Biomarkers for brain disorders

Biomarkers, be they genetic traits, biochemical changes or alterations in structural or functional features, are required to help the diagnosis of a variety of neurological disorders and to detect the progression of these diseases. As new medicines and therapeutic strategies are developed, biomarkers will also be required to measure the efficacy of these treatments. The importance of biomarkers in this field should not be underestimated, particularly considering the huge social and economic burden presently attributed to these diseases. This article explains the biomarker development process and aims to describe the current status of biomarker research in association with prevalent neurological disorders such as stroke, motor neuron disease, Alzheimer's disease, Parkinson's disease and Huntington's disease.

KEYWORDS: AD ■ Alzheimer's disease ■ biomarkers ■ cerebrospinal fluid ■ CSF ■ HD ■ Huntington's disease ■ Parkinson's disease ■ PD ■ plasma ■ stroke

Malcolm Ward[†1] & Emma Louise Schofield[1]

[1]Proteome Sciences plc, South Wing Laboratory (PO 045), Institute of Psychiatry, London, SE5 8AF, UK
[†]Author for correspondence:
Tel.: +44 207 848 5112
Fax: +44 207 848 5114
malcolm.ward@proteomics.com

The human brain is the most complex biological organ in the living world. However, as with all living things we are not invincible and we remain susceptible to a host of medical disorders, some of which are related to the malfunction of our brains. Examples of common neurological illnesses include stroke, motor neuron disease (MND), Alzheimer's disease (AD) and other dementias, Parkinson's disease (PD) and Huntington's disease (HD). Ideally, each of these conditions would have to exhibit a unique pathology to allow clinicians to distinguish particular conditions and give a reliable diagnosis and treatment. In reality, however, many neurodegenerative diseases share similar symptoms and features and the task of diagnosis is often challenging. Therefore, much research has been undertaken to explore both the clinical features and the molecular mechanisms that cause these illnesses in order to identify characteristics to aid diagnosis. This review explains the biomarker development process and aims to describe the current status of biomarker research in association with neurological disorders including stroke, MND, AD, PD and HD.

Biomarker features

A biomarker is a measurable attribute associated with the clinical status of a patient. The National Institutes of Health (NIH) defines a biomarker as: "a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes or pharmacologic responses to a therapeutic intervention" [1]. For brain disorders, biomarkers are urgently needed to aid diagnosis, monitor disease progression and as new medicines are introduced, detect the patient's response to treatment.

To meet this definition, a biomarker must detect a fundamental feature of neuropathology and be validated in confirmed cases. The biomarker test must have the appropriate sensitivity and specificity such that cases can be distinguished from healthy individuals and any particular disease can be differentiated from other brain disorders. Importantly for predictive utility a very low false-positive rate is required, whereas a biomarker for progression must display a measurable degree of change over a short timeframe. Biomarkers for monitoring the efficacy of a medicine must capture the beneficial effect of the therapy. In clinical trials biomarkers can be used to: enable the characterization of patient populations, quantify the extent to which new drugs reach intended targets or indeed alter proposed disease mechanisms to achieve clinical outcomes. Biomarkers also have utility in preclinical drug development where they can be used to monitor efficacy or screen for adverse effects in model systems prior to testing in man. Finally, biomarker tests must be reliable, reproducible and inexpensive, as well as noninvasive and simple to perform.

A biomarker may be a practical exercise where the ability of a patient to perform a physical task is measured; for example, hand tapping can be used to assess the extent of motor dysfunction in HD [2]. Genomics and molecular biology approaches can be used to designate candidate biomarker genes and genetic traits. Biochemical

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markers such as proteins, peptides and metabolites can be detected by mass spectrometry experiments typically employed in proteomic and metabolomic studies. In addition, the post-translational modification status of disease associated proteins affords enormous potential for biomarker utility. Structural features associated with disease can also be visualized using imaging technology such as magnetic resonance (MR) and positron emission tomography (PET).

The emphasis on biomarker utility is very much dependent on the particular brain disorder and this will be discussed in detail in later sections of this article. However, it is unlikely that a single biomarker will have value in diagnostic and prognostic use or measuring response to treatment. Hence, it is expected that a panel of several biomarkers will be required to serve these different tasks.

Biochemical techniques for biomarker discovery

Given that biomarkers can arise from genes, proteins, peptides and metabolites, the global biochemical approaches for biomarker discovery have been dubbed the 'omics' and there are a variety of technologies spanning genomics, proteomics and metabolomics, all of which are currently being applied to biomarker research. Similar to the drug development process, the progression of a biomarker from initial discovery through to clinical utility can also be considered as a pipeline (Figure 1).

Typically biomarkers enter the pipeline as putative candidates, which have been noted because their measurement is different in a particular experimental paradigm. For diagnostic markers this is usually a case versus control study. It is extremely important to consider the intended use of the biomarker prior to commencing the discovery phase as this will have a direct impact on the study design and the nature of the specimens required. Once interesting changes in gene expression, metabolite concentration, protein concentration or post-translational modification status have been observed and considered to warrant further investigation, additional experiments have to be undertaken to evaluate the candidate biomarker to provide extra data in support of the original discovery. Typically, this biomarker evaluation stage involves measuring the target analyte(s) using an orthogonal approach. For proteins, this can be either western blotting or a suitable immunoassay [3]. Mass spectrometry methods involving multiple reaction monitoring or multiple selective reaction monitoring have also begun to emerge as an alternative means of testing [4]. To be considered as a qualified biomarker, the candidate has to emerge at the end of the third phase of the pipeline having undergone extensive testing in a large number of clinical samples and ideally with replicate studies involving independent laboratories.

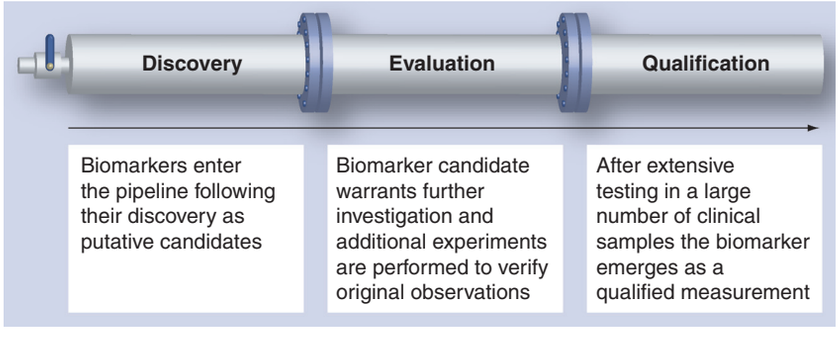

Figure 1. The biomarker development pipeline.

Samples

There are several fundamental issues regarding the choice of sample material that need to be carefully considered prior to commencing a new biomarker discovery study. Large numbers of clinical samples are needed to perform biomarker experiments because of our extensive biological variability. Ideally, all biomarker experiments should be undertaken using samples that have been obtained following well documented and controlled protocols. After procurement the samples need to be carefully curated. Clinical information should be available as this can influence the choice of specimens to be included in the study.

The use of biopsy or postmortem tissue samples to define biomarkers may have the most clinical relevance, in brain disorders. However, obtaining brain biopsy samples from living patients is invasive and not routinely carried out in diagnosis. Furthermore, ethical considerations often impose limitations with regard to availability of materials postmortem and despite initiatives such as Brains for Dementia [201], postmortem brains remain in short supply. The utility of postmortem tissues for diagnostic biomarker discovery is complicated by end-stage disease effects and postmortem protein alterations. Peripheral fluids such as cerebrospinal fluid (CSF) and plasma offer tractable alternatives and sampling is less intrusive. These fluids are however, more remote from the main site of disease and so the target biomarker molecules will be less concentrated and more difficult to detect. Furthermore, the extent of proteolysis may be greater and the final composition of the analyte may be difficult to predict.

Cerebrospinal fluid is in close anatomical contact with the brain and spinal cord where biochemical changes related to a chronic neurodegenerative disease are likely to be reflected and accumulate. Therefore, CSF is the most common sample examined in neurodegenerative studies. The collection of CSF by lumbar puncture is highly invasive and healthy controls are very rarely acquired in CSF biomarker discovery studies. Control samples usually include patients with other neurological or non-neurological disease, where lumbar puncture maybe used in diagnosis.

Plasma would be the most convenient source for biomarkers as it is more readily available and less invasive to obtain than CSF or tissue. Where speed of diagnosis is crucial to enabling rapid treatment and improved outcome for the patient (e.g., in cases of stroke), development of diagnostic tests that utilize plasma biomarkers is of huge importance. However, the use of plasma biomarkers in the clinical diagnosis and progression monitoring of brain and motor diseases makes the assumption that a CNS-based pathological process will be indicated in the peripheral fluids. The existence of the blood–brain barrier may limit the utility of brain cell-specific molecules as plasma biomarkers, because they are unable to enter into the blood stream in sufficient quantities to be reliably detected. A further analytical challenge is that the plasma proteome is highly complex, with approximately 12 orders of magnitude concentration difference between the highest and lowest abundance proteins [5]. Hence, key biomarkers may go undetected if they are masked by more abundant components.

Urine represents the most accessible biofluid with the most noninvasive type of collection for a biomarker research program. Urine contains fewer proteins but higher concentrations of metabolites and peptides than blood. Given how removed urine is from the brain and CNS, the discovery of specific and sensitive clinical biomarkers for brain diseases in this fluid is unlikely, although notably, one study detected increased collagen metabolites and levels of markers for oxidation in the urine of amyotrophic lateral sclerosis (ALS) patients [6]. However, the potential of urine biomarkers in brain diseases such as AD, MND and stroke will not be discussed in detail in this review.

Animal models may provide a useful alternative to human specimens because they are designed to mimic particular aspects of a disease pathology or mechanism. In addition, studies will not be confounded by environmental factors such as diet, social circumstances and concomitant medications.

Imaging techniques as surrogate biomarkers

Structural and functional characteristics of the brain can be measured using a variety of quantitative MR techniques. The noninvasive, nonradioactive, quantitative nature of these approaches makes them ideal for monitoring changes associated with brain disorders. Anatomical, biochemical and microstructural features as well as blood flow changes can be investigated using MR techniques.

Several major proton-containing metabolites present in the brain can be measured simultaneously using proton MR spectroscopy, which is sensitive to changes in the brain at the cellular level. The metabolite N-acetyl aspartate (NAA) is a marker of neuronal integrity and NAA levels have been shown to decrease in a variety of neurological disorders including AD [7]. Levels of the metabolite myoinositol (mI) have also been shown to be consistently abnormal in AD patients [8]. Elevated levels of mI correlate with glial profileration in inflammatory CNS demyelination and it is thought that the increase in mI signal is due to glial cell profileration and activation of astrocytes [9]. Both NAA and mI levels are normally normalized to creatine (Cr) levels and subsequently expressed as ratio measurements NAA/Cr and mI/Cr, respectively. Proton MR spectroscopy can also be used to detect changes in choline (Cho) in the brain of AD patients, although there are conflicting reports as to the behavior of this metabolite, with studies indicating elevated Cho and Cho/Cr ratios as well as normal levels [10].

Functional MRI has been used to measure activation of the brain. These experiments utilize a variety of activation criteria such as visual and motor responses, semantic processing and memory. Activation patterns have been shown to be different in AD patients compared with normal elderly people [11] and a detailed review of the various MR techniques with utility to measure surrogate markers of AD was published by Kantarci and Jack [12]. The authors emphasized that since different structural markers for disease progression may vary with the pathological state of AD, the choice of MR-based regional measurements needs to be tailored depending on the severity of the disease.

Novel PET ligands have been introduced and this has enabled imaging of amyloid *in vivo*. This is an exciting advance because the direct measurement of one of the key pathologic features of AD is now possible. Visualization of the amyloid-β (Aβ) plaques is based on the accelerated T2* relaxation times due to the elevated

levels of metal ions present within them [13]. This measurement is not specific to plaques so additional enhancements (e.g., contrast agents such as gadolinium-diethylenetriaminepenta-acetic acid [DTPA]), are required to increase the specificity for Aβ plaques [14]. High resolution imaging of gadolinium-DTPA-labeled Aβ plaques would enable the pathological progression of AD to be monitored noninvasively.

Recently, the findings of extensive imaging studies associated with AD have been published [15]. Here the authors report the development and design of AddNeuroMed, a cross European, public/private consortium developed for AD biomarker discovery. Furthermore, data acquired through the AD Neuroimaging Initiative (ADNI) study is available to the general scientific community [202]. The ADNI study is a large 5-year research project commenced in 2004 to study the rate of change of cognition, function, brain structure and function, and biomarkers in 200 elderly controls, 400 subjects with mild cognitive impairment (MCI) and 200 with AD. Public access to the clinical and imaging data is available via the ADNI website [202].

Specific brain disorders

Stroke

Stroke is the most common cause of a sudden acute neurological deficit in adults and children. Ischemic stroke, caused by the infarction of a vessel supplying blood nutrients to the brain, comprises 85% of all strokes in Europe and North America. Typically, a stroke is diagnosed by an experienced neurologist based on clinical symptoms with imaging technologies including MRI and computed tomography, being used to help determine stroke type. Computed tomography scans are used to diagnose hemorrhagic stroke but they are relatively ineffective at detecting ischemic stroke, particularly minor strokes of small volume [16,17]. However, MRI is more sensitive at detecting small-volume ischemic stroke but is still not 100% sensitive or specific [18].

The diagnosis of stroke can be complicated, as a patient presenting with a sudden acute neurological deficit may be suffering from a stroke but they may also be suffering from a 'stroke mimic' condition. Conditions that mimic stroke include; aura of migraine, postictal deficits following a seizure, hypoglycemia, an anamnestic spell, tumors or a functional psychogenic spell. Of the stroke mimic conditions, only tumors can be ruled out by imaging techniques. A further, complicating factor of stroke diagnosis is the poor correlation between volume of damaged tissue and the severity of the clinical deficit. Owing to the localized function of certain brain regions, small areas of injury can cause dramatic clinical syndromes with a high risk of long-term disability. Alternatively, large areas of injury in some brain regions may cause subtle clinical symptoms.

At the core of stroke infarction there is a region of brain cells that have died as a result of loss of blood supply. Surrounding the core is the penumbra, a region that contains cells that have a limited blood supply to remain viable. Cells in the penumbra are potentially salvageable if treatment is provided at an early stage. Thrombolytic agents have been shown to improve the outcome of patients with ischemic stroke, who have evidence of salvageable tissue, if administered within 6 h of stroke onset [19–21]. However, the presence of intracranial hemorrhage must be ruled out first.

Given that many smaller hospitals in rural locations do not have access to the sophisticated imaging technology required for rapid stroke diagnosis, there is a real need for a biomarker that is diagnostic of stroke type and has the ability to rule out stroke mimic conditions. A diagnostic stroke biomarker should be available in small medical centers, would not need interpretation by a consultant neurologist, should be complementary to imaging techniques, should be diagnostic within the first few hours after stroke onset and correlate with volume of brain cell injury. Given the pathology of ischemic stroke there are three main categories of molecules to consider as potential biomarkers for stroke diagnosis:

- Biomarkers of brain cell injury
- Biomarkers of blood vessel injury
- Biomarkers of inflammation

As discussed previously, the most likely source of reliable, specific and sensitive biomarkers for stroke in terms of its proximity to the infarction site is CSF. Monocyte chemoattractant protein-1, an inflammation-related protein, has been shown to be increased in CSF but not blood of stroke patients [22]. However, the collection of CSF from stroke patients carries some risk of hemorrhage and the procedure therefore requires the skills of trained doctors. As such CSF biomarkers are likely to be reliable, specific and sensitive but they may not meet the requirement of a rapid diagnostic stroke biomarker that could be available in a small centre without the need for a consultant neurologist.

During a stroke the blood–brain barrier is compromised, which increases the potential for brain-derived proteins from injured brain cells to be released into circulating blood. Evidence of a breakdown of the blood–brain barrier has been demonstrated in rodent models [23] and humans [24]; however, the timescale for the breakdown of the blood–brain barrier is unknown. Therefore, much effort has been given to the identification and validation of diagnostic biomarkers of stroke in plasma. One candidate that has shown promise is the astroglial protein, S-100B (see TABLE 1 for references). S-100B is a cytosolic calcium-binding protein and a marker of cellular activation. It has been found in plasma in several studies to correlate to the extent of tissue damage and neurological outcome [25,26]. However, levels of S100B in plasma were not significantly different within 6 h of stroke onset, limiting the utility of S-100B as a marker for stroke diagnosis. Other proteins, including neuron-specific enolase, myelin basic protein and glial fibrillary acidic protein, are found in the brain and have shown some potential as diagnostic stroke biomarkers and are shown in TABLE 1.

Potential diagnostic stroke plasma biomarkers of nonbrain origin have also been reported, although most candidates have been identified in relatively small studies and have yet to be validated. Candidates include markers of vascular injury and thrombosis including von Willebrand factor, fibrinogen and D-dimer, and markers of stroke-related inflammation including matrix metalloproteinase-9 and C-reactive protein (TABLE 1). In particular, markers of stroke-related inflammation have shown some potential in being able to identify a patient's suitability for thrombolytic therapy. Matrix metalloproteinase-9 has been shown to predict hemorrhagic transformation in patients of ischemic stroke, for example [27].

No one candidate diagnostic stroke biomarker has yet been validated in a larger study across multiple research groups and, given that many candidates have also been identified as potential diagnostic markers of other brain disease, it is likely that a specific diagnostic assay for stroke will consist of a panel of several markers. One study of interest undertaken by Reynolds and colleagues created a panel of markers for the diagnosis of stroke [28]. Here, a panel of five proteins, S100b, B-type neurotrophic growth factor, von Willebrand factor, matrix metalloproteinase-9 and monocyte chemotactic protein 1, were used in combination and showed increased correlation with the diagnosis of stroke compared with any one protein on its own within the first

Table 1. Candidate protein biomarkers for stroke.

Candidate biomarker	Type of marker	Diagnosis and/or prediction	Ref.
Neuron-specific enolase	Brain cell injury	Diagnosis	[25,96,97]
S100b	Brain cell injury	Diagnosis	[25,28,97–99]
Myelin basic protein	Brain cell injury	Diagnosis	[97]
Glial fibrillary acidic protein	Brain cell injury	Diagnosis	[97]
B-type NGF	Brain cell injury	Diagnosis	[28]
NMDA receptor autoantibodies	Brain cell injury	Diagnosis	[100]
Park 7	Brain cell injury	Diagnosis	[101]
Nucleotide diphosphate kinase A	Brain cell injury	Diagnosis	[101]
von Willebrand factor	Vascular injury	Diagnosis	[28,99]
Cellular fibronectin	Vascular injury	Diagnosis	[102]
Soluble VCAM-1	Vascular injury	Diagnosis	[102]
D-dimer	Thrombosis	Diagnosis and prediction	[98,103]
Fibrinogen	Thrombosis	Diagnosis and prediction	[29–31]
Soluble glycoprotein V	Thrombosis	Diagnosis	[32]
C-reactive protein	Inflammation	Diagnosis and prediction	[29–37]
TNF-α	Inflammation	Diagnosis and prediction	[104]
IL-6	Inflammation	Diagnosis and prediction	[104]
Matrix metalloproteinase-9	Inflammation	Diagnosis	[28,98,99]
Monocyte chemotactic protein-1	Inflammation	Diagnosis	[28]
VCAM	Inflammation	Diagnosis	[99]

NGF: Nerve growth factor; NMDA: N*-methyl-*D*-aspartic acid; TNF: Tumor necrosis factor; VCAM: Vascular cell adhesion molecule.*

6 h after stroke onset. Further testing in a larger cohort of patients is required to further validate this panel of markers.

In the UK stroke is the third largest cause of death. With over 111,000 people each year suffering from a stroke [203], the ability to be able to identify those individuals at risk and initiate preventative treatment would be of extreme benefit. Potential biomarkers of stroke risk are less likely to be molecules indicating brain injury but rather molecules relating to the presence of an atherosclerotic plaque in ischemic stroke, for example. One protein that has been shown to predict the risk of plaque rapture and thrombus formation in myocardial infarction as well as stroke in several studies is high sensitivity C-reactive protein [29–37]. Other molecules, including those involved in coagulation and inflammation that could also have potential utility in identification of active atherosclerotic plaques are TNF-α, IL-6, D-dimer and fibrinogen (see Table 1 for references).

MND & ALS

Motor neuron disease is a group of fatal neurodegenerative diseases that includes sporadic and familial ALS, spinal muscular atrophy, hereditary spastic paraplegia, primary lateral sclerosis and spinobulbar muscular atrophy. These heterogenous syndromes typically manifest clinically by weakness, spastic paralysis or both. ALS, the most common form of MND, is characterized by dysfunction and/or loss of both upper and lower motor neurons. The etiology and pathological mechanisms of MND are still not well understood.

Amyotrophic lateral sclerosis is familial in approximately 10% of cases, with 20% of all genetically inherited ALS being caused by mutations in superoxide dismutase 1 (SOD1) [38,39]. The potential mechanisms by which mutations in SOD1 cause motor neuron degeneration have yet to reveal the full story in MND pathology. These mechanisms have been covered in many reviews and, although not discussed in detail here, the reader is directed to articles published by Cleveland [39], Pasinelli and Brown [40] and Rothstein [41].

While the genetic alterations that lead to familial forms of these disorders aid in diagnosis, diagnosis of sporadic cases by clinical features and imaging technologies is often not defined until the advanced stages of the disease. This delay in diagnosis, often up to 1 year from onset of symptoms, prevents early treatment with potential disease-modifying drugs. By this time distal muscle wasting is visible and at least 30% of anterior horn neurons are thought to have degenerated [42]. Therefore, the reliance on clinical examination and imaging technologies to trigger intervention may not be adequate if degeneration is no longer salvageable at the time of diagnosis. Therefore, the need for a clinical diagnostic biomarker that is capable of identifying those at risk of developing MND before the onset of symptoms is of great importance. Furthermore, sensitive and specific biomarkers that discriminate between clinical phenotypes associated with shortened or prolonged survival rate and indicative of disease progression would be extremely beneficial as they would enable optimum treatment and appropriate planning of care.

A successful MND biomarker discovery and validation program, that aims to identify a single biomarker or panel of biomarkers in CSF or plasma that provides specific phenotype diagnosis and enables monitoring of disease progression, is likely to require a longitudinal study involving a large cohort of patients over many years. An alternative approach that enables longitudinal studies of disease progression in a shorter time frame than in humans is to first identify potential biomarker candidates in transgenic mouse models. Proteomic profiling of the SOD1 mouse model has been carried out comparing transgenic to nontransgenic animals at several time points early and late in the disease, identifying several candidate proteins [43,44]. However, further validation is required to evaluate the utility of these potential biomarker candidates in human disease phenotypes.

Inflammatory processes, especially activation of microglia in the cortex and spinal cord, appear to play a role in cell death in neurodegenerative disease including MND. Levels of IL-1β, IL-6 and TNF have been found in the plasma of ALS patients [45], and monocyte chemoattractant protein-1 has been detected in CSF but not plasma [46]. Due to their essential role in neuronal survival and regeneration, growth factors have also been studied as potential MND biomarkers. Levels of insulin-like growth factor have been found to be increased while levels of the regulatory binding protein insulin-like growth factor binding protein were decreased in plasma of ALS patients [47]. One particular growth factor that has gained much interest is VEGF. Some homozygotic haplotypes of VEGF have been linked to increased risk of ALS, which may be caused by decreased levels in plasma [48]. Further investigation of VEGF as a potential plasma biomarker and possible therapeutic target is warranted.

Another protein that has gained recent interest is the transactivator responsive region DNA-binding protein (TARDBP or TDP-43). TDP-43 is normally present in the nucleus and has a function in RNA processing, but has been identified as the main ubiquitinated protein present in the cytoplasmic inclusions that are characteristic of ALS and some types of frontotemporal dementia [49,50]. Furthermore, missense mutations in TDP-43 have also been linked to familial and sporadic ALS cases [51–58]. The pathological mechanisms by which TDP-43 contributes to ALS are not yet understood and further investigations are currently required. Table 2 provides an overview regarding current candidate biomarkers for MND.

Muscle biopsies are not a routine step in ALS diagnosis, however Jokic and colleagues have shown increased levels of proteins Nogo A and B in muscle biopsies of ALS patients [59]. The protein Nogo is known to inhibit axonal outgrowth in the spinal cord and peripheral nerves and as such may play a role in the pathophysiology of ALS [60]. Further studies are required to validate the utility of Nogo A and B as potential biomarkers of ALS progression.

AD & other dementias

Alzheimer's disease is the single largest cause of dementia and the fourth highest cause of death in the UK, with 821,884 people currently living with dementia [204]. Worldwide dementia affects 30 million people a figure that is anticipated to rise to 65.7 million by 2030 (AD International Report 2009 [205]). Therefore, AD is both common and devastating and there are currently no readily available biomarkers to aid diagnosis or to monitor disease progression. In addition to its high incidence and mortality, AD has a major impact on the economy through direct health costs and lost of production from patients, their families and careers at £21 billion [204]. Authors refer the reader to the following websites for further statistics on the economic burden of AD [206,207].

Two critical molecular pathologies in the brain contribute to the neurodegenerative process of AD that then result in dementia. These are described as senile neuritic plaques and neurofibrillary tangles. The formation of senile neuritic plaques with a central core of an extracellular deposit of Aβ peptide is well accepted as a key feature of AD pathology. Aβ is generated from metabolism of amyloid precursor protein (APP)

Table 2. Candidate protein biomarkers for motor neuron disease.

Candidate biomarker	CSF/plasma	Diagnosis and/or progression	Ref.
Cystatin C	CSF	Diagnosis	[105,106]
Neurosecretory protein VGF	CSF	Diagnosis and progression	[106,107]
Tau	CSF	Disease progression	[108]
S100b	CSF	Disease progression	[108]
Insulin-like growth factor-1	CSF	Diagnosis	[109]
Neurofilament light chain	CSF	Diagnosis and progression	[110,111]
Neurofilament heavy chain	CSF	Diagnosis and progression	[112]
Panel of five cytokines: IL-10, IL-6, GM-CSF, IL-2 and IL15	CSF	Diagnosis	[113]
Glial cell-line derived neurotrophic factor	CSF	Diagnosis	[114]
VEGF	CSF	Diagnosis	[115]
Erythropoietin	CSF	Diagnosis	[112]
Matrix metalloprotease-9	Plasma	Diagnosis	[116]
Angiogenin	Plasma	Diagnosis	[117]
Creatine kinase	Plasma	Disease progression	[108]
ApoE	Plasma	Disease progression	[118]
Fibrinogen	Plasma	Diagnosis and progression	[119,120]
C-reactive protein	Plasma	Disease progression	[120]
IL-6	Plasma	Diagnosis and progression	[109]
Plasma transforming growth factor-β1	Plasma	Disease progression	[121]
Monocyte chemoattractant protein-1α	Plasma	Diagnosis and progression	[122]
4-hydroxy-2,3-noenal	Plasma	Diagnosis and progression	[122]
Interleukin 13-positive T cells	Plasma	Diagnosis and progression	[123]
Insulin-like growth factor and insulin-like growth factor binding protein	Plasma	Diagnosis and progression	[47]

CSF: Cerebrospinal fluid; GM-CSF: Granulocyte-macrophage colony-stimulating factor.

by cleavage, first by β-secretase (BACE1) and then by γ-secretase (the activity of which is contained in multiprotein complexes that include presenilin-1) to generate the amyloidogenic Aβ fragment through the β-cleavage pathway. Aβ is secreted from neurons and this highly aggregating peptide then forms insoluble, extracellular deposits as plaques in the AD brain. APP can also be metabolized to nonamyloidogenic fragments by cleavage, first by α-secretase and then by γ-secretase through the α-cleavage pathway. A few families with familial AD harbor mutations in APP and model transgenic animals as well as cellular systems expressing mutant forms of APP have been found to produce elevated levels of CSF and plasma Aβ. Presenilin mutations linked to families with AD also appear to cause an increase in the ratio of $A\beta_{42/40}$. Furthermore, the Swedish mutation in APP also leads to an increase in total Aβ, which shows the importance of Aβ generation in disease pathogenesis.

Along with neuritic plaques, the other key feature of AD pathology is the presence of intraneuronal neurofibrillary tangles and neuropil threads composed of a highly phosphorylated form of the protein tau. Hyperphosphorylated tau in the tangles is aggregated into filaments known as paired helical filaments (PHFs) and this has given rise to the term PHF-tau, which refers to this pathological form of tau. The presence of tau in the intraneuronal neurofibrillary tangles characteristic of the AD brain, combined with the identification of tau mutations in frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17), serve to confirm the importance of this protein in the pathogenesis of neurodegenerative disease. Furthermore, there are several reports of a correlation between cognitive decline in AD and both neuronal loss and number of neurofibrillary tangles present [61–63].

Although plaques and tangles are distinct pathological signatures of AD they are also commonly found in individuals who are not clinically demented and, typically, AD manifests only after a certain threshold has been reached; therefore, by the time an individual is diagnosed with AD a significant loss of synaptic function and neurons has already occurred. Impaired memory is the earliest symptom of AD yet many elderly individuals with impaired memory do not meet the clinical criteria for dementia and this syndrome is defined clinically as MCI [64]. Patients with MCI have a higher risk of developing AD and although the conversion rate to AD is only 10–15% per year, most patients with MCI go on to develop AD during their lifetime. People with MCI therefore represent an important clinical group for the evaluation of biomarkers for early diagnosis and monitoring disease progression particularly at the early stages of the disease.

Relatively few biochemical biomarker tests are currently available for AD and those that are most established are measured in CSF (Table 3). Presently, Aβ1–42, T-tau and P-tau181 are the most useful tests and these are commercially available as a combined immunoassay [65]. Plasma is a more attractive specimen because it can be sampled less intrusively for the patient but it is even more remote from the main site of disease than CSF and the target biomarker molecules are therefore less concentrated and more difficult to detect. For example, p-Tau 231 levels in AD CSF range from 300 to 900 pg/ml but the anticipated concentration in plasma is much lower and estimated to be in the region of 15 pg/ml [66]. Hence, the analytical methodology appropriate for the analysis of tissue samples may not be directly transferable to biofluids.

The phosphorylation status of tau represents an exciting opportunity for biomarkers, not only for AD but also progressive supranuclear palsy and other tauopathies. Considering the limited availability of suitable antibodies, there is a sound rationale for the development of multiplexed quantitative mass spectrometry methods for tau phosphorylation screening. Nevertheless, the liquid chromatography tandem mass spectroscopy analysis of PHF-tau has revealed many sites of phosphorylation on serine and threonine residues [67,68]. In addition, phosphorylation of Tyr394 was shown to be

Table 3. Candidate protein biomarkers for Alzheimer's disease.

Candidate biomarker	CSF/plasma	Diagnosis and/or progression	Ref.
$A\beta_{1-42}$	CSF	Diagnosis	[65]
Tau, total tau, phospho-tau	CSF	Diagnosis	[65]
Complement factor H	Plasma	Progression	[69]
α-2-macroglobulin	Plasma	Progression	[69]
Clusterin	Plasma	Progression	[3]

Aβ: Amyloid-β; CSF: Cerebrospinal fluid.

present [68]. For a complete up to date listing of tau phosphorylation sites authors refer the reader to the following website [208].

Plasma proteins have also been described as potential biomarkers for AD (TABLE 3). Hye and colleagues reported changes in several proteins in AD plasma including complement factor H and α-2-macroglobulin [69]. These two proteins are particularly promising candidates because their plasma levels show a significant positive correlation with the hippocampal metabolite ratio NAA/mI, a biochemical measure that is associated with cognitive decline in early AD, suggesting that these proteins may reflect disease progression in early AD [70].

Clusterin is another interesting biomarker candidate, which colocalizes with Aβ peptide in neuritic plaques, is found reversibly complexed with Aβ in CSF and inhibits the aggregation of Aβ *in vitro* [71]. Clusterin is found in almost all mammalian tissues and biofluids but is differentially expressed by certain cell types and tissue-specific isoforms exist. It interacts with a wide range of molecules and its expression is upregulated in a variety of physiological and pathological states including apoptosis and response to injury. It is implicated in diverse mechanisms of cytoprotection, membrane recycling and regulation of membrane attack complex formation. A unified role has been proposed as a heat-shock or chaperone protein with cytoprotective properties [72].

Clusterin has been implicated in neurodegeneration: the number of clusterin-immunoreactive neurons in rat brain increases progressively with normal aging, and clusterin mRNA and protein are detectable in both human and rat brain, and increase with injury or with neurodegeneration [71]. Complement components are synthesized locally in the AD brain, and brain-derived clusterin is an inhibitor of membrane attack complex formation [71]. Clusterin levels are elevated in CSF in AD [73]. Brain-derived clusterin has been shown to cross the blood–brain barrier into plasma [74]. Furthermore, a recent publication by Güntert and colleagues has shown that plasma gelsolin is decreased and correlates with rate of decline in AD [3].

Parkinson's disease

In 1817, the English surgeon, James Parkinson, first described 'shaking palsy' in association with the progressive neurodegenerative movement disorder we now know as PD. PD is the most common form of motor system degeneration and second most common neurodegenerative disorder affecting 1% of the population over the age of 65 years old [75–77]. Figures published in 2007 estimated that the number of individuals in the UK affected by PD at 100–180 per 100,000 [78]. This number is expected to increase rapidly with the aging population. Similarly to AD, PD has a major impact on the UK economy through direct health costs and loss of production from patients, their families and careers estimated at GB£449 million–3.3 billion [78].

Parkinson's disease is characterized by rigidity, tremor, bradykinesia and postural instability. Like MND and dementia, PD represents a group of parkinsonism disorders (idiopathic PD [IPD], progressive supranuclear palsy, multiple system atrophy, corticobasal degeneration, frontotemporal dementia with parkinsonism and vascular parkinsonism) that frequently show clinical overlap. Currently, diagnosis relies almost entirely on medical history and neurological examination using the Unified Parkinson's Disease Rating Scale. Definitive diagnosis is performed posthumously with the hallmark pathology including degeneration of neurons within the pars compacta of the substantia nigra region of the midbrain and Lewy bodies containing the protein α-synuclein. In contrast to AD, imaging studies have not proved particularly useful at distinguishing patients with different forms of neurodegenerative parkinsonism [79]. As such, there is a great need for a diagnostic biomarker that is sensitive and specific to the various subtypes of parkinsonism disorder.

Although the etiology of PD is not completely understood, a number of genetic risk factors have been associated with the disease. Mutations in α-synuclein, parkin, UCH-L1 and DJ-1 are associated with familial forms of PD, which commonly lead to oxidative stress and malfunction of the mitochodrial and ubiquitin–proteasome system [80,81].

Similarly to MND and AD, the proximity of CSF to the pathology of the disease makes CSF biomarkers the most attractive biofluid for biomarker discovery studies in PD. Eller and Williams recently reviewed the current status of biological fluid markers relating to neurodegenerative parkinsonism [79]. The most promising assays relate to the measurement of α-synuclein and tau forms in CSF. α-synuclein is a particularly interesting candidate diagnostic biomarker for PD as it is a major component of Lewy bodies. CSF levels of α-synuclein have been shown to be significantly lowered in IPD or dementia with lewy bodies compared with AD patients of controls [82]. Furthermore, in pathologically confirmed cases of IPD, CSF levels of α-synuclein rapidly rise after death. Authors suggest

that this lower level of CSF α-synuclein during earlier stages of the disease is due to higher levels of α-synuclein in the brain, presumably sequestering in Lewy bodies. As brain cells die α-synuclein is released into the CSF [79]. As well as α-synuclein and tau isoforms, other interesting candidate CSF biomarkers including $A\beta_{42}$ and neurofilament proteins are detailed in Table 4.

Generally, the results of diagnostic CSF biomarker discovery studies seem to be encouraging but it is still necessary to perform further validation. Fewer studies have investigated the utility of diagnostic plasma biomarkers in PD. One study worth mentioning found decreased levels of uric acid and increased levels of gluthathione were predictive of IPD compared with controls [83]. However, this study was carried out on a relatively small number of patients and controls and further validation of these findings is required. Ultimately, the primary importance of CSF and plasma biomarkers in PD will be to aid the diagnosis of the less common parkinsonism conditions including progressive supranuclear palsy, multiple system atrophy, corticobasal degeneration and frontotemporal dementia.

Huntington's disease

Huntington's disease results from genetically programmed degeneration of neurons, in certain areas of the brain. Some early symptoms of HD are mood swings, depression, irritability or trouble driving, learning new things, remembering a fact, or making a decision. As the disease progresses, concentration on intellectual tasks becomes increasingly difficult and the patient may have difficulty eating and swallowing [84].

Huntington's disease is autosomally inherited and is caused by an excess of CAG codon repeats in a gene on chromosome 4. The gene encodes huntingtin protein, which is ubiquitously expressed in the CNS. The CAG codon expansion results in the production of a polyglutamine amino acid sequence within the protein. This poly-glutamine region within the huntingtin protein is highly polymorphic ranging between 10 and 35 repeats in the normal population and this is expanded to approximately 36–120 repeats in HD patients [85]. HD is one of nine inherited neurodegenerative disorders that are caused by this type of mutation. Other disorders include dentatorubral pallidoluysian atrophy, spinal and bulbar muscular atrophy, and the spinocerebellar ataxias 1, 2, 3, 6, 7 and 17. The mutant proteins are unrelated except for the polyQ tract, and aggregated polyQ is a major component of the proteinaceous deposits that are found in patients' brains for all of these diseases [86].

The disease is typically associated with progressive and severe degeneration of the striatum of the brain and widening of the intercaudate distance but there are other widespread changes in the CNS and systemic abnormalities have been identified including endocrine dysfunction [87] and immune activation [88].

The diagnosis of HD currently involves a genetic test, which has 100% sensitivity, coupled with a complete medical history and neurological tests. Presymptomatic testing is available for individuals who are at risk of carrying the HD gene. As such, there is no requirement for biomarkers to aid diagnosis but there is a real need for new biomarkers with utility in tracking progression of the disease and also the response to drug treatment or in clinical trials of new drug entities, because although there are a number of medications to help control emotional and movement problems associated with HD, most drugs used to treat these symptoms have side effects such as fatigue, restlessness or hyper-excitability and, at this time, there is no way to stop or reverse the course of HD. However, numerous approaches to disease-modifying treatments have been shown to have efficacy in animal models of HD and many candidates await clinical trials in humans [89]. HD progresses slowly and its clinical manifestations are highly variable. Moreover, standard clinical tools for assessing progression such as the Unified Huntington's Disease Rating Scale, initially

Table 4. Candidate protein biomarkers for Parkinson's disease.

Candidate biomarker	CSF/plasma	Diagnosis and/or progression	Ref.
α-synuclein	CSF	Diagnosis and progression	[82,124]
Tau, tau forms, total tau, phospho-tau	CSF	Diagnosis	[125,126]
$A\beta_{42}$	CSF	Diagnosis	[126,127]
Neurofilament light chain	CSF	Diagnosis	[128]
Neurofilament heavy chain	CSF	Diagnosis	[112]
Metabolic profile – uric acid and glutathione	Plasma	Diagnosis	[83]
Proteomic profile – panel of eight markers	CSF	Diagnosis	[129]

Aβ: Amyloid-β; CSF: Cerebrospinal fluid.

published by the Huntington Study group in 1996, are subject to problems with inter- and intra-relater reliability, and are also unuseful for distinguishing between symptomatic benefit and disease modification. Thus, there is a need for objective measures that are easy to quantify reliably in accessible tissue or fluid, track linearly with disease progression and change in response to disease-modifying therapeutic interventions. Several candidate biomarkers in plasma have been proposed for HD [90,91] but, as yet, these have not been evaluated longitudinally in a cohort of patients and control subjects.

Fang and colleagues recently reported the analysis of distinct sets of proteomics data profiling the constituents of CSF derived from HD affected and unaffected individuals [92]. The proteomics datasets were also integrated with genomics data profiling of various human and mouse tissues, including human HD brain. Based on this integrated analysis, the authors found that brain-specific proteins are more likely to be observed in CSF than in plasma and that brain-specific proteins tend to decrease in HD CSF compared with unaffected CSF. Furthermore, the majority of brain-specific proteins had quantitative changes concordant with transcriptional changes identified in different regions of HD brain.

The observed changes in clusterin reported by Dalrymple and colleagues are of particular interest [93]. Clusterin mRNA is known to be upregulated in the striatum of HD brain, along with that of complement components [74]. In a large microarray study of human HD brain, clusterin and complement component mRNA were upregulated, particularly in caudate [94]. The importance of neuroinflammation in HD is underscored by human PET studies demonstrating microglial activation in early HD and premanifest HD patients [95]. Thus, clusterin represents a biologically plausible HD biomarker that may be a surrogate marker of the underlying neurodegenerative process. Therefore, clusterin warrants further evaluation alongside other biomarker candidates in large longitudinal cohort studies, as well as in concert with potential disease-modifying interventions.

Conclusion & future perspective

There is a clear need for biomarkers to support the diagnosis of a variety of brain disorders and to help detect progression and response to therapies as they are introduced into mainstream clinical practice during the coming years. Biomarker studies are likely to involve both new discovery experiments as well as evaluation of previously identified molecules. Successful biomarker studies will continue to require multidisciplinary teams and continued collaboration between academia, the biotech industry and the pharmaceutical sector. Initially, it is envisaged that biomarkers will find increased utility in clinical trials and in preclinical drug development. Ultimately, if we are to achieve the primary objective of biomarker-related translational research initiatives, namely to transfer breakthroughs in medical research to the benefit of patients, sets of biochemical markers will be measured in readily accessible biofluids (e.g., blood), by clinical testing laboratories.

At first glance it appears challenging to predict exactly how the biomarker field will evolve over the next 5–10 years. Nevertheless, if we refer back to the concept of the biomarker development pipeline in Figure 1, we can postulate that a few key biomarkers, perhaps even a subset of those molecules currently contained within a larger collection of candidate molecules at the very early stages of discovery, will progress positively through the development process and emerge as clinically relevant measurements. One may choose to think of the biomarker development pipeline as a funnel (Figure 2) much like the drug development process, whereby many candidates may be initially described as putative or prospective candidates, but very few reach the desired end point and enter routine clinical practice. This could lead to a view that to date, efforts to identify biomarkers have been disappointing rather than considering that, in reality many recently described biomarkers have still to progress through the next phase of the development

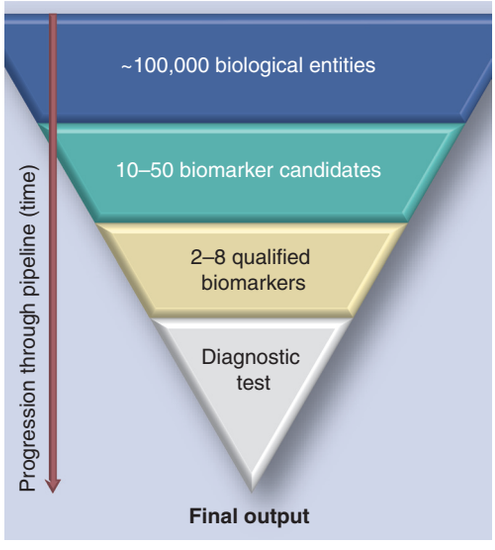

Figure 2. The intrinsic funnel effect of the biomarker development pipeline.

process. Hence, we need analytical tools and methods to rapidly filter out those candidates that will not survive further scrutiny whilst also enabling the most promising biomarkers to progress quickly and efficiently through the pipeline. Importantly, extensive bioinformatics and strict statistical testing should be undertaken before any molecule first receives the recognition as a potential biomarker. With continued collaboration between multidisciplinary teams from both clinical and academic centers of excellence and industry-based partnerships, biomarkers for brain disorders are a realistic ambition. It is anticipated that the current knowledge base within the research community will transfer tangible benefits to patients within the next 5–10 years.

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Executive summary

- Biomarkers are required to aid diagnosis, to detect disease progression and monitor the effect of new drugs and therapeutic strategies as they are developed.
- Many neurological disorders share common features therefore it is likely that multiple biomarkers will be required to adequately differentiate specific diseases and their sub-types. Integration of data from various sources will be necessary to strengthen the impact of biomarker combinations.
- Genetic mutations, changes in protein expression or post-translational modifications, levels of metabolites and imaging of the brain presently provide the main source of candidate biomarkers. Further benefits will be realized when these measurements are correlated to additional clinical information.
- Advances in technology, such as mass spectrometry and imaging procedures, will enable faster evaluation of large numbers of biochemical markers and structural changes in the brain. This will streamline the progression of the most relevant molecules towards preclinical and clinical utility.

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