Cathleen Lutz (Jackson Laboratory) described ALS as a "complex disease and consists of a group of conditions unified by a common theme, resulting in motor neuron degeneration and in many cases, convergent TDP-43 pathology. Importantly, the future for ALS therapeutics is no longer relegated to the concept of a single drug as a defining treatment, but rather opens the door for therapeutics that are stratified based on individual genes, pathways, or mechanisms." A follow up thought is that the important issues for testing the efficacy of treatments are knowledge of the mechanisms or pathways one wants to impact, as well as a model to test the treatment prior to testing safety in people.

There are over two dozen distinct gene mutations that can be found in patients with no known family history of ALS. And, if it wasn't complicated enough, patients may harbor more than one ALS-relevant mutation. The genetic revolution in ALS allows researchers to link a gene to a pathology, and logically, a therapy.

After developing a drug, one must identify the right model to test the effect on ALS pathology. There is increasing interest in cell models, however no model, cell or animal, has predictability when moved to human trials. Search Jackson Laboratory's web site for "mouse and ALS" and you will return *one hundred and five* results. What one is looking for is an "end-stage phenotype" within the lifespan of a mouse. And then consider how the result will translate to the human. We wrestled with the model selection dilemma and settled with sage advice from two colleagues: use two models!

One might want to consider *in vitro* cells before jumping into mice. Some researchers use iPSC's, induced pluripotent stem cells to have a motor neuron phenotype (iMN) to recapitulate neurodegenerative ALS processes. That seems like a direct approach and a way to test the effects of drugs.

One fascinating experiment[1] muddies these waters…iMN cells were created from a C9orf72 patient and a healthy control. The cells were cultured and examined for survival. When the cells were cultured in a basic medium supplemented with neurotrophic factors the control and the C9orf72 patient iMN's survived equally well. Because human C9orf72 patients have elevated glutamate levels in their cerebrospinal fluid[2] *and* glutamate excitotoxicity is associated with ALS, the scientists stimulated the patient and healthy iMN cultures with a high-glutamate pulse. Glutamate pulse initiated a robust degenerative response in the patient cells, but not the control iMN's. This effect was repeated in multiple patient and control cell lines. The environment plays a significant part in the cell's wellbeing. What would one expect the results to be, taking cells from a patient and creating iMN stem cells, expanding them in a culture tube with media and neurotrophic factors and selecting the morphologically healthy cells…and reintroducing them into the patient? Could one predict that the iMN cells would react to the elevated glutamate in the CNS, if the patient had glutamate excitotoxicity.

[1] Justin Ichidda Nature Medicine 2019

[2] Possibly triggered by DPR-mediated aberrant splicing of the astrocytic excitatory amino acid transporter 2 EAAT2

Treatment with glutamate receptor antagonists during glutamate administration prevented patient iMN degeneration in the flask. Would treating a patient given his own iMN cells and glutamate receptor antagonists have a better outcome?

Back to animal models of ALS. In 1993, Cu/Zn superoxide dismutase 1 (SOD1) was identified as the first causative gene in ALS. The transgenic mouse, SOD 1-G93A, was the first true ALS model to emerge and remains the most extensively used mouse line in the study of ALS. This transgenic mouse express high levels of altered SOD1 under the control of the endogenous SOD1 promoter. These mice show motor neuron loss, axonal denervation, progressive paralysis, reduced lifespan, and protein aggregation. This model contributed to understanding glutamate toxicity, astroglia involvement, axonal transport defects, mitochondrial abnormalities, protein misfolding and other cellular events related to ALS pathology.

The SOD 1-G93A mouse rapidly develops motor neuron degeneration resulting in paralysis and death by 120-150 days. There are things to keep in mind, including the genetic background of the mice and the number of copies of SOD1 induced in the transgenic mouse. We are cautioned that randomization and statistical powering of the experiments are critical to interpreting the data, that said Riluzole and Edavarone used this mouse for preclinical data. Predictably, the antisense oligonucleotide that targets SOD1 makes these mice live the longest.

The TAR DNA-binding protein 43 (TDP-43) was identified as the major pathological protein found in motor neuron inclusions in sALS. This protein is found in the nucleus of healthy motor neurons and is depleted with cytosolic aggregations in most forms of ALS. It may be that loss of the function of TDP-43 in the nucleus or toxic accumulations in the cytoplasm, or both, result in ALS pathology. Possibly, TDP-43 nuclear clearing is an end-stage cellular event, resulting from multitudes of other biochemical changes in ALS patients. The end game is that mice, engineered to aggregate cytosolic TDP-43, called transgenic mice, may be used to generate neurological phenotypes. Some of the TDP-43 transgenic animals have a decreased grip, apparent in a rotarod test. It is interesting that mutant TDP-43 protein in a cell may recruit wildtype TDP-43 to induce disease.

And then things get complicated in this mouse model. A genetically created mouse containing the defect, usually a breeder mouse (male) is crossed with a wild type female to make a transgenic animal. Not all wild type mice are the same. When crossed with some wild type mice (background), the mice die quickly. On the C57BL/6 background, transgenic mice exhibit a progressive neurodegeneration in the myenteric plexus of the colon, reducing intestinal motility that ultimately results in a bowel ileus and death. A high fat, gel- based diet can extend the survival of the mice. An accommodating diet can allow the development of motor neuron disease, but what eventually kills these mice is the bowel dysfunction.

There are different transgenic TDP-43 models. In one model, fragments from human genomic BAC containing libraries took advantage of the endogenous TDP-43 promoter and generated the transgenic A3215T mouse, properly called the TDP-43Prp-TDP43^{A315T} . Three times elevation of TDP-43 was expressed in the spinal cord compared to the wildtype mouse in this model. By 42 weeks, mice develop impaired rotarod performance and age-related neuroinflammation. The TDP-43Prp-TDP43^{A315T} transgenic mice express a progressive and fatal neurodegenerative disease reminiscent of both ALS and frontotemporal lobar degeneration with ubiquitin aggregates.

It's complicated, when taken together, and in the final analysis of choosing models, one must consider co-morbidities in these mice. For example, in the TDP-43 transgenic mouse, is one treating the gut-associated neurodegeneration in the myenteric plexus that may result in some signs that are centrally acting? In choosing a model, one must jump in and take a shot.

It is no secret that to combat the many pathological processes in symptomatic ALS patients it will take an army of compounds. Our goal is a single compound with multiple effects in the body, previously demonstrated as safe in people, and available. Armed with two possible treatments, we took our chance with model selection. We chose the SOD 1-G93A *and* TDP-43Prp-TDP43^{A315T} models. Our intent was to test levamisole HCl, formerly marketed as Ergamisol and thymosan, formerly marketed as Zadaxin. We already know these products are absorbed and metabolically active in mice and people. If biologic activity was unknown, a pK study would be required. Experiments could fail if the drug never reaches the intended target or if the human drug requires an enzyme that isn't conserved in the mouse. Mice don't have all human enzymes, although specific *humouses*[3] could be generated.

We settled on testing levamisole HCl and thymosan in both transgenic models. Thymosan is a molecule that is metabolized to provide the active 5 amino acids that bind thymopoietin receptors. We call it T5. Levamisole HCl is an imidothiazole with multiple actions and mimics some, but not all, of those initiated by T5.

Thymopoietin is an immune modulator that has some factors that cross the blood brain barrier. The molecules have varied biological properties that may act individually, sequentially, or in concert to influence the development of T cell subsets. Thymosan has actions on the acetyl choline receptor (AchR) and interferes with αBgt binding sites, decreases neuromuscular transmission; blocks neuromuscular transmission by accelerating desensitization of the nicotinic AchR (cholinergic-induced inactivation of nicotinic receptors); may act as an endogenous ligand for nicotinic AchR desensitization; induces myelin repair, induces neuronal protection; initiates, via Tβ4, neuroprotection by reduced apoptosis of neural progenitor cells that are subjected to oxygen glucose deprivation. Thymopoietin is an anti-inflammatory molecule; suppresses NF-kB activation; inhibits microglial activation by reducing secretion of inflammatory mediators; increases production of new oligodendrocytes generated from oligodendrocyte progenitor cells and assists their differentiation into mature myelinating oligodendrocytes; induces regulatory T cells and inhibits activated B cell differentiation and more…look to Singh 1998 for a review.

Peripherally, levamisole HCl is active on levamisole sensitive nicotinic AchR, it is an agonist, stimulating muscle contraction with a primary action on parasitic (Ca^{++}) ion channels. In mammals, levamisole reportedly isn't an agonist of cholinergic transmission, the nematode receptors are homologous to ganglionic AchR receptors in mammals. Levamisole can inhibit or facilitate ganglionic Ach receptors; it has strong immunomodulatory activity acting only on activated cells; it has anti-cancer activity; it acts as an antidepressant because it is a serotonin uptake inhibitor; it has action on dendritic cells; enhances cytokines IL12, p40, IL 10, IFNϒ; it is an antioxidant in redox systems glutathione, superoxide dismutase, catalase, and possibly against some glutathione related enzymes; it inhibits TNFα and IL6; its apoptotic action inhibits the cell cycle and increases endothelial cell adhesion. Levamisole is an anti-angiogenic; it

[3] A humouse is our term for a mouse with human genes, like the transgenic A3215T mouse that contains the human endogenous TDP 43 promoter

blocks succinate dehydrogenase[4] that converts succinate to fumarate; increases CD80, CD86, CD83, CD40, MHC class II, and HLA-DR.

Levamisole is a small molecule and crosses the blood brain barrier where it may have 4 targets in the nervous system. Inhibition of TNAP[5] may protect the blood brain barrier. Levamisole has multiple consequences resulting from interference with numerous pyridoxal phosphate-dependent enzymes, GABA synthesizing enzymes, or modifications of the extracellular concentrations of ATP and adenosine. It modulates responses mediated by ganglionic AchR, blocks nicotine uptake mechanisms, and blocks voltage-dependent sodium channels. It may inhibit acetylcholinesterase and monoamine oxidase, and blocks opiate receptors. Levamisole reduces neuronal response amplitudes and decreases axonal conduction by blocking voltage-dependent sodium channels. The effects of blocking voltage-dependent sodium channels may be suppression of axonal growth, myelination, and synaptic plasticity. Levamisole affects noradrenergic transmission in the peripheral nervous system by inhibiting noradrenergic reuptake.

We can't evaluate all the effects of these compounds in the transgenic mice and are limiting our observations to standard analysis. The data is provided in Issue 7 DATA November 2020.

[4] Oxaloacetate inhibits succinate dehydrogenase; reverses NADH production; reduces ubiquinone in the mitochondria (ubiquinone is an antioxidant)

[5] TNAP is tissue non-specific alkaline phosphatase that has a protective role at endothelial barriers and show increased survival and decreased severity scores compared to controls in mouse studies.