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Review Article

Application of "omics" to Prion Biomarker Discovery

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The advent of genomics and proteomics has been a catalyst for the discovery of biomarkers able to discriminate biological processes such as the pathogenesis of complex diseases. Prompt detection of prion diseases is particularly desirable given their transmissibility, which is responsible for a number of human health risks stemming from exogenous sources of prion protein. Diagnosis relies on the ability to detect the biomarker PrP^{Sc}, a pathological isoform of the host protein PrP^{C}, which is an essential component of the infectious prion. Immunochemical detection of PrP^{Sc} is specific and sensitive enough for antemortem testing of brain tissue, however, this is not the case in accessible biological fluids or for the detection of recently identified novel prions with unique biochemical properties. A complementary approach to the detection of PrP^{Sc} itself is to identify alternative, "surrogate" gene or protein biomarkers indicative of disease. Biomarkers are also useful to track the progress of disease, especially important in the assessment of therapies, or to identify individuals "at risk". In this review we provide perspective on current progress and pitfalls in the use of "omics" technologies to screen body fluids and tissues for biomarker discovery in prion diseases.

1. Introduction

Prion diseases, or Transmissible Spongiform Encephalopathies (TSEs), are invariably fatal neurodegenerative diseases associated with the conversion of the normal host cellular prion protein (PrP^{c}) into the abnormal protease-resistant isoform (PrP^{Sc}) [1]. They occur in a wide range of host species including humans, the most common of which is sporadic CJD (sCJD), occurring at a rate of approximately 1 case per million a year worldwide and accounts for greater than 80% of CJD cases [2]. Amino acid changes, which include point or insertional mutations in the normal (cellular) prion protein (PrP^{C}) encoded by the *PRNP* gene, are linked to genetic prion diseases such as Gerstmann-Strausler-Sheinker (GSS) disease, fatal familial insomnia (FFI), and genetically associated Creutzfeldt-Jakob disease (CJD). Acquired forms of disease are caused by ingestion of, or exposure to, contaminated biological material via food or during medical procedures. Kuru, found amongst the Fore tribe in Papua-New Guinea, was the first known human transmissible spongiform encephalopathy and resulted from exposure to infected material during ritualistic cannibalism. More recently a new human prion disease has emerged, variant CJD (vCJD), which is associated with exposure to the BSE agent in beef. Cases of iatrogenic transmission have also occurred through the use of improperly sterilized surgical instruments, the use of human growth hormone derived from cadaveric pituitaries, and transplantation of corneas and dura mater from infected patients [3]. Recently, human-to-human transmission of vCJD has been reported through blood transfusion [4]; human-adapted prions are more readily transmitted from human to human via this route than via ingestion of BSE prions from contaminated meat products [5].

Animals affected by TSEs include sheep (Scrapie), cattle (BSE) and mule, deer, elk (CWD). The impact of animal TSEs is twofold; firstly, there is a risk of transmission to humans, and secondly, the economic impact on animal production has been substantial. Although scrapie has been endemic for hundreds of years in many parts of the world its transmission to humans has never been reported. However, when vCJD in humans was determined to be associated with consumption of contaminated food there was concern as to what extent the population has been exposed. In the recent

years, the incidence of CWD has increased markedly within North America and although it has not been linked to CJD either epidemiologically, or by laboratory confirmation, there is concern about the possibility for cross-species transmission [6, 7]. TSEs in animals have caused huge economic loses. Since the BSE epidemic began in 1986, millions of cattle have been slaughtered and bans on the importation of beef have affected many countries and cost billions of dollars.

The threat posed to public health by dietary and medical exposure to prions has driven tremendous efforts to develop sensitive methods of detection of prions to control the spread of human and animal TSEs. All the commercially available diagnostic tests for TSEs rely on the direct detection of the proteinase K resistant, misfolded form (PrP^{Sc}) of cellular prion protein in the central nervous system (CNS). Although methodologies are sensitive and specific for postmortem diagnosis, the use of PrP^{Sc} as a preclinical or general biomarker for surveillance is difficult, due to the fact that it is present in extremely small amounts in accessible tissues or body fluids such as blood, urine, saliva, and cerebrospinal fluid (CSF). Recently, amplification techniques have been developed which have enabled increased sensitivity. These are based on the ability of the disease-related abnormal isoform, PrP^{Sc}, to convert a pool of normal PrP^{C} to a proteinase K resistant form thus "amplifying" the original infectious seed. Amplification can be increased by breaking down the resulting aggregated seeds of PrP^{Sc} to smaller units which in turn act as seeds for further replication until levels of PrP^{Sc} detectable by Western blot or ELISA are produced. These developments may provide the sensitivity necessary for a blood or food screening test useful for some of the transmissible TSEs. However, it has recently been reported that proteinase K sensitive, pathological isoforms of PrP may have a significant role in the pathogenesis of some prion diseases [8]. Novel PrP^{Sc} isoforms with unique biochemical properties may be generated in sporadic or acquired disease that exhibit increased sensitivity to PK digestion. Therefore, conventional tests may show significant discordance between the amounts of PrP^{Sc} detected and the infectivity observed. Accordingly, the development of new diagnostic tests that do not rely on PK digestion is desirable. Another challenge for diagnosis and surveillance is that hosts can incubate infectious prion agents for many months or years, during which time they exhibit no overt symptoms. Incubation periods for some human prion diseases can be as long as 40 years and given the recent cases of vCJD transmitted by blood transfusion the need for development of a test for screening blood has increased. Furthermore, a noninvasive test to identify the early stages of CJD would be valuable in the development of treatment strategies for TSEs.

A biomarker is defined as a discriminative feature that can be measured objectively and used as an indicator of biological processes such as normal health, pathogenic processes, or pharmacological responses to a therapeutic treatment. Biomarkers include physical traits such as temperature or blood pressure, imaging of pathological features such as amyloid deposition or ventricular volume changes in the brain, and the presence of biological molecules in tissues and body fluids such as blood or urine. One aim of biomarker discovery is the detection of molecular correlates of disease that can be used as early diagnostic tools. However, this type of marker has a crucial requirement for high sensitivity and specificity. Few markers of this type have emerged from omics studies, not only for prion diseases but also for the multitude of other diseases that have been investigated. Biomarkers with broad specificity for neurodegeneration (not only in TSEs) may also be useful as general indicators of disease pathology; identification of biomarkers to follow the progression of disease would significantly impact the time and cost required to evaluate the efficacy of therapeutic interventions.

The search for biomarkers (other than PrP^{Sc}) as tools for diagnosis of prion diseases has a long history; in fact there are several protein markers in cerebrospinal fluid (CSF) that are useful for diagnosis of human prion diseases. In 1980, two proteins were identified by 2D electrophoresis in the CSF of sporadic CJD patients. One of these, a 30 kDa polypeptide, was identified as a member of the 14-3-3 family of proteins, a normal neuronal protein that is released into the CSF after neuronal insult. The CSF detection of 14-3-3 protein by Western blot is widely used as diagnostic evidence of CJD, in conjunction with clinical indicators of prion disease [9]. The detection of 14-3-3 in CSF is a highly sensitive marker for sCJD, iCJD, and the genetic form of CJD, however, is much less sensitive for diagnosis of vCJD, GSS, and FFI [10]. A number of other proteins are also increased in the CSF of CJD patients including Tau and phospho-Tau, S-100ß, and neuron-specific enolase (NSE). Levels of the tau protein are raised in patients affected by all forms of CJD including vCJD. A recent study determined that the detection of tau in CSF has a sensitivity of 80% and specificity of 94% for vCJD, higher than any of the other markers tested. Testing for the presence of multiple markers, 14-3-3 protein in CSF plus tau, results in the highest sensitivity for the use of these biomarkers in diagnosis of human TSEs [11]. These CSF biomarkers have proven to be extremely useful in confirmatory diagnostics of CJD cases, and their widespread use illustrates an important role for surrogate marker detection in prion disease. They do not, however, have comprehensive value for surveillance of transmitted TSEs and are useful only when the disease is already at an advanced state. Ideally biomarkers able to detect all TSEs even at preclinical stages of infection are desirable. This paper will focus on recent efforts to harness the plethora of omic technologies to identify not only potentially diagnostic biomarkers, but also markers to follow disease progression or which have risk determining potential.

2. Tools for Biomarker Identification in TSEs

In the last few years technologies to study all the genes and proteins expressed in an organism or cells simultaneously have become accessible for most laboratories, and these provide a platform for biomarker discovery. Experimental strategies to detect biomarkers generally involve comparisons of mRNA, protein, peptide, and metabolite abundances

between samples collected from infected versus control tissues. The most commonly used technologies are described here, followed by discussion of approaches that are being used in the identification of useful biomarkers in relation to prion disease, as well as potential directions for future research.

2.1. Differentially Expressed mRNA Biomarkers. High-throughput genomic techniques, most commonly DNA microarrays and subtractive hybridization approaches, are the most frequently reported methodologies used for the identification of deregulated genes in tissues and cells [12]. These expression profiles, or "signatures," can themselves be used grossly as biomarkers, are relatively easy to generate, and the techniques can be readily adapted to high throughput. Signatures can be compared across multiple time points, tissues, or experimental populations to look for molecules predictive of disease. With the advent of ultra-high-throughput sequencing technologies, researchers are increasingly turning to deep sequencing for gene expression studies [13–15]. Advantages over microarray approaches are that different variants or "isoforms" of mRNA generated by differential splicing, alternative termination, and alternative transcriptional start sites are all identified. Additionally, these methodologies are also well suited for the identification and profiling of small RNAs, such as miRNAs, which are increasingly thought to play important roles in neurodegenerative diseases and may be useful biomarkers [16]. One recent innovation that has been applied to high-throughput sequencing is the purification of RNA from ribosome complexes prior to deep sequencing to capture those templates actively undergoing translation. This method has been found to be more reflective of protein abundance than are traditional microarray or sequenced mRNA profiles and may improve the ability to infer protein biomarkers from RNA profiles [17].

Changes in mRNA profiles in brain tissue from CJD patients are infrequently studied due to the rarity of cases. Only one report has been published using tissues isolated from the postmortem brain samples of sporadic CJD patients; transcriptional changes pointed to alterations in neuronal dysfunction pathways including the cell cycle, cell death, and the stress response [18]. A number of genomic analyses of brain tissue from rodent adapted models of prion diseases including CJD, scrapie and BSE have been performed, as well as investigation of samples from larger animals, sheep infected with scrapie and cattle infected with BSE [19–27]. These studies have revealed widespread alterations of multiple cellular pathways correlating with the onset of pathological disease including cholesterol homeostasis, ion homeostasis, and regulation of apoptosis, stress response, and metal ion homeostasis. The most consistent finding between experimental models, relates to the onset of neuroinflammation, a process common to many neurodegenerative diseases that is likely induced by damage and death of neurons. Accordingly, many of these transcriptional changes have been consistently identified in multiple neurodegenerative diseases and a selection of these is listed in Table 1. Although differential expression of these genes may not be specific to prion diseases, neuroinflammation-related gene expression may be an excellent choice as candidate biomarkers to track the stage of development of the neurodegenerative process and to predict the response to therapy.

Studies to correlate the temporal changes in neuronal health during disease have not yet been reported in prion-infected neurons; however, hippocampal neurons from Alzheimer's disease (AD) patients have revealed a transcriptional response comprising thousands of genes that significantly correlates with AD markers [31, 32]. It is possible that at least in part, these biological processes may be common to degenerating neurons in multiple degenerative conditions. In this case these biomarkers may well be broadly applicable to track the progression of neurodegeneration. As the vast majority of human samples are collected post-mortem, animal models may be the only practical way of assessing early markers of neuronal status prior to obvious clinical symptoms. A number of studies have attempted to use genomics to determine transcriptional changes at preclinical stages of disease; however, due to the cellular complexity of brain tissue only modest fold changes are revealed. Subtle alterations occurring in a small number of neurons at the onset of disease are likely masked. To get the best results from this type of study it is essential to use large sample numbers for statistical significance. Neuron specific expression changes may be masked even at late stages of disease by the extensive astrocytosis and gliosis that accompany neuronal degeneration. Neurons, and therefore their genetic material, are outnumbered 10 or 20 to 1. Laser capture microdissection to excise specific cell populations is set to overcome many of the limitations of whole tissue analysis and will undoubtedly provide a new "layer" of information regarding specific cellular responses to prion replication.

2.2. Differentially Expressed Protein Biomarkers. Protein biomarkers are particularly well suited for measuring and detecting phenotypic characteristics of disease processes. Proteomic technologies enable the exhaustive analysis of the protein content of a tissue or bodily fluid sample. Only in the recent years has technological advances facilitated the differential measurement of protein abundance levels between multiple conditions at a given time, and just as importantly, provided sufficient "through-put" to attach statistical significance to protein biomarker detection. Proteomics of prion infected tissues also suffer from some of the same drawbacks of genomics studies; samples are often very heterogeneous due to cellular complexity and the stage of disease. One of the major caveats of proteomics for prion disease discovery is that the commonly used rodent models provide very small sample volumes, especially in terms of bodily fluids such as blood, from which only the most abundant proteins can be identified. The laser capture microdissection techniques hold much promise for genomic studies to reduce cell heterogeneity as the small amounts of nucleic acid can be amplified by polymerase chain reaction

Table 1: Genes with differential abundances in prion disease and other neurodegenerative disorders.

Gene	Description	Reference for Prion Disease	Other Neurodegenerative Disorder*
ABCA1	ATP-binding cassette, subfamily A (ABC1), member 1	[20, 22, 23]	AD
APLP1	Amyloid beta (A4) precursor-like protein 1	[20]	AD
APOD	Apolipoprotein D	[20, 20, 23, 25, 28]	AD, NPC
APOE	Apolipoprotein E	[20, 22, 25]	AD, PD and MTS
B2M	Beta-2-microglobulin	[20, 22, 25, 29]	AD, Tay-Sachs, Sandhoff disease, and MTS
CD9	CD9 molecule	[20, 22, 23, 25]	SSPE, CMT
CLU	Clusterin	[20, 25, 30]	AD, PD
CST3	Cystatin C (amyloid angiopathy and cerebral hemorrhage)	[20, 25, 30]	AD, MTS
CTSB	Cathepsin B	[20, 22, 25]	AD, Seizures, Tay-Sachs, and Sandhoff disease
CTSS	Cathepsin S	[20, 22, 23, 25, 28]	AD
GFAP	Glial fibrillary acidic protein	[20, 22, 23, 25, 28]	Tay-Sachs, Sandhoff disease, MTS, and AD
SPARC	Secreted protein, acidic, cysteine-rich (osteonectin)	[20]	Tay-Sachs, Sandhoff disease, and MTS
SPP1	Secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)	[20, 22]	PD

*AD: Alzheimer's Disease; PD: Parkinson's Disease; NPC: Niemann-Pick type C; MTS: Mesial temporal sclerosis; SSPE: Subacute sclerosing panencephalitis, CMT: Charcot-Marie-Tooth disease.

(PCR). However, proteins cannot be amplified and ultrasensitive techniques must be developed in order to perform similar proteomic studies. New techniques for labelling small amounts of protein such as fluorescence saturation labelling may be one step in the right direction to overcome these limitations. This technique has enabled proteomic analysis of hippocampal CA1 neurons in an Alzheimer's mouse model; however identification of such small amounts of protein requires increased sensitivity of mass spectrometry techniques [33]. Given these problems and the scarcity of samples from large animal models and human TSE cases, it is not surprising that only a very small number of prion-related proteomic studies have been reported. Table 2 provides a general summary of these, and some examples from similar studies of neurodegenerative diseases with similar aetiologies as proof of principle.

Current methodologies for proteomics follow two principal steps. Firstly proteins are separated to provide a sample with decreased complexity and then mass spectrometry (MS) is used for protein identification. Separation techniques include surface-enhanced laser desorption ionizing time of flight (SELDI-TOF), two-dimensional gel electrophoresis (2D-GE) and the recently developed two-dimensional differential gel electrophoresis (2D-DIGE) for differential protein analysis, and liquid chromatography (LC). All of these methodologies are not inherently quantitative but have been adapted to allow the user to identify qualitative changes between samples, for example, differential abundances of proteins between diseased and control tissues.

Surface-enhanced laser desorption ionizing time of flight (SELDI-TOF) is a mass spectrometry technique that is based on a combination of techniques, chromatography, and matrix-assisted laser desorption ionization time of flight (MALDI-TOF) [44]. In the first step of SELDI-TOF MS proteins are captured using different platform chemistries such as absorption, electrostatic interaction, and affinity chromatography which reduce the complexity of the original sample. This happens on a small "protein-chip" surface which enables multiple separations to be performed on very small sample volumes at high throughput. Following this, the bound proteins are cocrystallized with an energy absorbing matrix (EAM) which is then vaporized propelling the ionized proteins down the flight tube through an electric field based upon the particles mass/charge (m/z) ratio. Contact of complexes with the detector at the end of the flight tube results in a unique peak resolved by m/z ratio of the original protein or protein isoform. The advantages of this method are the ability to perform high-throughput analysis of hundreds or thousands of samples, resolving power of the captured proteins, and the ease of analysis using dedicated user friendly software. However, protein peaks of interest must be experimentally isolated for identification using peptide mass fingerprinting along with protein purification techniques. This step requires much larger sample volumes than used in the initial discovery stage and can be arduous and time-consuming.

In 2D gel electrophoresis separation of proteins is performed in a polyacrylamide gel according to their size and their charge thus enabling resolution of multiple isoforms of an individual protein. The spot intensities can be used to calculate differences in protein abundance between different samples and individual protein spots can then be excised

Table 2: Potential Biomarkers of Neurodegenerative Diseases Identified by Mass spectrometry (MS) and 2D-Gel Electrophoresis.

Marker	Fluid	Disease*	Reference
10 kDa subunit of vitronectin	Serum	AD	[34, 35]
alpha 1-acid glycoprotein	Serum	AD	[34, 35]
alpha1-antichymotrypsin	Urine	CJD	[36]
Apolipoprotein B100	Serum	AD	[34, 35]
Apolipoprotein E	Serum and CSF	AD and PD	[29, 34, 35]
Cathelicidin antimicrobial peptide (Bos taurus)	Urine	BSE	[37]
Clusterin	Urine, CSF, blood, and plasma	BSE and AD	[37–39]
Complement C3 component C3dg	Serum	ALS and PD	[40]
Complement C3 components of C3c family	Serum	ALS, PD, and AD	[34, 35, 40]
Complement C4	Serum	AD	[34, 35]
Complement Factor H	Serum and Plasma	ALS, PD, and AD	[34, 35, 40, 41]
Fragment Bb of Complement Factor B	Serum	PD	[40]
Haptoglobin α-2 chain	Serum and CSF	AD and PD	[29, 34, 35]
Heart-type fatty acid binding protein (H-FABP)	Plasma and CSF	CJD and AD	[42]
Hemoglobin α-2 chain	Serum	AD	[34, 35]
Histidine-rich glycoprotein	Serum	AD	[34, 35]
Ig Gamma-2 chain C region (Bos taurus)	Urine	BSE	[37]
Transthyretin	Serum and CSF	CJD, AD, PD	[29, 34, 35, 43]
Uroguanylin	Urine	BSE	[37]
Vitronectin precursor	Serum	AD	[34, 35]
α-2-macroglobulin (α-$_2$M)	Plasma and Serum	AD	[34, 35, 41]

*AD: Alzheimer's Disease; PD: Parkinson's Disease; ALS: Amyotrophic lateral sclerosis.

from the gel and identified using MS. Practical issues such as variations in sample preparation make it very difficult to get consistently reproducible gels making this methodology labour intensive. Recent adaptations to this methodology have improved the situation somewhat, with the most significant innovation being labelling of samples using fluorescence dyes (2D-DIGE). In this way it is possible to include three samples per gel, control and infected samples plus an internal standard (pooled samples). This creates a standard for each protein in the analysis resulting in the user being able to make comparisons across different gels with a high degree of confidence [45].

In LC the sample components interact to a varying extent with a chromatographic packing material in a column (stationary phase). A pump moves the mobile phase through the column, and the sample is separated based on a retention time through the column which varies depending on the interactions between the stationary phase, the molecules being analyzed, and the solvent used. Generally protein samples are enzymatically digested prior to loading on the column. This differs from both SELDI and 2D-GE in which it is generally intact proteins that are separated. In one dimension the peptide mixtures are generally too complex to separate; however, in combination with chromatography or 2D gel electrophoresis the methodology provides a means to perform large-scale proteomic analysis with good dynamic range. Labelling of peptides with isotopes enables this methodology to be used to identify the differential abundance of peptides between samples. To do this a stable isotope is used to label peptides. The labelled peptide is chemically identical to its native counterpart, so it behaves in an identical fashion during chromatographic separation, however it is distinguishable by MS, therefore variation in abundance between a tagged and untagged sample can be determined. A number of approaches using this methodology have been described including Isotope Coded Affinity Tagging (ICAT) and the recently described Isobaric Tagging for Relative and Absolute Protein Quantification (iTRAQ) quantitative proteomic approach [46]. iTRAQ is ideally suited for biomarker applications, as it provides both quantification and allows some degree of multiplexing in a single reagent. The isotopic tag can be incorporated either during sample labelling or in vivo (stable isotope labelling, SILAC), further increasing its scope [47].

MS to identify the mass/charge ratio of the peptide/protein of interest or to determine the primary sequence of the peptide is the final step in all proteomic approaches. This step involves ionization of the sample; MALDI and electrospray ionization (ESI) are the most commonly used technologies for this. In MALDI the sample is mixed with a matrix, applied to a target surface and inserted into a vacuum chamber and a laser is used to activate sample ionization. In ESI the sample is dissolved in a solvent and pumped through a narrow, stainless steel capillary. A high

voltage is applied to the tip of the capillary, which is situated within the ionisation source of the mass spectrometer, and as a consequence of this strong electric field, the sample emerging from the tip is dispersed into an aerosol of highly charged droplets. A gas, usually nitrogen, helps this process and directs the spray emerging from the capillary tip towards the mass spectrometer. The ionized sample is generally resolved based on the m/z ratio in a time-of-flight (TOF) analyser. However, a number of choices for ion sources are available and these can be combined with different spectrometers. One promising innovation is fourier transform mass spectrometry (FTMS), a popular tool for discovery due to its high resolving power, mass measurement accuracy, multistage MS/MS potential, and extended dynamic range [48]. When accompanied by 1D or 2D electrophoresis, FTMS has demonstrated an excellent ability to deal with sample complexity for biomarker discovery [49].

One further innovative method sometimes used in biomarker discovery is the antibody microarray, the proteomic equivalent of gene microarrays. In these arrays specific antibodies are spotted onto glass or membranes, or bound to beads in fluidic arrays. Target proteins are then captured from samples of plasma or disrupted tissue and detected using an ELISA type approach using labelled secondary antibodies. The use of these arrays has not been described in prion disease; however a study to screen the abundance of 120 signalling proteins in plasma from Alzheimer's patients was recently reported. A total of 259 samples were analysed with the antibody panel, and 18 proteins were identified as potential biomarkers. These proteins were used to classify blinded samples from Alzheimer's and control subjects with close to 90% accuracy as well as identifying patients with mild cognitive impairment that progressed to Alzheimer's disease 2–6 years later. The 18 proteins are involved in biological processes known to be disrupted in neurodegeneration including deregulation of haematopoiesis, immune responses, apoptosis and neuronal support [50].

3. Searching the Body for Prion Related Biomarkers

The complexity of prion-induced neurodegenerative diseases along with their unique molecular mechanisms poses huge challenges to understand their biology and to identify antemortem biomarkers. In addition the diseases are aetiologically heterogeneous. Prion diseases are unique in that they can occur in one of three ways, spontaneously, via genetic changes, or acquired through oral or iatrogenic transmission of the infectious agent. Spontaneous or genetic forms of the disease arise and progress solely in the brain, with minimal to no agent replication in the periphery. Only in the case of a TSE transmitted by digestion or blood transfusion, such as in vCJD, does the initial replication of the agent take place in the periphery. Implications of these aetiologies are that diagnostic biomarkers, especially for preclinical stages of disease, will unlikely encompass all forms of prion disease.

Prions acquired from different sources, strains, or different genetic origins present with differing symptoms, incubation periods, and pathobiological features will result in ambiguities in biomarker detection. Accordingly, the tissues and bodily fluids chosen for biomarker selection need to be tailored to the TSE under study and the specific aim of biomarker selection. For example, blood or lymphoid tissue may be the sample of choice for selection of a preclinical marker of vCJD infection, brain tissue for indicators of prognosis, and blood or urine to follow disease progression or perhaps to identify individuals more susceptible to disease or particular treatments.

While specificity to prion disease would be a requirement for identifying preclinical cases or screening donated blood, for example, progression of disease could be followed using markers of broader specificity such as indicators of CNS damage and neuronal death. To further complicate the selection of biomarkers in prion disease the long incubation period prior to development of clinical symptoms, from many months to many years, may well result in temporal differences in marker expression. Therefore disease stage, as well as target tissue, needs to be taken into account when deciding on a sampling strategy and evaluating biomarkers. The greatest public health risk accompanies those TSEs that can be transmitted in food, medical products, and blood such as vCJD or any future novel outbreaks. A closer look at prion pathogenesis in these instances may lead to the identification of appropriate tissues and body fluids for early detection of prion diseases. A summary of these potential tissues and body fluids is given in Figure 1(a). In Figure 1(b) a schema illustrating the incubation period of a typical TSE indicating disease stages optimal for identification of biomarkers for different purposes is provided. In the next section we describe some of these tissues and bodily fluids that are potential reservoirs for biomarkers in more detail, and review related biomarker studies.

3.1. Biomarkers in Lymphoid Tissues. Following ingestion of contaminated foodstuffs, PrP^{Sc} must be transported from the gut to the brain. Current data suggests that PrP^{Sc} crosses the gut epithelium, possibly through M cells, and rapidly accumulates in the gut-associated lymphoid tissues (GALTs), mainly in the mesenteric lymph nodes, and then in the spleen early in the preclinical phase prior to neuroinvasion. Two studies of gene expression changes have been described using tissue from infected and control Peyers patches. In the first samples from cattle orally infected with BSE revealed 90 genes and 16 ESTs to be differentially expressed. Of these genes, five were found to be related to immune function. These were major histocompatibility complex (MHC) class II, MHC class II DQ alpha, L-RAP, and two hypothetical proteins. Other differentially expressed genes identified related to cellular and metabolic processes including the development and maturation of cells [51]. In the second study the mRNA level of a pancreatitis-associated protein (PAP)-like protein was found to be elevated in the ileal Peyer's patch of lambs during the early phase of scrapie infection [52]. Although the first study tissue analysed was

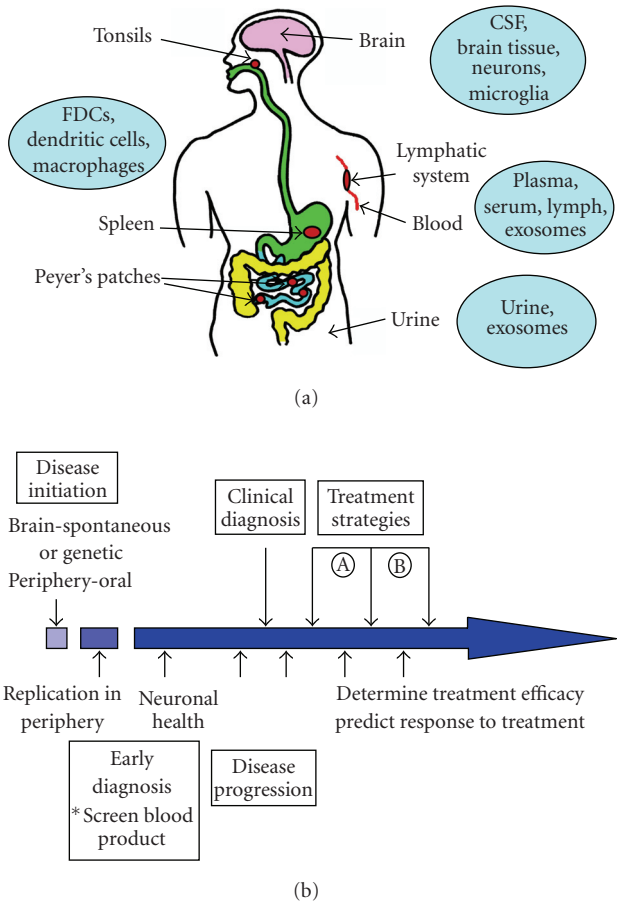

Figure 1: Summary of the tissues, cell populations, and bodily fluids that provide a source of discovery for biomarkers of prion infection (a). Schema to illustrate the stage-specific diagnostic and therapeutic windows for biomarker identification for diagnosis, disease progression, and monitoring pharmacological interventions (b).

12 months following oral inoculation Peyers Patches may be sources of very early disease specific markers.

In B cell follicles, PrP^{Sc} is detected on follicular dendritic cells (FDCs) networks and macrophages within germinal centres (GCs) [53–55] of the spleen and lymph nodes. Although prion infection still occurs in the absence of FDCs, the infection is severely delayed suggesting that FDCs are significant cellular sites of peripheral replication [53, 56, 57]. The lymphoinvasion step is an appealing point in the disease process for the identification of biomarkers as it occurs prior to neuroinvasion in those TSEs with ingestion aetiologies and is thus the optimal time point for early diagnosis or therapeutic intervention [58]. In addition lymphoid tissues are also more accessible than brain tissue for sampling purposes. Despite this, very few studies describing gene or protein biomarkers associated with prion replication in spleen or lymph nodes have been published. A recent study has identified changes in the expression of *St6gal1*, *St3gal5*, *Man2a1*, *Hexb*, *Pigq*, glycosylation-related genes, in the spleens of scrapie infected mice [59]. The authors suggest that this indicates modification of the splenic metabolism of glycosphingolipids associated with prion disease.

FDCs themselves express high levels of PrP^{C} and are able to retain antigens for relatively long periods of time, including replicating PrP^{Sc}, making them good cell candidates for the identification of biomarkers. However, FDCs make up less than 1% of the total cells within the spleen or lymph node which likely means that most disease associated expression changes are masked when looking at whole tissues. They are also tightly associated with other cell types, especially B cells, which along with the gap in knowledge regarding their lineage and molecular characteristics make them difficult to isolate for independent analysis. One study, however, has shown an increase in clusterin expression in association with abnormal PrP accumulation expression on FDCs during TSE disease, particularly human vCJD cases [60].

Lymph fluid passes through lymph nodes and contains a mixture of proteins and antigens picked up from the interstitial tissues which it drains; it therefore reflects changes associated with any immunoinflammatory response within the node itself. The protein composition therefore emulates that of blood as well as being highly reflective of the host response to mucosal challenge. Given the route of transmission of acquired prion infections following ingestion and the subsequent preclinical replication in lymph nodes, lymph fluid may be an excellent, as yet untested source, from which biomarkers that accompany preclinical prion disease progression can be identified. In the human genetic and sporadic forms of prion diseases, the disease occurs spontaneously within brain tissue and there is no preliminary involvement of peripheral tissues, and so FDCs and macrophages do not play a role in disease pathogenesis. Indeed PrP^{Sc} is most often absent in the lymphoid tissues, although, secondary infection of lymph nodes can occur in some instances, as recent studies show that PrP^{Sc} can be detected in spleens of patients with sCJD [61]. Biomarkers specifically expressed in lymphoid tissue or FDCs would therefore be useful for the detection of TSEs acquired specifically by peripheral exposure.

3.2. Biomarkers in CSF. Following neuroinvasion and establishment of prion disease in the brain of transmitted TSEs and following the onset of sporadic or genetic forms of disease, the CSF has been the tissue of choice for diagnosis and biomarker identification, due to its obvious association with the CNS and the fact that it is somewhat more accessible than CNS tissue itself. CSF is ideal for use in protein and gene expression profiling techniques to identify biomarkers both to track progression of neurodegeneration, as well as having the potential to contain biomarkers specific to prion replication.

Studies using 2D gel electrophoresis to profile proteins in CSF have the longest history in biomarker identification for CJD. A number of studies have identified cystatin C, transferrin, ubiquitin, Apoliprotein J, lactate dehydrogenase, 14-3-3 proteins plus other as yet unidentified polypeptides as potential biomarkers [43, 62, 63]. In addition a study employing SELDI-TOF analysis of CSF revealed

a 13.4 KDa protein. Further analysis using cationic exchange chromatography, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and liquid chromatography-tandem mass spectrometry (LC-MS/MS) revealed this protein to be cystatin C. Immunoblotting confirmed the significantly increased abundance of cystatin C in all eight CJD-affected patients included in this preliminary study [64]. Interestingly many, if not all, of the genes mentioned above have also been identified as differentially expressed in brain tissue of rodents infected with prions. A study by Brown et al. also links the levels of differentially expressed genes to protein levels in the CSF, an observation that suggests that candidate gene panels identified from animal studies could be used for prediction of disease-associated CSF biomarkers [30, 65–67]. The detection of biomarkers in the CSF appears promising; however, all of the above-mentioned proteins have been observed to increase in abundance in the brains and/or CSF in other neurodegenerative conditions such as Alzheimer's disease or in traumatic brain injury. This finding suggests that the proteins identified to date are not specific markers for prion disease but general biomarkers of neurodegeneration disease or trauma. However, the use patterns of expression of panels of these markers may confer specificity, or alternatively, these markers could well be used as useful indicators of disease progression.

Recently, a proof of principle study revealed that the combination of MALDI-FTMS, in addition to machine learning for the classification of mass spectral features, is able to identify preclinical protein signatures from the CSF of prion infected animals with reasonable predicative accuracy [68]. In this study CSF was isolated from 21 infected and 22 control hamsters at a time-point when approximately 80% of the expected incubation period had been completed. CSF was isolated and subjected to trypsin digestion without further fractionation and subjected to MALDI-FTMS, a methodology described earlier in the chapter. Peptide profiles were identified and the peaks compared using IonSpec peak picking software; a number of peptide peaks exhibiting differential abundances were identified. It was reported that these peaks were amongst the least abundant peptides detected in the study, highlighting the need for improved methodologies to target low abundance proteins and peptides in biomarker studies. A linear support vector machines (SVM) and 10-fold leave-one-out cross validation was used to evaluate the predictive accuracy of the peptide peaks showing the greatest differences in abundance between infected and non-infected hamsters. The predictive accuracy was determined to be 72%; true positive rate of 73% and false positive rate of 27% using a 10-fold leave-one-out cross validation demonstrated a potential for the use of proteomic profiling of CSF for the identification of multiple biomarkers with diagnostic value. However, the identity of these peptides was not resolved in this study. Although specificity was fairly low, as mentioned by Herbst et al. a disease-specific protein signature clearly exists in the CSF. This type of approach combined with a prefractionation step to improve the accuracy of biomarker detection in the range of low abundance proteins could well result in identification of a panel of markers with diagnostic potential. In this case the small size of hamsters and small volumes of CSF precludes this approach so larger animal models or human samples would be required. The comparison of protein profiles with other forms of neurodegenerative disorders would be the next step in increasing specificity to prion diseases.

3.3. Biomarkers in Blood. Blood is the ideal reservoir for markers indicative of the progression of disease processes in the body, samples are easy to obtain and noninvasive to the patient, and as it circulates throughout the whole body, is a repository for biomarkers of general health and disease. Although no secondary transmission of the sporadic or genetic forms of CJD has been reported, secondary transmission of vCJD from "human-to-human" can occur via blood transfusion. The infectious agent itself is present in the blood in this instance probably following replication in peripheral lymphoid tissue. An in vitro test to detect vCJD prion contamination in human blood or blood products is therefore one of the priorities for the development of sensitive and specific tests. Diagnostic signatures of BSE have been identified in serum of infected cattle by multivariate analysis of infrared spectra, at a sensitivity of 85% and a specificity of 90%, strongly supporting the hypothesis that infection with prion agents leads to specific changes in the molecular content of serum [69]. However, no predictive tests for CJD or other prion disease have yet been validated in blood, including detection of the prion specific biomarker PrP^{Sc}.

A number of studies have identified differential abundances of a handful of proteins in the blood of patients with CJD. One study has shown an increase of the S-100ß protein and another, an increase in cystatin C [70]. Another recent study found elevated levels of heart fatty acid binding protein (H-FABP) in the serum of patients with CJD [42]. Fatty acid binding proteins are located within the cell and are responsible for the shuttling of fatty acids in the cytosol and are released from the cell in response to cell damage [42]. However, again, high levels of this protein have also been observed in acute myocardial infarctions, in stroke patients, and Alzheimer's patients, implying that this is not a specific prion disease biomarker.

An early genomics study using differential display reverse-transcriptase PCR (DDRT-PCR) to determine differentially expressed genes in blood identified lower levels of the erythroid differentiation-related factor (ERAF) in the spleen, bone marrow and blood of scrapie infected mice [71]. The same group also observed differential expression of other erythroid-related genes (KEL, GYPA) in the spleens of infected mice as a common feature of murine scrapie [72]. However, these genes were found to be expressed at highly variable levels between individuals, thus precluding their usefulness as accurate markers for diagnosis.

Blood (serum and plasma) is one of the most difficult tissues to analyse using omic technologies. Blood is a highly complex tissue that displays a huge dynamic range of protein abundances challenging the identification of the less abundant species; these rare proteins and peptides likely include the majority of disease specific biomarkers.

Prefractionation steps are absolutely necessary to deplete the most abundant proteins [73]. The most common of these are immunodepletion, used extensively for the specific removal of high abundance proteins, based on the action of specific antibodies. More recently, saturation protein binding to a random peptide library has been proposed as an alternative method [38]. Not surprisingly, given these difficulties and the scarcity of samples from CJD patients and large animal models, no proteomic screens for biomarkers of prion disease have been reported to date. Protein profiling of plasma has been reported in a number of studies of Alzheimer's disease and these show some success as evidenced by cross-study reproducibility and validation (albeit fairly low sensitivity and specificity) in patient cohorts. In one study, mass spectrometric analysis of the changes observed in two-dimensional electrophoresis from the plasma of 50 Alzheimers patients and 50 matched controls identified a number of proteins previously implicated in the disease pathology. These included complement factor H (CFH) precursor and alpha-2-macroglobulin (alpha-2M). Although the specificity and sensitivity was fairly low, elevation of CFH and alpha-2M was shown to be specific for Alzheimer's disease and to correlate with disease severity [38]. Two other studies also identified these proteins as upregulated in the plasma of Alzhiemers patients; given that prion diseases have similar aetiologies it is likely that plasma may well be a rich source for biomarkers to monitor disease progression, and potentially for use in diagnosis [34, 35, 74].

Blood contains a number of circulating cells such as lymphocytes, macrophages, dendritic cells and platelets. Another approach in the search for biomarkers is to isolate specific cell populations and use these as a basis for gene or protein profiling studies. Targeting cells that may be involved in prion replication may increase the chance of picking up disease specific changes; however, no such studies have yet been done. Circulating immune cells such as macrophages and dendritic cells can carry infectious PrP^{Sc} and may therefore traffic infectious prion agent around the body. Macrophages have been found to contain PrP^{Sc} even in the absence of FDCs, thus leading to the speculation that they might serve as alternative sites of prion accumulation and replication when there are no functional FDCs [56]. Dendritic cells are also mobile cells that can retain endocytosed particles without degradation for long periods of time, therefore ideal candidates for propagating prion proteins throughout the body [75].

Activation of the innate immune system in the brain is a general response during neurodegeneration, including that induced by prions. Studies have identified inflammatory genes that are significantly induced or suppressed in microglia isolated from CJD infected brains and these may be a source for potential candidate markers. In one study the CJD expression profile obtained contrasted with that of uninfected microglia exposed to prototypic inflammatory stimuli such as lipopolysaccharide and IFN-gamma, as well as PrP amyloid. Transcript profiles unique for microglia and other myeloid cells involved in neurodegeneration provide opportunities for the discovery of disease specfic biomarkers [19]. A second study also describes the expression of a number of potentially neuroprotecive genes in macrophages/microglia from CJD infected patients [76]. The serum levels of immunomarkers may reflect the inflammatory process in the brain so that monitoring the levels of a panel of these in the serum of infected individuals may track the progress of the neurodegenerative process in patients. Whether or not the inflammatory process is reflected in the serum in prion diseases has not been investigated and is an area for further work. However, a number of studies in other diseases support this possibility including the identification of upregulated neuroinflammatory markers in the blood of Parkinson's affected individuals [34, 35] and a study by Ray et al to identify plasma biomarkers of Alzheimer's disease using antibody arrays [48]. These arrays were used to identify 18 plasma protein biomarkers that one able to discriminate Alzheimer's disease with 90% accuracy, the majority of which were immune related cytokines and growth factors.

3.4. Biomarkers in Urine. Urine is commonly used for diagnostic testing in many different conditions and being somewhat less complex than serum is amenable to exploratory biomarker analysis. Two recent studies have applied proteomics for the identification of prion-induced biomarkers. In the first study, the urine of infected cattle over the time course of disease was examined using a combination of 2D-DIGE and mass spectrometry analysis [37]. Four classifier proteins were identified, two of these proteins, immunoglobulin Gamma-2 chain C region and clusterin significantly increased in abundance over time. Increase in the abundance of immunoglobulins has also been reported previously in the urine of scrapie-infected hamsters [77]. Levels of an isoform of clusterin were found to predict with 100% accuracy during infection with BSE, however, the study size was extremely small and limited to a single sample group and so requires validation. Clusterin is a multifunctional glycoprotein found ubiquitously expressed throughout the body and is abundant in astrocytes, CSF, and blood plasma [39, 78]. Its expression has been found to increase in the brains of prion infected mice as well as in other neurodegenerative diseases, and on insult to the brain. A recently reported identified clusterin as a blood borne biomarker following plasma profiling in Huntington's disease patients and additionally saw its upregulation in the CSF of affected individuals [79]. These studies suggest clusterin could have general utility as an inflammatory associated marker for multiple conditions including neurodegeneration.

In the second study, urinary alpha1-antichymotrypsin was found to be dramatically increased in urine of patients suffering from sporadic Creutzfeldt-Jakob disease and a number of other animal models of prion disease [36]. Alpha1-antichymotrypsin, like clusterin, has been identified as a potential disease marker in many disparate diseases including the response to renal and other injuries, and deregulation of expression in many cancers and is therefore not specific to TSEs. It is likely that as both clusterin and alpha1-antichymotrypsin levels are highly responsive to multiple diseases and trauma that the levels in a normal population would preclude utility as a diagnostic marker. However, as

levels of both clusterin and alpha1-antichymotrypsin were reported to increase incrementally during the course of disease, this type of marker could potentially be used to monitor the progress of degeneration in individuals during treatment.

3.5. Exosomes. Exosomes have been investigated for their value as "repositories" for biomarker detection. Exosomes are small (50–90 nm) microvesicles that originate in the cell and following release are thought to be able to migrate and interact with or on other cells [80]. They are often released by cells undergoing stress or other stimuli and may therefore act as carriers of potential biomarkers. Additionally, exosomes are easily isolated from multiple biological fluids and have a much less complex protein component than whole blood, serum, CSF or urine; during the formation of intraluminal vesicles in exosomes extensive sorting of proteins and lipids occurs at the membrane of endosomes which results in them containing a specific group of proteins [80]. Recently the presence of both PrP^{C} [81] and PrP^{Sc} [82] on exosomes has been demonstrated. In prion infection, exosomes may be ideal candidates for biomarker discovery as they have been reported to be released from several cell types that are involved in prion infection, including intestinal epithelial cells, neurons, neuroglial cells, and DCs.

PrP^{Sc} is associated with exosomes from neuroglial and epithelial cells and these may provide a means of cell-to-cell transfer of infectious prions [82]. FDCs which are actively involved in peripheral prion replication release significant numbers of exosomes on stimulation and it is possible that these are involved in the extracellular transport of PrP^{Sc} to nerve endings, although the mechanism by which prions travel from FDCs to the nervous system is presently unknown. It has, however, been shown that the topographical location between FDCs and nerve endings plays a key role in determining the efficiency of neuroinvasion; the process is accelerated when FDCs are in closer contact with the nerve endings [83]. As exosomes in human plasma may have a multitude of cellular origins including release from human platelets, epithelial cells and hemopoetic cells such as mast cells we believe they could be both a source of biomarkers for early detection of PrP^{Sc} in peripheral infection or for the identification of biomarkers specific to prion replication. Alternatively, they may be reservoirs of inflammation- or stress-related biomarkers that could be detected in plasma, CSF or urine. Although these avenues have yet to be explored in prion diseases, other studies to identify exosomal biomarkers provide proof-of-principle, such as the identification of Fetuin-A as a potential biomarker from urine in patients with acute kidney injuries and glioblastoma [84, 85].

4. Future Perspectives in Prion Biomarker Research

Techniques for protein profiling are rapidly evolving as are techniques for rapid genome scale sequencing for gene expression profiling. Other novel methodologies can be applied to the fractionation and isolation of pertinent cell types from which to isolate more specific markers of prion disease and neurodegenerative processes. One technique that shows promise in this regard is laser-capture microdissection (LCM) capable of isolating individual cells from cut tissue sections, thus allowing identification of RNA and protein changes specifically in prion-replicating cells. These biomarkers may well be too scarce to pick up on examination of whole tissues or body fluids. It is a useful tool for either markers of peripheral infection in cells from the spleen, gut mucosa, and lymphoid tissue such as tonsils, or to identify prion-replication associated markers, or neuronal health related markers in brain tissues which may well translate to markers in CSF or blood. So far LCM biomarker research on prion diseases and other neurodegenerative disorders is in its infancy but is a promising area for future research.

A burgeoning area for biomarker research is the identification of dysregulated small noncoding RNAs, especially the recently identified family of microRNAs (miRNAs) which are involved in post-transcriptional regulation of gene expression in both plants and animals [86]. These short RNAs have been determined to have regulatory roles that are vital to many cellular processes and appear to be particularly active in controlling complex functions in the nervous system such as neurodevelopment and neuronal function. Recently, compelling evidence for the involvement of microRNAs (miRNAs) in neurodegenerative diseases including Alzheimer's, Parkinson's and prion diseases, has been published [87–89]. Indeed two miRNAs exhibiting increased expression in the brains of rodent models of scrapie were similarly upregulated in the brains of BSE infected macaques illustrating the potential for consistency across species [90]. The potential of miRNAs as biomarkers for diagnosis and prognosis has also been endorsed by studies showing that expression of miRNAs in various cancers can be highly specific and discriminatory profiles between diseased and non-diseased tissues can be readily identified [91].

5. Conclusion

Significant advances in recent years in technologies for high-throughput sequencing and proteomics mean that the future is bright for biomarker discovery in relation to prion diseases. Of particular note are the ability to obtain transcriptional profiles from homogeneous cell populations at different stages of disease, advances in prefractionation methods for proteomic studies, and the possibility of high-throughput proteomics to identify ever increasing numbers of individual proteins from a single sample. However, a number of unique hurdles and pitfalls remain in relation to prion diseases; these include the very small number of clinical cases for validatory studies, the long incubation period, and the variability of pathogenesis between strains and routes of infection. It is this heterogeneity among prion disease phenotypes that requires careful choice of tissues and time points to use as starting materials for biomarker

discovery. Given these factors it may well be impossible to, for example, identify a single preclinical biomarker congruent to the diagnosis of all prion diseases. Conversely, similarities between molecular mechanisms leading to damage and death of neurons in multiple degenerative conditions may allow the broad utility of biomarkers to track disease progression or to predict the onset of disease between prion and other neurodegenerative conditions. Another factor of note that contributes to the relatively slow progress of research in this area relates to the physical properties of the agent itself. The resistance of prions to conventional chemical and physical procedures designed to inactivate viruses and bacteria means that infected tissues must be analysed under biocontainment conditions. Analysis equipment must in many instances be dedicated to TSE biomarker discovery following contamination with potentially infectious prions. These issues often preclude the use of the most up-to-date techniques that rely on expensive, often core-facility-based, apparatus.

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