Systemic Epigenetic Biomarkers for ALS Improve Early Diagnosis, Treatment and Trials

Amyotrophic lateral sclerosis (ALS), or Lou Gehrig's disease, is a devastating, rapidly progressive and invariably fatal neurological disease that attacks the nerve cells responsible for controlling voluntary muscles and for which there is no diagnostic or prognostic test. The disease belongs to a group of disorders known as motor neuron diseases (MNDs), characterised by the gradual degeneration and death of motor neurons. ALS is one of the most common neuromuscular diseases worldwide, affecting around 2 in 100,000 people each year. About 5 to 10 per cent of all ALS cases are inherited (familial ALS). However in 90 to 95 per cent of all ALS cases, the disease occurs apparently at random and is not gene-linked. There is increasing evidence that the most common form of ALS (known as sporadic ALS) is the result of the long-term influence of environmental factors on the genome[1] and, specifically, epigenetic alterations[2,3]. Most people with ALS die from respiratory failure, usually within three to five years from the onset of symptoms. However, about 10 per cent of those with ALS survive for 10 or more years. These differences in disease progression can be linked to epigenetics.

Oxford BioDynamics (OBD) is developing and validating diagnostic and prognostic epigenetic biomarkers based on its proprietary, innovative EpiSwitch™ biomarker platform technology. The tests would enable early detection and prognostic stratification of ALS patients into groups with fast and slow progressing disease, in direct correlation with their ALSFRS-R scores, the revised Amyotrophic Lateral Sclerosis Functional Rating Scale, which is used today to assess disability in patients with MNDs. The resultant prognostic epigenetic test could be used to inform patient recruitment into clinical trials, to determine therapeutic impacts on rates of progression and as a companion test for novel targeted therapeutic approaches. Additionally, the test would have value as a standalone diagnostic and prognostic test to inform strategies for the clinical management of patients.

Currently, to deliver a confident diagnosis of ALS, a comprehensive diagnostic workup is needed that requires many different procedures, including electro diagnostic tests such as electromyography and nerve conduction velocity, blood and urine studies including high-resolution serum protein electrophoresis, thyroid and parathyroid hormone levels and 24-hour urine collection for heavy metals, lumbar puncture x-rays, including magnetic resonance imaging, myelogram of cervical spine, muscle and/or nerve biopsy, and a thorough neurological examination. These processes require hours of patient cooperation, and days of laboratory time. In contrast, the diagnostic tests being developed by OBD are non-invasive, quick to administer, and can be analysed in four hours.

The Value of Systemic Epigenetic Biomarker Tests

Diagnosing and treating ALS is currently expensive, and fails to match the critical time needs of patients, placing an economic burden on the patient, the payer and the community. Direct costs include those owed mainly to the healthcare sector for treatment of the disease, including GP contacts, outpatient specialist contacts, physical, speech and occupational therapy, complementary healthcare, hospitalisations, prescribed and over-the-counter medications and medical interventions to support nutrition and respiratory functions, e.g. percutaneous endoscopic gastronomy (PEG) or bi-level intermittent positive pressure (Bipap) ventilation. On top of these direct medical costs come non-medical costs, including adaptations to the patient's home, and adapted means of transportation, aids and appliances needed to perform life's daily activities. Indirect costs are those representing lost resources including productivity losses due to absence from work, whether that is for the patient or for family members who take time off to care for them; these can be substantial if the disease affects workers earning high rates near the end of their careers. Intangible costs are virtually impossible to measure but encompass the reduction in the quality of life.

Despite intensive research into the biological basis of ALS, and numerous well-powered multicentre clinical trials, the disease remains untreatable. A major impediment to identifying drugs of therapeutic benefit in ALS is the absence of adequate biomarkers that can provide objective evidence of a favourable alteration in biological pathways known to be important in the pathogenesis. It is entirely plausible that drugs that could have an impact on the disease process for a specific cohort of patients have been systematically discarded, based on crude clinical endpoints in clinical trials. Importantly, it is likely that combinatorial approaches to diseases like ALS will be required and therefore it is critical to find improved ways of measuring the effect of drugs directly on disease relevant pathways.

The OBD programme of work will directly contribute to knowledge, both within the UK and internationally, by increasing the biological understanding of disease heterogeneity and thus informing clinical and basic science studies of disease natural history. It will improve basic laboratory research by identifying key biological pathways underpinning motor neuron degeneration, and aid the pharmaceutical industry and its academic partners to select drugs of potential therapeutic effect and to allow targeting of specific sub-groups of patients. Academic researchers working on other neurodegenerative diseases will also benefit from the expansion of our understanding of epigenetic effects in these conditions and in understanding potential overlaps in pathogenesis. These benefits will not be limited only to ALS or UK-based researchers, as biomarker and therapeutic research in this area is necessarily a collaborative activity in a rare disease. As an example of this collaborative activity, today OBD is actively engaged in its efforts with the US North East ALS consortium (NEALS), Massachusetts General Hospital, Boston and Nuffield Department of Clinical Neurosciences, University of Oxford. It counts Professor Merit Cudkowicz,

Professor of Neurology, Harvard Medical School Director, MGH MDA ALS Clinic Chief, Neurology Service Co-Director of Neurological Clinical Research Institute, and Professor Kevin Talbot, University of Oxford, among its collaborators.

OBD tests will have value clinically as they support diagnosis and prognosis, scientifically as they back up clinical research, and commercially as they add value to the marketability of drug manufacturers' products. The tests will enable earlier patient diagnosis and accelerate clinical decision-making, allowing patients earlier access to wider clinical support for the manangement of their disease, improving quality of life. Additionally, the ability to exclude a diagnosis of ALS early in presentation will enable alternative diagnostic testing to be indicated, and appropriate therapeutic relief to be delivered sooner. OBD analysis will improve clinicians' ability to offer a prognosis, by giving healthcare practitioners objective insight into individualised patient trajectories (e.g. fast vs. slow progressing disease), and would provide, according to key opinion leaders, a desperately needed tool for long-term patient management, enabling patient-centred effective care planning.

For managers of clinical trials, applications in clinical trial recruitment will improve their ability to stratify patients into groups, such as those more likely to respond to treatment due to the stage of disease (earlier is better) and those more likely to show effects of therapy over a shorter trial duration due to the fast rate of progression. Such stratification could potentially improve clinical trial outcomes. In clinical trial monitoring, changes in prognostic biomarkers could provide a surrogate for earlier therapeutic efficacy, irrespective of drug mechanism of action. This could dramatically improve the way trials are designed, outcome measures being currently based on rate of decline in ALSFRS-R and survival, which is lengthy and costly. This would offer a great benefit to therapeutic developers, who could run trials more quickly and more quickly with lower patient numbers, and for patients who would neither get recruited into inappropriate trials, nor have to stay on ineffective experimental therapies for longer than is necessary. By further refining the biomarker panel together with a novel therapy, drug developers could deploy an approach combining a companion diagnostic with a therapeutic. This combination enables the identification of specific ALS patient populations for trial recruitment that are most likely to respond to therapy based on specific epigenetic or targeted molecular mechanisms, and provide a mechanism to measure early response to therapy. This work could also provide insights for preclinical researchers in ALS, to identify further epigenetic mechanisms involved in disease onset and progression, enabling the development of novel targeted therapeutic approaches.

Stratifying Patients Using Chromosome Conformation Signatures

Our work focuses on developing for personalised medicine in disease areas where epigenetic changes are implicated in disease phenotype. OBD's platform technology, EpiSwitch™, is based on the identification of a specific class of epigenetic biomarkers – chromosome conformation signatures (CCSs) which serve as early markers of gene deregulation and precede other epigenetic changes. This innovative technology offers the ability to rapidly develop simple, sensitive and affordable diagnostic products. CCSs have much to recommend themselves among the many different classes of biomarkers[4]:

- They offer informative stratification of complex phenotypes, where genetic differences are strongly modulated by epigenetics,
- Have high biochemical and physiological stability,
- Have a binary nature, for ease of statistical analysis and construction of strong classifiers,
- Offer a signal that is detected early in the disease process due to its primary position in the eukaryotic cascade of gene regulation,
- Are detectable in blood samples.

These advantages mean chromosome conformation signature biomarkers are an excellent, innovative choice for the screening, early detection, monitoring and prognostic analysis of major diseases associated with aberrant and responsive gene expression, including ALS.

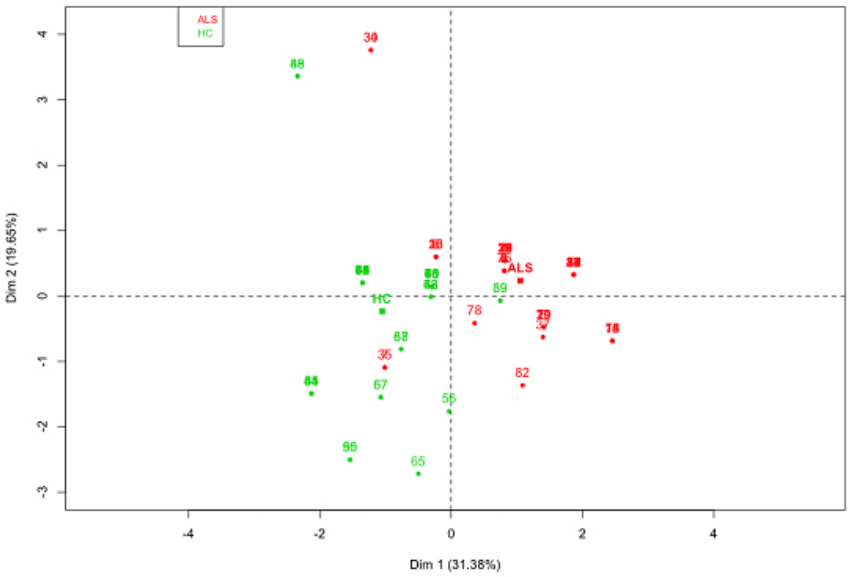
Using its custom-designed EpiSwitch™ Array platform, OBD has conducted initial discovery and evaluation of potential systemic epigenetic biomarkers. Extensive analysis of epigenetic regulation on over 296 genes and 14,000 EpiSwitch™ chromosome conformation signatures has already identified 927 epigenetic ALS-specific biomarkers with significant fold change against controls and $p<0.1$. This offers a rich pool of potential biomarker leads for further evaluation. Significant epigenetic deregulations have been identified within the most relevant main loci: for their roles in familial type ALS, SOD1 and C9orf 72; for its role in sporadic type ALS, TDP43; and for their roles in neurodegeneration PANX1 and DNM3; T Cell Receptor Signalling and Toll-like Receptor Signalling Pathways.

Evaluation analysis performed on a cohort of 74 ALS patients and controls has identified a signature of five EpiSwitch™ biomarkers. In the pipeline of statistical analysis, the attribute space was reduced using either logistic regression or Random Forest approaches with linear forward selection. The libraries under the Weka package were used for these steps. The resultant EpiSwitch™ panel of five markers was the best for discerning the classes under investigation (Figure 1). When tested for validation in an independent blinded cohort of peripheral blood samples, the signature stratified ALS patients with 87.5% sensitivity.

Biomarker Discovery Technology

The custom 15k EpiSwitch™ Array is used to analyse around 300 genetic loci (up to 50 candidate markers per loci), that are interrogated with the EpiSwitch™ Biomarker discovery technology. The array is built on the basis of the Agilent SurePrint G3 Custom CGH microarray platform, and each EpiSwitch™ probe is presented as a quadruplicate, thus allowing for statistical evaluation of reproducibility. The probes that are spotted onto the arrays are the chimeric fragments predicted to exist at the loci, as predicted by the EpiSwitch™ pattern recognition software. Samples representing ALS and control are labelled with Cy5 or Cy3 and assessed by competitive hybridisation for the prevalence of individual EpiSwitch™ marker candidates. The resultant data is analysed with proprietary processing scripts using Bioconductor in R: Limma. The normalisation of the arrays is done using the normalise within arrays function in Limma with Agilent and EpiSwitch™ controls. Probes that pass the $p<0.01$ FDR p-value are used for further screening. To reduce the probe set further, multiple factor analysis is performed using the FactorMineR package in R. The top

Figure 1. Factor analysis of 45 ALS patients and 45 healthy controls stratified with the 5 EpiSwitch™ markers' signature. Red - ALS, Green – Healthy Controls



statistical and discerning EpiSwitch™ markers from the R analysis are then taken forward to screening and reduction using the EpiSwitch™ PCR assay.

The EpiSwitch™ assay and its PCR platform present a simple, robust test that is readily transferable to healthcare or pharma R&D settings. Specialised test technology has already been transferred to several parties in Asia. OBD reference facilities in Oxford and Penang run EpiSwitch™ tests under industry quality control standards, with a current throughput capacity of up to 50,000 sample readouts per quarter. OBD is planning to commercialise its technologies primarily through licensing agreements in the US, where OBD is conducting a number of collaborative biomarker projects with leading pharmaceutical partners, and Asia, where OBD operates its second reference facility and is working with leading private hospitals.

Extended evaluation of diagnostic markers starts on the EpiSwitch™ array platform using a custom-designed array based on loci of interest. If a diagnostic signature is confirmed (CV value of <30% and p<0.01 FDR p-value) the top performing markers are taken forward for validation using the PCR platform in an iterative process involving more than 100 ALS samples and 100 age-matched controls. The patients will be a mix of incident and prevalent, the latter being enriched for those patients within three years of symptom onset.

The markers confirmed in the diagnostic work packages will also be evaluated for prognostic signatures using a pared-down version of the custom array from the earlier work package and tested using longitudinal samples from the fast and slow progressing patients (assessed at point of recruitment and at three and six months). Once the prognostic signature is confirmed (CV value of <30% and p<0.01 FDR p-value) it will be taken forward for marker refinement to improve the analytical qualities of the test, and then validation using the EpiSwitch™ PCR platform and longitudinal samples from all available ALS patients. Determination of fast versus slow progression is based on the classification using the formula 48-ALSFRS-R score at presentation/ months since symptom onset.

Subsequently, blinded validations of the diagnostic and prognostic tests will be performed on independent cohorts.

Apart from the diagnostic and prognostic tests, OBD is also planning to explore the potential for sub-stratification of ALS patients in clinical trials: a correlation of changing epigenetic profiles during treatment with primary endpoint outcomes will help the development of response biomarkers; a correlation of epigenetic profiles of patients at base line with primary endpoint outcomes following the treatment will help the development of predictive biomarkers for identification of potential responders and non-responders to treatment.

An Impact Summary

The identification of an epigenetic biomarker could have a major impact in a short to medium timescale of 3-5 years across all parts of the drug development industry, from investigators and trial patients to drugs companies and industry regulators, as well as having a spill-over effect on other areas of neurodegenerative research. Investigators would be able to streamline clinical trials, allowing drugs of potential effect to be pre-screened prior to entering full Phase II and III studies, and potentially saving considerable costs for the pharmaceutical industry that could be invested into the development of new therapies. People living with ALS would benefit from a more precise prognostication and personalised care planning because a sub-stratification of patients according to the biological mediators of clinical heterogeneity would identify fast/slow progressors. ALS is a heterogeneous disease and clinical trials do not generally distinguish between subtypes of the disease, which partly

may contribute to the failure of pharma to identify treatments that might benefit sub-groups of patients. The identification of biomarkers would improve efficiency of clinical trial design and reduce waste. It would reduce the number of patients exposed to avoidable risk by restricting trials to appropriate groups based on biological characteristics.

The wider community of researchers investigating the causes of neurodegenerative disease (Parkinson's and related conditions, dementia and degenerative ataxias), patients living with such diseases, and the pharmaceutical industry, would benefit by a greater understanding of the overlaps between ALS and other neurodegenerative diseases (already established for ALS and frontotemporal dementia). Drug regulators would benefit from a greater objectivity in clinical trial outcomes that would come from the identification of epigenetic biomarkers. Providers of healthcare, policy-makers and public health organisations would benefit from a greater understanding of the biological heterogeneity of ALS, so they can plan and streamline services for patients, especially if therapies alter the natural history of the disease. In addition to these benefits to the research and development community working in neurodegeneration, the research has the potential to improve the efficiency with which pharmaceutical industry resources are used, freeing them up to support further growth in the biopharma industry, and creating opportunities for training and career development in this important disease area.

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