

PLL Therapeutics driving a new approach to ALS diagnosis and therapy

PLL Therapeutics is developing a new paradigm for the early diagnosis and treatment of patients with amyotrophic lateral sclerosis. Our pioneering therapy, currently undergoing a phase I/II study, is based on emerging insights into the role of the gut-brain axis in the pathophysiology of the condition, while our investigational diagnostic identifies patients based on a unique serum antibody signature.

Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease, characterized by injury to and loss of motor neurons in the brain and spinal cord. Initial symptoms can include muscle weakness and cramping or difficulties in swallowing or speaking. As these gain in severity and spread to voluntary muscles throughout the body, patients lose the ability to perform everyday activities, experience impaired mobility, and eventually develop almost total paralysis. The condition, although highly heterogeneous, has a poor prognosis and a rapid course. Patients typically die from respiratory failure within two to five years of their diagnosis¹.

Clinical manifestations of the disease vary from patient to patient, but most exhibit one of two distinct types of onset. Spinal or limb onset ALS, which accounts for about 70% of cases, starts with muscle weakness or stiffness, often affecting a hand, arm or leg. Bulbar-onset ALS, so-called because of the involvement of the bulbar region of the brainstem, starts with slurred speech or problems with swallowing. It usually follows a more aggressive course. About 15% of ALS patients also develop frontotemporal dementia (FTD)², while up to 60% of patients exhibit less severe cognitive and behavioural changes.

Rising prevalence

ALS is an Orphan disease. Its prevalence varies across different populations. In the U.S., the estimated prevalence in 2022 was 9.9 cases per 100,000; this is projected to rise to 10.5 cases per 100,000 by 2030³. About 500,000 people are currently living with the condition – and this number is expected to grow by 6% annually over the next fifteen years. In Europe and the U.S. combined, about 30,000 new cases are diagnosed every year. Most cases are sporadic, having no known cause, but about 10% are inherited. Mutations in over thirty genes have been linked with ALS⁴, but a small fraction of these – notably hexanucleotide expansions in *chromosome 9 open reading frame 72* (*C9orf72*) and mutations in the genes encoding superoxide dismutase 1 (SOD1), TAR DNA-binding protein 43 (TARDBP), fused in sarcoma (FUS) and TANK-binding kinase 1 (TBK1) – account for a majority of familial cases. Epidemiological studies suggest a range of environmental risk factors may also influence an individual's susceptibility, including exposure to pesticides, metals, air pollution, neurotoxins, infectious agents, trauma, diet, and electromagnetic fields⁵. In addition, occupational and lifestyle factors may also play a role.

Despite extensive efforts, progress in developing effective therapies for ALS has been minimal. Riluzole, an inhibitor of the neurotransmitter glutamate, gained approval in 1995. It slows disease progression and may offer a modest survival benefit⁶. Radicava (edaravone), an antioxidant which gained FDA approval in 2017, slows the loss of physical function⁷. Qalsody (tofersen), an antisense oligonucleotide that causes degradation of *SOD1* mRNA, gained FDA approval in 2023 for treating patients with *SOD1*-mutated familial ALS. It slows disease progression and extends survival⁸.

Dysbiosis implicated in ALS

The pathophysiology of ALS is complex. Inflammation, mitochondrial dysfunction, DNA repair defects, toxic protein aggregation, and impaired axonal transport within neurons have all been implicated in disease initiation or progression⁹. So, too, has blood-brain barrier dysfunction¹⁰. In addition, there is a growing body of evidence linking dysbiosis – or perturbations in the gut microbiome – with ALS. The gut microbiome, a highly diverse microbial community, typically comprises about 10^{14} bacterial, archeal, fungal, and protozoan cells. Its composition is not fixed – it can vary greatly among different individuals and populations and, within a given individual, over time¹¹. Because of its size – it weighs approximately the same as the human brain – and its essential role in human physiology, it is often referred to a ‘virtual organ’¹². Its key functions include digestion, particularly of dietary fiber; provision of nutrients, such as short chain fatty acids (SCFAs); immune modulation; protection against pathogens; and maintenance of the gut barrier¹³. Gut dysbiosis arises from alterations in the composition of the microbiome, which compromises its functions. It can result from inappropriate proliferation of ‘pathobionts’, potentially pathogenic species that are normally present in low numbers; loss of commensals, species that are normally resident and provide essential functions; or a general loss of diversity, which damages the overall health of the system¹⁴. These disturbances have been associated with myriad conditions, including gastrointestinal disorders, autoimmune disease, obesity, metabolic disease, infection, and neurodegenerative diseases.

Most of the evidence linking gut dysbiosis to ALS comes from studies in mice. One study in transgenic mice expressing multiple copies of a mutated human *SOD1* gene suggests that certain bacterial species in the microbiome can improve ALS symptoms, whereas others exacerbate them. Moreover, by manipulating the composition of the microbiome, the study authors were able to vary the severity of the disease¹⁵. A separate study in *C9orf72*-mutant mice suggests that the *C9ORF72* gene product suppresses inflammation induced by gut bacteria, which suggests a direct link between dysbiosis and the most common form of familial ALS¹⁶. Additional evidence comes from an analysis of the microbiomes of ALS patients – they contain lower levels of several species of butyrate-producing bacteria than do those of healthy controls¹⁷. Moreover, constipation has a much higher rate of incidence in ALS patients than it does in the general population¹⁸. This can result from the physical changes that accompany the condition, including reduced physical activity, loss of mobility, and muscle weakness, as well as dietary changes and side effects from medications used to manage

different symptoms of the condition, including pain, excess saliva production, muscle spasms, and depression or emotional lability. There are a range of options for managing the problem, but constipation may not just be a secondary complication of ALS. It may also be implicated in its pathophysiology, as a manifestation of wider gastrointestinal disturbances linked to autonomic nervous system dysfunction¹⁹. One recent observational study reported that the severity of constipation in an ALS cohort was linked with more rapid disease progression and reduced survival²⁰.

Critically, these microbiome changes and gastrointestinal symptoms often precede motor symptoms, which further suggests a potentially causative rather than a merely consequential role²¹⁻²². The relationship appears bidirectional: dysbiosis impairs intestinal barrier integrity leading to endotoxemia and systemic inflammation, which damages the blood-brain barrier and allows lipopolysaccharide (LPS) and inflammatory cells to enter the central nervous system (CNS). Conversely, ALS progression itself worsens dysbiosis, creating a vicious cycle of neuroinflammation and neurodegeneration²³⁻²⁵.

The involvement of the gastrointestinal tract, as well as the CNS, in the pathology of ALS implies a role also for the vagus nerve, given its function as the principal conduit of sensory information along the gut-brain axis. The vagus nerve is a critical regulator of systemic inflammation through the cholinergic anti-inflammatory pathway. Efferent vagal nerve fibers release the neurotransmitter acetylcholine, which binds $\alpha 7$ nicotinic acetylcholine receptors expressed on macrophages and other immune cells. This inhibits the release of pro-inflammatory cytokines, including TNF- α , IL-1 β and IL-6. Atrophy of the vagus nerve has been widely documented in ALS patients, which impairs CAP pathway function and results in a shift toward a pro-inflammatory milieu²⁶.

Braak's theory of Parkinson's disease development offers a potential model for how the ALS pathology unfolds. First proposed by the German scientist Heiko Braak more than two decades ago, it hypothesises that the initial trigger for Parkinson's occurs in the enteric nervous system and/or the olfactory nerve²⁷. Exposure to an environmental toxin or biological pathogen results in the formation of globular Lewy bodies and thread-like Lewy neurites containing misaggregated alpha-synuclein protein at these sites. These can travel along the vagus nerve, which links the enteric nervous system to the brain, or the olfactory nerve, which connects the nasal cavity with the olfactory bulb, and 'seed' the pathology into the brain through alpha-synuclein's prion-like properties. There is a wealth of *in vitro*, *in vivo* and clinical evidence to suggest the theory explains the disease course in some, but not all, Parkinson's patients²⁸. As yet, the evidence for a similar mechanism of disease spread in ALS is circumstantial rather than definitive. The main protein species that misfold and form neurotoxic aggregates in ALS, such as TAR DNA binding protein 43 (TDP-43), superoxide dismutase 1 (SOD1), and fused in sarcoma (FUS), also exhibit prion-like behaviour, a self-amplifying process in which misfolded proteins bind to and cause other proteins of their type to adopt a conformation that favors misfolding²⁹. Whether this seeding process takes place along the vagus nerve as well as within the

CNS has not been resolved. Vagus nerve stimulation, which has been approved for drug-resistant epilepsy, depression, stroke rehabilitation, and drug-resistant rheumatoid arthritis, has not been tested in ALS.

PLL Therapeutics is at the forefront of efforts to further evaluate the role of dysbiosis in ALS in patients, by conducting clinical trials of a candidate therapy, PLL001, which is designed to halt systemic and neural inflammation and restore gut health. Under normal physiological conditions, the gut microbiome helps to maintain the integrity of the intestinal barrier. Resident microbes stimulate endogenous expression of the proteins that form tight junctions, which limit the passage of macromolecules between epithelial cells of the gut lining. They also influence the production of mucus, which maintains a physical barrier between the epithelial cells and the bacteria present in the gut. And they also promote immune homeostasis throughout the gastrointestinal tract.

Pleiotropic mechanism

SCFAs play a key role in the maintenance of gut health. Derived from microbial digestion of dietary fibre, they act as the principal energy source for the epithelial colon cells that form the intestinal barrier, including colonocytes, which absorb nutrients, moisture, and electrolytes, mucus-producing goblet cells, hormone-secreting enteroendocrine cells, and the stem cells that constantly replenish the gut lining, which turns over every five to seven days. They also act as signalling molecules, through free fatty acid receptors (FFARs), to suppress innate and adaptive immune responses and broadly influence gut physiology³⁰. Their effects are not limited to the gut. They also counter the neuroinflammatory signals induced by toll-like receptor 2 (TLR2) and TLR4 activation in the CNS. These pattern recognition receptors are highly expressed on microglia, the principle immune effector cells within the CNS, and astrocytes, which play multiple roles in maintaining brain homeostasis. TLR activation, which is triggered by their binding to pathogen-associated molecular patterns or damage-associated molecular patterns, switches on gene expression programs regulated by the transcription factors NF- κ B and AP-1, resulting in the production of the pro-inflammatory cytokines interleukin-1 beta (IL-1 β), IL-6 and tumor necrosis factor alpha (TNF- α)³¹⁻³². TLR activation also impairs the blood brain barrier by down-regulating the production of several proteins involved in forming tight junctions, thereby permitting immune cell infiltration of the brain and the entry of toxins such as bacterial lipopolysaccharide, which cause further disruption and inflammation. In microglia, SCFAs influence epigenetic regulation of gene expression by inhibiting histone deacetylase enzymes, leading to the down regulation of IL-1 β , IL-6 TNF- α , as well as up regulation of the anti-inflammatory cytokines transforming growth factor beta (TGF β) and IL-4. In astrocytes, SCFAs suppress activation through a distinct mechanism involving the tryptophan-aryl hydrocarbon receptor (AhR)-aquaporin-4 (AQP4) signaling axis³³.

Bacteroides and *Firmicutes* are the microbiome's main sources of SCFAs. Declines in their activity can lead to a reduction in SCFA availability and an increase in gut permeability, immune activation,

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and chronic systemic and CNS inflammation, as antigens and toxins normally held within the gut lumen spill out into the circulation and cross the blood-brain barrier. PLL001 alleviates this shortage directly. It comprises four active drug substances, the SCFAs acetate, butyrate, lactate and propionate, each conjugated to a poly-L-lysine (PLys) carrier to improve cellular uptake. Each PLys comprises a linear peptide containing 70 lysine residues – to which an average of seven individual SCFA molecules are attached by an amidation reaction. These enter cells by endocytosis, and the SCFA moieties are released within the cytoplasm following deamidation. They then undergo metabolism or exert their pharmacological effects, depending on the cellular context.

Given that it comprises four distinct active drug substances, PLL001 has multiple activities – in the CNS as well as in the gut. In a range of different animal models, butyrate, the main source of energy for epithelial colon cells, restores blood-brain barrier integrity, reverses neuroinflammation and blocks neuronal apoptosis³⁴⁻³⁵. In the widely used SOD1-G93A transgenic mouse model of ALS, butyrate supplementation delayed the onset of ALS symptoms, slowed disease progression, and improved survival³⁶. Propionate has antioxidants, neuroprotective and neurodegenerative effects³⁷, and it also helps to maintain the integrity of the blood-brain barrier³⁸. Lactate has both signaling and metabolic actions in the brain, with effects on energy availability, neuronal excitability and neuronal plasticity³⁹. Acetate attenuates neuroinflammation by regulating the activity of the CNS-resident innate immune cells microglia and astrocytes⁴⁰.

In silico modelling studies indicate that the hydrophilic PLys carrier ensures efficient systemic transport of the molecules, while the lipophilic SCFAs then facilitate attachment to and transport across cell membranes. A third-party study illustrates the efficiency of the approach – a similar poly-L-lysine carrier boosted cellular uptake of a conjugated enzyme 900-fold as compared with uptake of the naked enzyme⁴¹. A different form of polylysine, ϵ -polylysine, has a long history of human use as a food preservative in Japan and Korea, due to its anti-microbial properties. It has had GRAS (generally regarded as safe) designation in the U.S. for over two decades⁴². But the PLys backbone is not just a passive SCFA transporter. At physiological pH, it is positively charged, and it can bind negatively charged lipopolysaccharide and remove it from circulation⁴³.

Previous Clinical History

PLL's patent-protected PLys, the first poly-L-lysine to be produced under Good Manufacturing Practice (GMP) conditions, is structurally distinct from food-grade poly-L-lysine. It has a linear rather than a branched structure and, unlike ϵ -poly-L-lysine, it is not cytotoxic. A prior entity previously tested a different formulation of PLys in two trials, which recruited patients with rheumatoid arthritis and secondary progressive multiple sclerosis, respectively. The conjugated active pharmaceutical ingredients employed also differed from those of PLL001, but each study confirmed the safety and tolerability of the constructs, while also showing preliminary signs of clinical efficacy.

Building an evidence base

Human data on the therapeutic potential of SCFAs in ALS are lacking. Most of the evidence the field has generated to date is based on experiments conducted in animal models, particularly in the SOD1-G93A transgenic mouse model. No model captures all of the features of the human disease, however, and the biological relevance of animal data may often be limited to a small minority of patients with the associated genetic mutation. PLL Therapeutics has, therefore, prioritized clinical studies in ALS patients, in order to build more substantial evidence base relevant to the broad patient population. Before opening that study, however, it commissioned over a dozen *in vitro* cytotoxicity and *in vivo* toxicology studies to establish the preclinical safety of PLL001. The *in vitro* studies included standard transepithelial electrical resistance (TEER) measurements of gut barrier integrity, which showed that PLL001 had a positive effect on this physiological parameter. In a SOD1 mouse model, PLL001 demonstrated improvements in mobility and in behaviour. These effects, moreover, were positively correlated with greater abundance of *Lachnospiraceae* bacterial species, which are important butyrate producers, and negatively correlated with the presence of *Desulfovibrionaceae* and *Prevotellaceae* bacteria. These findings constitute preliminary *vivo* proof of concept.

Since then, PLL Therapeutics has initiated a multicentre phase I/II randomized, double-blinded, placebo-controlled clinical trial of PLL001 in patients in Australia and New Zealand (NCT06513546). It has already completed part 1 of the trial, a single-ascending dose study, in which twelve patients were assigned to one of three dose cohorts. In each cohort, participants were randomized, in a 3:1, ratio to receive a single subcutaneous dose of PLL011 or placebo. The therapy was safe and well tolerated – no serious adverse events and no treatment-related adverse events resulting in study discontinuation were reported.

Part 2 of the study will recruit 140 patients, who will receive a daily injection of either PLL011 or placebo over a six-month treatment period. The primary efficacy endpoint comprises a change from baseline in participants' Amyotrophic Lateral Sclerosis Functional Rating Scale – Revised (ALSFRSR) scores, which will be measured every 2 months during Part 2 as well as blood, stool, urine and, for some of them, cerebrospinal fluid to obtain a comprehensive picture of how the biomarker changes over time and of the therapy's efficacy. The effect of PLL011 on survival will also be evaluated. In addition, the effect of the therapy on patients' quality of life, respiratory function, upper and lower limb muscle strength will also be followed, and patients and physicians will also complete questionnaires designed to capture their subjective assessments of PLL011's effects. Patients' microbiota and serum antibody profiles will also be assessed during the treatment period. At the conclusion of Part 2, all study participants will be invited to enrol in Part 3 of the trial, an open-label extension study, which will involve an additional six months of therapy.

Diagnosis and Monitoring

Management of ALS is complicated by the protracted nature of the diagnostic process. Because of a lack of reliable, disease-specific biomarkers, it typically takes a year or more to arrive at a definitive diagnosis⁴⁴. In parallel with our therapeutic development efforts, PLL Therapeutics is developing a

companion diagnostic that could substantially reduce the time required to diagnose ALS accurately and provide ongoing insights into the progression of the condition in each patient.

It is based on the same underlying principle that is guiding our drug development efforts – that dysbiosis is a significant driver of ALS. As well as offering opportunities for therapeutic intervention, perturbation of the gut microbiome and the consequent impairment of intestinal barrier integrity also opens up diagnostic possibilities, as a ‘leaky’ intestine introduces bacterial components and metabolites to circulation. Outside the gut’s tolerogenic milieu⁴⁵ this results in both innate and adaptive immune responses.

Our investigational diagnostic captures the resulting crosstalk between the gut microbiome and the systemic immune system. It comprises a panel of antibodies that detects specific antibody isotypes, which recognize small-molecule haptens – bound to endogenous proteins – that are associated with the ALS pathology. Unpublished data from screens against sera from two unrelated patient cohorts indicate that several of these candidate biomarkers can distinguish with a high degree of accuracy between female patients with bulbar-onset ALS and female patients with either Alzheimer’s disease (AD) or Parkinson’s disease (PD). In this particular patient segment, the area under the receiver operating characteristic (ROC) curve – a statistical method for analyzing the relationship between true and false positives – was 0.9 (a score of 1 implies a perfect test). The same metric was ≥ 0.85 for the same biomarkers in distinguishing female patients with spinal onset ALS from female patients with either AD or PD and in distinguishing male patients with spinal or bulbar onset ALS from male patients with either AD or PD. Further refinement of the panel is now underway in order to improve its utility.

Conclusion

There is an urgent need for more timely diagnosis of ALS and for therapies that can slow or even halt the condition’s devastating loss of neuronal function and muscle control. The two agendas are interdependent. Achieving progress in drug development will remain challenging under the current diagnostic paradigm, as it precludes the possibility of early intervention to arrest the overwhelming pathological cascade that characterises disease progression. And it is essential that newly diagnosed patients have access to a wider range of therapeutic options than is currently available. That is why PLL Therapeutics is committed to a dual strategy of developing novel therapy and a companion diagnostic. Because they are based on the same underlying biological principle, they have the potential to work in tandem: the diagnostic may select for those patients most likely to benefit from PLL001, although this has to be demonstrated prospectively.

Elsewhere, genetics studies have galvanized research into the pathophysiology of ALS, opening up new drug targets and new therapeutic possibilities. Not all these insights will be clinically actionable, however, and those that are may not necessarily apply to the majority of patients with sporadic disease. In any case, PLL001, if it can be shown to be safe and effective, has the potential to

become an ideal combination partner for any targeted therapy that may emerge over the next decade. And PLL's diagnostic could revolutionize the diagnosis and management of this intractable condition.

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