at codon 129 and presented with the same clinical and pathological characteristic of BSE-derived vCJD, the neurologically asymptomatic patient had the 129MV genotype and presented with only splenic and lymph node infection [51].

Differentiating iCJD from sCJD cases is important for the identification and prevention of human-to-human prion transmission. Despite no significant differences
identified in gel profiles, stability and PrP<sup>Sc</sup> infectivity between the two CJD forms, protein misfolding cyclic amplification (PMCA) conversion efficiency is greater for iCJD in comparison to sCJD [66]. Additionally, iCJD (as is the case for vCJD) often lacks the C-terminal 12-13kDa fragment present in sCJD cases, suggesting that acquired and spontaneous prion diseases may have distinct prion formation pathways [66].

**Prion-like diseases**

Clinical outcome of prion disorders, such as a high percentage of sporadic cases and late onset of the inherited forms, are also shared by many other neurodegenerative diseases, such as Alzheimer’s diseases, Parkinson’s diseases and amyotrophic lateral sclerosis (ALS) (Table 3). This suggests that certain molecular events may occur with aging that lead disease-specific proteins to become pathogenic. More than two decades ago, Prusiner suggested that this event involves the stochastic refolding of the etiologic protein into a misfolded infectious state known as “prion” that, when it exceeds a certain threshold, impairs the degradation pathways, thereby enabling the prion to self-propagate [67, 68]. This issue has been recently addressed by a number of groups that proposed different definitions for these disorders in relation to the particular behavior of their disease causing agent [69]. Fernandez-Borges and colleagues proposed the term “prion-like diseases”, highlighting the similarities with prion protein self-perpetuating aggregation and spreading characteristics [70]. Aguzzi and Rajendran coined the term “prionoids” given that, unlikely prions, none of the other misfolded proteins presents high infectivity under natural conditions [71]. The lack of global scientific consensus on a unique definition is considerably dependent on the meaning of “infectivity”. Very recently, Castilla proposed to consider “infection” as a process by which and exogenous or
spontaneously generated self-propagating agent interacts with the host – with or without causing damage and/or disease - as a consequence of its intrinsic capacity to replicate itself through diverse mechanisms [69].

Regardless the definitions used, there is much evidence that points out to the observation that many neurodegenerative diseases should effectively be considered prion-like disorders, notwithstanding the involvement of misfolded proteins being bona fide prions. For example, many of the mutant proteins causing heritable neurodegenerative diseases are found in insoluble aggregates known as amyloid deposits, such as amyloid-β plaques and neurofibrillary tangles (NTs) in AD, and Lewy bodies in PD. Moreover, both seeding and cell-to-cell transmission has been shown for AD, PD, ALS and AA amyloidosis [69]. As regards the latter, evidence exists for transmission of serum amyloid A (SAA) protein in a prion-like manner. SAA, an acute phase apolipoprotein reactant, can undergo N-terminal cleavage leading to the formation of amyloid protein A (AA) that is then deposited extracellularly and systemically as amyloid in vital organs. In humans, this process appears as a complication of chronic inflammatory diseases -such as rheumatoid arthritis- in which the plasma SAA levels are consistently high [72]. This stimulus is supposed to trigger an autonucleation-dependent process in which AA protein interacts with AA-derived fibril seeds, possibly leading to the formation of SAA oligomeric conformers. Regarding mice, it has been shown in secondary reactive amyloidosis that small quantities of misfolded AA aggregates can be orally transmitted between individuals and cause disease [73]. In addition, bovine AA-fibrils demonstrated to be able to induce AA- amyloidosis in mice, but with a reduced efficiency if compared to murine AA-fibril, a phenomenon reminiscent of the species barrier observed for prions [72].
Also, Huntington’s disease (HD), an autosomal-dominant neurodegenerative disorder caused by an increased number of CAG repeats in the huntingtin gene, is characterized by the accumulation of abnormal aggregates largely composed of N-terminal fragments of the mutant huntingtin protein (mHTT) [74]. Furthermore, seeded aggregation occurs in mammalian cells. Indeed, internalized fibrillar polyglutamine peptide aggregates were able to selectively recruit soluble cytoplasmic sequence-homologous proteins [75]. In addition, transneuronal propagation of mHTT occurs in neural networks, both in cell models and in mice [74]. However, despite that sequence-specific nucleated protein aggregation constitutes the molecular basis of prion formation, one key feature of all prions is that they do not need to polymerize into fibrils, but can undergo self-propagation as oligomers, which are increasingly thought to represent the toxic species [68, 76].

<table>
<thead>
<tr>
<th>Neurodegenerative diseases</th>
<th>Causative prion proteins</th>
<th>Percent inherited</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creutzfeldt-Jakob disease</td>
<td>PrPSc</td>
<td>10–20</td>
</tr>
<tr>
<td>Gerstmann-Sträussler-Scheinker</td>
<td></td>
<td>90</td>
</tr>
<tr>
<td>Fatal insomnia</td>
<td></td>
<td>90</td>
</tr>
<tr>
<td>Bovine spongiform encephalopathy</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Scrapie</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Chronic wasting disease</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Alzheimer’s disease</td>
<td>Aβ → tau</td>
<td>10–20</td>
</tr>
<tr>
<td>Parkinson’s disease</td>
<td>α-synuclein</td>
<td>10–20</td>
</tr>
<tr>
<td>Fronotemporal dementia (FTD)</td>
<td>tau, TDP43, FUS, C9orf72 (progranulin)</td>
<td>10–20</td>
</tr>
<tr>
<td>Posttraumatic FTD, called chronic traumatic encephalopathy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amyotrophic lateral sclerosis</td>
<td>SOD1, TDP43, FUS, C9orf72</td>
<td>10–20</td>
</tr>
<tr>
<td>Huntington’s disease</td>
<td>huntingtin</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 3. Neurodegenerative diseases caused by prions or prion-like proteins (from Prusiner 2013)
Alzheimer's Disease (AD)

AD, the most common human neurodegenerative disease, is clinically defined by a progressive decline in memory and cognitive functions and neuropathologically by atrophy and the accumulation of extracellular amyloid plaques made of aggregated amyloid-β (Aβ) peptide as well as intracellular neurofibrillary tangles composed of aggregated and hyperphosphorylated tau protein in the brain [77]. The Aβ peptide is generated by the subsequent cleavage of the amyloid precursor protein (APP) by β- and γ-secretase enzymes. The amyloid cascade hypothesis suggests that the accumulation and subsequent deposition of Aβ in the brain are the initiating pathological events in AD that lead to the downstream aggregation of tau [78]. In order to assess transmissibility of the disease, some years ago AD brain homogenate was intracerebrally inoculated into marmosets, leading to the development of Aβ amyloid plaques after ≈4 years and thus showing that the disease may be transmissible [79]. Similar results have been obtained using APP transgenic mice and rats (Table 4) [80-82]. Ultimately, using Aβ synthetic peptides, it has been shown that the disease agent consists solely of Aβ prions [83]. Like prions, Aβ seeds range in size from small, soluble, protease-sensitive aggregates to large, insoluble, protease-resistant fibrils. Moreover, the recent finding of the existence of “strain-like” Aβ morphotypes which retain their properties after repeated passaging between animals might at least partly explain the heterogeneous morphology, pathogenicity and progression of Aβ lesions and associated pathologies in AD [84]. Additionally, it further supports the concept of prion-like templated misfolding of Aβ [4].

Parkinson Disease (PD)

PD is the second-most common neurodegenerative disease whose symptoms include progressive bradykinesia and additionally one among rigidity, resting tremor
or gait disturbance [85]. PD is characterized by the widespread degeneration of subcortical structures of the brains, particularly dopamine neurons in the *substantia nigra pars compacta*, coupled with the accumulation of α-synuclein within Lewy bodies (LBs) in neurons [77]. The cause of cell death in PD is still not known, but proteolytic stress with the accumulation of misfolded proteins has been implicated [86]. α-synuclein, a 140-amino acid protein that is highly enriched in presynaptic nerve terminals, usually seems to be in an unstructured form when in aqueous buffers or in a α-helical-rich form when bound to membranes. However, α-synuclein can also adopt a β-sheet-rich conformation that easily polymerizes into fibrils when present in high concentration or in a mutant form and that is able to seed aggregation of the soluble protein [68, 87]. Furthermore, α-synuclein is a key player also in the pathogenesis of a group of other neurodegenerative diseases defined as synucleinopathies, comprised of dementia with Lewy bodies (DLB) and multiple system atrophy (MSA) [88]. It is likely that β-sheet-enriched α-synuclein can transmit through a cell-to-cell mechanism from neurons of patients with PD into transplanted fetal mesencephalic dopaminergic neurons inducing the formation of *de novo* Lewy bodies in the grafted cells [89]. Moreover, it has been shown that intracranial inoculation of brain extracts from DLB affected patients into wild-type mice is sufficient to cause the appearance of LBs/neurite-like α-synuclein *in vivo* [90]. Further evidence for α-synuclein “prions” come from studies with recombinant α-synuclein fibrils able to induce self-propagation as well as aggregation in transgenic mice [91] and in cell culture models [88] (Table 4). Moreover, in both wild-type mice and macaques, intranigral or intrastriatal inoculations of PD-derived LB extracts resulted in progressive nigrostriatal neurodegeneration together with intracellular and presynaptic accumulation of pathological α-synuclein in different brain areas, processes reminiscent of PD neuropathology [92]. Similar results were observed
after inoculation of TgM83(+/−) mice heterozygous for a mutant A53T alpha-synuclein transgene with brain homogenate from MSA affected patients [93]. Recently, the existence of distinct α-synuclein strains that differentially promote tau inclusion in neurons has been reported. Strains are a feature typical of prions, and this may explain the tremendous heterogeneity of synucleinopathies [94].

**Amyotrophic lateral sclerosis (ALS)**

ALS is a motor neuron degenerative disease affecting two out of 100,000 individuals worldwide, with death occurring within 3 years after diagnosis in over than 80% of cases [95]. The etiology of sporadic ALS is still unknown, however at least two proteins associated with this disease have to date demonstrated multiple aspects of prion-like activity: SOD1 and TDP-43 [96]. After the identification of SOD1 mutations in familial cases (less than 10% of the total) [97], some years later misfolded SOD1-containing inclusions were identified in both familial and some sporadic cases [98]. Moreover, it has been shown that the vast majority of sporadic ALS cases present TDP-43 cytosolic inclusion [99]. There is increasing evidence that all types of ALS are associated with misfolded and aggregated SOD1 [100, 101], which also seems to have the ability to impose its misfolded conformation to the native protein [102, 103] in a sequence/structure-dependent way, reminiscent of the species barrier phenomenon observed in classical prion diseases [103]. Similarly, hyperphosphorylated and aggregated TDP-43 is found mislocalized in cytoplasmic inclusions both in frontotemporal dementia (FTD) and ALS [104]. In addition, TDP-43 displays a C-terminal prion-like domain prone to misfolding which is essential for aggregation and toxicity [105] and also self-propagation and seeded aggregation properties have been observed (Table 4) [106, 107].
**Tauopathies**

Tau is a cytoplasmic protein that normally stabilizes microtubules, but it becomes hyperphosphorylated and prone to aggregation during the course of a variety of neurodegenerative diseases such as Pick's disease, progressive supranuclear palsy (PSP), argyrophilic grain disease (AGD) and corticobasal degeneration (CBD), collectively called tauopathies [68, 108]. In human adult brain, six tau isoforms ranging from 352 to 441 amino acids are produced through the alternative mRNA splicing from \textit{MAPT} gene, but only the three forms containing four repeats are present in PSP, CBD and AGD [77].

Hyperphosphorylated tau is a component of the neurofibrillary tangles present in several neurodegenerative diseases, including AD. Also, tau mutations cause familial forms of FTD, indicating that tau protein dysfunction is sufficient to cause neurodegeneration and dementia. Evidence for mutant tau transmissibility also exists; indeed, the injection of brain homogenate from mutant P301S tau-expressing mice into transgenic wild-type expressing animals induced the formation of tau aggregates one year after inoculation (Table 4) [108]. More recently, intracerebral injections of brain extracts from various human tauopathies (AGD, PSP, CBD) have been shown to cause filament formation and neurodegeneration in mutant tau-overexpressing mice, similar to what happens in the human diseases. Importantly, the induced formation of tau aggregates could be propagated between mouse brains [109]. As in the case of prions, tau seeds are of many sizes with small, soluble assemblies being the effective seeds [110]. Assembled tau can behave like prion; in the human AD brain, neurofibrillary lesions appear to spread along neural pathways from one brain region to another, following the opposite direction to Aβ plaques. Even though tau deposition is probably necessary, but not sufficient for AD
development, it is still unclear whether tau inclusions and Aβ plaques formation is independent from each other [77].

Table 4. Summary of studies showing the transmissibility of non-prion protein aggregates. (modified from Guo 2014)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Type of seed</th>
<th>Seeded aggregation in different model systems</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Non-neuronal cells</td>
</tr>
<tr>
<td>Aβ</td>
<td>Synthetic fibrils</td>
<td>Not tested</td>
</tr>
<tr>
<td></td>
<td>Mouse brain lysates</td>
<td>Not tested</td>
</tr>
<tr>
<td></td>
<td>Human brain lysates</td>
<td>Not tested</td>
</tr>
<tr>
<td>Tau</td>
<td>Synthetic fibrils</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Mouse brain lysates</td>
<td>Not tested</td>
</tr>
<tr>
<td></td>
<td>Human brain lysates</td>
<td>Not tested</td>
</tr>
<tr>
<td>α-Syn</td>
<td>Synthetic fibrils</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Mouse brain lysates</td>
<td>Not tested</td>
</tr>
<tr>
<td></td>
<td>Human brain lysates</td>
<td>Not tested</td>
</tr>
<tr>
<td>TDP-43</td>
<td>Synthetic fibrils</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Human brain lysates</td>
<td>Not tested</td>
</tr>
<tr>
<td>SOD1</td>
<td>Synthetic fibrils</td>
<td>Not tested</td>
</tr>
<tr>
<td>PolyQ</td>
<td>Synthetic fibrils</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Non-human primate models

Several animal models have been employed to investigate neurodegenerative diseases, from invertebrates such as *Caenorhabditis elegans* or *Drosophila melanogaster* to numerous mouse strains and transgenics and several primate species as marmosets or macaques [111]. The transmissibility of human prion diseases to non-human primates was first shown in 1966 when kuru was successfully transmitted to chimpanzees and, a couple of years later, the same results were obtained for CJD [112, 113]. Later on, prion diseases were efficiently transmitted also to marmosets, macaques, gibbons and other primate species. In the 80’s, oral transmission to primates was reported for kuru, CJD (both sporadic and
iatrogenic) and scrapie: the latter, however, to date has never been reported to be transmitted to humans [114]. Evidence also exists for the transmissibility of both GSS and FFI to primates. In general, all these studies indicated that efficiency of transmission depends on the primate species (host), the origin of the inoculated material (donor) as well as the inoculation route. These studies also indicated that incubation times varied greatly among different animals even within the same species [115]. After the BSE epidemic and the following characterization of vCJD, a second round of experiments involving primates was initiated, with the main objective to assess the zoonotic potential and risk of transmissibility of BSE and vCJD [116]. However, due to ethical considerations, the use of non-human primates was drastically reduced and therefore the number of animals used in these studies, mainly cynomolgus and rhesus macaques, is usually small [117]. Importantly, rhesus and cynomolgus macaques are evolutionary very close to humans, have a high degree of amino acid sequence identity in the PrP sequence and bear also the polymorphism at codon 129 [118]. In 1996 BSE was successfully transmitted by intracerebral inoculation, leading to a pathology which resembled vCJD: “florid” plaques in brain tissue, identical vCJD PrP<sup>Sc</sup> type, and the same clinical presentation [119]. Years later, secondary transmission of macaque adapted BSE, both via intravenous or intracerebral inoculation, lead to a shortening in incubation time. In particular, as shown in figure 5, both routes of inoculation led to similar immunopathological lesions such as severe vacuolation and astrocytosis in the thalamus, together with a patchy distribution of vacuolation in the cortex with presence of dense plaques harboring, in some cases, a florid morphology. Importantly, the pattern of PrP deposition and plaque morphology were identical to those observed at first passage of BSE in adult inoculated animals, reminiscent of human vCJD immunopathology [53].
In 2004 oral transmission of BSE was accomplished, showing however a considerably longer incubation time [120]. The recent discovery of atypical forms of BSE, L-BSE/BASE and H-BSE, gave rise to questions about their transmissibility to humans. This has been shown for BASE both via intracerebral and oral inoculation, to macaques and lemurs, respectively [30, 121]. Showing that the BSE agent behaves similarly in humans and macaques, all these studies indicate that non-human primates are an excellent model for studying human prion diseases, since they reproduce all the main characteristics in terms of incubation time, clinical symptoms and neuropathological signs.

**Figure 5. Immunopathology of BSE in cynomolgus macaques.**
All panels show the pattern of PrP deposition with the 3F4 antibody, except a, which depicts glial fibrillary acidic protein immunohistochemistry. (a) Thalamus in BSE, ×10. (b) Cerebral cortex in BSE (i.v.), ×2.5. (c) Cerebral cortex in BSE (i.c.), ×2.5. (d) Immature florid plaque with a dense core of PrP surrounded by few vacuoles in the cerebral cortex (BSE, i.c.), ×20. (modified from Lasmezas 2001)

**Diagnosis**
Concerning sporadic CJD, the diagnosis is complicated by the fact that the clinical presentation is highly variable, often with a subacute onset. Most diagnostic criteria
are generally based on the recognition of progressive dementia together with two of the following symptoms: myoclonus, visual/cerebellar dysfunction, pyramidal/extrapyramidal signs, akinetic mutism. These criteria have been developed primarily for epidemiological purposes and therefore are not particularly sensitive early in the disease course as many of the symptoms are common also among the other neurodegenerative diseases. Brain MRI is currently the most accurate diagnostic criterion for early detection of sCJD, through the identification of characteristic hyperintensity patterns. Cerebrospinal fluid (CSF) instead is typically normal in sCJD, with only a mild and low specific increase in neuron-specific enolase 14-3-3 and total tau protein [47, 122]. Also, EEG generally shows relatively specific sharp waves in about two-thirds of sCJD patients [123].

Even for genetic prion disease, the diagnosis poses some issues. Indeed, a single PRNP mutation can be associated with different phenotypes and up to 60% of the genetic cases do not have a positive family history, either due to previous AD or PD misdiagnosis or to incomplete penetrance [124]. Recently, CSF RT-QuIC showed modest sensitivity but high specificity for sCJD and, more interestingly, RT-QuIC using nasal brushings from 15 sCJD and 2 fCJD patients was able to detect PrPSc in all of the samples [125-127].

Acquired prion diseases, mainly iCJD and vCJD, are less common in comparison to sporadic cases but, due to their higher potential for human-to-human transmission, their diagnosis is of particular relevance. Concerning iCJD, the main indication would come from the report of the use of cadaveric-derived hormones or tissues from afterwards diagnosed CJD patients. Variant CJD is typically distinguished from sCJD by its younger age of onset and prominent early psychiatric symptoms that often precede neurologic symptoms. While EEG rarely shows anomalies, brain MRI usually shows the characteristic “pulvinar sign”, a highly specific hyperintense signal
in the posterior thalamus [128]. Recently, both a novel stainless steel powder-based blood test and a PMCA-based urine test were able to detect vCJD with high specificity [129, 130]. Nevertheless, it is unclear whether these tests can be used for preclinical diagnosis of asymptomatic CJD patients. Nonetheless, for all CJD cases, current criteria for definite diagnosis require brain biopsy or autopsy for evidence of positive PrP\textsuperscript{Sc} tissue immunoreactivity.

**Therapy**

Despite all active efforts, there are no currently available drugs to change disease progression of prion disorders. Symptomatic treatment is the only available option, including antipsychotics such quetiapine, clonazepam to treat myoclonus and selective serotonin re-uptake inhibitors (SSRIs) to treat depression [131]. Clinical trials have been done with flupirtine and pentosan polysulfate, but both have failed to show consistent benefits. Two recently published randomized, double blind placebo-controlled trials with quinacrine and doxycycline were, as well, unable to demonstrate significant improvements [132, 133]. Despite this, doxycycline is currently being tested in presymptomatic patients from a family with FFI in order to assess whether the drug is able to delay the onset of the disease [47].

Currently, drug discovery for the identification of compounds for the treatment of prion diseases follows two main strategies. One is the so-called small molecule design, whose rationale relies in the ability of a given compound to interfere with PrP\textsuperscript{C} conversion into PrP\textsuperscript{Sc} thereby blocking self-amplification of misfolded proteins and, hopefully, halting the pathology spreading [134]. The other option is to investigate the molecular mechanism underlying the onset and the progression of prion disorders, identifying the specific altered pathways that may be therefore become potential targets for new drugs [135].
Functional genomic approach in prion disorder

For early ante-mortem diagnosis of prion diseases, neither specific biomarkers nor effective therapeutic targets have been identified. Increasing evidence suggests that other genes in addition to PRNP also contribute to the genetic susceptibility of acquired TSEs [26]. In this context, microarray-based gene expression analysis offer unique tools to approach neurodegenerative disorders. In particular, transcriptome profiling can be used to find altered transcripts in response to pathogens and select potential targets for novel therapeutic approaches [136]. In the prion biology field, several studies have been performed in order to identify differentially expressed genes (DEGs) in healthy and diseased systems, such as cell cultures and mouse models in addition to sheep and cattle [26]. These analyses identified molecular pathways that are altered upon prion infection, including cholesterol synthesis, apoptosis, lysosomal pathway, immune and inflammation response [136]. The commonalities among prion diseases and PD and AD disorders highlight either similar neurodegenerative molecular mechanisms or similar pathological secondary events. Comparative analyses of altered pathways in prion diseases, PD and AD could expose characteristic gene targets that could be used as potential diagnostic biomarkers for risk determination and also as general indicators of disease progression [26, 136].

AIM OF THE WORK

To date, a number of studies have been carried out to investigate the gene expression alterations occurring in prion-infected organisms (reviewed in [26, 135]), but most involved animal models such as mice [137-142], sheep [143-147] and cattle
Some studies have been performed on non-human primates [37, 152-155], but none have investigated the genomic outcome of prion infection. In this study, I performed a whole transcriptome gene expression analysis on BSE-infected cynomolgus macaques (Macaca fascicularis), which are an excellent model for studying human acquired prion disease [154-158]. Indeed, BSE can be transmitted either intracranially or orally to these animals leading to a disease that is very close to the human one with regards to preclinical incubation time, clinical symptoms and pathophysiology [120, 156].

The initial objectives of the present work were to identify the main genes that are differentially expressed in the frontal cortex of intracranially infected monkeys compared to non-infected ones. This analysis would help to shed some light on the biological processes underlying the pathogenesis of human prion diseases that may therefore become potential targets for both diagnostic and therapeutic strategies. Following the encouraging results obtained in monkeys, I decided to further confirm the dysregulation pattern in human prion disorders. For this second analysis I investigated the specificity of the identified gene signature for CJD in comparison to both healthy subjects and to other neurodegenerative diseases such as AD and PD. This approach could shed some light not only on prion disease molecular mechanisms, but also on neurodegeneration processes in general.

**MATERIALS AND METHODS**

**Ethics statement**

Ethical approval for the animal study was released by the Lower Saxony Ministry for consumer protection and food safety (509.42502/08/07.98). Animal experimentation was performed in accordance with section 8 of the German Animal Protection Law in
compliance with EC Directive 86/609. Ethical committee authorization for human samples is currently under approval.

**Animals**

Cynomolgus macaques (Macaca fascicularis) were obtained from the Centre de Recherche en Primatologie, Mauritius and maintained in social groups of six monkeys housed in a microbiological containment BSL3 facility. Six 1-year old female cynomolgus macaques (A1-A6), all MM homozygous at codon 129, were intracerebrally inoculated with 50 mg of bovine BSE-positive brain stem material derived from an EU-standard inoculum. The inoculum was a pool of homogenized bovine brain stems from 11 naturally infected, histopathologically and immunohistochemically confirmed cases of BSE. The BSE stock tested positive for PK-resistant prion protein by Western blotting. Inoculation was performed via slow injection of 250 µl of a 20% BSE brain homogenate (w/v) diluted in sucrose into the right hemisphere through the dura mater into the caudo-putamen region. The preclinical incubation time was on average 1,100 days. One additional cynomolgus macaque (B6) was orally challenged with the same material; inoculation was performed *per os*, as single dose. The preclinical incubation time of this animal was 1950 days. Brain material from age-and sex-matched non-infected cynomolgus macaques (CovA, CovB, CovC, CovDI, CovDII) was obtained from Covance Laboratory Münster GmbH and processed with the same protocol. It should be taken into account that the diet and housing conditions of the experimental and the control animals were not perfectly identical. These differences may have led to some inter-individual variations among the animal groups.
**Patient samples**

A total of 37 brains were collected. 16 of them were from sCJD affected patients, 11 were Type 1, 5 were Type 2. 6 brains were from vCJD patients (one from Italy and five from France), 5 were from iCJD ones (growth hormone treatment), 6 were from non-CJD neurodegenerative disease affected patients (NEG), 4 were from healthy subjects (CTRL). All vCJD patients were MM homozygous at codon 129, while iCJD cases were either MM homozygous (3) or MV heterozygous (2). All the samples were kindly provided by Fondazione I.R.C.C.S Istituto Neurologico Carlo Besta, (via Celoria 11, Milan, Italy) with the exception of 5 vCJD and all iCJD samples that were provided by ICM-Hôpital Pitié-Salpêtrière (47, Boulevard Hôpital, 75013 Paris – France) and two control samples that were kindly provided by University of Verona, Department of Neurological and Movement Sciences, Policlinico G.B. Rossi (P.le L.A. Scuro 10, Verona, Italy).

**Tissues and RNA extraction**

Seven BSE-infected cynomolgus macaques at advanced stage of disease and five non-infected control animals were sacrificed, and for each animal one hemisphere of the brain was dorso-ventrally sliced and immediately frozen on dry-ice. The *girus frontalis superior* region was macroscopically identified on the frozen tissue and removed via a biopsy stamp. Tissues were manually homogenized with micro pestles (Kisker Biotech GmbH) and total RNA was isolated using TRIZol (Invitrogen). Following RNA isolation, DNase I digestion was performed using 1 unit of enzyme per µg RNA (Fermentas) for 30 min at 37°C and then heat inactivated for 5 min at 95°C followed by precipitation with Sodium Acetate / Ethanol. RNA was checked for quantity and purity on a Spectrophotometer 2000 (PEQLAB) and for integrity of the
18S and 28S ribosomal band by capillary electrophoresis using the 2100 Bioanalyzer (Agilent Technologies).

Regarding human samples, total RNA was isolated from about 100mg of frontal cortex from each of the following individuals: vCJD (n=6), iCJD (n=5), sCJD T1 (n=11), sCJD T2 (n=5) affected patients; CJD negative patients affected by other neurodegenerative diseases (NEG) (n=6) and controls (n=4). The brain material was manually homogenized with pestles and glass vessels using TRIzol (Invitrogen). Then, RNA was extracted with PureLink RNA Mini Kit (Life Technologies) and on-column DNA digestion was performed using PureLink DNase Set (Life Technologies). RNA was checked for quantity and purity on a NanoDrop 2000 spectrophotometer (Thermo Scientific) and integrity on a 2100 Bioanalyzer (Agilent Technologies).

**Immunoblot analysis**

PK-treated (50 µg/mL for 1 hour at 37°C) and untreated brain homogenates were separated on 12% Bis/Tris Acrylamide gels (NuPAGE, Invitrogen) and transferred to nitrocellulose membranes (Protran, Schleicher & Schüll, Germany). Detection of macaque PrP\textsuperscript{Sc} was performed using the monoclonal anti-PrP antibody 11C6 and a HRP-conjugated anti-mouse IgG-antibody (Sigma-Aldrich, Germany). Signal was visualized using a chemiluminescent substrate (Super Signal West Pico, Pierce) and high sensitivity films (Amersham). Densitometric analysis of PrP\textsuperscript{Sc} was performed using the Image J program 1.37v.
Microarray analysis using the GeneChip® Rhesus Macaque genome array

Microarray analyses using the GeneChip® Rhesus Macaque genome array samples were labeled using the GeneChip® 3’IVT Express Kit (Affymetrix®). Reverse transcription of RNA was achieved using 500 ng of total RNA to synthesize first-strand cDNA. This cDNA was then converted into a double-stranded DNA template for transcription. In vitro transcription included a linear RNA amplification (aRNA) and the incorporation of a biotin-conjugated nucleotide. The aRNA was then purified to remove un-incorporated NTPs, salts, enzymes, and inorganic phosphate. The labeled aRNA of each animal was fragmented (50–100 bp) and hybridized to a GeneChip® Rhesus Macaque Genome Array (Cat N° 900656; Affymetrix®). The degree of fragmentation and the length distribution of the aRNA were checked by capillary electrophoresis using the Agilent 2100 Bioanalyzer (Agilent Technologies). The hybridization was performed for 16 h at 1 × g and 45°C in the GeneChip® Hybridization Oven 640 (Affymetrix®). Washing and staining of the arrays was performed on the Gene Chip® Fluidics Station 450 (Affymetrix®) according to the manufacturer's recommendations. The antibody signal amplification, washing and staining protocol were used to stain the arrays with streptavidin R-phycoerythrin (SAPE; Invitrogen). To amplify staining, SAPE solution was added twice with a biotinylated anti-streptavidin antibody (Vector Laboratories, Burlingame, CA, USA) staining step in-between. Arrays were scanned using the GeneChip® Scanner 3000 7G (Affymetrix®).

Microarray data analysis

Intensity data from the CEL files were imported to the Partek® software including a quality control based on internal controls. All chips passed the quality control and
were analyzed using the Limma package [159] of Bioconductor [160, 161] and the Partek® software. The microarray data discussed in this paper were generated conforming to the MIAME guidelines and are deposited in the NCBI’s Gene Expression Omnibus (GEO) database [162]. They are accessible through GEO series accession number GSE52436 (see section: Availability of supporting data).

The microarray data analysis consisted of the following steps: 1. quantile method normalization, 2. global clustering and PCA-analysis, 3. fitting the data to a linear model, 4. detection of differential gene expression and 5. overrepresentation analysis of differentially expressed genes. Quantile-normalization was applied to the log2-transformed intensity values as a method for between array normalization to ensure that the intensities had similar distributions across arrays. For cluster analysis, we used a hierarchical approach with the average linkage-method. Distances were measured as 1 - Pearson's Correlation Coefficient. The PCA was performed using the princomp-function in the Partek® software. To estimate the average group values for each gene and assess differential gene expression, a simple linear model was fitted to the data, and group-value averages and standard deviations for each gene were obtained. To find genes with significant expression changes between groups, empirical Bayes statistics were applied to the data by moderating the standard errors of the estimated values [159]. P-values were obtained from the moderated t-statistic and corrected for multiple testing with the Benjamini–Hochberg method [163]. The p-value adjustment guarantees a smaller number of false positive findings by controlling the false discovery rate (FDR). For each gene, the null hypothesis, that there is no differential expression between degradation levels, was rejected when its FDR was lower than 0.05. Because no candidates appeared using FDR 0.05, we made the selection using another p-value (unadjusted p-value ≤ 0.005) and a fold change ≥ 2.
**Reverse transcription and qPCR**

Quantitative reverse transcription real-time PCR (RT-qPCR) was performed using gene-specific primer pairs. For macaque samples, cDNA synthesis was accomplished using 100ng RNA, 10ng random hexamer primer, 2 mM dNTPs, 0.5 U RNase inhibitor and 5 U reverse transcriptase (Bioline) in 1 x reaction buffer. For human samples, cDNA was obtained using 3µg RNA, 1µl 50µM oligo(dT)$_{20}$, 1µl 10mM dNTP mix, 40 U RNAse inhibitor and 200 U Superscript III Reverse Transcriptase (Life Technologies).

For each sample a negative control was performed by omission of the reverse transcriptase (-RT control). The cDNA was diluted 1:10 (macaques) or 1:15 (humans) prior to RT-qPCR. The reaction mix included 2x iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Inc.) and 400 nM of the corresponding forward and reverse primer (Sigma). Technical triplicates were quantified on an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.). All primers used for RT-qPCR are listed in Table 5 and 6.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Chromosome</th>
<th>Primer and probe sequence</th>
<th>Amplicon length (bp)</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTB</td>
<td>3</td>
<td>F: GTTGCCTTACACCTTTCTTG</td>
<td>146</td>
<td>NM_001033084.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CTGTACCTTCACCGTCTCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>P: ACAAGATGAGATGGCATGGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>11</td>
<td>F: CCTGACCACCAACTGCTTA</td>
<td>74</td>
<td>NM_001195426.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CATGAGTCCTCACAAGTACA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>P: CTGGCCAGTGTACCCATGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AKR1C1</td>
<td>9</td>
<td>F: CCGCATATTGATTTTGTCA</td>
<td>132</td>
<td>NM_001195574.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: TGGAAATTGCACCAAGTCTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCAM1</td>
<td>14</td>
<td>F: GAGCAAGAGGAAGTACGAGG</td>
<td>150</td>
<td>XM_001083697.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GACTTTGAGGGTGTGGTACG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NR4A2</td>
<td>12</td>
<td>F: CCACTGAGGAGGTAACACATC</td>
<td>145</td>
<td>NM_001266910.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: AGGAGAGGGCAAGAATTCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>USP16</td>
<td>3</td>
<td>F: GCAGAACACTGCACAAACACC</td>
<td>146</td>
<td>NM_001260999.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CTAAGAAAGGAGGCGCTTACG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAP18</td>
<td>17</td>
<td>F: GGAAATGTACCGTAGGAGG</td>
<td>109</td>
<td>NM_001261034.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: TGCCCTCTCTTCTACTCTTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IRF3</td>
<td>19</td>
<td>F: TGGGTTGTTTGAGCAAGAGG</td>
<td>90</td>
<td>NM_001135797</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GAAAGATCCCAACTTCTAGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SERPINA3</td>
<td>7</td>
<td>F: GCTGGCATTGAGGAGTCT</td>
<td>123</td>
<td>NM_001195350.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GTGCCCTCCTCAGACACATC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>P: TTTCCCTGGCAGTACCATCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HBA2</td>
<td>20</td>
<td>F: CGACAAGAGCAAGTCAAGG</td>
<td>126</td>
<td>NM_001044724.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: TCAGAAGGAGGGAAGTAGGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>P: TGGCCGATATGGTCGGAGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HBB</td>
<td>14</td>
<td>F: GTCCCTCTCTGATGCTTATG</td>
<td>102</td>
<td>NM_001164428.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: TTAAGGTGTCAGGCTATTAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>P: AAGTGCCGGGCTGCTTATGAGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>APOC1</td>
<td>19</td>
<td>F: TTCTGTGATGTCTTTGGAAG</td>
<td>138</td>
<td>AK240617.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CACTCTGTTGATGCGTGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>P: TGGAGGACAGGCGTTGGAAGTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TTR</td>
<td>18</td>
<td>F: TCACCTGGCATCTCCCCATT</td>
<td>114</td>
<td>NM_001261679</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GGTGGAATAGGAGTAGGGGCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>P: ATCGTTGGCGTGAATACCCCTCTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALAS2</td>
<td>X</td>
<td>F: TCCCTTCATGCCTGGGAAC</td>
<td>108</td>
<td>XM_002806252.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GAGGTACAGGATCTTGGTAAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RHAG</td>
<td>4</td>
<td>F: AGGCAGAGCAGATCTTTGGTC</td>
<td>87</td>
<td>NM_001032815.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GGTTGAAATTGCCATATCCGC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5. Genes analyzed in macaques by RT-qPCR
Primers (F: forward and R: reverse) and probes (P: probe) used for gene amplification, amplicon length, and GenBank accession numbers of the macaque cDNA sequences used for primer design. All primers were designed according to the genome sequence of *Macaca mulatta*. *Apolipoprotein C-I (APOC1)* primers and probe were designed according to the genome sequence of *Macaca fascicularis* because the *Macaca mulatta* mRNA sequence was not annotated (TSA *Macaca mulatta* Mamu_450725, accession number: JV045807.1). Homology between the two sequences was 99%.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Chromosome</th>
<th>Primer sequence</th>
<th>Amplicon length (bp)</th>
<th>Accession number</th>
</tr>
</thead>
</table>
| ACTB     | 7          | F: AGAGTACGAGCTGCGTGAC  
R: AGCAGTCGTGGGGGCTGACAG | 184 | NM_001101.3 |
| RPL19    | 17         | F: CTAGTGTCCTCCGCTGTTG  
R: AGGCTTTTTCCGGCATAC | 169 | NM_000981.3 |
| HBB      | 11         | F: AAGGAGAAGTCTCCCGCTGACTG  
R: CCGACACTTTTCTGCCATGA | 190 | NM_000518.4 |
| SERPINA3 | 14         | F: TGCCAGGCGACCTCTCCAT  
R: TGTCCTCTGCTGATGTCCTCC | 167 | NM_001085.4 |
| HBA1/    | 16         | F: GCTCTGCCAGGTGTAAGGG  
R: CAGTGCTTGGAGGCTTGAAG | 160 | NM_000558.4 |
| HBA2     | 16         | F: GTCACCTTCAAGTCTCTCAT  
R: AACACAGAGGAGAAGGCC | 145 | NM_001645.3 |
| APOC1    | 19         | F: AAACACTGGAGGGAGAAGCC  
R: CTTACCTGGAAGGCCACTGGC | 131 | NM_000371.3 |
| TTR      | 18         | F: AGGCCGTGTTGGAATAGGAG  
R: CTTACTGGAAGGAGGACCACTGGC | 113 | NM_000311.3 |
| PRNP     | 20         | F: CTTCTTGCAGAGGCCAGG  
R: CGAAGCTTCTCTCCTCCTCAC | 150 | NM_001037968.3 |
| ALAS2    | X          | F: TGTCCTGAGTGTAGTAATGGA  
R: GGAAGCTTCCAAGGACTGAATG | 87 | NM_000324.2 |

**Table 6. Genes analyzed in humans by RT-qPCR**

Primers (F: forward and R: reverse) used for gene amplification, amplicon length, and GenBank accession numbers of the human cDNA sequences used for primer design.

The cycling conditions included an initial denaturation of 3 min at 95°C then 45 cycles at 95°C for 15 sec and 58°C for 1 min. Differential gene expression of candidates was normalized to GAPDH and ACTB (macaques) or ACTB and RPL19 (humans) expression. –RT controls were included in the plates for each primer pair and sample. The relative expression ratio was calculated using the $2^{-\Delta\Delta C_T}$ method [164]. Statistical significance was calculated with the unpaired student t-test ($p<0.05$). Fold change values smaller than 1 were converted using the equation $-1/fold$ change, for ease of representation. Melting curve analysis and gel electrophoresis of amplification products was performed for each primer pair to verify that artificial
products or primer dimers were not responsible for the obtained signals. Some results on macaque samples were further confirmed using TaqMan® MGB probes and iQ™ MGB probes (Bio-Rad Laboratories, Inc.). The primer sequences, the reaction setup and the cycling conditions were the same described above. The FAM-probes used are listed in Table 5.

**Immunohistochemistry on human brain slices**

Immunohistochemistry analysis was performed on alcolin-fixed paraffin-embedded 7µm-thick frontal cortex brain slices. In order to eliminate paraffin, glass slides were kept at 56° C for 20 min and then immersed in xylene for 20 min. After this, slides were re-hydrated using decreasing ethanol concentrations (from 100% to 70%) for 5 min each and lastly put in water for 10 min. Antigen retrieval was achieved with pH6 citrate buffer (100° C for 20 min). Endogenous peroxidase blocking was accomplished by treating slides with 6% H₂O₂ for 20min. Goat serum (1:20 in PBS-Tween) was incubated for 20 min at RT, and then primary antibodies were incubated overnight. After washing the slides with PBS-tween (3 times, 2 min each) secondary anti-rabbit HRP-polymer Ab EnVision™ (Dako) was incubated for 1h at RT. After washing, DAB was added to each slide and, after development, it was blocked in water. Counterstaining with hematoxylin was also performed in some cases. Antibodies and dilutions used were: Anti-TTR (1:200), Anti-APOC1 (1:100), Anti-SERPINA3 (1:1000), Anti-HBB (1:100), from Sigma, Anti-HBA2 (1:50) from Thermo Scientific.

**Availability of supporting data**

The microarray data set supporting the results illustrated here is available in the Gene Expression Omnibus (GEO) repository.
The DEGs were analyzed for their functions, pathways and networks using Ingenuity Pathways Analysis (IPA) [http://www.ingenuity.com/products/ipa/try-ipa-for-free].

RESULTS

PrPSc content in brain tissue of macaques

PrPSc presence in brain homogenate of 6 BSE-infected macaques was assessed by Western Blot (Figure 6).

Figure 6. PrPSc profile of macaque-adapted BSE in comparison to human CJD.
Brain homogenates from human sCJD type 1, sCJD type 2, vCJD, and BSE-infected macaques were subjected to PK treatment, separated on 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and blotted onto nitrocellulose membranes. PrPSc for human and macaque brain was detected with the widely used monoclonal antibody 3F4 or with 11C6. (from Montag 2013)

Densitometric analysis of the monoglycosylated band revealed that the relative amount of PrPSc strongly differed between individual macaques. We hypothesized that this variation was dependent either on the preclinical incubation time or rather it corresponded to the gradual accumulation of PrPSc during the clinical phase of disease as reported for sCJD [165, 166]. As anticipated, we found a significant
correlation between PrP<sup>Sc</sup> content and the duration of the symptomatic phase (Figure 7).

![Figure 7. Correlation between PrP<sup>Sc</sup> content and duration of clinical phase.](image)

This correlation analysis included only the 6 intracranially inoculated macaques. Given that these animals were housed within the same social group, environmental factors, which could influence the disease course and duration, were identical. These factors can slightly differ for the orally inoculated animal, which was therefore omitted from this analysis. All infected animals were sacrificed at an advanced stage of prion disease; the details of their clinical course have been previously described [155]. Briefly, animal A1 showed the shortest duration of disease (17 days) and a short pre-clinical incubation time (931 days) together with the lowest PrP<sup>Sc</sup> content, while animal A5 showed the longest survival period (143 days), compared to an average clinical phase of about 90 days, together with the highest PrP<sup>Sc</sup> content and the second longest pre-clinical phase (1340 days). In general, animals A1, A3, A4 and A6 were considered as early onset, while A2 and A5 were late onset, the difference
between the two groups being statistically significant. Early symptoms included coordination impairment and ataxia, followed by dysmetria and dementia [155]. As regards B6, the only orally inoculated animal, limited data are available. However, from another study involving animals orally infected with the very same inoculum as the one used in the present study, it is known that the main neurological symptoms (gait ataxia, disturbed social behavior or loss of social status ranking, panic-stricken responses provoked by optical stimuli, reduced escape reactions in the presence of humans, abnormal shrieking, decreased competitiveness, restlessness) are present in BSE-infected monkeys regardless of the route of infection, and the same for spongiosis and PrPSc deposits [158]. Of note, no florid plaques were observed; neither in IC nor in orally inoculated animal brains, in contrast with what has been shown in a similar study involving IV and IC infected monkeys [53, 119]. It should be considered however that in Yutzy et al. the IC inoculum was a low-dose one (5mg) while in our case (and in Lasmezas et. al one too) it was ten times higher. Nevertheless, some evidence (orally infected animals showed absence of the “stage 1” characterized by disturbed body growth, no neurological signs, CSF-negative for 14-3-3 proteins) exist that the clinical course may be influenced by the inoculation route [158].

**Microarray analysis of brain gene expression in cynomolgus macaques**

To investigate differential mRNA expression in BSE-infected macaques (*Macaca fascicularis*) we used brain samples from 6 animals that were intracranially challenged [155]. One macaque that was orally infected with 50 mg BSE-homogenate was also included in our study. For comparison purposes, we used 5 brain samples derived from non-infected age- and sex-matched control macaques.
RNA was isolated from the *gyrus frontalis superior* of all animals and checked for quality by nano-scale electrophoresis, which resulted in an overall RNA Integrity Number (RIN) of about 6. This value suggests that the RNA was at least partially degraded; one possible explanation for the limited RNA integrity in the samples may reside in the procedure used to remove the *gyrus frontalis superior* region from the frozen tissue slice. Indeed, the biopsy stamp was attached to a cordless screwdriver that was used to drill a borehole in the frozen tissue block of +/- 1 cm height. This technique was preferred in order to ensure that the tissue did not thaw; however, the local heat induced by the rotating biopsy stamp may have led to substantial degradation of the RNA. Nevertheless, human brain material showing a comparable RIN value was successfully used for similar studies [167]. All samples were analyzed using the GeneChip® Rhesus Macaque Genome Array (Affymetrix®) that contains more than 50,000 rhesus probe sets to enable gene expression studies of *Macaca mulatta* transcriptome interrogating more than 47,000 transcripts. The genomes of *M. mulatta* and *M. fascicularis* display a small genetic divergence of around 0.4% [168] [169], which confidently allows the detection of homologue transcripts with a high specificity.

Raw data were quality checked and analyzed using Affymetrix® proprietary analysis tools, a hierarchical clustering performed and a heat map generated. Then the signals were aligned to the annotation library and a spreadsheet containing gene symbols, *p*-values and expression fold changes was created. Microarray data were submitted to Gene Expression Omnibus (GEO). The bioinformatics analysis identified 300 probe sets that were up- or down-regulated about twofold (≥ |1.95|). Since among them no candidate appeared using FDR 0.05, we chose as criteria an unadjusted *p*-value of ≤0.005 together with a fold change ≥ |2.0|. Additional table 1
lists the resulting 86 probe sets that were then used to generate the heat map shown in Figure 8.

**Figure 8. Condition trees of the clustering analysis**
The cluster analysis was performed using a hierarchical approach with the average linkage-method (R and Partek® Software, Partek® Inc.): 86 probe sets showed a differential expression with FC ≥ 2. The color represents the level of expression (red: up-regulation, blue: down-regulation) and the sample information is listed across the bottom. The names of the known genes are indicated. More details on all genes are reported in Additional file

### Functional classification of differentially expressed genes (DEGs) in macaques

We used the Ingenuity Pathways Analysis (IPA®, see section: Availability of supporting data) to annotate genes according to their functional relationships and to determine potential regulatory networks and pathways. Among the 300 differentially expressed (about twofold, ≥| 1.95|) probe sets identified, 105 were associated to mapped IDs. Of them, 53 were identified as network eligible genes, while 86 were identified as function eligible genes. We would like to clarify that the designation of
functional classes in this study cannot be considered definitive or exclusive, given that the annotation of gene function is incomplete and that the same multifunctional gene products can be involved in several different cellular pathways.

As a first step we identified key biological functions and/or diseases that contain a disproportionately high number of genes from the DEGs list compared to the total gene population from the microarray. The analysis was initiated by identifying the top categories \( (p<0.01) \) of DEGs within three main classes (Table 7). In the “Diseases and Disorders” class the top scoring categories were cancer and developmental disorder, while within the “Molecular and Cellular functions” class most genes were involved in cellular development and cell death/survival. The two main categories for the “Physiological System Development and Function” class were tissue morphology together with nervous system development and function.

<table>
<thead>
<tr>
<th>Diseases and Disorders</th>
<th>p-value</th>
<th># Molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer</td>
<td>1.13E-05 - 9.19E-03</td>
<td>58</td>
</tr>
<tr>
<td>Developmental Disorder</td>
<td>2.10E-05 - 9.19E-03</td>
<td>24</td>
</tr>
<tr>
<td>Hematological Disease</td>
<td>2.10E-05 - 6.83E-03</td>
<td>7</td>
</tr>
<tr>
<td>Organismal Injury and Abnormalities</td>
<td>6.76E-05 - 9.19E-03</td>
<td>22</td>
</tr>
<tr>
<td>Reproductive System Disease</td>
<td>7.30E-05 - 9.19E-03</td>
<td>22</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Molecular and Cellular Functions</th>
<th>p-value</th>
<th># Molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellular Development</td>
<td>4.22E-06 - 9.19E-03</td>
<td>35</td>
</tr>
<tr>
<td>Cellular Assembly and Organization</td>
<td>3.30E-05 - 9.19E-03</td>
<td>13</td>
</tr>
<tr>
<td>DNA Replication, Recombination, and Repair</td>
<td>3.30E-05 - 1.57E-03</td>
<td>10</td>
</tr>
<tr>
<td>Cell Death and Survival</td>
<td>5.67E-05 - 9.19E-03</td>
<td>35</td>
</tr>
<tr>
<td>Lipid Metabolism</td>
<td>1.17E-04 - 9.19E-03</td>
<td>19</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Physiological System Development and Function</th>
<th>p-value</th>
<th># Molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue Morphology</td>
<td>9.70E-08 - 9.19E-03</td>
<td>30</td>
</tr>
<tr>
<td>Skeletal and Muscular System Development and Function</td>
<td>4.22E-06 - 9.19E-03</td>
<td>17</td>
</tr>
<tr>
<td>Nervous System Development and Function</td>
<td>1.04E-05 - 9.19E-03</td>
<td>26</td>
</tr>
<tr>
<td>Organ Morphology</td>
<td>2.10E-05 - 9.19E-03</td>
<td>17</td>
</tr>
<tr>
<td>Hematological System Development and Function</td>
<td>5.31E-05 - 9.19E-03</td>
<td>22</td>
</tr>
</tbody>
</table>

Table 7. Top three classes of key biological functions and/or diseases.
Subsequently, genes were clustered in relation to the main pathways they belong to: the top three canonical pathways ($p$-value < 0.005) in our DEGs list were LXR/RXR activation, which is associated with lipid metabolism and transport, acute phase response signaling and the neutral pathway of bile acid biosynthesis, which is also involved in lipid metabolism and is a major route of cholesterol catabolism (Figure 9).

**Figure 9.** Top 10 significant canonical pathways over-represented in the DEGs list

**Identification of biologically relevant networks in macaques**

To further investigate the global expression response to BSE infection and to define interactions among the identified specific pathways containing the regulated genes, potential networks of interacting DEGs were identified using IPA®. All potential networks with score >9 (a score ≥3 was considered significant, $p$<0.001) are listed in
Table 8, with information on network genes, score, focus molecules and top functions associated with the focus genes in each network.

<table>
<thead>
<tr>
<th>ID</th>
<th>Molecules in Network</th>
<th>Score</th>
<th>Focus Molecules</th>
<th>Top Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ACVR1C, AKR1D1, Alp, AMPK, Ap1, APOC1, Calcineurin protein(s), CARTPT, caspase, CD3, CHI3L1, Creb, cytochrome C, DACH1, DLK1, ERK, ERK1/2, F13A1, Focal adhesion kinase, GNRH1, HBA1/HBA2, HBB, HDL, hemoglobin, HEY2, HINT1, HIPK2, ikk (family), IL1, IRF3, Jnk, KDELRA2, LDL, LGALS1, Mapk, MEF2C, Mek, MET, MT2A, N4BP1, NADPH oxidase, NGFR, NR4A2, OTX2, P38 MAPK, p85 (pi3K), Pdgf (complex), PDGF BB, PI3K (complex), PI3K (family), PIK3R3, Pkc(s), PLC gamma, PON3, Pro-inflammatory Cytokine, Ras, SERPINA1, SERPINA3, Shc, SHOC2, SLCO1A2, Sox, ST4K, TCF, TCR, TNSF10, TTR, TWIST1, Vegf, WSB1</td>
<td>71</td>
<td>35</td>
<td>Tissue Morphology, Cell Death and Survival, Developmental Disorder</td>
</tr>
<tr>
<td>2</td>
<td>ABR, ACTL6B, ARMC6, ASB6, C10orf137, C6orf211, CAMKV, CHMP2A, CLIC4, CLPP, CSNK1G3, CTBP2, DCLRE1A, DDX19B, DGKE, ECT2, FHL3, FLVCR1, GALNTL5, GLD4, HEATR6, HSP90AA1, HSPA12A, ITFG1, KLF3, KPN6, MCTS1, MEIG1, METTL7B, MRPL44, MXD3, MYBPc1, NCLN, NIPB1, NOL4, OSBP1L0, PCDH11X, PLEXH2, PMM2, POLR2J, PPAP2C, PRCP, PROSC, RAI2, SAP18, SCAND1, SEPT6, SGTB, SMRCC1, SMc3, SPATA22, SPSB3, SRPK3, SSU72, STAG1, TATDN1, TESPA1, TM7SF3, TNN1, TNN3K, TP53BP1, TRAPPc2L, TRIP12, TUFM, TXNL4A, UBC, ZNF131, ZNF235, ZNF397, ZNF420</td>
<td>54</td>
<td>28</td>
<td>Developmental Disorder, Hereditary Disorder, Hematological Disease</td>
</tr>
<tr>
<td>3</td>
<td>26s Proteasome, ADCY, AKR1C1/AKR1C2, Akt, APP, ARL6C, Amlt-Clock, AVP, AVPR1B, CACNA1B, CAMKV, CARPT, CBLN2, CEACAM6, CLDN10, CLOCK, COX4I2, CTF1, DNAJC12, endocannabinoid, estrogen receptor, FAM46A, FSH, GABRE, GNA15, GPR15B, GPX1, GPX2, GSK3A, Histone h3, HMGCY, HNF4A, HSPA12A, Insulin, JPH3, KCNC3, KCNS1, LINGO1, LPAR1, LXR, MGAT2, miR-125b-5p (and other miRNAs w/seed CCCUGAG), Mmp, MST1, NFkB (complex), Npff, OPN1LWT, PDX1, PI3K, Pka, Pkm, PLc, Proinsulin, RAB39A, RAI2, RIOK2, RUFY3, SERPINA3, SMAD5, SMC4, SOX7, SYT17, TCF19, Tnfrsf22/Tnfrsf23, TOR2A, tretnoin, trypsinin, TXNL4B, ZBTB44, ZFHX3</td>
<td>36</td>
<td>21</td>
<td>Cellular Development, Neurological Disease, Skeletal and Muscular System Development and Function</td>
</tr>
</tbody>
</table>

Table 8. List of 3 Ingenuity networks generated by mapping the focus genes that were differentially expressed between non-infected and BSE-infected samples. Names in lowercase are genes/molecules that are not from the DEGs list but are associated with some of them within pathways identified by Ingenuity Pathway Knowledge Base (IPKB).
The highest ranked network identified by IPA® (Figure 10) was associated with tissue morphology (specifically the determination of cell quantity), developmental disorder and biological processes controlling cell death and survival (Table 8).

Figure 10. Identification of biologically relevant networks
Top ranking network generated by mapping the genes that were differentially expressed in infected animals vs controls. Pathway analysis based on the Ingenuity Pathway Knowledge Base (IPKB) is shown. Color shading corresponds to the type of dysregulation: red for up-regulated and green for down-regulated genes according to the microarray fold change calculation method. White open nodes are not from the list of 300 DEGs, but are transcription factors that are associated with the regulation of some of these genes identified by IPKB. The shape of the node indicates the major function of the protein. A line denotes binding of the products of the two genes, while a line with an arrow denotes 'acts on'. A dotted line denotes an indirect interaction.
This network contained genes that are known to be involved in several neurological diseases and nervous system functions, as shown in Figure 11.

Figure 11. Schematic representation of nervous system-related functions for selected DEGs according to IPKB

Validation of differentially expressed genes in macaques by RT-qPCR

To further confirm the array results using an independent and more sensitive technique, we performed RT-qPCR analyses for a subset of differentially expressed genes. This group (Additional table 2) was selected in the subsequent steps. First, among the 86 probe sets identified in the microarray analysis (Additional table 1) we selected the top 36 with fold change ≥ |2.5| and p ≤ 0.005. Then, we noticed that many were not annotated or did not have a known function and therefore we extended the selection to additional 29 probe sets having fold change ≥ |2.5| but 0.005 ≤ p ≤ 0.05; however, even with these lower stringent criteria we still had few
eligible genes and so in the end we decided to consider for our analysis some additional probe sets with a consistent fold change of $\geq |2.5|$ but $p>0.05$ (24 candidates). At this point, having identified only 13 feasible candidate transcripts, we further added seven probe sets (corresponding to 5 additional transcripts) selected among those with a slightly lower fold change (FC$>2$ for at least 1 probe) but possessing an interesting function as indicated by the IPA® analysis or according to the literature. Finally, \textit{HBA2} was added to the list because of its tight relationship with one of the previously selected genes of the hemoglobin complex (\textit{HBB}), as revealed by the top ranking network from the IPA® analysis (Figure 10). Summarizing, we designed RT-qPCR assays for 19 genes (Table 9), most of which were already known to be involved in neurodegenerative disorders or nervous system regulation, even though very few had been implicated in prion diseases.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession number</th>
<th>Known relation with PrP/nervous system</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKR1C1</td>
<td>NM_001195574.1</td>
<td>putative role in myelin formation</td>
<td>[170]</td>
</tr>
<tr>
<td>HBB</td>
<td>NM_001164428.1</td>
<td>putative role in intraneuronal oxygen homeostasis, reduced in Alzheimer's and Parkinson's disease</td>
<td>[171]</td>
</tr>
<tr>
<td>NCAM1</td>
<td>XM_001083697.2</td>
<td>PrP/N-CAM complexes found in prion infected N2a cells</td>
<td>[172]</td>
</tr>
<tr>
<td>NR4A2</td>
<td>NM_001266910.2</td>
<td>Mutations related to dopaminergic dysfunction, including Parkinson schizophrenia and depression</td>
<td>[173]</td>
</tr>
<tr>
<td>USP16</td>
<td>NM_001260999.2</td>
<td>depletion of USP16 prevented ATMi from restoring transcription after DSB induction</td>
<td>[174]</td>
</tr>
<tr>
<td>CALB1</td>
<td>XM_001085269.2</td>
<td>plays a protective role in neurodegenerative disorders (depleted in HD)</td>
<td>[175]</td>
</tr>
<tr>
<td>DACH1</td>
<td>XM_001082371.2</td>
<td>required for normal brain development</td>
<td>[176]</td>
</tr>
<tr>
<td>LXXN</td>
<td>NM_001266988.1</td>
<td>marker for the regional specification of the neocortex</td>
<td>[177]</td>
</tr>
<tr>
<td>PIK3R3</td>
<td>NM_001266826.1</td>
<td>involved in β-amyloid plaque formation and regulatory pathways in the AD brain</td>
<td>[178, 179]</td>
</tr>
<tr>
<td>SAP18</td>
<td>NM_001261034.1</td>
<td>possibly related to AD</td>
<td>[180]</td>
</tr>
<tr>
<td>SERPNA3</td>
<td>NM_001195350.1</td>
<td>increased in schizophrenia, SNPs affecting onset and duration of AD</td>
<td>[181, 182]</td>
</tr>
<tr>
<td>TNFSF10</td>
<td>NM_001266034.1</td>
<td>implicated in pathogenesis of MS (causing demyelination)</td>
<td>[183]</td>
</tr>
<tr>
<td>HBA2</td>
<td>NM_001044724.1</td>
<td>putative role in intraneuronal oxygen homeostasis, reduced in Alzheimer's and Parkinson's disease</td>
<td>[171]</td>
</tr>
<tr>
<td>GNRH1</td>
<td>NM_001195436.1</td>
<td>key regulator of the reproductive neuroendocrine system in vertebrates</td>
<td>[184]</td>
</tr>
<tr>
<td>IRF3</td>
<td>NM_001135797</td>
<td>putative protective role against prion infection</td>
<td>[185]</td>
</tr>
<tr>
<td>APOC1</td>
<td>AK240617.1</td>
<td>binds to ApoE, risk factor for Alzheimer's disease</td>
<td>[186]</td>
</tr>
<tr>
<td>TM7SF3</td>
<td>XM_001099269.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MYBPC1</td>
<td>XM_001091952.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TTR</td>
<td>NM_001261679</td>
<td>amyloid neuropathies, interaction with Aβ</td>
<td>[187]</td>
</tr>
</tbody>
</table>

Table 9. Candidate genes for validation
List of 19 identified genes selected on the basis of FC value and known relevance for neurodegeneration. Because of very low signal (LXXN, PIK3R3, TNFSF10, GNRH1) or lack of reliable sequence data (CALB1, DACH1, TM7SF3, MYBPC1), only 11 genes (in bold) were successfully analyzed. *Macaca fascicularis* transcript.

Among these, we were able to successfully analyze only 11 genes (reported in bold Table 9) together with 2 housekeeping genes (ACTB and GAPDH), since all the other RT-qPCR assays either showed too low expression (C_T >35) or amplification of trace amounts of gDNA. In addition, because several gene names have changed
since the first annotation was done, updated names from the latest Affymetrix® annotated library are provided in Additional table 2, together with the old ones.

In order to achieve optimal RT-qPCR conditions we performed titration of template and primers as well as optimization of cycling conditions using human cDNA from SH-SY5Y neuroblastoma cells, in order to save the scarce macaque cDNA available for definitive assays. To assess the specificity of the chosen primer pairs prior to performing the final quantitative assays, some experiments were performed using macaque cDNA obtained from a different brain region of control animals, in order to check the correct amplicon length. Two housekeeping genes, GAPDH and ACTB [188], were selected as reference genes to normalize RT-qPCR data. Both genes were monitored across infected and control macaques in order to evaluate their expression stability, yielding very similar results (Figure 12).

![Figure 12. Evaluation of reference gene expression stability across non-infected and BSE-infected samples.](image)

After these preliminary optimizations, we performed the quantitative analysis and in general we observed a large intra-assay variability for most genes across different samples, both for infected (Figure 13) and for control animals (Figure 14).
Interestingly, we found a completely different expression pattern for B6, the only orally-infected sample available, in comparison to the intracranially infected animals,
for three genes (*USP16, NR4A2, HBB*), suggesting that the route of infection might play a substantial role in determining the gene expression regulation (Figure 15).

![Figure 15](image)

**Figure 15.** Comparison between the intracranially infected samples (A1-A6) and the orally infected one (B6)

In light of these results, we decided to carry out another microarray clustering analysis excluding this animal in order to assess its influence on the final results. As shown in Figure 16, the comparison of the two clustering analysis with (panel A) or without (panel B) the orally challenged animal (B6) does not show marked differences.

![Figure 16](image)

**Figure 16.** Cluster analysis
Cluster analysis was performed using a hierarchical approach with the average linkage-method for all animals (panel A) or excluding the orally infected one, B6 (panel B).
Using SYBR® Green-based RT-qPCR we were able to confirm the statistically significant up-regulation of TTR (FC= 7.11), SERPINA3 (FC= 18.73) and APOC1 (FC= 6.33) as well as the down-regulation of HBB (FC= 5) and HBA2 (FC= -4.5), normalizing the data against GAPDH. Very similar results were obtained against ACTB (Figure 17).

Regarding all the other genes analyzed, the RT-qPCR results confirmed the regulation pattern observed in the microarrays, but without statistical significance (p-value> 0.05).

**Figure 17.** SYBR Green-based RT-qPCR validation of microarray results
Relative expression levels of 11 genes normalized against GAPDH o ACTB in BSE-infected cynomolgus macaques.

In order to further confirm the SYBR® Green-based results we performed additional RT-qPCR analyses using FAM-labeled TaqMan® probes, therefore providing higher sensitivity and specificity for those genes that showed a significant fold change. Using this approach we confirmed the regulation of SERPINA3, APOC1, HBB and
HBA2, but not of TTR, which showed a comparable fold change but lost statistical significance (Figure 18).

Figure 18. Comparison between microarray, SYBR Green-based and TaqMan probe-based results for the five selected genes. Expression levels for each transcript are normalized against GAPDH. All three technologies yielded similar results.

This may depend on the higher variability among triplicates, due to C_T values higher than 35 obtained with the TaqMan® probe chemistry compared to SYBR® Green detection system (Figure 19).
In summary, we were able to confirm the results of the array platform obtaining consistent fold change values for all genes analyzed, even though we validated with statistical significance using the specific TaqMan® detection system only four of them: *HBB, HBA2, APOC1, SERPINA3* (see Table 10 for details on p-values and FC).

Figure 19. Comparison between SYBR-Green and TaqMan-based C_T values for TTR
<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Microarray fold change</th>
<th>RT-qPCR fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Min</td>
<td>Max</td>
</tr>
<tr>
<td>AKR1C1</td>
<td>2.3</td>
<td>2.9</td>
</tr>
<tr>
<td>HBB</td>
<td>-2.2</td>
<td>-2.6</td>
</tr>
<tr>
<td>NCAM1</td>
<td>-1.1</td>
<td>2.5</td>
</tr>
<tr>
<td>NR4A2</td>
<td>1.1</td>
<td>-2.1</td>
</tr>
<tr>
<td>USP16</td>
<td>-1.2</td>
<td>-5.5</td>
</tr>
<tr>
<td>SAP18</td>
<td>-1.2</td>
<td>-2.6</td>
</tr>
<tr>
<td>SERPINA3</td>
<td>10.0</td>
<td>16.0</td>
</tr>
<tr>
<td>HBA2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IRF3</td>
<td>2.0</td>
<td>2.1</td>
</tr>
<tr>
<td>APOC1</td>
<td>4.3</td>
<td>-</td>
</tr>
<tr>
<td>TTR</td>
<td>3.1</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 10. RT-qPCR confirmation of microarray results
Differential expression of selected genes analyzed by microarray and RT-qPCR. For microarray analysis, the lowest (Min), the highest (Max) and the average (Mean) fold change values of all the respective probes are shown. For RT-qPCR analysis, fold change (FC) and statistical significance (p-value) for both SYBR Green and TaqMan results are shown. In bold, genes validated with statistical significance. HBA2 did not have a related probe set on the microarray. *normalization performed only against ACTB.

In addition, given that the autopsy procedure for these animals could have led to the presence of some blood in the brain material, and considering that some recent studies have highlighted the existence of active transcription within human red blood cells [189], we decided to analyze our samples also for the expression of two erythrocyte markers, ALAS2 and RHAG, in order to ensure that the RT-qPCR signals of both hemoglobin-related genes (HBB and HBA2) reflected the actual brain gene expression dysregulation and not a blood contamination. In particular, ALAS2 (5'-aminolevulinate synthase 2) gene product specifies an erythroid-specific
mitochondrially located enzyme, which catalyzes the first step in the heme biosynthetic pathway. *RHAG* (Rh-associated glycoprotein) instead, codes for an erythrocyte-specific protein which is thought to be part of a membrane channel that transports ammonium and carbon dioxide across the blood cell membrane and also it is thought to interact with Rh blood group antigens. The microarray data for these genes suggested a negligible but identical presence of blood in control and infected samples, RT-qPCR analysis revealed a small blood contamination (*C*ₜ ≥ 34 for *ALAS2*, *C*ₜ ≥ 36 for *RHAG*) within two samples, one control (CovDI) and one infected sample (A4) (Figures 20 and 21).

![Graph](image)

**Figure 20. Blood erythrocyte markers expression across BSE-infected and non-infected macaque samples**

Absolute *Cₜ* values for Rh-associated glycoprotein gene (*RHAG*)
In light of these results, we performed an additional gene expression analysis for \( HBB \) and \( HBA2 \) excluding these two samples. As expected, we obtained slightly different results (FC\( \sim \)0.3 for \( HBB \) and FC\( \sim \)0.2 for \( HBA2 \) using TaqMan\textsuperscript{®} probes), but a relevant down-regulation still persisted with statistical significance. Given some recent reports about neuroinflammation involvement in sCJD patients [190], we wondered if immune system genes dysregulation occurred in BSE-infected macaques. However, analysis of the microarray data did not reveal relevant deregulation of cytokines that might be involved in neuroinflammation and/or immune response. Indeed for GFAP, a known marker of microgliosis, only one probe gave an indication of up-regulation (FC\( \sim \)1.97). For IL6 and TNF\( \alpha \), all the probes showed FCs around 1 with and CD68, another microglial marker, the single probe available showed a border-line up-regulation (FC\( \sim \) 1.65). These data suggest that inflammatory responses may not be particularly relevant in this primate model or in the specific area of the frontal cortex that was examined.
Furthermore, one of our first candidates (TNFSF10), which is a member of the TNF family that triggers the release of pro-inflammatory cytokines [191], was down-regulated (FC= -2.7 and -2.08) for only two out of four probes. It was then analyzed by RT-qPCR, but the assay was not robust due to primer design difficulties. Therefore, this target was not validated.

Also, we noticed that a couple of genes coding for subunits of mitochondrial complexes, namely MT-CYTB and MT-ND4, were significantly dysregulated in our microarray data (Additional table 1). However, the very high FC value (> 300) observed for one of the probes led us to assume that probably it could have been a spurious result. Furthermore, we considered that the array probe was designed on *Macaca mulatta* genome which does not have a strong similarity with the *Macaca fascicularis* one in this region. Indeed, it is known that mitochondrial DNA displays substantial sequence diversity even between subpopulations of the same species, mainly due to high mutation rate [192]. Therefore, given also the very limited amount of cDNA available, we decided not to further investigate mitochondrial transcripts in macaque samples. Analyses on human samples are currently ongoing.

**Validation of the gene signature in humans by RT-qPCR**

Given the encouraging results found in macaques, we wondered if BSE-infection gene signature was reliable also in discriminating CJD patients from healthy ones. One of the closest human diseases to that of BSE-infected macaques is vCJD. However, given the limited numbers of definitive diagnosed vCJD patients (slightly more than 200 worldwide, two from Italy) and considered their reduced accessibility, we decided to extend our analysis including also sCJD patients. This would also allow us to shed some light on the possible differences in gene regulation
mechanisms between acquired and sporadic human prion disorders. In addition, to better investigate the influence of different etiologies, we also included some patients with iatrogenic CJD (iCJD), an acquired prion disease -as vCJD- but with a different origin, in this case treatment with growth hormone derived from prion contaminated cadavers. Regarding control samples, there is a limited availability of brain samples from healthy subjects, either age-matched with vCJD (around 30 years) or with sCJD (around 65 years). This is because for young individuals, the relatively rare deaths usually occur as a consequence of accidents (mainly unintentional injuries) or known diseases -all cases that generally do not contemplate autopsies since the cause of death is already established- while for aged people, in general it is likely that they present some neuropathological sign. Therefore, we decided to introduce in our study some samples from patients with non-CJD neurodegenerative disorders as an additional “control” group. This would also enable the identification of possible prion-specific gene expression alterations. Two additional common issues with postmortem human brain samples that we had to deal with were the stability of endogenous reference genes and the RNA integrity [193]. A number of different variables have been supposed to negatively correlate to different extents with RNA integrity: for example, higher degradation is observed in samples subjected to prolonged thawing [194] or storage [195] as well as in samples of patients showing longer agony before death (probably due to brain acidosis) [196]. Clearly, in studies involving postmortem human tissue is not possible to control all the variables that might impair RNA quality. This inevitably leads to non-homogenous sample collections with high degree of biological variance. However, it is widely accepted that moderate degradation does not preclude reliable analyses of small amount of RNA. Indeed, it has been shown that gene expression profiles from partially degraded RNA samples with still visible ribosomal bands are highly similar to that of intact samples [195], especially if the
RT-qPCR amplified products are smaller than 250bp [197]. This is not particularly surprising considering that reference genes transcripts most likely degrade gradually and in parallel with target ones [193]. For all these reasons, a minimum RIN ≥ 3.95 was suggested as including threshold for human brain tissue [198]. In order to ensure a sufficient reliability of the samples, in our study we applied a slightly more stringent threshold; indeed, only RNA that showed RIN around 4.5 or higher were selected for further analysis. As regards housekeeping genes, there is a lack of consensus about the optimal reference genes in postmortem brain tissues. Indeed, while some issues have been reported about GAPDH involvement in human neurodegenerative processes [199], ACTB and the ribosomal protein family seem to show good stability across different RIN values [196] and brain samples [199]. Therefore, we decided to use ACTB and RPL19 as reference genes to normalize RT-qPCR data, also given that in our preliminary optimizations GAPDH showed sensibly higher C_T values in comparison to the two other housekeeping genes analyzed.

As for macaque samples, the relative mRNA levels were calculated using the 2^{-ΔΔC_T} method and two reference genes (ACTB and RPL19, CV < 0.5, M value < 1 for both), obtaining very similar results. Firstly, the two housekeeping genes were analyzed across diseased and control patients in order to assess their expression stability (Figure 22).
Figure 22. Comparison between RPL19 and ACTB stability across control, CJD and non-CJD samples.
For each group, average values of absolute C\textsubscript{T}s among the samples for RPL19 (dark grey) and ACTB (light grey) are shown. Each sample was analyzed in triplicates.

Fold change values smaller than 1 were converted using the equation -1/fold change, for ease of representation.

In general, the gene expression trend observed in macaques was statistically confirmed, with consistent FC values, for four out of five genes. HBA1/2 (FC ~ -5.0) is down-regulated in both Type 1 (T1) and Type 2 (T2) sCJD patients, but not in vCJD and iCJD cases. As regards patients affected by other non-CJD neurodegenerative diseases (NEG), a trend of down-regulation (FC ~ -3) was observed as well, but without statistical significance. A similar trend was found also for HBB, which indeed showed a slight down-regulation in sCJD and NEG patients, even though without statistical significance (Figure 23, upper panel).

SERPINA3 is up-regulated in both sCJD T1 and T2 patients (FC ~6) as well as in vCJD (FC ~ 8.5) and iCJD (FC ~ 19) cases. APOC1 shows an up-regulation in both sCJD T1 and T2 patients (FC ~7) and in iCJD cases (FC ~4.5), but not in vCJD patients. Both the two genes are not regulated in patients affected by other
neurodegenerative diseases (NEG), as they show levels of expression similar to that of the controls (FC < |2|) (Figure 23, lower panel).

Figure 23. RT-qPCR validation of gene signature
Relative expression levels of HBB, HBA1/2 (upper panel), APOC1, SERPINA3 (lower panel) normalized against RPL19 or ACTB in sCJD, vCJD, iCJD and NEG patients. sCJD T1 and T2, showing similar results, were grouped for ease of representation.
Regarding *TTR*, an up-regulation which is consistent with the results obtained in BSE-infected macaques was found in both iCJD (FC~18) and sCJD cases (FC~2.5), but not in vCJD and NEG patients (Figure 24).

![Figure 24. RT-qPCR analysis of TTR in human samples](image)

Relative expression levels of *TTR* normalized against *RPL19* or *ACTB* in sCJD, vCJD, iCJD and NEG patients. sCJD T1 and T2, showing very similar results, were grouped for ease of representation.

For some genes, statistical significance was obtained only in a few cases (*), mainly due to the lack of appropriate sample size of control samples (N=4) and to the high variability of the samples within certain groups. Nevertheless, when comparing BSE-infected macaques with human vCJD, iCJD and sCJD patients, we observed consistent results between macaques and humans for all the genes, with the exception of *HBB* (Figure 25).
Therefore, we could confidently assume that our results on human patients may be reliable, despite the lack of statistical significance in some cases.

With regards to other neurodegenerative diseases (NEG), both HBB and HBA were found down-regulated similarly to BSE-infected macaques and CJD patients, while for APOC1, SERPINA3 and TTR no significant dysregulation was observed. This last evidence prompted us to perform an additional analysis for these three genes, normalizing the data on NEG samples as a surrogate “control” group, in order to highlight a potential role of specific CJD markers in comparison to other

Figure 25. Comparison between BSE-infected macaques and CJD patients
Relative expression levels of HBB, HBA1/2, APOC1, TTR and SERPINA3 normalized against ACTB in BSE-infected macaques, sCJD, vCJD, iCJD and NEG patients. sCJD T1 and T2, showing similar results, were grouped for ease of representation.
neurodegenerative diseases for these genes. In this case, the up-regulation of all genes in CJD samples was even stronger, in terms of FC values and/or statistical significance, suggesting that the dysregulation of these three genes might be specific to CJD (Figure 26).

![Graph showing RT-qPCR results for SERPINA3, APOC1 and TTR in sCJD, vCJD and iCJD patients in comparison with NEG group.](image)

Figure 26. RT-qPCR results for SERPINA3, APOC1 and TTR in sCJD, vCJD and iCJD patients in comparison with NEG group. Relative expression of SERPINA3, APOC1 and TTR normalized against ACTB or RPL19 in sCJD, iCJD and vCJD patients. In this analysis, FC for sCJD, iCJD and vCJD group was calculated normalizing ΔC_T average of each group on the ΔC_T average of NEG group. sCJD T1 and T2, showing similar results, were grouped for ease of representation.

In addition to this, we analyzed all the samples for expression of two erythrocyte markers, ALAS2 and RHAG, to exclude any major blood contamination that may be present as a consequence of the autopsy procedure. RT-qPCR analysis of RHAG
revealed a negligible blood contamination ($C_T \geq 35$) within some samples, even though $ALAS2$ $C_T$s were slightly lower ($\geq 30$) (Figure 27). However, the difference in the expression levels of $ALAS2$ between brain and blood samples (used as control) was $\geq 10$ $C_T$, confirming the very limited influence of blood contamination in our analysis. In addition, blood traces were almost uniformly present in all the groups; therefore, we can conclude that our results were not significantly affected by blood presence.
Lastly, we investigated the gene expression pattern of \textit{PRNP}. No significant change of \textit{PRNP} expression was observed in any of the groups analyzed. However, a different behavior clearly emerged between vCJD and iCJD: while iCJD samples showed a down-regulation of \textit{PRNP}, vCJD samples showed an up-regulation. Indeed, the difference between the two groups was statistically significant, suggesting a role of the infection route in the modulation of PrP mRNA levels (Figure 28).
We are also conducting immunohistochemistry analyses on these brains to check the expression of the proteins encoded by the five genes. Preliminary analyses have been already performed for TTR, APOC1 and SERPINA3 on AD and sCJD brain slices in order to establish a suitable IHC protocol and check the specificity of the selected antibodies. So far, all the tested antibodies displayed a specific reactivity that mirrored the localization reported in literature for each of the related proteins (Figure 29). Further analyses on these and the remaining antibodies in other patient samples are ongoing, both in IHC and Western blot.
Figure 29. Immunohistochemistry analysis on human brain slices. APOC1 antibody staining (upper boxes) in AD brain slice shows endothelial reactivity and co-localization with Aβ plaques. These slices were counterstained with hematoxylin. SERPINA3 antibody staining in sCJD brain slice (lower left box) shows endothelial/perivascular reactivity and extracellular/cytoplasmic localization. TTR antibody staining in sCJD brain slice shows endothelial reactivity and subpial mater localization. Magnification: 10X

DISCUSSION

To date, an in-depth knowledge of the molecular processes that lead to neurodegeneration onset and progression in TSEs is still missing. In this context, genomic approaches are unbiased and powerful tools to investigate the molecular basis of these complex diseases. In addition, since neither effective therapy nor specific diagnosis is available yet, genomics may also allow the identification of new potential biomarkers. Many investigators in past years have performed genomic analyses of brain tissues from different models of animal TSE; some of the studies involved the mRNA profiling of cattle BSE [27, 149-151] or ovine scrapie [143-147,
but obviously the vast majority employed rodent-adapted models of prion disease [137-142, 200, 201]. In a number of these prion-infected mouse models, genomic expression profiling showed increased oxidative and endoplasmic reticulum (ER) stress, activated ER and mitochondrial apoptosis pathways together with induction of cholesterol biosynthesis in the CNS of preclinical mice [201].

We present here the first large-scale transcriptome study of the superior frontal gyrus of BSE-infected macaques. This specific region was selected due to its histopathological and functional relevance in the majority of neurodegenerative disorders [179] and ultimately because it corresponds to Broadmann areas 10 and 11, known to be involved in strategic processes in memory recall, diverse executive functions (domain-general function in “cognitive branching”) as well as in planning, reasoning, and decision making [202], all processes known to be affected by neurodegeneration.

As starting point, the microarray analysis revealed 300 transcripts that were differentially regulated in BSE-infected animals versus controls. Among these, 97 probe sets were selected as candidates for validation with RT-qPCR, a technique well known for its high sensitivity and wide dynamic range [203].

Due to the shortage of cDNA availability and to the technical difficulties in designing the RT-qPCR primers for some of the genes -the macaque genome has been recently sequenced, but it’s far from being completely validated [10]- only 11 DEGs were actually analyzed by RT-qPCR.

In general, variations of the expression levels observed with RT-qPCR were slightly larger than those seen in the microarray analysis. This is an obvious consequence of the different detection ranges of the two techniques. Microarray platform tends to have a lower dynamic range in comparison to RT-qPCR, the latter is used to validate array results [203]. RT-qPCR results confirmed the regulation trend seen in the
microarray platform for all the analyzed genes, providing very similar FC values using either GAPDH or ACTB for normalization. For five of them (HBB, HBA2, TTR, SERPINA3, APOC1) we obtained statistical significance with one or both qPCR detection systems we used (SYBR Green® and TaqMan® probes). As highlighted from functional classification of the identified DEGs reported in the Results section, some genes were involved in the top three canonical pathways: APOC1 and TTR are part of the LXR/RXR activation pathway, which is associated with lipid metabolism and transport, and are also involved in the neutral pathway of bile acid biosynthesis, which is a major route of cholesterol catabolism. Indeed, the liver X receptors (LXRs) are central players in cholesterol homeostasis throughout the periphery, where they function as sterol sensor to induce expression of genes involved in the regulation of cholesterol and lipid metabolism: APOC1 is one of the several apolipoproteins involved in reverse cholesterol transport, and is also a transcriptional target for LXRs [204, 205]. Both subtypes of LXR, LXRα and LXRβ, are expressed and functional in the CNS, the second being present 2 to 5 fold higher than in the liver, demonstrating the importance of these receptors in CNS cholesterol metabolism [205]. Interestingly, these pathways have been previously related to prion infection, particularly at the preclinical stage, suggesting a potential involvement of these two genes in the onset of the disease [201, 206-208]. On the other hand TTR, as well as SERPINA3, are also involved in the acute phase response signaling pathway, a systemic reaction to inflammation that may arise also in response to the extracellular protein deposition. This has been associated with prion and in general neurodegenerative diseases in the literature [209-211]. All the other genes analyzed seemed to reside in the grey zone of both platforms and therefore their FC values could not be considered reliable. Nevertheless, it would be premature to conclude that no other DEGs are present in this BSE model, since the sample size we analyzed was modest for the
large animal-to-animal variability that characterized this study and therefore weak effects (FC < |2|) could have not been detected with statistical significance [212].

Analyzing the clinical and histological data available for the macaques, we observed that animal A1 showed the shortest preclinical incubation period, a very short duration of disease [155] and the lowest deposition of PrP$^{Sc}$ in brain tissue (Figure 7). This may be explained considering that PrP deposits are more stable and hence less pathogenic/infective than smaller oligomers [68]. Thus, since this sample exhibits a low amount of deposited protein, a higher amount of free PrP seeds might be present, and it is known that this form is more prone to trigger a rapidly progressive disease. Moreover, sample A5 behaves in quite the opposite way, showing the highest PrP$^{Sc}$ deposition, the longest survival time and nearly the longest preclinical incubation time among intracranially infected animals [155], supporting the above mentioned hypothesis.

When coming to validate the microarray data with RT-qPCR, the first evidence we noticed was a considerable variability among samples, both within controls and infected groups. A reason for this inhomogeneity could be that, unlike other common animal models, non-human primates involved in this study were not inbred. Therefore, different genomic backgrounds may have substantially contributed to the inter-individual variations among the animals, leading to a remarkable variation in time of disease onset [155] and in gene expression within the same group. Curiously, for some of the most strongly regulated genes (APOC1, HBB, HBA2) the variability resulted even more prominent within the control group than within infected group. One possible explanation might be that BSE infection plays such a dramatic influence on the host organism that the normal biological differences in gene expression within healthy animals are somehow flattened toward up- or down-regulation in diseased ones, as a consequence of the powerful effect of the
pathology. In addition, infected and control animals were kept in different animal facilities, leading to possible differences in diet and/or housing conditions that may have contributed to these diversities.

Concerning the orally infected sample (B6), we observed a peculiar dysregulation pattern for a number of genes, which displayed a completely opposite trend compared to intracranially infected animals (Figure 15). Even though we have no data for PrP\textsuperscript{Sc} deposition in any tissue of this animal, the significantly longer incubation period (1950 days vs 1100 days) could indicate a correlation between the gene expression profile and the route of infection [183]. Of particular interest, \textit{HBB} seemed to be expressed at a higher level in the orally infected animal compared to intracranially infected ones, both in the array platform and in RT-qPCR (FC\textasciitilde+6 in B6, FC \textasciitilde -6 in A1- A6 animals). This might give some clues on a possible protective role of hemoglobin in prion infection, which may lead to a slower progressing disease and maybe even to a milder phenotype. This model of oral transmission of BSE is particularly reliable since the old-world monkeys, besides being MM homozygous at 129 codon of \textit{PRNP}, show a digestive physiology similar to that of human beings [119, 120, 156]. Of course, we should have had some gene expression data about the preclinical phase of these monkeys in order to conclude that the higher level of \textit{HBB} found in B6 is due to the root of infection and not to a pre-existing higher expression of hemoglobin of the animal itself. Also, the different age at euthanasia should be taken into account while considering this different transcription pattern; the orally infected animal was almost 10 years old while, on average, intracranially infected animals were around 7 years old. Clearly, we are aware that one single sample is not enough to make any conclusion, however this striking difference is anyhow interesting.
Several of our DEGs were already found to be somehow associated with neurodegenerative diseases. Regarding hemoglobin (Hb), some years ago it was unexpectedly found to be expressed in mesencephalic dopaminergic neurons of different mouse strains, as well as in rats and humans. Later, its presence was also confirmed in substantia nigra (SN) of human Parkinson's disease (PD) postmortem brains and in multiple sclerosis (MS) postmortem brains, suggesting the intriguing hypothesis of a role for Hb in brain physiology and PD pathogenesis, presumably related to the respiration processes [213-215].

It has been already established that Hb expression decreases in neurons of PD, Alzheimer's disease (AD), argyrophilic grain disease (AGD) and dementia with Lewy bodies (DLB) brains [171] and that it can be regulated by both histone acetylation and CpG methylation of regulatory regions in erythroid cells [216, 217]. Furthermore, one interesting study suggests that HBB gene transcription is susceptible to (down-) regulation by histone deacetylase (HDAC) inhibition in the cortex of thricostatin (TSA)-treated mice, providing evidence for epigenetic mechanisms in the regulation of hemoglobin expression in neurons as well [218]. Substantial evidence already exists for hemoglobin and Hb-related proteins’ involvement in neurodegenerative disorders in different animal models. Hba-a1 and Hbb-y were found to be down-regulated both in the preclinical and clinical phase in the CNS of scrapie-infected mice [137, 138] confirming the importance of these genes as pre-clinical neurodegeneration markers.

Interestingly, haptoglobin (Hpt), an Hb-binding protein involved in hemoglobin catabolism, exhibited late up-regulation (45 mpi) in the medulla of orally BSE-infected cattle [27]. In addition, an antioxidant role for Hpt has been proposed in human CSF [219]. Haptoglobin precursor has been found to be up-regulated also in the medulla oblongata of symptomatic naturally infected scrapie sheep [19]. Furthermore, since
hemoglobin is the most abundant source of peripheral iron in humans, one clear implication of its dysregulation would be iron homeostasis alteration. An expanding body of evidence exists about iron dysregulation in the brain of many neurodegenerative disease affected patients: iron accumulation in AD brains [220], iron deposition in CNS and SN in PD patients [221, 222], and iron imbalance in prion disease affected brains [223]. Moreover, a possible iron-dependent translational control of human α-synuclein mRNA [224]. Also, in regards to AD, it is well known that hypoxic conditions, which may be present as a consequence of hemoglobin down-regulation, up-regulate APP mRNA and protein expression. In addition, it has been shown that Hb is able to bind to Aβ enhancing its aggregation and this in turn co-localizes in amyloid plaques in AD brains [225]. Therefore, if we envision a similar mechanism for prion diseases in which Hb interacts with β-rich PrP\textsuperscript{Sc} isoforms, we could hypothesize that down-regulated Hb fails to promote aggregation of the prion protein, determining an increased presence of toxic species such as oligomers [226]. In addition, the decrease in Hb would determine an increased amount of free iron which is highly toxic due to its ability to generate reactive oxygen species via the Fenton and Haber-Weiss reactions [227]. This released iron would be bound by ferritin, creating a redox-active and cytotoxic complex with PrP\textsuperscript{Sc}, ultimately lead to iron imbalance (deficiency phenotype) and to an increase of PrP\textsuperscript{Sc} toxic species [227], supporting the progression of the disease. This hypothesis is supported by the finding of redox-active iron in association with PrP\textsuperscript{Sc} plaques in vCJD brains [228], α-synuclein aggregates in Lewy bodies in PD brains [229] as well as an increase of total iron coupled with a paradoxical iron deficiency in CJD brains [230]. Furthermore, in PD it has been proposed that Hb may act as storage molecule for oxygen which, if necessary, could be released from oligodendrocytes to neighboring neurons in order to counteract hypoxia conditions and maintain the aerobic metabolism [213, 214].
Therefore, if down-regulated, Hb could not exert this function and neuronal functionality would be impaired by defective oxygen homeostasis.

In our study, we show a consistent down-regulation of both HBB and HBA, either in late-stage BSE-infected macaques or in post-mortem sCJD and neurodegenerative affected patients. The robustness of the assay was proved through a stringent analysis that excluded any relevant influence of potential blood contamination: small traces of erythrocyte-specific markers were inevitably detected in some samples; however, this negligible blood presence was homogeneously distributed among healthy and diseased groups. These results provide compelling evidence for a common role of Hb and iron dysregulation in neurodegeneration, possibly related to an alteration of O₂ homeostasis and oxidative metabolism [193]. These pathways seem to be shared among different diseases and species, reinforcing the emerging hypothesis of a prion-like mechanism for most—if not all—neurodegenerative disorders [55]. Additional investigations are needed to assess whether this down-regulation occurs as an early/late consequence of the disease, or if it represents a pre-existing condition that may act as a susceptibility factor for the onset of the pathology. One possible explanation for this Hb mRNA down-regulation could reside in a more general alteration of brain energy metabolism due to extensive neurodegeneration occurring in late/end stage disease, when our samples were collected. However, it is known that the specific region we selected for our analysis—superior frontal gyrus— is only moderately affected by CJD-related neurodegenerative processes. This would allow us to identify gene expression alterations which are directly related to early/moderately advanced changes occurring in CJD, and not to secondary effects of cell death occurring with disease progression.

Furthermore, given that our study was performed on whole frontal cortex tissue, we cannot discriminate if this down-regulation occurs in neurons or in glial cells.
However, as discussed previously, array data about some glial markers such as GFAP and CD68 does not suggest any relevant deregulation in this cell population. One other molecule that appears to be central in neurodegeneration, APOC1, was found to be significantly up-regulated in BSE-infected macaques as well in iCJD and sCJD patients. APOC1 is part of the APOE/C-I/C-IV/C-II gene cluster and codes for apolipoprotein C-I (apoC-I), a small 6.6 kDa apolipoprotein component of lipoproteins (mainly HDL) that is known to inhibit receptor-mediated lipoprotein clearance, in particular particles containing apoE, via direct blockade of the low density lipoprotein (LDL) and VLDL receptors and LDL receptor-related protein [231]. Increasing evidence indicates a role for this gene in neurodegenerative disorders, especially AD and MS. APOC1 has been previously found to be in linkage disequilibrium with APOE 4 in AD [232]. In particular, the H2 allele of APOC1 is a susceptibility factor for LOAD [233] and its mRNA was found to be down-regulated in the frontal cortex – similar to what we found in our NEG samples- especially in H2-allelic individuals [208]. Moreover, its AA genotype has been shown to determine a younger age-of-onset in African American female multiple sclerosis patients, together with a higher progression index and MS severity score [234]. Impairment in lipid metabolism and signaling is one of the early alterations present in many neurodegenerative diseases, including prion diseases [210, 235]. Several studies focus on cholesterol metabolites with the aim of identifying early biomarkers for neurodegenerative disorders [236-238]. A number of genes involved in cholesterol metabolism and lipid biosynthesis have been found to be up-regulated in preclinical scrapie-infected mice [201]. ApoCI, together with ApoE and ApoAI, able to activate cholesterol esterification via lecithin-cholesterol acyltransferase (LCAT) [239], a pathway that contributes to excessive cholesterol elimination from the brain [219]. Indeed, brain cholesterol is mostly converted into an oxysterol (24S-hydroxycholesterol) that can freely diffuse to blood
but, upon esterification, it can also be exported to the liver embedded within lipoproteins throughout circulation [240]. Under normal conditions, this second pathway could be of minor relevance in regulating the efflux of cholesterol from the brain [241], but it could play an important role in triggering the increase of cholesterol accumulation – and particularly the consequent increase of the neurotoxic oxysterol– within neuronal cells upon prion infection [207]. If we add to this scenario the fact that Hpt, an above-mentioned hemoglobin-binding protein, has shown to be able to bind also ApoE and ApoAI [219] and that ApoAI has been found to interact with ApoCl [242], we can try to put together in the same pathway both the up-regulation of APOC1 and the down-regulation of Hb. As already mentioned, it is known that prion infection reduces cholesterol efflux from neuronal cells leading to an increase in cellular cholesterol [207, 236], the main component of lipid rafts where PrP\(^C\) conversion into PrP\(^Sc\) is thought to occur [243, 244]. With this mechanism, prions might promote an increase of cellular cholesterol content in order to have more favorable environment to allow/enhance their conversion [207]. This increase in cholesterol would lead to a simultaneous activation of Hpt that in case of oxidative stress -presumably triggered by increased oxysterol- is expected to work in the CNS [245] preventing oxidative injuries to ApoE and ApoAI, thus saving their esterification function [246]. This Hpt-ApoAI complex could in turn stimulate ApoCl, further raising the esterification of cholesterol in order to increment its displacement from the brain toward the liver, where cholesterol is then catabolized into bile acids [219]. In addition, to increase Hpt available sites to bind apoliproteins, Hb is down-regulated (or due to a negative feedback because Hpt binds less Hb and therefore free Hb levels increase). This decrease in Hb would result in an increase in free iron that, upon binding to ferritin, stimulates the formation of a toxic ferritin-PrP\(^Sc\) complex which ultimately leads to iron deficiency and increase in PrP\(^Sc\) toxic species [227].
Consistent with this hypothesis, some *in vitro* studies have shown that depletion of cellular cholesterol reduces the conversion of PrP<sup>C</sup> to PrP<sup>Sc</sup> [243]. Taken together, these data suggest that both Hb and ApoCI could represent extremely powerful drug targets as well as potentially useful biomarkers to assess the efficacy of possible therapies.

Another gene that we found highly up-regulated in BSE-infected monkeys—as well as in vCJD, iCJD and sCJD patients—is SERPINA3, a serpin peptidase inhibitor extensively reported to be regulated in several neurodegenerative disease models. The encoded protein is involved in acute phase response pathways and it is well known for its interaction with APP in promoting amyloid plaque formation—a hallmark of AD [247]. Indeed, increased levels of SERPINA3 protein have been found in the brain and peripheral blood of AD patients [248], mainly due to persistent inflammation [249]. Furthermore, a polymorphism in its gene promoter has been identified as a disease modifier both for AD and PD: the TT genotype resulted as a risk factor for EOAD [250], while the AA genotype has been suggested to play a modest protective role against early-onset PD in women, especially in the Taiwanese population [251]. In prion disease models, SERPINA3 was increased in urine and cerebrospinal fluid of CJD patients and also in brains of scrapie-infected mice [235, 252]. Moreover, it is up-regulated after 10 weeks post inoculation (prior to clinical sign manifestation) in mice infected with RML prion strain, probably due to activation of astrocytes and microglia [253]. Being an acute phase protein, its up-regulation is generally explained by the onset of an inflammation condition, particularly as a response of the innate immunity to infection [254]. However, inflammation and immune system activation seem not to be a hallmark of prion diseases themselves, and is more likely an eventual consequence of massive late-stage neuronal degeneration [209]; therefore, an alternative role for SERPINA3 in prion disorder
should be considered. Of particular interest, two β-sheets of SERPINA3 exhibit a polymorphism mimicking changes in the serpin structure that normally occur during the formation of its stable complex with the target proteinase. In this conformation, SERPINA3 can bind Aβ, thus imposing a β-strand conformation that upon dissociation leads to a faster formation of fibrils [247]. Therefore, an intriguing hypothesis may be proposed in which PrP conversion into β-sheet conformation can be assisted by the “chaperone” SERPINA3, which would then accelerate the formation of toxic species like PrP oligomers. In addition, SERPINA3 expression in the brain can be regulated in vivo by ApoE [255]. Also, some in vitro studies indicated that SERPINA3 is able to bind cholesterol [256]. Therefore we hypothesize that abnormal cholesterol levels might trigger SERPINA3 up-regulation as a supplementary reinforcement for cholesterol binding and transport when ApoE and other lipoproteins are saturated and therefore no longer available. Nevertheless, due to its presumable capability to bind β-sheet enriched PrPSc, the SERPINA3 increase would lead to a sustained conversion of the prion protein, therefore supporting the disease progression. Despite SERPINA3 protein has been found up-regulated in several neurodegenerative disease, in addition to CJD, we did not observe up-regulation at the mRNA level in non-CJD affected patients. Therefore, we suggest that transcriptional regulation is a unique mechanism occurring in CJD disorder and that, as well as APOC1, represents a specific prion disease marker that could be considered as a potential diagnostic and therapeutic tool.

In the same pathway of acute phase response, also the transthyretin gene was up-regulated in our BSE-infected macaques, even though we were not able to confirm the statistical significance with the more specific TaqMan assay. Nevertheless, we found similar results also in human samples; indeed, both sCJD and especially iCJD patients showed an upregulation for TTR. TTR is a prealbumin, best known as a
carrier of the thyroid hormone thyroxine (T4) in serum and CSF. Also, when associated with retinol-binding protein (RBP) in the serum, it can transport retinol as well. TTR is associated with systemic amyloidosis characterized by deposition of aggregated protein in various tissues, but it has been also associated with an anti-amyloidogenic effect in regard to Aβ. Like SERPINA3, TTR binds to beta amyloid, but in this case the binding seems to prevent Aβ deposition. In fact, TTR not only inhibits Aβ fibril formation, but also neutralizes the toxicity of pre-formed oligomers by promoting their further assembly into larger aggregates, as shown in neuroblastoma cells and primary rat neurons [257]. Moreover, increased mRNA and protein levels have been reported in neurons from the AD mouse model ‘APP23’ and in human AD brain. This suggests that TTR transcription and synthesis may be up-regulated and sustained by the increased production of Aβ or its precursors. The subsequent interaction between TTR and Aβ and the formation of TTR- Aβ complexes with reduction of toxic aggregates may be a protective mechanism in response to AD [258]. Furthermore, silencing the endogenous TTR gene accelerated the disease pathogenesis in the well validated AD mouse model APP23, and TTR overexpression suppressed the behavioral symptoms of AD [76], confirming the neuroprotective role of this protein. Even in prion models TTR levels have been found strongly increased in the cortex of scrapie-infected mice [139]. Our study now provides evidence that upregulation of TTR is also found in BSE-infected macaques and CJD affected patients, further expanding the view that this is an important player in human neurodegeneration. If we hypothesize a common mechanism in AD and TSEs, we can speculate a possible up-regulation of TTR, promoted by the presence of PrP toxic oligomeric species, as a “protective” mechanism that would facilitate the formation of larger non-toxic PrP aggregates. Taken together, the data on SERPINA3 and TTR suggest an implication of innate immune system activation and
inflammatory response, as suggested by some authors [259, 260]. Fratini et al. also reported a clear increase of seven proteins all belonging to the acute phase response processes in a study of plasma samples from sporadic CJD patients [261]. This suggests not only a localized inflammatory condition at brain level, but also a possible generalized systemic inflammation in sCJD. However, analysis of our microarray data did not reveal any relevant deregulation of other genes typically involved in neuroinflammation and/or immune response. Indeed, cytokines and other mediators such as IL6, TNFα, GFAP and CD68 showed FC < |2|, indicating that inflammatory responses seem not be of particular relevance in this prion model or in the area of the CNS that was examined. However, since we found a trend of down-regulation of TTR in non-prion neurodegenerative diseases -for which involvement of inflammation has been proposed [247, 262, 263]- we can consider that the TTR dysregulation in this case may be actually related to activation of inflammatory responses. It is known that as soon as the inflammation process starts, the synthesis of the negative acute phase proteins, such as TTR, decrease in order to provide more amino acids for the synthesis of positive acute phase ones. Nevertheless, we could suggest a different explanation for TTR involvement in neurodegeneration that regards, again, cholesterol. It has been reported that TTR is able to bind membrane lipids via electrostatic interactions [264] and this binding appears to be influenced by the concentration of cholesterol and the lipid composition of the membrane. This membrane binding is also correlated with the cytotoxic effect of TTR oligomers [265]. Therefore, we could hypothesize that TTR in neurodegenerative diseases behaves to some extent similarily to PrP in prion disorders, binding preferentially to lipid rafts in the plasma membrane where it promotes the formation of toxic oligomers that ultimately lead to neuronal degeneration.
Concerning the other genes we analyzed, microarray and RT-qPCR platforms indicated a concordant expression trend. Some of these genes, e.g. NR4A2 and IRF3, have been previously reported in association with prion infection and other neurodegenerative disorders. Our data showed down-regulation for NR4A2 and slight up-regulation for IRF3 and, even though we did not obtain statistically significant results, they are in line with the findings of these reports [144, 185, 266-268].

One further interesting point that remains to be elucidated regards the expression of the prion protein gene itself in the different diseases models we studied. In literature, some contradictory results are present; indeed, while some studies reported no PRNP alterations during BSE infection in cattle [191], in a recent work Llorens and colleagues found that sCJD MM1 patients displayed a significant slight decrease in PRNP mRNA levels which was not found in sCJD VV2 patients [259]. Due to shortage of macaque cDNA, we were not able to investigate PRNP levels in BSE-infected samples; nevertheless, our microarray data suggested that, at least at the mRNA level, no changes occur between control and infected animals. However, in humans we were able to analyze PRNP mRNA levels across the different groups. Also in this case, no significant alterations were observed between controls and CJD affected patients. Given that genetic analysis was available only for a limited number of the analyzed patients, we were not able to discriminate the influence of the codon 129 genotype and/or the PrPSc type on the PRNP mRNA levels. However, we were able to highlight a difference between the two acquired CJD groups. Indeed, vCJD patients showed a significantly higher level of PRNP mRNA levels in comparison with iCJD ones. In light of these findings, we could hypothesize that the route of infection might somehow influence the mRNA levels of PrP. As regards cellular prion protein, its levels have been investigated little due to the concurrent presence of PrPSc, which
is often present in molar excess and complicates the analysis. Reduced levels of total prion protein has been detected in the CSF of a number of neurodegenerative diseases, including CJD, AD, DLB, PD and MS [269]. However, by using velocity gradient centrifugation and CDI, Safar and colleagues recently highlighted a down-regulation of PrP<sup>C</sup> in end stage WT mice infected with scrapie, CJD and CWD prion strains. They also found this down-regulation in preclinical WT mice infected with RML, suggesting the occurrence of proteostasis as a host protective response triggered by the presence of PrP<sup>Sc</sup> [270].

Summarizing, our results suggest that while hemoglobin seems to be involved in unspecific pathways shared among many different neurodegenerative diseases, APOC1, TTR and particularly SERPINA3 seem to represent specific CJD alterations. Interestingly, for TTR, APOC1 and PRNP we observed a significantly different behavior between vCJD and iCJD patients, indicating that the route of infection plays a major role in the regulation of these genes and presumably also of the related pathways.

One other main consequence of these results is that these genes, in particular APOC1 and SERPINA3, upon further validation, could be exploited as potential biomarkers for both diagnostic purposes and also as promising targets for therapy. All together, these data strongly suggest the crucial role of impaired cholesterol metabolism in prion disease onset and progression, as well as iron imbalance in general neurodegeneration processes.

**CONCLUSIONS**

As far as we know, this is the first genome-wide expression study in the frontal cortex of cynomolgus macaques inoculated with BSE. In our work we used both microarray
and RT-qPCR technologies that allowed us to identify a gene signature able to distinguish BSE-infected macaques from control animals. The identified genes are involved in oxygen transport and iron metabolism (\textit{HBB, HBA2}), cholesterol metabolism and lipid transport (\textit{APOC1, SERPINA3}) as well as acute phase response (\textit{SERPINA3, TTR}).

Importantly, the dysregulation of four of these genes (\textit{HBA2, APOC1, TTR, SERPINA3}) have been validated with consistent FC values also in CJD affected human samples, confirming the reliability of our previous analysis in BSE-infected monkeys and providing important hints on some prion-specific alterations in CJD disease. These results could be extremely helpful in understanding the mechanism underlying the progression of the disease, possibly leading to the identification of some key players which, even if not the cause of onset, could be some of the target genes affected by the disease. In addition, the two genes related to hemoglobin were found similarly (down-)regulated also in non-CJD neurodegenerative disorders, shedding some light on common pathways underlying neurodegeneration. Therefore, some of our findings support the hypothesis of a potential shared mechanism underlying the onset and the development of all neurodegenerative disorders. This is in concordance with very recent data supporting the idea of a unifying role of prions in these diseases in general and maybe a prion-like behavior for most neurodegenerative disorder [68].

Moreover, all the DEGs we validated were also expressed also in blood. It is reasonable to believe that the genome is equivalent in sequence and structure in all cells and tissues of the same organism [271]. Indeed, in most instances the genome observed in peripheral blood is highly similar to that in the brain – with an overlapping of about 70-80% [272-274]-, allowing for investigation of transcriptional alterations in blood that would reflect brain disorders. Therefore, validating their consistent
dysregulation in blood, would open the interesting possibility to employ these transcripts as "readily available" biomarkers for both diagnostic and therapeutic purposes. Even more important, this could allow to investigate the gene expression pattern of these biomarkers also in earlier stages of the disease, providing an invaluable insight about the pathogenic mechanism underlying the onset of these disorders and, therefore, also a potential tool for early diagnosis and eventual therapy monitoring.

In conclusion, our data suggest that, in order to identify diagnostic and/or therapeutic approaches for prion diseases and other neurodegenerative disorders, a combination of various pathways has to be targeted, including oxygen and iron homeostasis, inflammation response and in particular cholesterol and lipid metabolism.
## APPENDIX

<table>
<thead>
<tr>
<th>Probe ID</th>
<th>Gene Symbol</th>
<th>Gene name</th>
<th>RefSeq Transcript ID</th>
<th>P-value</th>
<th>FC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mmu10512.1.1_s_at</td>
<td>—</td>
<td>—</td>
<td>1.47E-09</td>
<td>394.064</td>
</tr>
<tr>
<td>2</td>
<td>MmSTS-2122.1.1_s_at</td>
<td>LOC578106</td>
<td>alpha-f-antichymotrypsin</td>
<td>XM_001106847</td>
<td>2.76E-02</td>
</tr>
<tr>
<td>3</td>
<td>Mmu10512.1.1_s_at</td>
<td>LOC578106</td>
<td>alpha-f-antichymotrypsin</td>
<td>XM_001106847</td>
<td>4.36E-03</td>
</tr>
<tr>
<td>4</td>
<td>MmSTS-1188.1.1_s_at</td>
<td>LOC703401</td>
<td>similar to rhodanese-like 1</td>
<td>XM_001105376</td>
<td>2.44E-03</td>
</tr>
<tr>
<td>5</td>
<td>MmmuNA-20462.1.1_s_at</td>
<td>—</td>
<td>—</td>
<td>2.64E-04</td>
<td>3.820</td>
</tr>
<tr>
<td>6</td>
<td>MmmuNA-31051.1.1_s_at</td>
<td>—</td>
<td>—</td>
<td>1.06E-05</td>
<td>3.677</td>
</tr>
<tr>
<td>7</td>
<td>MmmuNA-21071.1.1_s_at</td>
<td>—</td>
<td>—</td>
<td>3.76E-03</td>
<td>3.385</td>
</tr>
<tr>
<td>8</td>
<td>MmmuNA-16064.1.1_s_at</td>
<td>—</td>
<td>—</td>
<td>3.17E-04</td>
<td>3.196</td>
</tr>
<tr>
<td>9</td>
<td>MmmuNA-23711.1.1_s_at</td>
<td>PROSC</td>
<td>Protein synthesis ce-1, transcript variant</td>
<td>XM_001109837</td>
<td>7.66E-04</td>
</tr>
<tr>
<td>10</td>
<td>MmmuNA-10792.1.1_s_at</td>
<td>—</td>
<td>—</td>
<td>1.26E-03</td>
<td>2.949</td>
</tr>
<tr>
<td>11</td>
<td>MmmuNA-2891.1.1_s_at</td>
<td>MARK4AG</td>
<td>major histocompatibility complex, class I, AG</td>
<td>XM_001134230</td>
<td>3.06E-04</td>
</tr>
<tr>
<td>12</td>
<td>MmmuNA-6291.1.1_s_at</td>
<td>—</td>
<td>—</td>
<td>2.62E-03</td>
<td>2.917</td>
</tr>
<tr>
<td>13</td>
<td>MmSTS-1104.1.1_s_at</td>
<td>LAIR88</td>
<td>Leukocyte immunoglobulin-like receptor, suddnly R, member b</td>
<td>XM_001088664</td>
<td>6.20E-04</td>
</tr>
<tr>
<td>14</td>
<td>MmmuNA-10586.1.1_s_at</td>
<td>PAPRA</td>
<td>papain, prionglucylase-like subfamily, papain-like</td>
<td>XM_001384580</td>
<td>1.16E-03</td>
</tr>
<tr>
<td>15</td>
<td>MmmuNA-26076.1.1_at</td>
<td>—</td>
<td>—</td>
<td>2.06E-03</td>
<td>2.369</td>
</tr>
<tr>
<td>16</td>
<td>MmSTS-242.1.1_s_at</td>
<td>LOC716524</td>
<td>hypothetical protein LOC716524</td>
<td>XM_001088170</td>
<td>4.61E-04</td>
</tr>
<tr>
<td>17</td>
<td>MmmuNA-26086.1.1_at</td>
<td>—</td>
<td>—</td>
<td>4.07E-03</td>
<td>2.260</td>
</tr>
<tr>
<td>18</td>
<td>MmmuNA-15806.1.1_at</td>
<td>—</td>
<td>—</td>
<td>1.67E-03</td>
<td>2.244</td>
</tr>
<tr>
<td>19</td>
<td>MmmuNA-15851.1.1_at</td>
<td>—</td>
<td>—</td>
<td>5.16E-04</td>
<td>2.161</td>
</tr>
<tr>
<td>20</td>
<td>MmmuNA-56517.1.1_at</td>
<td>LOC686207</td>
<td>similar to rhodanese-like 2</td>
<td>XM_001109405</td>
<td>2.36E-03</td>
</tr>
<tr>
<td>21</td>
<td>MmmuNA-42711.1.1_s_at</td>
<td>LOC709349</td>
<td>hypothetical protein LOC709349</td>
<td>XM_001108640</td>
<td>9.04E-04</td>
</tr>
<tr>
<td>22</td>
<td>MmmuNA-30481.1.1_at</td>
<td>LOC712270</td>
<td>similar to TACE domain family, member 1C</td>
<td>XM_001083607</td>
<td>2.28E-04</td>
</tr>
<tr>
<td>23</td>
<td>MmmuNA-54869.1.1_at</td>
<td>IRF3</td>
<td>interferon regulatory factor 3</td>
<td>XM_001135797</td>
<td>3.38E-04</td>
</tr>
<tr>
<td>24</td>
<td>MmmuNA-69017.1.1_at</td>
<td>FLJ48941</td>
<td>hypothetical protein LOC716524</td>
<td>XM_001088358</td>
<td>1.12E-03</td>
</tr>
<tr>
<td>25</td>
<td>MmmuNA-20661.1.1_at</td>
<td>—</td>
<td>—</td>
<td>2.33E-03</td>
<td>2.079</td>
</tr>
<tr>
<td>26</td>
<td>MmmuNA-30540.1.1_at</td>
<td>—</td>
<td>—</td>
<td>2.92E-03</td>
<td>2.073</td>
</tr>
<tr>
<td>27</td>
<td>MmmuNA-15191.1.1_at</td>
<td>—</td>
<td>—</td>
<td>8.05E-04</td>
<td>2.098</td>
</tr>
<tr>
<td>28</td>
<td>MmmuNA-48671.1.1_at</td>
<td>LOC521957</td>
<td>similar to transcriptional repressor LT34</td>
<td>XM_001135799</td>
<td>1.23E-02</td>
</tr>
<tr>
<td>29</td>
<td>MmmuNA-26042.1.1_at</td>
<td>CSNK1G3</td>
<td>casein kinase 1, gamma 3</td>
<td>XM_001083839</td>
<td>4.27E-03</td>
</tr>
<tr>
<td>30</td>
<td>MmmuNA-24218.1.1_at</td>
<td>LOC703401</td>
<td>hypothetical protein LOC716524</td>
<td>XM_001109832</td>
<td>9.92E-04</td>
</tr>
<tr>
<td>31</td>
<td>MmmuNA-27791.1.1_at</td>
<td>LOC703401</td>
<td>hypothetical protein LOC716524</td>
<td>XM_001109832</td>
<td>4.95E-03</td>
</tr>
<tr>
<td>32</td>
<td>MmmuNA-20292.1.1_at</td>
<td>LOC521957</td>
<td>casein kinase 1, gamma 3</td>
<td>XM_001083839</td>
<td>1.26E-04</td>
</tr>
<tr>
<td>33</td>
<td>MmmuNA-22221.1.1_at</td>
<td>—</td>
<td>—</td>
<td>2.52E-03</td>
<td>-2.068</td>
</tr>
<tr>
<td>34</td>
<td>MmmuNA-30667.1.1_at</td>
<td>ACVR1C</td>
<td>activin A receptor, type IC</td>
<td>XM_001086925</td>
<td>4.64E-03</td>
</tr>
<tr>
<td>35</td>
<td>MmmuNA-34812.1.1_at</td>
<td>LOC711550</td>
<td>similar to GDP-Galactose:peptidoglycan N-acetylglucosaminyltransferase, peptidoglycan</td>
<td>XM_001101991</td>
<td>2.64E-03</td>
</tr>
<tr>
<td>36</td>
<td>MmmuNA-24846.1.1_at</td>
<td>TNAK4A</td>
<td>Thrombopoietin-like 4A</td>
<td>XM_001089626</td>
<td>3.07E-03</td>
</tr>
<tr>
<td>37</td>
<td>MmmuNA-71431.1.1_at</td>
<td>—</td>
<td>—</td>
<td>4.25E-03</td>
<td>-2.163</td>
</tr>
<tr>
<td>38</td>
<td>MmmuNA-67410.1.1_at</td>
<td>—</td>
<td>—</td>
<td>3.45E-04</td>
<td>-2.163</td>
</tr>
<tr>
<td>39</td>
<td>MmmuNA-61360.1.1_at</td>
<td>FLVCR</td>
<td>feline leukemia virus subgroup C cellular receptor</td>
<td>XM_001107014</td>
<td>5.05E-04</td>
</tr>
<tr>
<td>40</td>
<td>MmmuNA-65277.1.1_at</td>
<td>—</td>
<td>—</td>
<td>1.16E-03</td>
<td>-2.188</td>
</tr>
<tr>
<td>41</td>
<td>MmmuNA-26064.1.1_at</td>
<td>KPNMB</td>
<td>kynureninase alpha 6</td>
<td>XM_001101084</td>
<td>5.16E-04</td>
</tr>
<tr>
<td>42</td>
<td>MmmuNA-30671.1.1_at</td>
<td>TMSF3</td>
<td>transmembrane 7 superfamily member 3</td>
<td>XM_001088280</td>
<td>4.76E-04</td>
</tr>
<tr>
<td>43</td>
<td>MmmuNA-9334.1.1_at</td>
<td>—</td>
<td>—</td>
<td>2.34E-03</td>
<td>-2.217</td>
</tr>
<tr>
<td>44</td>
<td>MmmuNA-23662.1.1_at</td>
<td>—</td>
<td>—</td>
<td>2.08E-03</td>
<td>-2.218</td>
</tr>
<tr>
<td>45</td>
<td>MmmuNA-61791.1.1_at</td>
<td>—</td>
<td>—</td>
<td>1.41E-03</td>
<td>-2.228</td>
</tr>
</tbody>
</table>
Additional table 1. List of 86 differentially expressed probe sets with p values ≤ 0.005 and FC ≥ 2. Probe ID, Gene Symbol, Gene Name and RefSeq Transcript IDs annotation as of release 29 of the Affymetrix® Rhesus Annotation library (01/July/09). P-values and fold changes are reported for all 86 probe sets.
<table>
<thead>
<tr>
<th>Probe ID</th>
<th>Previous gene symbol</th>
<th>Current gene symbol</th>
<th>Gene name</th>
<th>RefSeq Transcript ID</th>
<th>P-value</th>
<th>FC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mmu.78.1.51_x_at</td>
<td>MT-CYP3A01</td>
<td>—</td>
<td>MT-147A1-588B(1)</td>
<td>2.7E-6</td>
<td>23.15</td>
</tr>
<tr>
<td>2</td>
<td>Mmu.04A.1.51_x_at</td>
<td>NCIH6</td>
<td>NCIH6</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>3</td>
<td>Mmu.03A.1.51_x_at</td>
<td>GLT12OS</td>
<td>NCIH6</td>
<td>—</td>
<td>—</td>
<td>9.2E-3</td>
</tr>
<tr>
<td>4</td>
<td>Mmu.03A.1.51_x_at</td>
<td>SLC35A1</td>
<td>NCIH6</td>
<td>—</td>
<td>—</td>
<td>5.4E-3</td>
</tr>
<tr>
<td>5</td>
<td>Mmu.132.1.51_x_at</td>
<td>GRM7</td>
<td>NCIH6</td>
<td>—</td>
<td>—</td>
<td>3.1E-3</td>
</tr>
<tr>
<td>6</td>
<td>Mmu.04A.1.51_x_at</td>
<td>GATN1</td>
<td>NCIH6</td>
<td>—</td>
<td>—</td>
<td>9.6E-3</td>
</tr>
<tr>
<td>7</td>
<td>Mmu.uti.944.1.51_x</td>
<td>TNNT2</td>
<td>NCIH6</td>
<td>—</td>
<td>—</td>
<td>4.4E-3</td>
</tr>
<tr>
<td>8</td>
<td>Mmu.2091.1.51_x_at</td>
<td>MARCKS-A1</td>
<td>NCIH6</td>
<td>—</td>
<td>—</td>
<td>9.6E-3</td>
</tr>
<tr>
<td>9</td>
<td>Mmu.130.1.51_x_at</td>
<td>RPL22A</td>
<td>NCIH6</td>
<td>—</td>
<td>—</td>
<td>3.8E-3</td>
</tr>
<tr>
<td>10</td>
<td>Mmu.130.1.51_x_at</td>
<td>RPL22A</td>
<td>NCIH6</td>
<td>—</td>
<td>—</td>
<td>3.8E-3</td>
</tr>
<tr>
<td>11</td>
<td>Mmu.uti.944.1.51_x</td>
<td>TNNT2</td>
<td>RPL22A</td>
<td>—</td>
<td>—</td>
<td>3.8E-3</td>
</tr>
<tr>
<td>12</td>
<td>Mmu.uti.944.1.51_x</td>
<td>TNNT2</td>
<td>RPL22A</td>
<td>—</td>
<td>—</td>
<td>3.8E-3</td>
</tr>
<tr>
<td>13</td>
<td>Mmu.130.1.51_x_at</td>
<td>MARCKS-A1</td>
<td>RPL22A</td>
<td>—</td>
<td>—</td>
<td>3.8E-3</td>
</tr>
<tr>
<td>14</td>
<td>Mmu.130.1.51_x_at</td>
<td>MARCKS-A1</td>
<td>RPL22A</td>
<td>—</td>
<td>—</td>
<td>3.8E-3</td>
</tr>
<tr>
<td>15</td>
<td>Mmu.uti.944.1.51_x</td>
<td>TNNT2</td>
<td>RPL22A</td>
<td>—</td>
<td>—</td>
<td>3.8E-3</td>
</tr>
<tr>
<td>16</td>
<td>Mmu.uti.944.1.51_x</td>
<td>TNNT2</td>
<td>RPL22A</td>
<td>—</td>
<td>—</td>
<td>3.8E-3</td>
</tr>
<tr>
<td>17</td>
<td>Mmu.uti.944.1.51_x</td>
<td>TNNT2</td>
<td>RPL22A</td>
<td>—</td>
<td>—</td>
<td>3.8E-3</td>
</tr>
<tr>
<td>18</td>
<td>Mmu.uti.944.1.51_x</td>
<td>TNNT2</td>
<td>RPL22A</td>
<td>—</td>
<td>—</td>
<td>3.8E-3</td>
</tr>
<tr>
<td>19</td>
<td>Mmu.uti.944.1.51_x</td>
<td>TNNT2</td>
<td>RPL22A</td>
<td>—</td>
<td>—</td>
<td>3.8E-3</td>
</tr>
<tr>
<td>20</td>
<td>Mmu.uti.944.1.51_x</td>
<td>TNNT2</td>
<td>RPL22A</td>
<td>—</td>
<td>—</td>
<td>3.8E-3</td>
</tr>
<tr>
<td>21</td>
<td>Mmu.uti.944.1.51_x</td>
<td>TNNT2</td>
<td>RPL22A</td>
<td>—</td>
<td>—</td>
<td>3.8E-3</td>
</tr>
<tr>
<td>22</td>
<td>Mmu.uti.944.1.51_x</td>
<td>TNNT2</td>
<td>RPL22A</td>
<td>—</td>
<td>—</td>
<td>3.8E-3</td>
</tr>
<tr>
<td>23</td>
<td>Mmu.uti.944.1.51_x</td>
<td>TNNT2</td>
<td>RPL22A</td>
<td>—</td>
<td>—</td>
<td>3.8E-3</td>
</tr>
<tr>
<td>24</td>
<td>Mmu.uti.944.1.51_x</td>
<td>TNNT2</td>
<td>RPL22A</td>
<td>—</td>
<td>—</td>
<td>3.8E-3</td>
</tr>
<tr>
<td>25</td>
<td>Mmu.uti.944.1.51_x</td>
<td>TNNT2</td>
<td>RPL22A</td>
<td>—</td>
<td>—</td>
<td>3.8E-3</td>
</tr>
<tr>
<td>26</td>
<td>Mmu.uti.944.1.51_x</td>
<td>TNNT2</td>
<td>RPL22A</td>
<td>—</td>
<td>—</td>
<td>3.8E-3</td>
</tr>
<tr>
<td>27</td>
<td>Mmu.uti.944.1.51_x</td>
<td>TNNT2</td>
<td>RPL22A</td>
<td>—</td>
<td>—</td>
<td>3.8E-3</td>
</tr>
<tr>
<td>28</td>
<td>Mmu.uti.944.1.51_x</td>
<td>TNNT2</td>
<td>RPL22A</td>
<td>—</td>
<td>—</td>
<td>3.8E-3</td>
</tr>
<tr>
<td>29</td>
<td>Mmu.uti.944.1.51_x</td>
<td>TNNT2</td>
<td>RPL22A</td>
<td>—</td>
<td>—</td>
<td>3.8E-3</td>
</tr>
<tr>
<td>30</td>
<td>Mmu.uti.944.1.51_x</td>
<td>TNNT2</td>
<td>RPL22A</td>
<td>—</td>
<td>—</td>
<td>3.8E-3</td>
</tr>
<tr>
<td>31</td>
<td>Mmu.uti.944.1.51_x</td>
<td>TNNT2</td>
<td>RPL22A</td>
<td>—</td>
<td>—</td>
<td>3.8E-3</td>
</tr>
<tr>
<td>32</td>
<td>Mmu.uti.944.1.51_x</td>
<td>TNNT2</td>
<td>RPL22A</td>
<td>—</td>
<td>—</td>
<td>3.8E-3</td>
</tr>
<tr>
<td>33</td>
<td>Mmu.uti.944.1.51_x</td>
<td>TNNT2</td>
<td>RPL22A</td>
<td>—</td>
<td>—</td>
<td>3.8E-3</td>
</tr>
<tr>
<td>34</td>
<td>Mmu.uti.944.1.51_x</td>
<td>TNNT2</td>
<td>RPL22A</td>
<td>—</td>
<td>—</td>
<td>3.8E-3</td>
</tr>
<tr>
<td>35</td>
<td>Mmu.uti.944.1.51_x</td>
<td>TNNT2</td>
<td>RPL22A</td>
<td>—</td>
<td>—</td>
<td>3.8E-3</td>
</tr>
<tr>
<td>36</td>
<td>Mmu.uti.944.1.51_x</td>
<td>TNNT2</td>
<td>RPL22A</td>
<td>—</td>
<td>—</td>
<td>3.8E-3</td>
</tr>
<tr>
<td>37</td>
<td>Mmu.uti.944.1.51_x</td>
<td>TNNT2</td>
<td>RPL22A</td>
<td>—</td>
<td>—</td>
<td>3.8E-3</td>
</tr>
<tr>
<td>38</td>
<td>Mmu.uti.944.1.51_x</td>
<td>TNNT2</td>
<td>RPL22A</td>
<td>—</td>
<td>—</td>
<td>3.8E-3</td>
</tr>
<tr>
<td>39</td>
<td>Mmu.uti.944.1.51_x</td>
<td>TNNT2</td>
<td>RPL22A</td>
<td>—</td>
<td>—</td>
<td>3.8E-3</td>
</tr>
<tr>
<td>40</td>
<td>Mmu.uti.944.1.51_x</td>
<td>TNNT2</td>
<td>RPL22A</td>
<td>—</td>
<td>—</td>
<td>3.8E-3</td>
</tr>
<tr>
<td>41</td>
<td>Mmu.uti.944.1.51_x</td>
<td>TNNT2</td>
<td>RPL22A</td>
<td>—</td>
<td>—</td>
<td>3.8E-3</td>
</tr>
<tr>
<td>42</td>
<td>Mmu.uti.944.1.51_x</td>
<td>TNNT2</td>
<td>RPL22A</td>
<td>—</td>
<td>—</td>
<td>3.8E-3</td>
</tr>
<tr>
<td>43</td>
<td>Mmu.uti.944.1.51_x</td>
<td>TNNT2</td>
<td>RPL22A</td>
<td>—</td>
<td>—</td>
<td>3.8E-3</td>
</tr>
<tr>
<td>44</td>
<td>Mmu.uti.944.1.51_x</td>
<td>TNNT2</td>
<td>RPL22A</td>
<td>—</td>
<td>—</td>
<td>3.8E-3</td>
</tr>
<tr>
<td>45</td>
<td>Mmu.uti.944.1.51_x</td>
<td>TNNT2</td>
<td>RPL22A</td>
<td>—</td>
<td>—</td>
<td>3.8E-3</td>
</tr>
<tr>
<td>46</td>
<td>Mmu.uti.944.1.51_x</td>
<td>TNNT2</td>
<td>RPL22A</td>
<td>—</td>
<td>—</td>
<td>3.8E-3</td>
</tr>
<tr>
<td>47</td>
<td>Mmu.uti.944.1.51_x</td>
<td>TNNT2</td>
<td>RPL22A</td>
<td>—</td>
<td>—</td>
<td>3.8E-3</td>
</tr>
<tr>
<td>48</td>
<td>Mmu.uti.944.1.51_x</td>
<td>TNNT2</td>
<td>RPL22A</td>
<td>—</td>
<td>—</td>
<td>3.8E-3</td>
</tr>
<tr>
<td>49</td>
<td>Mmu.uti.944.1.51_x</td>
<td>TNNT2</td>
<td>RPL22A</td>
<td>—</td>
<td>—</td>
<td>3.8E-3</td>
</tr>
</tbody>
</table>
Additional table 2. List of 97 differently expressed probe sets selected as RT-qPCR candidates. Probe IDs and Previous Gene Symbol annotation as of release 29 of the Affymetrix Rhesus Annotation library (01/July/09). Current Gene Symbol annotation as of the latest Affymetrix®
Rhesus Annotation library (release 32 - 09/June/11). Gene Name and RefSeq Transcript IDs as of Ensembl release 72 (June 2013). Annotation using alignment with the human genome has been performed (as stated in the gene name column) for the most highly regulated probe sets with unknown macaque annotation. P-values and fold changes are reported for all genes.
Bibliography


ACKNOWLEDGEMENTS

In these four years spent as PhD student in SISSA, I had the great opportunity of working in the Prion Biology Lab of Giuseppe Legname. I am really grateful to him for having introduced me to the world of prions with all its fascinating aspect of molecular and cellular biology, which has been the source of my motivation over the years. The encouragement, reassurance and the active support I received from Prof. Legname have been important in establishing my confidence and persistency in carrying out my research work. Grazie GL!!

I would like to express my sincere thanks to Dr. Maura Barbisin who has worked with me step-by-step in these years. Her knowledge, patience and attention to the even smallest details have been an invaluable help in addressing every practical and technical issue of this work. The results of this thesis would have not been achieved without the uncountable days she spent working with me and without the precious advices she gave me.

I would like to give my true appreciation to Dr. Fabio Moda and Prof. Fabrizio Tagliavini from the “Istituto Neurologico Carlo Besta” in Milan for hosting me on several occasions in the Institute’s in order to carry out the human part of this work. It has been a great experience for me to enter a BSL3 lab and learn how to work with human brain samples.

In addition, I would like to thank Prof. Jason Bartz and Prof. Isidro Ferrer for their very useful comments and suggestions for the improvement of this thesis.
I want to thank Prof. Gianluigi Zanusso for providing human control samples and the DPZ for providing macaque samples and initial analysis.

A sincere thank also to Helena Krmac for her help in analyzing hundreds of RNA and to Dr. Paolo Vatta, Dr. Raffaella Calligaris and Prof. Gustincich for the useful discussions we had and for kindly sharing with me their knowledge.

I want to thank also all the SISSA technicians, in particular Jessica Franzot and Andrea Tomicich, for being always available every time I got lost in the lab looking for reagents or for internet connectivity 😊

A huge thank to all my present and past colleagues of Legname’s lab who were always ready to help me when I had questions or problems... or even existential doubts! In particular I want to thank the “seniors” Gabriele, Diana and Lisa for their advices and for the time they spent training me in my first steps inside the lab.
Also, I have to thank a lot Suzana, Ludovica and Marcella for supporting me in the “obscure” world of proteins... a lot of endless WB and ELISA would have been a real nightmare without you all!

I wish to thank Elena for so many reasons that I would need to write another thesis... but I can sum up all of these just saying thank you for being a real friend. I definitely can say you’re one of the best people I’ve ever met.

Many thanks also go to Ilenia, Anna and Marika for their much-needed support in this last year of work... and life. I will never forget you ladies!! :*

Last but not least, a big thank to my parents, who always believed in me and pushed me to not get discouraged by difficulties and bad experiments 😊
Gene expression profiling of brains from bovine spongiform encephalopathy (BSE)-infected cynomolgus macaques

Maura Barbisin1†, Silvia Vanni1†, Ann-Christin Schmädicke2, Judith Montag2,4, Dirk Motzkus2, Lennart Opitz3, Gabriela Salinas-Riester3 and Giuseppe Legname1*

Abstract

Background: Prion diseases are fatal neurodegenerative disorders whose pathogenesis mechanisms are not fully understood. In this context, the analysis of gene expression alterations occurring in prion-infected animals represents a powerful tool that may contribute to unravel the molecular basis of prion diseases and therefore discover novel potential targets for diagnosis and therapeutics. Here we present the first large-scale transcriptional profiling of brains from BSE-infected cynomolgus macaques, which are an excellent model for human prion disorders.

Results: The study was conducted using the GeneChip® Rhesus Macaque Genome Array and revealed 300 transcripts with expression changes greater than twofold. Among these, the bioinformatics analysis identified 86 genes with known functions, most of which are involved in cellular development, cell death and survival, lipid homeostasis, and acute phase response signaling. RT-qPCR was performed on selected gene transcripts in order to validate the differential expression in infected animals versus controls. The results obtained with the microarray technology were confirmed and a gene signature was identified. In brief, HBB and HBA2 were down-regulated in infected macaques, whereas TTR, APOC1 and SERPINA3 were up-regulated.

Conclusions: Some genes involved in oxygen or lipid transport and in innate immunity were found to be dysregulated in prion infected macaques. These genes are known to be involved in other neurodegenerative disorders such as Alzheimer’s and Parkinson’s diseases. Our results may facilitate the identification of potential disease biomarkers for many neurodegenerative diseases.

Keywords: Prion diseases, BSE, Non-human primates, Neurodegeneration, Transcriptome, Microarray, RT-qPCR, Biomarker, Serpina3, Hemoglobin

Background

Prion diseases, or transmissible spongiform encephalopathies (TSEs), are incurable and fatal neurodegenerative disorders that affect both humans and animals; their origin may be sporadic, acquired or genetic [1,2]. TSEs include Creutzfeldt-Jakob Disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS), kuru and fatal familial insomnia (FFI) in humans [2], bovine spongiform encephalopathy (BSE) in cattle [3], scrapie in sheep and goats [4], chronic wasting disease (CWD) in cervids [5], transmissible mink encephalopathy, and feline spongiform encephalopathy (FSE) [6].

A major event that leads to the development of prion diseases is the conversion of the cellular form of the prion protein (PrP\textsuperscript{C}) into an abnormally folded, β-sheet enriched and protease resistant isoform (PrP\textsuperscript{Sc}). PrP\textsuperscript{Sc} is prone to accumulate and aggregate in the brain of affected individuals [1,2,4] leading to neuronal loss, spongiosis and astrogliosis, which are hallmarks of neurodegeneration. The underlying conversion mechanism of PrP\textsuperscript{C} into PrP\textsuperscript{Sc} is poorly understood and it is further complicated by the existence of several different strains characterized by distinct
ternary and quaternary structures as well as different clinical patterns [7,8]. Several hypotheses exist about the contribution of unknown molecules other than PrP to prion propagation [9-11]. To address this issue, several animal studies have investigated the host response to prion infection of different origin and strain. The differential transcription profile after prion infection has been extensively explored (reviewed in [6,12]); however, most of the studies involved animal models such as mice [13-18], sheep [4,19-22] and cattle [23-28], all not closely related to humans. Some expression analyses have been conducted in non-human primates focusing mainly on the susceptibility to the infection and the variety of clinical symptoms [29-33], but none has investigated large-scale transcriptome changes due to prion infection. All these investigations suggest that besides the PrP-encoding gene (PRNP in humans), other genes are key players and contribute to the genetic susceptibility to acquired TSEs [6,34]. The main genes identified so far are related to oxidative stress, mitochondrial apoptotic pathways, endosome/lysosome function, immunity, synapse function, protease inhibitors, calcium binding proteins, regulation of the actin cytoskeleton, ion transport, cell adhesion, and transcription processes [6]. Dysregulation of these genes seems to cause increased oxidative stress that in turn determines oxidation of proteins, lipids and DNA as well as mitochondrial dysfunction and ER stress [6]. Apart from TSEs, transcriptional changes of these genes are common to other neurodegenerative pathologies [12] and, together with functional proteomics data, may help to identify novel selective biomarkers of prion diseases and neurodegeneration in general.

To accomplish that, we performed a large-scale transcriptional profiling in BSE-infected cynomolgus macaques (Macaca fascicularis). They are known to be an excellent model for studying human acquired prion diseases [32,33,35-37], as shown by BSE infection via the intracranial and oral routes, which lead to a disease pattern comparable to that of human maladies in terms of preclinical incubation time, clinical symptoms and pathophysiology [35]. The objective of this study was to identify genes that are differentially expressed in brain tissue of intracranially infected monkeys compared to non-infected ones using an unbiased genomic approach such as expression microarrays with subsequent data validation by RT-qPCR. Our study aims at revealing biological processes that are relevant to the pathogenesis of human prion diseases using a systematic approach that connects the identified DEGs into potential networks of interacting pathways. This may allow us to discover novel selective markers as potential targets for diagnostic and therapeutic strategies.

Results
PrPSc content in brain tissue
The relative amount of PrPSc in brain homogenate of 6 BSE-infected macaques was examined by Western Blot. Densitometric analysis of the monoglycosylated band revealed that the relative amount of PrPSc strongly differed between the individual macaques. We wondered whether this discrepancy might be due to the preclinical incubation time or rather correspond to the gradual accumulation of PrPSc during the clinical phase of disease as reported for sCJD [38,39]. As anticipated, we found a significant correlation between PrPSc content and the duration of the symptomatic phase (Figure 1). The correlation analysis includes only the 6 intracranially inoculated macaques. Since these animals were housed in one social group, environmental factors, which may influence the disease course and duration, are identical. Such factors can be different for the orally inoculated animal, which was therefore omitted from the analysis. The infected animals were at an advanced stage of prion disease and the details of their clinical course have been previously described [33]. Briefly, animal A1 showed the shortest duration of disease (17 days) and a short pre-clinical incubation time (931 days) together with the lowest PrPSc content, while animal A5 showed the longest survival period (143 days), compared to an average clinical phase of about 90 days, together with the highest PrPSc content and the second longest pre-clinical phase (1340 days).

Microarray analysis of brain gene expression in cynomolgus macaques
To investigate differential mRNA expression in BSE-infected macaques we used brain samples from 6 animals that were intracranially challenged [33]. One macaque that
was orally infected with 50 mg BSE-homogenate was also included in our study. For comparison purposes, we used 5 brain samples derived from non-infected age- and sex-matched control macaques.

RNA was isolated from the *gyrus frontalis superior* of all animals and checked for quality by nano-scale electrophoresis, which resulted in an overall RNA Integrity Number (RIN) of about 6. This value is indicative of at least partially degraded RNA within the sample; one possible reason for the reduced RNA integrity may be the procedure utilized to remove the *gyrus frontalis superior* region from the frozen tissue slide. The biopsy stamp was plugged into a cordless screwdriver that was used to drill a borehole in the frozen tissue block of +/− 1 cm height. This method was chosen to ensure that the material did not thaw; however, the local heat induced by the rotating biopsy stamp may have led to substantial degradation of the RNA. Nonetheless, human brain material exhibiting a comparable RIN value was successfully used for similar studies [40]. All samples were analyzed using the GeneChip® Rhesus Macaque Genome Array (Affymetrix®) that contains 52,024 rhesus probe sets to enable gene expression studies of *Macaca mulatta* transcriptome interrogating more than 47,000 transcripts. The genomes of *M. mulatta* and *M. fascicularis* exhibit a small genetic divergence of approximately 0.4% [41,42] that presumably allows for the detection of homologue transcripts with high specificity.

Raw data were quality checked and analyzed using Affymetrix® proprietary analysis tools, a hierarchical clustering was performed and a heat map was generated. Then the signals were aligned to the annotation library and a spreadsheet containing gene symbols, p-values and expression fold changes was created. Microarray data were submitted to Gene Expression Omnibus (GEO). The bioinformatics analysis identified 300 probe sets that were up- or down-regulated about twofold (≥ 1.95). Because among them no candidate appeared using FDR 0.05, we chose as criteria an unadjusted p-value of ≤ 0.005 together with a fold change ≥ 2.0. Additional file 1 lists the resulting 86 probe sets that were then used to generate the heat map shown in Figure 2.

### Functional classification of differentially expressed genes (DEGs)

We used the Ingenuity Pathways Analysis (IPA®, see section: Availability of supporting data) to annotate genes according to their functional relationships and to determine potential regulatory networks and pathways. Among the 300 differentially expressed (about twofold, ≥ 1.95) probe sets identified, 105 were associated to mapped IDs; 53 of the latter were identified as network eligible genes, while 86 were identified as function eligible genes. It should be emphasized that the designation of functional class in the present study is neither definitive nor exclusive, as annotation of gene function is incomplete, and

---

**Figure 2 Condition trees of the clustering analysis.** The cluster analysis was performed using a hierarchical approach with the average linkage-method (R and Partek® Software, Partek® Inc.). 86 probe sets showed a differential expression with FC ≥ 2. The color represents the level of expression (red: up-regulation, blue: down-regulation) and the sample information is listed across the bottom. The names of the known genes are indicated. More details on all genes are reported in Additional file 1.
multifunctional gene products can be involved in several cellular pathways. First, we identified key biological functions and/or diseases that contain a disproportionately high number of genes from the DEG list compared to the total gene population from the microarray. The analysis was started by identifying the top categories (p < 0.01) of DEGs within three main classes. In the “Diseases and Disorders” class the categories were cancer and developmental disorder, while within the “Molecular and Cellular functions” class most genes were involved in cellular development and cell death/survival. The main categories for the “Physiological System Development and Function” class were tissue morphology as well as nervous system development and function. As a second step, genes were clustered in relation to the main pathways they belong to: the top two canonical pathways in our DEG list were LXR/RXR activation, which is associated with lipid metabolism and transport, and acute phase response signaling.

Identification of biologically relevant networks

To further investigate the global expression response to BSE infection and to define interactions among the identified specific pathways containing the regulated genes, potential networks of interacting DEGs were identified using IPA®. All potential networks with score > 9 (a score ≥ 3 was considered significant, p < 0.001) are listed in Table 1 with information on network genes, score, focus molecules and top functions associated with the focus genes in each network. The highest ranked network identified by IPA® was associated with tissue morphology (specifically the determination of cell quantity), developmental disorder and biological processes controlling cell death and survival (Figure 3a). This network contained genes that are known to be involved in several neurological diseases and nervous system functions, as shown in Figure 3b.

Validation of differentially expressed genes by RT-qPCR

To further confirm the array results using an independent and more sensitive technique, we decided to perform RT-qPCR for a subset of differentially expressed genes. This subset (Additional file 2) was selected in subsequent steps: first, among the 86 probe sets identified during the microarray analysis (Additional file 1) we selected the top 36 with fold change ≥ 2.5 and p ≤ 0.005. Then, after realizing that many were not annotated or did not have a known function, we extended the selection to additional 29 probe sets having fold change ≥ 2.5 but 0.005 ≤ p ≤ 0.05; for the same reasons stated above, we extended the list of candidates one more time using as criteria fold change ≥ 2.5 and p > 0.05 (24 candidates). At this point, having still some cDNA available and only 13 feasible candidate transcripts, we added seven probe sets, corresponding to 5 additional transcripts, selected among the ones with a slightly lower fold change (FC > 2 for at least 1 probe) but possessing an interesting function as revealed by the IPA® analysis or according to the literature. Lastly, HBA2 was added to the list because of its tight

Table 1 List of 3 Ingenuity networks generated by mapping the focus genes that were differentially expressed between non-infected and BSE-infected samples

<table>
<thead>
<tr>
<th>ID</th>
<th>Molecules in network</th>
<th>Score</th>
<th>Focus molecules</th>
<th>Top functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ACVR1C, AGR1D1, Alp, AMPK, Ap1, APOC1, Calcineurin protein(s), CANTPT, caspase, CD3, CHIC1L1, Clec, cytochrome C, DACH1, DLX1, ERK1/2, F13A1, Focal adhesion kinase, GNIR1, HBA1/HBA2, HBB, HDL, hemoglobin, HEY2, HINT1, HIPK2, Ikk (family), IL1, IRF3, Jnk, KDELQR2, LDL, LGALS1, Mapk, MEF2C, Mek, Met, MT2A, N4BP1, NADPH oxidase, NGFR, NRA42, OTX2, P38 MAPK, p85 (pik3r), Pdgf (complex), PDGF BB, PI3K (complex), PIK3R1, Pkc(s), PLC gamma, PON3, Pro-inflammatory Cytokine, Ras, SERPINA1, SERPINA3, Shc, SHOC2, SLC10A2, Sox, STK4, TCF, TCA, TNFSF10, TTR, TWIST1, Vegf, WSB1</td>
<td>71</td>
<td>35</td>
<td>Tissue Morphology, Cell Death and Survival, Developmental Disorder</td>
</tr>
<tr>
<td>2</td>
<td>ABR, ACTL6B, ARMC6, ASB6, C1orf137, C6orf211, CAMKV, CHMP2A, CLIC4, CLPP, CSNK1G3, CTBP2, DCLRE1A, DDX19B, DGKE, ECT2, FH13, FLVCR1, GALNT5, GLOD4, HEATR6, HSPI00A1, HSPA1A2, IFITG1, KL3, KPN6, MCTS1, MEIG1, METTL7B, MRPL44, Mxd3, MYBPC1, NCLN, NPB1, NOL4, OSBP1L10, PCEB3, PLEKH4B, PMM2, POLR2J, PPAP2C, PRCP, PRSC, RA2, SAPIB2, SCAD1, SEPT6, SGTB, SMARCC1, SMC3, SPATA22, SPB3, SRPK, SSU72, STAG1, TATDN1, TESP1, TNSF53, TNP, TNNS6, TP53BP1, TRAPC21, TRIP21, TUFM, TXNL4A, UBC, ZNF131, ZNF235, ZNF397, ZNF420</td>
<td>54</td>
<td>28</td>
<td>Developmental Disorder, Hereditary Disorder, Hematological Disease</td>
</tr>
<tr>
<td>3</td>
<td>26 s Proteasome, ADCY, AKR1C1/AKR1C2, Akt, APP, ARL4C, Arnt-Clock, AVP, AVPR1B, CACNA1B, CAMKV, CANTPT, CBLN1, CEACAM6, CLDN10, CLOCK, COX4I2, CTF1, DNAJC12, endocannabinoid, estrogen receptor, FAM6A, FSH, GABRE, GNA15, GPR158, GPX1, GPX2, GSK3A, Histone h3, HMGR, HNF4A, HSPA1A2, Insulin, JPH3, KCN3, KCN51, LUNG01, LPR1, LNX1, MGAT2, miR-125b-5p (and other miRNAs w/seed CCCUGAG), Mmp, Mt1, NFH (complex), Nop, OMI11W, PAX1, PIK3R5, Pka, PKM, PLC, Proinsulin, RAB39A, RA1, ROK2, RUY3, SERPINA3, SMD5, SMCG, SOX7, SYT17, TCF19, Trnfr2/Tnftr23, TDR2A, trentin, trypsin, TXNL4B, ZBTB44, ZFH3</td>
<td>36</td>
<td>21</td>
<td>Cellular Development, Neurological Disease, Skeletal and Muscular System Development and Function</td>
</tr>
</tbody>
</table>
Figure 3 (See legend on next page.)
relationship with one of the previously selected genes of the hemoglobin complex (HBB), as revealed in the top ranking network from the IPA* analysis (Figure 3a).

In order to achieve optimal RT-qPCR conditions we performed titration of template and primers as well as optimization of cycling conditions using human cDNA from SH-SY5Y neuroblastoma cells (macaque cDNA was scarce). To assess the specificity of the chosen oligonucleotides prior to performing the quantitative assays, some reactions were carried out using macaque cDNA obtained from control animals to verify the correct amplicon length. Two housekeeping genes, GAPDH and ACTB [61], were used as reference genes to normalize RT-qPCR data. Both genes were monitored across samples derived from infected and control macaques in order to evaluate their expression stability, yielding very similar results (Additional file 3).

At this point we performed the quantitative analysis and in general we observed large intra-assay variability for most genes across different samples, both for infected

Table 2 Candidate genes for validation

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession number</th>
<th>Known relation with PrP/nervous system</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKR1C1</td>
<td>NM_001195574.1</td>
<td>Putative role in myelin formation</td>
<td>[43]</td>
</tr>
<tr>
<td>HBB</td>
<td>NM_001164428.1</td>
<td>Putative role in intraneuronal oxygen homeostasis, reduced in Alzheimer’s and Parkinson’s disease</td>
<td>[44]</td>
</tr>
<tr>
<td>NCAM1</td>
<td>XM_001083697.2</td>
<td>PrP/N-CAM complexes found in prion infected N2a cells</td>
<td>[45]</td>
</tr>
<tr>
<td>NR4A2</td>
<td>NM_001266910.2</td>
<td>Mutations related to dopaminergic dysfunction, including Parkinson schizophrenia and depression</td>
<td>[46, 47]</td>
</tr>
<tr>
<td>USP16</td>
<td>NM_001260999.2</td>
<td>Depletion of USP16 prevented ATMi from restoring transcription after DSB induction</td>
<td>[48]</td>
</tr>
<tr>
<td>CALB1</td>
<td>XM_001085269.2</td>
<td>Plays a protective role in neurodegenerative disorders (depleted in HD)</td>
<td>[49]</td>
</tr>
<tr>
<td>DACH1</td>
<td>XM_001082371.2</td>
<td>Required for normal brain development</td>
<td>[50]</td>
</tr>
<tr>
<td>LXN</td>
<td>NM_001266988.1</td>
<td>Marker for the regional specification of the neocortex</td>
<td>[51]</td>
</tr>
<tr>
<td>PKIR3</td>
<td>NM_001266826.1</td>
<td>Linked to β-amyloid plaque formation in AD brain</td>
<td>[52]</td>
</tr>
<tr>
<td>SAP18</td>
<td>NM_001261034.1</td>
<td>Possibly related to AD</td>
<td>[53]</td>
</tr>
<tr>
<td>SERPINA3</td>
<td>NM_001195350.1</td>
<td>Increased in schizophrenia, SNPs affecting onset and duration of AD</td>
<td>[54, 55]</td>
</tr>
<tr>
<td>TNFSF10</td>
<td>NM_001266034.1</td>
<td>Implicated in pathogenesis of MS (causing demyelination)</td>
<td>[56]</td>
</tr>
<tr>
<td>HBA2</td>
<td>NM_001044724.1</td>
<td>Putative role in intraneuronal oxygen homeostasis, reduced in Alzheimer’s and Parkinson’s diseases</td>
<td>[44]</td>
</tr>
<tr>
<td>GNRH1</td>
<td>NM_001195436.1</td>
<td>Key regulator of the reproductive neuroendocrine system in vertebrates</td>
<td>[57]</td>
</tr>
<tr>
<td>IRF3</td>
<td>NM_001135797</td>
<td>Putative protective role against prion infection</td>
<td>[58]</td>
</tr>
<tr>
<td>APOC1*</td>
<td>AK240617.1</td>
<td>Binds to ApoE, risk factor for Alzheimer’s disease</td>
<td>[59]</td>
</tr>
<tr>
<td>TM7SF3</td>
<td>XM_001099269.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MYBP1C</td>
<td>XM_001091952.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TTR</td>
<td>NM_001261679</td>
<td>Amyloid neuropathies, interaction with Aβ</td>
<td>[60]</td>
</tr>
</tbody>
</table>

List of 19 identified genes selected on the basis of fold change value and known relevance for neurodegeneration. Because of very low signal (LXN, PKIR3, TNFSF10, GNRH1) or lack of reliable sequence data (CALB1, DACH1, TM7SF3, MYBP1C), only 11 genes (in bold) were successfully analyzed. *Macaca fascicularis transcript.
Interestingly, we found a completely different expression pattern for B6, the only orally-infected sample, compared to the intracranially infected animals, except for a couple of genes (AKR1C1, NCAM1), suggesting that the route of infection might play a role in determining the gene expression changes (Additional file 6). Therefore we decided to rerun the microarray clustering analysis excluding this animal in order to verify its influence on the final results. As shown in Additional file 7, the comparison of the clustering analysis with (panel A) and without (panel B) the orally challenged animal B6 does not show marked differences.

Using SYBR® Green-based RT-qPCR we confirmed the statistically significant up-regulation of TTR (FC = 7.11), SERPINA3 (FC = 18.73) and APOC1 (FC = 6.33) as well as the down-regulation of HBB (FC = 0.19) and HBA2 (FC = 0.22), normalizing the data against GAPDH (Figure 4). Similar results were obtained against ACTB (Additional file 8). For all the other genes the RT-qPCR results confirmed the regulation trend of the microarrays, but without statistical significance (p-value > 0.05).

In order to confirm the SYBR® Green-based results we performed an additional RT-qPCR analysis using FAM-labeled TaqMan® probes, providing more sensitive and specific detection signals for those genes that showed a significant fold change. Using this approach we confirmed the regulation of SERPINA3, APOC1, HBB and HBA2, but not of TTR, which showed comparable trends in FC but lost statistical significance (Figure 5). This may be due to higher variability among triplicates, caused by C<sub>T</sub> values higher than 35 obtained with the TaqMan® probe chemistry compared to SYBR® Green detection system (Additional file 9).

### Table 3 Genes analyzed by RT-qPCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chromosome</th>
<th>Primer sequence</th>
<th>Amplicon length (bp)</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTB</td>
<td>3</td>
<td>F: GTGGCCTACACCTTCTCTGT</td>
<td>146</td>
<td>NM_001033084.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CTTGCACCCACACCTGCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>11</td>
<td>F: CCGACACCACCTGCTTAA</td>
<td>74</td>
<td>NM_001195426.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CAGTGCCTCCTCCACACAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AKR1C1</td>
<td>9</td>
<td>F: CAGCCCATATTGATCTTCTCAT</td>
<td>132</td>
<td>NM_001195574.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: TGGGAATCCACAAACGTTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HBB</td>
<td>14</td>
<td>F: GTCCCTCCTGTGGCTTATG</td>
<td>102</td>
<td>NM_001164428.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: TTAGGTGCTCCAGGTTTCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCAM1</td>
<td>14</td>
<td>F: GACGCAAGGAGAAAGATGAG</td>
<td>150</td>
<td>XM_001083697.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GACTTTGAGGTGGATGGTCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NR4A2</td>
<td>12</td>
<td>F: CCAGTGGAGGTTAAGTCTCAT</td>
<td>145</td>
<td>NM_001266910.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: AGGAGAAGGGGGAAGATGTCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>USP16</td>
<td>3</td>
<td>F: GGAGAATTGCACCCACAGCA</td>
<td>146</td>
<td>NM_001260999.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CTAAATGTAAGGGCCGTGAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAP18</td>
<td>17</td>
<td>F: GGAAATGTACCGTCCAGG</td>
<td>109</td>
<td>NM_001261034.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: TGCCCCCTTCTCTCAACTCTTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SERPINA3</td>
<td>7</td>
<td>F: GCTGGGCACTTTAGGAGCTT</td>
<td>123</td>
<td>NM_001195350.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GTGCCCTTCCTGACACATC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HBA2</td>
<td>20</td>
<td>F: CGAACAAGGCAAGCTCAAGG</td>
<td>126</td>
<td>NM_001044724.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: TCGAAGTGGGGAAGTATGTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IRF3</td>
<td>19</td>
<td>F: TGGGGTGTGTTTCAAGAGG</td>
<td>90</td>
<td>NM_001135797</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GAAAAGTCGCCCAACTCCTGAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>APOC1*</td>
<td>19</td>
<td>F: TTCTGTGCGATGTCCTGGAAG</td>
<td>138</td>
<td>AK240617.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CACCTGTTTGATGCGGTGTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TTR</td>
<td>18</td>
<td>F: TCACCTTGACACCCACATTCC</td>
<td>114</td>
<td>NM_001261679</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GGTGGAGAATGGGAGGAGGCAC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Primers (F: forward and R: reverse) used for gene amplification, amplicon length and GenBank® accession numbers of the macaque cDNA sequences used for primer design. All primers were designed according to the genome sequence of Macaca mulatta.

*Apolipoprotein C-I (APOC1) primers were designed according to the genome sequence of Macaca fascicularis because the Macaca mulatta mRNA sequence was not annotated (SRA Macaca mulatta Mamu_450725, accession number: JN045607.1). Homology between the two sequences was 99%.

(Additional file 4) and for control animals (Additional file 5). Interestingly, we found a completely different expression pattern for B6, the only orally-infected sample, compared to the intracranially infected animals, except for a couple of genes (AKR1C1, NCAM1), suggesting that the route of infection might play a role in determining the gene expression changes (Additional file 6). Therefore we decided to rerun the microarray clustering analysis excluding this animal in order to verify its influence on the final results. As shown in Additional file 7, the comparison of the clustering analysis with (panel A) and without (panel B) the orally challenged animal B6 does not show marked differences.

In order to confirm the SYBR® Green-based results we performed an additional RT-qPCR analysis using FAM-labeled TaqMan® probes, providing more sensitive and specific detection signals for those genes that showed a significant fold change. Using this approach we confirmed the regulation of SERPINA3, APOC1, HBB and HBA2, but not of TTR, which showed comparable trends in FC but lost statistical significance (Figure 5). This may be due to higher variability among triplicates, caused by C<sub>T</sub> values higher than 35 obtained with the TaqMan® probe chemistry compared to SYBR® Green detection system (Additional file 9).
Figure 4 SYBR® Green-based RT-qPCR validation of microarray results. Relative expression levels of 11 genes normalized against GAPDH in BSE-infected cynomolgus macaques.

Figure 5 Comparison between SYBR® Green-based and TaqMan® probe-based results. TaqMan® (white) versus SYBR® Green-based (grey) expression levels for each transcript. Both detection systems yielded similar results. Data are normalized against GAPDH. Similar results were obtained with normalization against ACTB (data not shown).
Table 4 RT-qPCR confirmation of microarray results

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Microarray fold change</th>
<th>RT-qPCR fold change</th>
<th>SYBR® Green</th>
<th>TaqMan®</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Min</td>
<td>Max</td>
<td>Mean</td>
<td>FC</td>
</tr>
<tr>
<td>AKR1C1</td>
<td>2.3</td>
<td>2.9</td>
<td>2.5</td>
<td>1.7</td>
</tr>
<tr>
<td>HBB</td>
<td>-2.2</td>
<td>-2.6</td>
<td>-2.4</td>
<td>0.2</td>
</tr>
<tr>
<td>NCAM1</td>
<td>-1.1</td>
<td>2.5</td>
<td>-0.3</td>
<td>0.5</td>
</tr>
<tr>
<td>NRA2</td>
<td>1.1</td>
<td>-2.1</td>
<td>-1.6</td>
<td>0.4</td>
</tr>
<tr>
<td>USP16</td>
<td>-1.2</td>
<td>-5.5</td>
<td>-2.6</td>
<td>0.5</td>
</tr>
<tr>
<td>SAP18</td>
<td>-1.2</td>
<td>-2.6</td>
<td>-1.7</td>
<td>0.8</td>
</tr>
<tr>
<td>SERPINA3</td>
<td>10.0</td>
<td>16.0</td>
<td>13.0</td>
<td>18.7</td>
</tr>
<tr>
<td>HBA2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.2</td>
</tr>
<tr>
<td>IRF3</td>
<td>2.0</td>
<td>2.1</td>
<td>2.0</td>
<td>1.3</td>
</tr>
<tr>
<td>APOC1</td>
<td>4.3</td>
<td>-</td>
<td>4.3</td>
<td>6.3</td>
</tr>
<tr>
<td>TTR</td>
<td>3.1</td>
<td>3.1</td>
<td>3.1</td>
<td>7.1</td>
</tr>
</tbody>
</table>

Differential expression of selected genes analyzed by microarray and RT-qPCR. For microarray analysis, the lowest (Min), the highest (Max) and the average (Mean) fold change values of all the respective probes are shown. For RT-qPCR analysis, fold change (FC) and statistical significance (p-value) for both SYBR® Green and TaqMan® results are shown. In bold are the genes validated with statistical significance. HBA2 was not present in the array chip.

*Normalization performed vs. ACTB only.

In general, we were able to confirm the results of the array platform obtaining consistent fold change values for all genes analyzed, even though we validated with statistical significance using the specific TaqMan® detection system only of four of them: HBB, HBA2, APOC1, SERPINA3 (see Table 4 for details on p-values and FC).

In addition, dealing with animals whose brain material isolation may be susceptible to blood contamination, and as several works in the last few years have shown the presence of active transcription within human red blood cells [62], we decided to analyze the samples also for expression of some erythrocyte markers, such as ALAS2 and RHAG, in order to verify the reliability of the results related to the regulation of both chains of hemoglobin (HBB and HBA2). Although the array data for these genes suggested a negligible and virtually identical presence of blood in both control and infected samples, RT-qPCR analysis revealed a small blood contamination (C\textsubscript{T} ≥ 34 for ALAS2, C\textsubscript{T} ≥ 36 for RHAG) within two samples, one control (CovD1) and one infected sample (A4) (Additional file 10 and Additional file 11). In light of these results, we performed an additional gene expression analysis for HBB and HBA2 excluding these two samples. As expected, we obtained slightly different results (FC ~0.3 for HBB and 0.2 for HBA2 using TaqMan® probes), but a relevant down-regulation still persisted with statistical significance.

Discussion

The precise mechanisms regulating the molecular processes that lead to neurodegeneration in TSEs remain unknown. Genomic approaches represent unbiased and powerful tools to uncover the molecular basis of these complex mechanisms and they may also contribute to discover new biomarkers for these diseases. Several studies have presented genomic analyses of brain tissues from animal models of TSE; a few of them involved the mRNA profiling of cattle BSE [23,25-27] or ovine scrapie [4,19-22,63] whereas the vast majority was performed on rodent-adapted models of prion disease [13-18,64]. In several of these prion-infected mice, genomic expression profiles revealed the induction of oxidative and endoplasmic reticulum (ER) stress, activated ER and mitochondrial apoptosis pathways as well as activated cholesterol biosynthesis in the CNS of preclinical mice [64].

We report here the first large-scale transcriptome analysis of the superior frontal gyrus of BSE-infected macaques. This region was selected based on its histopathological and functional relevance in the majority of neurodegenerative disorders [65] and because it corresponds to Brodmann areas 10 and 11, known to be involved in strategic processes in memory recall, various executive functions as well as in planning, reasoning, and decision making [66], all processes known to be disturbed by neurodegeneration. In general, RT-qPCR results confirmed the regulation trend seen in the microarray platform for all the 11 genes analyzed with very similar values using either GAPDH or ACTB for normalization. For five of them (HBB, HBA2, TTR, SERPINA3, APOC1) we obtained statistical significance with one or both qPCR detection systems utilized in this study (SYBR® Green and TaqMan® probes) and some of them were involved in the top two canonical pathways identified during the functional classification reported in the Results section: APOC1 and TTR are part of the LXRxRR activation pathway, which is associated with lipid metabolism and transport, whereas SERPINA3 and TTR are involved in the acute phase response signaling pathway. All the other genes seemed to fall in the grey zone of both platforms and therefore their FC values could not be considered reliable.

When validating the array results by RT-qPCR, the first evidence obtained was a marked variability among the samples of the same group, either control or infected animals. Unlike other animal models, nonhuman primates are usually not inbred. Therefore, differences in the genomic background of the animals in our study may have contributed to the variability in the time of disease onset [33] and in gene expression within the same group. Paradoxically, for some genes that resulted strongly regulated (APOC1, HBB, HBA2) the variability resulted even more accentuated within the control group.
if compared to that of the infected group. The experimental and control animals were housed in different animal facilities and this may have generated slight differences in diet and/or housing conditions that may have contributed to the above-mentioned effect.

We also reported a peculiar dysregulation pattern of the orally infected sample (B6) for several genes, showing a completely opposite trend compared to intracranially infected animals. Although no data are available for PrPSc deposition in brain or other tissues of this animal, the significantly longer incubation period (1950 days compared to an average of 1100 days for the other animals) could suggest a correlation between the mRNA expression profile and the route of infection [67]. Nonetheless, this different pattern may be due to the age difference at the time of euthanasia: 7.1 +/- 0.7 years for the intracranially infected macaques versus 9.9 years for the orally infected animal.

Concerning hemoglobin (Hb), a few years ago its expression was unexpectedly discovered in mesencephalic dopaminergic neurons of different mouse strains, as well as in rats and humans affected by Parkinson’s disease (PD) and multiple sclerosis (MS) [68-70].

Hb expression is known to decrease in neurons of PD, Alzheimer’s disease (AD), argyrophilic grain disease (AGD) and dementia with Lewy bodies (DLB) brains [44] as well as in the CNS of scrapie-infected mice [13,14]. Also, it has been shown that Hb binds to Aβ enhancing its aggregation and co-localizes in amyloid plaques in AD brains [71]. If we consider a possible similar interaction with β-rich PrPSc isoforms in prion diseases, we can hypothesize that in our animal model down-regulated Hb fails to promote aggregation of the prion protein, thus leading to a higher presence of toxic species like oligomers [72]. Moreover, in PD it has been hypothesized that Hb may act as oxygen storage molecule in oligodendrocytes [68]. Oxygen would be later released to neighboring neurons in hypoxia conditions to maintain the aerobic metabolism [68,69]. When down-regulated, Hb would not be available for this function and cells would be damaged by the defective oxygen homeostasis. Our results indicated a strong down-regulation (about 70-80% lower expression than normal) of both HBB and HBA2 in symptomatic advanced-stage BSE-infected macaques. The data were analyzed with a very stringent procedure after excluding any major effect of potential blood contamination, thus confirming the robustness of the results.

Taken together, all these data indicate a possible general role for hemoglobin in neurodegenerative disorders, possibly related to an alteration of O2 homeostasis and oxidative metabolism [68]. One point that needs further investigation is whether this alteration (down-regulation) occurs as an early/late consequence of the disease, or may act as a susceptibility factor that influences the onset of the pathology. Furthermore, future studies may investigate the localization of the observed down-regulation in terms of cell population: it could involve neurons as well as astrocytes or microglia.

Another crucial molecule, APOC1, was significantly up-regulated in BSE-infected brains samples compared to controls. Apolipoprotein C-I, whose gene APOCI is part of the APOE/C-I/C-IV/C-II gene cluster, is a small 6.6 kDa component of lipoproteins (mainly HDL) that is known to inhibit receptor-mediated lipoprotein clearance, especially particles containing apoE [73]. Increasing evidence indicates a role for this gene in neurodegenerative disorders, especially in AD and MS [74-76]. A disruption in lipid metabolism and signaling is one of the early alterations apparent in many neurodegenerative diseases, including prion diseases [77,78]; indeed, cholesterol metabolites are investigated by a number of studies aimed at the identification of early biomarkers for neurodegenerative disorders [79-81]. Several genes involved in cholesterol metabolism and lipid biosynthesis have been found to be up-regulated in preclinical scrapie-infected mice [64]. Since APOC1 is able to activate cholesterol esterification via lecithin-cholesterol acyltransferase [75], its up-regulation could lead to an increase in cholesterol biosynthesis, consistent with the concomitant presence of prion disease. In fact, in vitro studies have shown that depletion of cellular cholesterol reduces the conversion of PrPSc to PrPSc [82] and evidence exists also in AD, where altered cholesterol metabolism has been found [83]. Hypercholesterolemia has also been shown to influence amyloid precursor protein processing [84]. One explanation for altered cholesterol homeostasis affecting prion disease development could lie in the fact that PrP is localized in cholesterol-rich lipid rafts [85].

SERPINA3, a serpin peptidase inhibitor involved in acute phase response pathways, is another gene that we found highly regulated in our animal model. It is extensively reported to be regulated in other neurodegenerative disease models and in particular it is well known to interact with APP to promote amyloid plaque formation — a hallmark of AD [86]. Indeed, increased levels of SERPINA3 have been found in the brain and peripheral blood of AD patients [87], mainly due to persistent and almost chronic inflammation [88]. In prion disease studies, SERPINA3 was found increased in brains of scrapie-infected mice [77], in mice infected with RML prior to clinical onset [89] as well as in urine and cerebrospinal fluid of CJD patients [90]. Being an acute phase protein, its up-regulation is explained by the onset of an inflammatory condition, particularly as a response of the innate immune system [91]. Interestingly, two β-sheets of SERPINA3 exhibit a polymorphism mimicking changes in
the serpin structure that normally occur during the formation of its stable complex with the target protease. In this conformation, SERPINA3 can bind Aβ, thus imposing a β-strand conformation that upon dissociation leads to a faster formation of fibrils [86]. Therefore, an intriguing hypothesis may be envisioned in which PrP conversion into β-sheet conformation can be assisted by SERPINA3, which would accelerate the formation of toxic species like PrP oligomers.

Transthyretin, a protein in the same pathway of acute phase response as SERPINA3, was found to be up-regulated at the transcription level in our BSE-infected macaques according to the SYBR® Green assay. Even though we were not able to confirm the statistical significance using the TaqMan® assay, this gene seems to be of interest. Indeed, TTR, carrier of the thyroid hormone thyroxine (T₄) in serum and CSF, is associated with systemic amyloidosis in humans [92], but also with an anti-amyloidogenic effect preventing Aβ deposition in neuronal cell cultures [93]. Moreover, increased mRNA and protein levels have been shown in neurons from the AD mouse model APP23 and in human AD brain with a neuroprotective role [94,95]. Even in prion models TTR levels have been found strongly increased in the cortex of scrapie-infected mice [15]. Our study now provides indication that up-regulation of TTR may also be found in BSE-infected macaques, further reinforcing the hypothesis of a common mechanism in AD and TSEs. Taken together, these data may suggest innate immune system activation and inflammatory response in these diseases [96], leading to a sustained up-regulation of both SERPINA3 and TTR genes simultaneously: SERPINA3 as inflammation effect, TTR as attempt to neutralize the infectious agent preventing its deposition. However, analysis of the microarray data did not reveal relevant deregulation of other genes typically involved in neuroinflammation and/or immune response, such as cytokines and other mediators. Even though some authors have reported alteration of these pathways [97], in our array IL6, TNFα, GFAP and CD68 showed a fold change < [2], suggesting that inflammatory responses may not be particularly severe in this model.

One last point that remains to be addressed is the expression of the prion protein gene itself (PRNP in humans) upon infection. Because of shortage of cDNA, we were not able to validate its levels in our samples. Nevertheless, our microarray data did not identify any changes between control and infected samples, at least at the mRNA level. This is in agreement with findings reported for BSE-infected cattle [3], but differs from the situation in sporadic CJD patients who show reduced mRNA expression [97]. Whether this disagreement is related to the host or the infectious agent needs to be explored.

Conclusions
To our knowledge, this is the first genome-wide expression study in the gyrus frontalis superior region of cynomolgus macaques inoculated with BSE. Using microarray and RT-qPCR technologies we identified a gene signature able to distinguish infected macaques from control animals. These results could be extremely helpful in understanding the progression of the disease, allowing for the identification of some key players which, if not being the cause of the onset, could be some of the target genes affected by the disease. Therefore, after deeper investigations to validate these targets at the protein level and confirm their specificity for prion diseases, they may be exploited as potential biomarkers to set up pre-clinical diagnostic tests.

In particular, our findings support the hypothesis of a potential shared mechanism underlying the onset and the development of all neurodegenerative disorders, as the majority of our DEGs are known to be involved in other diseases such as AD or PD. This is in concordance with very recent data supporting the idea of a unifying role of prions in these diseases in general and maybe a prion-like behavior for most neurodegenerative disorders [98]. Furthermore, some of the DEG transcripts we found are present also in blood (hemoglobin, transthyretin, serpin peptidase inhibitor) and among them hemoglobin exhibited decreased expression throughout the entire course of the infection, including preclinical time points, in mouse models. Therefore, there is the intriguing possibility to employ these "readily available" biomarkers for diagnostic purposes, especially if additional studies will confirm the expression level of the proteins encoded by these DEGs in brain and/or blood tissue.

In general, our results suggest that, in order to identify potential biomarkers and drug targets for prion diseases and other neurodegenerative disorders, a combination of various pathways has to be targeted, including oxygen homeostasis, lipid metabolism and inflammation response.

In summary, large-scale transcriptome analyses of human TSEs are rare [97,99] and primate models are a valid approach to better understand the mechanisms of these fatal diseases. Even with all the limitations discussed above, our BSE-infected macaques are, to our knowledge, the closest available model for human vCJD and these results, obtained with an unbiased methodology as the gene expression microarray technology, are contributing to shed some light on the molecular basis of TSEs as well as neurodegeneration as a whole.

Methods
Ethics statement
Ethics approval for the study was issued by the Lower Saxony Ministry for consumer protection and food safety (509.42502/08/07.98). Animal experimentation
was performed in accordance with section 8 of the German Animal Protection Law in compliance with EC Directive 86/609.

Samples
Samples were derived from six BSE-infected macaques, Macaca fascicularis (A1 to A6) that were intracranially inoculated with a single dose of 50 mg brain homogenate (10% wt/vol) [33,37]. One cynomolgus macaque (B6) was orally inoculated with the same material; inoculation was performed per os, as single dose.

Brain material from five age- and sex-matched non-infected cynomolgus macaques (CovA, CovB, CovC, CovD1, CovD2) was obtained from Covance Laboratory Münster GmbH and processed in an equivalent manner.

Tissue and RNA extraction
At autopsy of seven BSE-infected cynomolgus macaques at advanced stage of disease and five non-infected control animals, one hemisphere of the brain was sliced dorso-ventrally and snap-frozen on dry-ice plates. The gyrus frontalis superior region was macroscopically identified on the frozen tissue and removed via a biopsy stamp. Total RNA (RNA > 200 bases) was isolated by manually homogenizing the material with micro pestles (Kisker Biotech GmbH) in TRIzol (Invitrogen). RNA isolation was performed according to the supplier’s instructions. Following RNA isolation, a DNease I digestion was performed using 1 unit of enzyme per μg RNA (Fermentas) for 30 min at 37°C, and heat inactivated for 5 min at 95°C followed by precipitation with Sodium Acetate/Ethanol. RNA was checked for quantity and purity on a Spectrophotometer 2000 (PEQLAB) and for integrity of the 18S and 28S ribosomal bands by capillary electrophoresis using the 2100 Bioanalyzer (Agilent Technologies).

Immunoblot analysis
PK-treated (50 μg/mL for 1 hour at 37°C) and untreated brain homogenates corresponding to 0.7 mg or 0.3 mg brain tissue, respectively, were separated on 12% Bis/Tris Acrylamide gels (NuPAGE, Invitrogen) and transferred to nitrocellulose membranes (Protran, Schleicher & Schüll, Germany). Detection of macaque PrPSc was performed using the monoclonal anti-PrP antibody 11C6 and a Peroxidase conjugated anti-mouse IgG-antibody (Sigma-Aldrich, Germany). Signal was visualized using a chemiluminescent substrate (Super Signal West Pico, Pierce) and high sensitivity films (Amersham). Densitometric analysis of PrPSc was performed using the Image J program 1.37v.

Microarray analysis using the GeneChip® Rhesus Macaque genome array
Samples were labeled using the GeneChip® 3’IVT Express Kit (Affymetrix®). Reverse transcription of RNA was performed using 500 ng of total RNA to synthesize first-strand cDNA. This cDNA was then converted into a double-stranded DNA template for transcription. In vitro transcription included a linear RNA amplification (aRNA) and the incorporation of a biotin-conjugated nucleotide. The aRNA was then purified to remove un-incorporated NTPs, salts, enzymes, and inorganic phosphate. The labeled aRNA of each animal was fragmented (50–100 bp) and hybridized to a GeneChip® Rhesus Macaque Genome Array (Cat No’ 900656; Affymetrix®). The degree of fragmentation and the length distribution of the aRNA were checked by capillary electrophoresis using the Agilent 2100 Bioanalyzer (Agilent Technologies).

The hybridization was performed for 16 h at 45°C in the GeneChip® Hybridization Oven 640 (Affymetrix®). Washing and staining of the arrays were performed on the Gene Chip® Fluidics Station 450 (Affymetrix®) according to the manufacturer’s recommendations. The antibody signal amplification and washing and staining protocol were used to stain the arrays with streptavidin R-phycocerythrin (SAPE; Invitrogen). To amplify staining, SAPE solution was added twice with a biotinylated anti-streptavidin antibody (Vector Laboratories, Burlingame, CA, USA) staining step in-between. Arrays were scanned using the GeneChip® Scanner 3000 7G (Affymetrix®).

Microarray data analysis
Intensity data from the CEL files were imported to the Partek® software including a quality control based on internal controls. All chips passed the quality control and were analyzed using the Limma package [100] of Bioconductor [101,102] and the Partek® software. The microarray data discussed in this paper were generated conforming to the MIAME guidelines and are deposited in the NCBI’s Gene Expression Omnibus (GEO) database [103]. They are accessible through GEO series accession number GSE52436 (see section: Availability of supporting data).

The microarray data analysis consisted of the following steps: 1. quantile method normalization, 2. global clustering and PCA-analysis, 3. fitting the data to a linear model, 4. detection of differential gene expression and 5. over-representation analysis of differentially expressed genes. Quantile-normalization was applied to the log2-transformed intensity values as a method for between-array normalization to ensure that the intensities had similar distributions across arrays.

For cluster analysis, we used a hierarchical approach with the average linkage-method. Distances were measured as 1 - Pearson’s Correlation Coefficient. The PCA was performed using the princomp-function in the Partek® software. To estimate the average group values for each gene and assess differential gene expression, a
Reverse transcription and RT-qPCR
Validation by quantitative reverse transcription real-time PCR (RT-qPCR) was performed using gene-specific primer pairs. cDNA synthesis was accomplished using 100 ng RNA, 10 ng random hexamer primer, 2 mM dNTPs, 0.5 U RNase inhibitor and 5 U reverse transcriptase (Bioline) in 1× reaction buffer. For each sample a negative control was carried along by omission of the reverse transcriptase (–RT control).

The cDNA was diluted 1:10 prior to RT-qPCR. Ten ng RNA equivalent was added to the reaction mix including 2× iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Inc.), 400 nM of the corresponding forward and reverse primer (Sigma), and quantified in technical triplicates on a qPCR (Bio-Rad Laboratories, Inc.), 400 nM of the corresponding forward and reverse primer (Sigma), and quantified in technical triplicates on an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.). All primers used for RT-qPCR are listed in Table 3.

After initial denaturation for 3 min at 95°C, 45 cycles were performed at 95°C for 15 sec and 58°C for 1 min. Differential gene expression of candidates was calculated using the pair and sample. The relative expression ratio was calculated using the unpaired student t-test (p < 0.05). Melting curve analysis and gel electrophoresis of amplification products were performed for each primer pair to verify that artificial products or primer dimers were not responsible for the signals obtained. Some results were further confirmed using TaqMan® MGB probes and iQ™ Multiplex Powermix (Bio-Rad Laboratories, Inc.). The primer sequences, the reaction setup and the cycling conditions were the same as described above.

The probe sequences used for the detection of specific targets were:

- GAPDH: 5’-FAM CGGCCAAAGGTACTCCATGA-3’
- ACTB: 5’-FAM-AACAGATGAGATGGCATCCG-3’
- HBB: 5’-FAM-AAGTCTTGGTCCTTATGATGG-3’
- HBA2: 5’-FAM-TGGCGAGTATGGGTCGGAGG-3’
- SERPINA3: 5’-FAM-TTCTGGCCTCTGTGATTCC-3’
- TTR: 5’-FAM-ATCGTGTGCTGTGAATACCACCTCCTG-3’
- APOCI: 5’-FAM-TGGAGGCAAGGCTTGGGAAGTG-3’

Availability of supporting data
The microarray data set supporting the results of this article is available in the Gene Expression Omnibus (GEO) repository, [http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=wnmjwqghzcpod&acc=GSE52436].

The DEGs were analyzed for their functions, pathways and networks using Ingenuity Pathways Analysis-IPA® [http://www.ingenuity.com/products/ips/try-ips-for-free].

Additional files
Additional file 1: List of 86 differentially expressed probe sets with p values ≤ 0.005 and FC ≥ |2|. Probe ID, Gene Symbol, Gene Name and RefSeq Transcript IDs annotation as of release 29 of the Affymetrix® Rhesus Annotation library (01/July/09). p-values and fold changes are reported for all 86 probe sets.

Additional file 2: List of 97 differentially expressed probe sets selected as RT-qPCR candidates. Probe IDs and Previous Gene Symbol annotation as of release 29 of the Affymetrix® Rhesus Annotation library (01/July/09). Current Gene Symbol annotation as of the latest Affymetrix® Rhesus Annotation library (release 32 - 09/June/11), Gene Name and RefSeq Transcript IDs as of Ensembl release 72 (June 2013). Annotation using alignment with the human genome has been performed (as stated in the gene name column) for the most highly regulated probe sets with unknown macaque annotation. p-values and fold changes are reported for all genes.

Additional file 3: Evaluation of reference gene expression stability across non-infected and BSE-infected samples. For each sample, average values of absolute C_{TTR} (+/- SD) of triplicate wells for GAPDH (grey) and ACTB (white) are shown.

Additional file 4: ΔC_{T} values for all genes showing variability among BSE-infected samples. ΔC_{T} values (+/- SD) normalized against GAPDH. Very similar results were obtained with normalization against ACTB (data not shown).

Additional file 5: ΔC_{T} values for all genes showing variability among non-infected samples. ΔC_{T} values (+/- SD) normalized against GAPDH. Very similar results were obtained with normalization against ACTB (data not shown).

Additional file 6: ΔΔC_{T} values of selected genes in the infected samples. ΔΔC_{T} values (+/- SD) for HBB, NR4A2, NCAM1, USP16 and ARFICT normalized against GAPDH. In the orally-infected animal B6 (white) compared to intracranially infected samples A1-A6 (grey). Only 5 genes were analyzed for animal B6 due to shortage of cDNA.

Additional file 7: Cluster analysis. Cluster analysis was performed using a hierarchical approach with the average linkage-method for all animals (panel A) or excluding the orally infected one, B6 (panel B).

Additional file 8: SYBR® Green-based RT-qPCR validation of microarray results. Relative expression levels of 11 genes in BSE-infected cynomolgus macaques normalized against ACTB as reference gene.

Additional file 9: Comparison between SYBR® Green -based and TaqMan® probe-based results for TTR. Average values of absolute C_{T} (+/- SD) of triplicate wells for TTR obtained with SYBR® Green (grey) and TaqMan® probe (white) detection methods in BSE-infected samples are shown.
Additional file 10: RT-qPCR analysis of blood specific marker RHAG. C1 values for the erythrocyte marker RHAG were monitored across BSE-infected (solid fill) and non-infected (dotted fill) samples. Human blood cDNA was used as positive control (gradient fill). Note that for almost all the samples, C1 values were ≥35 therefore indicating a very low expression level. Primer sequence (3′-5′): RHAG: F = AGGCAAGCTCAA CATGTGTC, R = GGGATGACGATCTGGTGGAA.

Additional file 11: RT-qPCR analysis of blood specific marker ALAS2. C1 values for the erythrocyte marker ALAS2 were monitored across BSE-infected (solid fill) and non-infected (dotted fill) samples. Human blood cDNA was used as positive control (gradient fill). Note that for almost all the samples, C1 values were ≥35 therefore indicating a very low expression level. Primer sequence (3′-5′): ALAS2: F = TCCCTCTACGCTGCGGAAAC, R = GAGCTACGGACACGTCTTGGAA.

Acknowledgements

The authors wish to thank Dr. Lisa Gasperini for the RT-qPCR initial technical support, Dr. Paolo Vatta for suggestions on primer design and useful discussions, and Prof. Stefano Gustincich for useful discussions. This work was supported by the European Regional Development Fund, Cross-Border Cooperation Italy—Slovenia, Programme 2007–2013, Strategic Project TRANSCARE to GL, and by the European Community supported by the European Regional Development Fund, Cross-Border support. Dr. Paolo Vatta for suggestions on primer design and useful discussions. This work was supported by the European Regional Development Fund, Cross-Border Cooperation Italy—Slovenia, Programme 2007–2013, Strategic Project TRANSCARE to GL, and by the European Community supported by the European Regional Development Fund, Cross-Border support. Dr. Paolo Vatta for suggestions on primer design and useful discussions. This work was supported by the European Regional Development Fund, Cross-Border Cooperation Italy—Slovenia, Programme 2007–2013, Strategic Project TRANSCARE to GL, and by the European Community supported by the European Regional Development Fund, Cross-Border support. Dr. Paolo Vatta for suggestions on primer design and useful discussions.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

MB conceived and designed the RT-qPCR validation studies, carried out the initial optimization experiments, supervised all the experiments as well as the data interpretation, and wrote the manuscript. SV carried out the functional classification of the DEGs, performed most of the RT-qPCR experiments with the related statistical analysis, and provided the initial draft of the manuscript. ACS performed RNA isolation and cDNA preparation of BSE-infected and non-infected brain material, and contributed to the drafting of the manuscript. JM performed the immunoblot analysis. DM provided tissues and contributed to the drafting of the manuscript. C1 was responsible for the statistical analysis, and provided the initial draft of the manuscript. MB conceived and designed the RT-qPCR validation studies, carried out the related statistical analysis, and provided the initial draft of the manuscript. ACS performed RNA isolation and cDNA preparation of BSE-infected and non-infected brain material, and contributed to the drafting of the manuscript. JM performed the immunoblot analysis. DM provided tissues and contributed to the drafting of the manuscript. C1 was responsible for the statistical analysis, and provided the initial draft of the manuscript. MB conceived and designed the RT-qPCR validation studies, carried out the related statistical analysis, and provided the initial draft of the manuscript.

Author details

1Department of Neuroscience, Scuola Internazionale Superiore di Studi Avanzati (SISSA), Via Bonomea 265, 34136 Trieste, Italy. 2Unit of Infection Models, German Primate Center, Kellnerweg 4, 37077 Göttingen, Germany. 3Microarray Core Facility, University Medical Center Göttingen, Justus-von-Liebig-Weg 11, 37077 Göttingen, Germany. 4Molecular and Cell Physiology, Hannover Medical School, Carl-Neuberg Str. 1, D-30625 Hannover, Germany.

Received: 13 January 2014 Accepted: 7 May 2014

Published: 5 June 2014

References


Cite this article as: Barbisin et al.: Gene expression profiling of brains from bovine spongiform encephalopathy (BSE)-infected cynomolgus macaques. BMC Genomics 2014 15:434.