Amyotrophic Lateral Sclerosis and Personalized Medicine

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Introduction & History

Amyotrophic lateral sclerosis (ALS) or "Lou Gehrig's disease" (coined due to the baseball player's public struggle with the disease in 1939) was first described by French neurologist Jean-Martin Charcot, who synthesized clinical and pathophysiologic observations of the disease in collaboration with colleague Alex Joffroy between 1865 and 1869 at the Salpêtrière Hospital in Paris. (Goetz 20000, Kumar 2011) Charcot and Joffroy identified patients with weakness in voluntary movement without sensory deficits, epilepsy or problems with involuntary movement, some with spastic contractures and others with amyotrophic atrophy. In 1865, Chatcot presented his findings in a female patient diagnosed with hysteria, who presented with profound weakness, increased muscle tone and contractures of the extremities, with no sensory abnormalities or bladder control problems in whom he found, "two brownish-gray streak marks produced by sclerotic changes…[beginning] outside the line of insertion of the posterior roots [of the spinal cord and continuing], though greatly thinning out, up to the widening point of the cervical cord," in the white matter only. (Charcot 1865, quoted in Goetz 2000). In 1869, he observed, "spinal lesions are systematically limited to the anterior horns of the gray matter" in a case of infantile paralysis. (ibid.) Charcot

synthesized from his observations that the motor system consisted of 2 parts, and that legions to each part caused differential clinical presentation due to damage in the white and grey matter of the spinal cord, respectively. After 1869, he observed that patients with anterior horn legions *and* bilateral sclerosis of the lateral spinal cord columns, supporting the synthesis of the clinical symptoms and underlying pathology he observed into a clinical disorder with specific pathologic correlates in the white and grey matter. In 1874, Charcot coined the anatomical term "amyotrophic lateral sclerosis" to describe the grey matter involvement (amyotrophy) and white matter damage (lateral sclerosis) in his clinical cases. (Goetz 2000)

Epidemiology

ALS is a rare disorder, affecting about 12,000 people in the United States at a rate of 2.9 cases per 100,000 persons in the general U.S. population (CDC Surveillance Summary 2014). The ALS Registry Act (110th Congress, 2008) spawned the first and only large scale analysis of ALS incidence, prevalence and risk factors in the United States, which led to a CDC report published in January 2014. The study resulted in identification of 12,187 persons with definite ALS, with an overall prevalence of 3.9 cases per 100,000 people that increased with age. The lowest prevalence was found in 18-39 year olds (0.5 per 100,000), and the highest prevalence rate was found in 70-79 year olds (17.0 per 100,000). There was also a higher prevalence rate in males (4.8 per 100,000) versus females (3.0 per 100,000), and high prevalence for whites, who accounted for 79.1% of all cases identified. The majority of ALS patients are either disabled (45%) or retired (31%) and only 15% are employed full time. Average overall survival time from diagnosis with ALS is approximately 3 years according to the analysis.

A study of global ALS epidemiology was conducted in 2009 by integration of 37 published articles, including 25 studies from Europe, 5 in North America, 6 in Asia and the Pacific, and 1 in Uruguay. (Chiò 2013) From this study, the highest incidence was found to occur in the Faroe Islands in the Norwegian Sea between Norway and Iceland (3.6 per 100,000), the Piemont/Valle d'Aosta region of northwestern Italy and Modena, Italy (both 2.9 per 100,000), and Ireland (2.6 per 100,000). Another European epidemiological study of ALS pooled data from six ALS registries in Italy, Ireland, Scotland and England, and found that the incidence of ALS across Europe to be 2.1 per 100,000 person-years, a result similar to studies done in the U.S. The authors of that study state that this, "uniform frequency of ALS suggests that the same environmental and/or genetic factors may underlie disease within the white population." (Logroscino 2010)

Genome wide association studies (GWAS) in Finland elucidated a mutated locus on chromosome 9p21 present in 40% of inherited ALS cases and 25% of all ALS cases. (Laaksovirta 2010) Finland is particularly useful for studying ALS genomics because it has the highest incidence of the disease in the world at 8.2 per 100,000 (Murros 1983), and the genetic heterogeneity of the Finnish population enables more efficient detection of disease loci. In examining Finnish population genetics, Laaksovirta et al.. identified significant signals at chromosome 21q22 corresponding to the *SOD1* gene, and a 232 kb run of linkage disequilibrium at chromosome 9p21 previously identified in familial ALS studies by GWAS. The 21q22 *SOD1* signal corresponded with homozygosity of the D90A allele in *SOD1* in cases of sporadic ALS in a recessive model, implicating the gene in both sporadic and familial disease causality.

Clinical Presentation

Amyotrophic lateral sclerosis is a neurodegenerative disease in which motor neurons responsible for transmitting movement signals (upper motor neurons), initiating, and controlling muscle movement (lower motor neurons) degenerate over time, causing muscular atrophy by scarring of the lateral spinal cord progressive paralysis that manifests as extreme weakness (ALS Association 2015). People with ALS progressively loose control of their ability to voluntarily move their muscles, experience severe loss of strength, and can loose their ability to breathe independently.

ALS has several clinical presentations. Limb-onset and bulbar onset are most common. (Kiernan 2011) Limb-onset al.S implies the presence of concurrent upper motor neuron (UMN) and lower motor neuron (LMN) signs. UMN "signs" include spasticity, exaggerated reflexes (including overactive gag reflex), while LMN signs include muscle weakness, atrophy, muscle cramps, and spontaneous brief muscle contractions ("fasciculations," i.e. Charcot's contractures). Patients presenting with limb onset al.S experience weakness in one or more limbs that progresses over time. As the disease progresses further, movement becomes more difficult until patients are essentially debilitated. (National Institute of Neurological Disorders and Stroke 2015)

Bulbar onset al.S implies damage to brainstem anatomic regions. Therefore, it most often presents with bulbar palsy, i.e. flaccid paralysis, muscular atrophy and spasms of the tongue. It also includes muscular weakness in the facial muscles that causes extreme difficulty with respiration, speech (dysarthria), repression of voluntary and involuntary coughing, spasms of the tongue and difficulty swallowing (dysphagia). (Kühnlein 2008) Most commonly, bulbar onset symptoms begin to present as speech difficulties in the early stages of ALS, and patients will experience involuntary slurring of their words and difficulty with articulation. Bulbar onset symptoms are the some of most common clinical manifestations of ALS, with dysarthria and dysphagia appearing in 93% and 86% percent of cases, respectively.

As the disease progresses, muscle wasting and neurodegeneration caused by ALS manifests as respiratory difficulty that progresses to respiratory failure and associated pulmonary complications that are fatal. (Gordon 2011) At present, clinicians must rely on clinical diagnostic criteria to make a definitive ALS diagnosis. (Kiernan 2011) The El Escorial Criteria (Table 1) were developed by a conference of neurologists to standardize clinical recognition of the disease. (Brooks 1994, Dengler 2010) The criteria are composed of "A" and "B" subsets of presentations observed in cases of ALS. (Table 1)

The A criteria evaluate upper motor neuron (UMN) and lower motor neuron (LMN) signs in 4 defined regions of the body. The B criteria introduce electrophysiological criteria that can be confirmed or denied through electromyography (EMG) testing as well as neuroimaging criteria that can be assessed by magnetic resonance imaging (MRI). The B criteria for electrophysiology and imaging essentially stipulate that other neurological disorders must be ruled out by these diagnostic modalities in order for an ALS diagnosis to be made in confidence. The body regions assessed by the criteria encompass the brain stem and 3 subsections of the spinal cord. MRI scans are also necessary to determine if the anatomic neuropathology associated with ALS is present.

Table 1: El Escorial Criteria (EEC) for the diagnosis of ALS. Adapted from Dengler 2010.

A criteria	**B Criteria**	**Body regions**
A1: Degeneration of the lower motor neuron approved by clinical, electrophysiological or neuropathological examination. **A2:** Degeneration of the upper motor neuron approved by clinical examination. **A3:** Progressive dissemination beyond typical nerve supply areas.	**B1:** Electrophysiological or neuropathological findings typical for other diseases which could explain the degeneration of the upper and lower motor neuron. **B2:** Findings in imaging studies which can explain the clinical symptoms	Bain stem (bulbar) Cervical (neck and upper extremities) Thoracal (trunk, abdominal wall) Lumbosacral (lumbar spine and lower extremities).

Disease Mechanism

The principle causes underlying the neuronal degeneration characteristic of ALS is unknown. The disease process contains multiple steps and involves multiple neuronal cell subtypes. (Neusch 2007) There are unresolved connections and interactions between the molecular and genetic levels of the disease, and the pathology of ALS has been called "multifactorial." (Kiernan 2011, Shaw 2005) One hypothesis is that the pathology of ALS results from a pathologic milieu of glutamate excitotoxicity, free radical accumulation, cytoplasmic protein aggregation, and SOD1 enzyme hyperactivity in conjunction with mitochondrial dysfunction and impairment of axonal transport by intracellular protein aggregates. (Kiernan 2011, Fig. 1)

Genetic defects in the poorly characterized gene *C9orf72* (chromosome 9, open reading frame 72) have been implicated in about 1/3 of familial ALS cases and a small percentage of sporadic cases, and alterations in *SOD1* (copper-zinc superoxide disputes 1) are implicated in an additional 20% of familial cases. (National Institute of Neurological Disorders and Stroke 2015) The pathophysiology of ALS has at present been resolved to a large number of variables that have proven difficult to synthesize into a succinct sub-anatomical pathology.

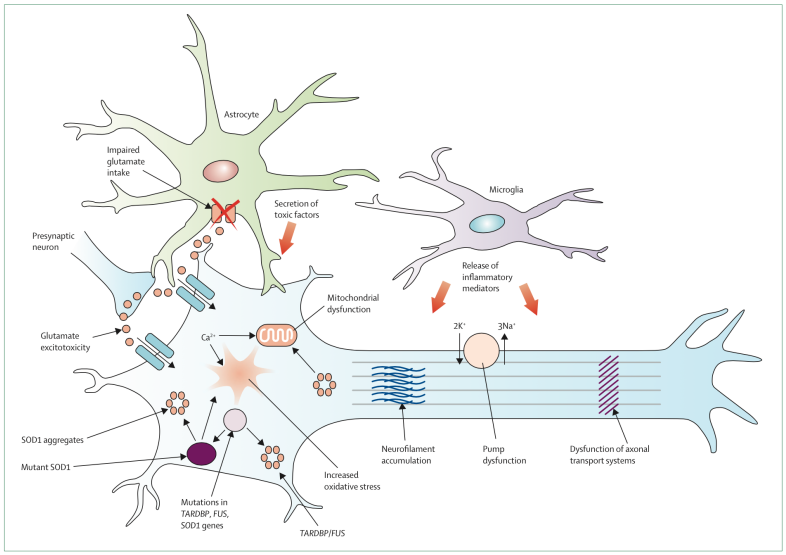

Figure 1: Hypothesized multifactorial cell and molecular pathology of ALS. Adapted from Kiernan 2011.

At the cellular level, the pre- and post-synaptic neurons as well as astrocytes and glia are thought to play a role in the pathology of ALS. Impaired glutamate uptake in astrocytes may, in conjunction with pre-synaptic excitotoxicity, create a pool of excitatory glutamate in the neuronal microenvironment. As a result, the post-synaptic cell experiences a calcium influx, which in turn may result in free radical formation and eventually neuronal death. (Mitchell 2007) Impaired glutamate reuptake mechanisms may also play a role in the spastic contractures characteristic of ALS. Outside the pre-and post-synaptic system, microglia are also hypothesized to play a role, particularly in the release of inflammatory mediators in response to motor neuron injury caused by pathological conditions in the post-synaptic neuron. (Kiernan 2011) Pathology in the post-synaptic neuron may be traceable to several mutations found to be characteristic of ALS.

SOD1 on chromosome 21q22.1 codes for a metalloenzyme that converts intracellular super oxide (O^{-}_{2}) toxic free radicals produced by mitochondrial oxidative phosphorylation by the action of a copper atom in its active site that is reduced and oxidized by intracellular superoxide. (Shaw 2005) *SOD1* has been identified as mutated in ALS populations. The D90A allelic variant is of interest in motor neuron disease and ALS, as it was discovered to be inherited as a homozygous recessive disease variant in Scandavavian populations, and is thought to be pathogenetic through a toxic gain-of-function mechanism. (Anderson 1995)

When taken in combination with the calcium influx caused by the pre-synaptic neuron, it may be the case that the influx of positive charge from excess calcium ions in the post-synaptic neuron adversely affects the ability of SOD1's copper-mediated active site to properly oxidize superoxide to a form that can be neutralized by free radical scavengers, resulting in superoxide accumulation and neuronal death. The incidence of a homozygous gain-of-function mutation in *SOD1* in ALS populations suggests that the calcium imbalance in the post-synaptic neuron may adversely affect the SOD1 enzyme such that its activity exerts some other effect of the intracellular

environment. Moreover, it has also been discovered that mutant SOD1 enzyme increases the sensitivity of motor neurons to glutamate toxicity (Kruman 1999), lending further strength to the link between mutant SOD1 and glutamate-mediated excitotoxicity in ALS.

Protein aggregation in ALS has been linked to alterations in *TARDBP*, a gene encoding TAR DNA-binding protein (TDP-43), and aggregates of TDP-43 are thought to play a role in the pathology of ALS as well as frontotemporal dementia. (Guo 2011, Shaw 2005) Work in chicken embryos, rats, mice, flies and cultured cells showed that overexpression of mutant or wild type TDP-43 can result in recapitulated motor neuron disease or proteinopathy. (Guo 2011) Guo and colleagues also discovered that TDP-43 peptides take the form of beta sheets and, when aggregated, form amyloid fibrils. They also found that treating cultured neurons with TDP-43 derived peptides increased rates of neuronal cell death. This suggests that TDP-43 peptides can cause neurotoxicity, and this is consistent with hypothesized ALS pathology. Later work (Lattante 2013) has elucidated that both *TARDBP* and *FUS* have functional similarity, and both play roles in motor neuron development and axonal transport. In sum, these elucidations regarding protein aggregation suggest that the proteinopathy underlying ALS is multifactorial in the same respect as the entire disease process, adding another layer of complexity to ALS pathology.

Mitochondrial dysfunction has also been implicated in ALS. The dysfunction has been hypothesized to be age related, but there are also reports indicating that mitochondrial dysfunction may be a hallmark of ALS and motor neuron disorder. (Shaw 2005) Calcium uptake by mitochondria is a condition for excitotoxicity, and increases in mitochondrial calcium are correlated with free radical generation, which are linked to cell death (Stout 1998) and also to the SOD1 mechanism discussed above. Integrating these findings, it may be the case that the influx of calcium caused by pre-synaptic transmission of glutamate alters the cytoplasmic environment of the cell such that mitochondria take up positive ions and thus prime the post-synaptic neuron for

excitotoxicity. Synergistic with this component of hypothesized ALS pathology could be mutated gain-of-function SOD1-mediated increases in intracellular free superoxide and protein aggregation. This mosaic of dysfunctional variables discussed above begins to paint the complicated picture of ALS pathology at the cell and molecular levels. The disease mechanism of ALS is presently unresolved due to the multivariate nature of this mosaic pathology.

Current Treatments

There is no established cure for ALS, and the only FDA approved drug for the management of the disease is oral Riluzole (Rilutek ®), a benzothiazole thought to inhibit neuronal glutamate release that showed increased overall survival time and time to tracheostomy in randomized clinical trials of patients with familial or sporadic ALS (MedLine Plus 2015, Sanofi-Aventis Prescribing Information 2008). Two randomized controlled clinical trials have been conducted using Riluzole to treat ALS, and results were only modest. Results indicated that Riluzole extended survival in ALS patients about 4 months versus placebo. (Gordon 2011, Bensimon 1994) These trials primarily measured survival and changes in functional status, and secondarily evaluated the effects of Riluzole on muscle strength, respiratory function, and patients' subjective perception of their symptoms, among other variables. In one trial, survival in the experimental group treated with Riluzole at 1 year was 74%, versus 58% in the placebo group, and mortality was reduced in the Riluzole group by 38.6%. A positive effect on the rate of deterioration of muscle function was also observed. (Bensimon 1994)

Respiratory issues caused by ALS may include weakened cough as a result of weakened muscles, which in turn can lead to secretory buildup and poor airway clearance that can cause aspiration pneumonia when food, saliva or other liquids are breathed into the airway and lungs.

These symptoms are difficult to manage as there is no solution to restore respiratory function. However, patients can be placed on night time ventilators to assist with breathing difficulty and promote more restful sleep. When respiratory symptoms are maximally severe, tracheostomy can be performed, however many patients forego this option due to the physical and emotional burden associated with the decision. The swallowing difficulty, and facial and arm muscle weakness experienced by ALS patients often deters them from eating, so nutrition is also a concern. Solutions for progressive weakness that circumvent a patient's inability to eat includes modification of diet consistency as well as the eventual use of a feeding tube. (Gordon 2011) Interventions for bulbar symptoms are not curative, but include speech therapy, use of communication assistance devices, compensatory techniques for swallowing, and dietary/feeding techniques to circumvent dysphagia. (Kiernan 2011)

For mental health symptoms, various anti-psychotics and other psychiatric drugs or approaches such as SSRIs or clinical psychotherapy may be used. Sialorrhea (hypersalivation) is also a debasing and socially embarrassing consequence of ALS that can be managed with mechanical (e.g., suction machines, cough-assist devices) and pharmacological (e.g., transdermal scopolamine, oral glycopyrrolate) means. (Gordon 2011) Muscle spasticity and other motor neuron symptoms can be managed with various agents, including Baclofen (a GABA analogue), dantrolene sodium, tizanidine, and benzodiazepines. Botulinum toxin injections are also an option for sialorrhea and spasticity. Urinary urgency becomes a problem for ALS patients, and can be managed with anti-cholinergics, oxybutinin and toldterodine tartrate. (Gordon 2011) Pain and fatigue are also common. Fatigue is also a side effect of Riluzole, so, if a patient is taking Riluzole, the drug can be stopped, however this is a complicated decision if the patient is deriving benefit from the drug. Other solutions include methylphenodate and modafinil. Pain may also be managed pharmacologically by use of non-steroidal anti-inflammatory drugs, narcotics or anxiolytics. Physical therapy is also an

option for patients. (Gordon 2011) The lack of understanding of the ALS disease mechanism means that there are no drugs available that ameliorate the disease effectively. Therefore paradigms of care applicable in ALS are ultimately palliative and clinical trial participation is commonplace.

Future Prospects with Personalized Medicine

There are huge challenges at multiple levels of biological organization challenging efforts to understand and effectively treat ALS. However, stem cell technology presents a number of possibilities for revolutionizing scientific understanding and treatment of ALS. Induced pluripotent stem cells (iPSCs) can be used to model the disease, which can to deeply enrich our understanding of ALS's basic biology and enable high throughput drug screening to search for new therapeutics. In combination with next-generation sequencing and bioinformatics, there is also great hope for pinning down crucial genetic alterations and synthesizing that knowledge with the downstream molecular and cellular events and phenotypes already known to be part of the disease process, and for identifying new causal variables. These scientific advancements will, in turn, enable personalized medical approaches to ALS, as they will strengthen our ability to understand individual cases of the disease in greater depth and generalize observations.

In 2008, Dimos et al.. created induced pluripotent stem cells (iPSCs) from an 82 year old patient with ALS (*SOD1* mutated) and subsequently differentiated them into patient-specific motor neurons and glia. They retrovirally transfected skin fibroblasts with *KLF4*, *SOX2*, *OCT4*, and *c-MYC* genes to produced viable iPS cells by reprogramming. The iPSCs then subsequently differentiated into embryoid bodies representing the 3 germ layers, leading the authors to attempt differentiation into motor neurons and glia. (The authors acknowledge evidence that glia produce factors toxic to motor neurons in the ALS disease state, which prompted them to pursue differentiation of both cell

types.) They generated spinal motor neurons and glia by treating the embryoid bodies that differentiated from the patient-derived iPSCs with an sonic hedgehog (SHH) signaling pathway agonist and retinoid acid (RA), and confirmed the identity of the derived cells through detection of neuronal and motor neuronal markers.

The work of the Dimos group establishes that patient-derived ALS cells can be generated with iPSC technology. This implies that the defects plaguing the degenerated neurons of a single ALS patient can be derived in the laboratory to recapitulate the cellular components of that patient's disease, enabling *in vitro* modeling of the ALS disease at an individual level. With such models in hand, it becomes possible to deeply interrogate the cellular, molecular and genomic/genetic variables contributing to motor neuron damage in a patient-specific manner. Combining this approach with high throughput drug screening means that it is now possible to not only create personalized models of ALS motor neuron dysfunction to interrogate their biology, but also to test an array of drugs for possible therapy. This was achieved in 2012 by Egawa et al.. with iPSCs recapitulating ALS cells with a *TDP-43* mutant genotype, and they discovered that anacardic acid, a histone acetyltransferase inhibitor, was able to rescue the ALS phenotype. In addition, they found that TDP-43 protein existed in these cells bound to a spliceosomal factor, SNRPB2. This reinforces the possibility of the paradigm discussed above as a viable approach in ALS research that can simultaneously pursue questions about ALS pathology and treatment concurrently.

There are at least two options for using stem cells to advance the treatment of ALS. One is to use iPSCs to do high throughput drug screening with existing compounds as expressed above. This approach beings with it several challenges. First, the multivariate nature of the ALS disease process suggests that simply having motor neurons and glia *in vitro* may not be sufficient to recapitulate the entire disease process. As articulated above, the ALS disease mechanism critically involves the release of glutamate from the presynaptic neuron and its effects on calcium

concentrations in the post-synaptic neuron. Since this is true of the disease state, it must be recapitulated in any laboratory model. That is, in addition to growing the defective cells, a true *in vitro* model of ALS disease process must include some semblance of a motor neural network as well as the glutamate-saturated microenvironment. Considerable effort would need to be expended to create such a model. However once created, the power of the model is undeniable.

If iPSCs from ALS patients can be used to create recapitulations of an individual's disease outside the body and the specific genetic and molecular dysfunctions sufficient for motor neuron degeneration can be identified and corrected *in vitro*, this implies that cell replacement therapy is one possible next-generation therapeutic approach for ALS patients. This granted, the challenges associated with this idea are immense. Cell replacement therapy in ALS is exceptionally difficult because even if new motor neurons or motor neuron precursors can be introduced into a patient, they must be coaxed to grow and innervate the appropriate muscles to relieve the patient's symptoms, which requires a detailed understanding of the sequence of molecular events of motor neuron growth and differentiation. (Dimos 2008, Cashman 2013)

Since ALS has both familial (inherited) and sporadic forms, the advent of personalized medicine affords possibilities for more more the development of effective ALS treatment due to greater focus on the individual patient. As discussed above there are also several different genes involved in the disease, and genomic epidemiology suggests that the frequency and gene dosage of the various identified ALS mutations in populations will scale down to the level of individual patients. In the future, next-generation sequencing will allow us to determine what specific genetic defects apply to particular ALS patients, and stem cell technologies in combination with high throughput drug screening and other functional assays will permit better resolution of the pathology that causes the disease and its potential treatments. The rate-limiting step in reaching this goal is *in vitro* modeling of human forms of the disease effectively enough to increase confidence in

experimental results over animal models. Therefore, the concurrent development of *in vitro* modeling techniques will also influence our ability to decipher ALS. Models of these approaches have been published (Richard and Margakis 2015), and it is now just a matter of time and effort to realize them.

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