23rd Annual International Symposium on ALS/MND, Chicago, Illinois

(http://www.alzforum.org/new/detail.asp?id=3340)

By Amber Dance

ALS Database Opens for Business

Data miners: Start your search engines. The largest collection of data from clinical trials in amyotrophic lateral sclerosis (ALS)—representing more than 8,500 individuals—is now live. Prize4Life, a Cambridge, Massachusetts, nonprofit working to speed up drug discovery for motor neuron disease (MND), announced the launch of the Pooled Resource Open-Access ALS Clinical Trials (PRO-ACT) database on 5 December 2012 at the International Symposium on ALS/MND in Chicago, Illinois. Prize4Life, which funds this reporter's position at Alzforum, developed the database in conjunction with the Neurological Clinical Research Institute at Massachusetts General Hospital in Boston.

The organizers of 18 Phase 2 or 3 trials for ALS offered up data. Contributors include Sanofi of Paris, France; Regeneron, headquartered in Tarrytown, New York; Teva Pharmaceuticals in Petah Tikva, Israel; and Novartis, based in Basel, Switzerland. Most of the longitudinal data, from both placebo and treatment participants, spans about a year, said Melanie Leitner of Prize4Life. However, it's been thoroughly scrubbed of identifying information, down to the particular treatment provided, to protect the anonymity of the participants and proprietary interests. The database includes several elements such as medical history and lab results. Over time, the organizers plan to add other features, for example, adverse events and the approximate date of the trial, Leitner said.

What veins of informational ore might data miners tap? With the right strategies, researchers might discover factors that influence disease progression, said Leitner. She anticipates researchers may hunt for subsets of people who responded to treatment, even when a trial failed overall. "You cannot do that unless you have a huge number of data points," she said. Should researchers find evidence of an effective treatment, they could contact Prize4Life to find out which drugs subjects received, Leitner said.

An abbreviated PRO-ACT database has already yielded a few gems. Prize4Life offered researchers access to about a quarter of the information represented in the final database and asked them to sift out predictors of a slow or rapid disease progression. The organization gave out $50,000 in November for algorithms that explained about half of the variability among people with ALS (see ARF related news story). Understanding the progression of ALS better can help researchers streamline clinical trials, reducing subject numbers and costs.

ALS Protein SOD1 Painted as Disease Template

More than 900 researchers and clinicians gathered in Chicago, Illinois, at the 23rd annual International Symposium on ALS/MND, held 5-7 December 2012.

Genetics was, as ever, a hot topic. As outlined in this multipart series, enthusiasm ran high over the discovery of C9ORF72 expansions as a cause of disease, and the potential of turning this knowledge into treatments. Recent clinical trial data also topped the agenda. Alas, most drugs failed efficacy, and researchers are now hoping that newer therapies in development may fare better. A new database of past trial results, announced at the meeting, may help speed progress toward therapy (see ARF related news story). Basic protein and cell biology were not neglected either. **Neil Cashman** of the University of British Columbia in Vancouver related a tale of how amyotrophic lateral sclerosis pathology might travel from one neuron to the next via templated protein misfolding.

The spread of protein pathology from molecule to molecule is popping up across the spectrum of neurodegenerative diseases. Both tau and β-amyloid, for example, misfold and act as templates to induce further conformational chaos (reviewed in Walker et al., 2012). Cashman argued that the ALS gene SOD1 produces a protein that can template misfolding even in its wild-type form. He described how SOD1 can transmit pathological misfolding from cell to cell, via liquid media. And early data indicate that the protein uses two modes of travel: as a free protein aggregate or as a passenger on exosomes.

Cashman believes SOD1 behaves like a prion. He told Alzforum that to prove a protein fits the prion definition, it must pass two tests. First, the infectious nature of the particle should be passed from molecule to molecule. He and Les Grad—formerly of Cashman's lab and now working at PrioNet Canada, a research network based in Vancouver—showed intermolecular transfer of SOD1 misfolding in 2011 (see ARF related news story on Grad et al., 2011). Second, the misfolded protein must pass from cell to cell and region to region. ALS pathology does seem to start in one anatomical location and spread throughout the nervous system (Ravits and La Spada, 2009), and in Chicago, Cashman reported that SOD1 indeed transfers from cell to cell.

A bona fide prion must be transmissible between individuals, Cashman said. He emphasized that no one is suggesting that ALS or other non-prion neurodegenerative conditions are contagious. In fact, many researchers prefer the term "templated protein misfolding" to distinguish the propagation of amyloidogenic proteins from that of truly infectious prions that cause Creutzfeldt-Jakob disease, bovine spongiform encephalitis, and scrapie (Hardy and Revesz, 2012).

Grad and colleagues addressed the cell-to-cell requirement with a media transfer experiment. They transfected HEK293FT human embryonic kidney cells with mutant SOD1, then transferred the conditioned media to cultures that expressed only the native, wild-type SOD1. That wild-type protein promptly converted to the misfolded, aggregated form. The researchers used misfold-specific antibodies to detect the conversion. The team was able to serially transmit the misfolded conformation from culture to culture, via the media, indefinitely, Cashman said.

One possible explanation for the transmission could be that the mutant SOD1 itself hopped from culture to culture, leaving wild-type protein untouched. That seems unlikely since the mutant protein would be diluted over time, but the

researchers wanted to definitively prove that the wild-type protein transmitted the pathological shape. By silencing SOD1 expression in recipient cells with RNA interference, they showed that wild-type SOD1 was required as raw material for more misfolding.

The team still had to prove that malformed SOD1 itself, and not some other factor in the conditioned media, was responsible for misfolding in the recipient cells. They did so by incubating the media with antibodies to SOD1 to pull out the protein, which abolished the transmission of misfolding. "The transmissible particle must contain misfolded SOD1," Cashman told Alzforum. "There is really no other explanation than a prion-like mechanism."

Researchers have focused on ALS genes and mutations for a long time, but Cashman's presentation suggested it is now time to pay close attention to protein-based disease mechanisms, even those concerning wild-type proteins, commented Hande Ozdinler of the Northwestern University Feinberg School of Medicine in Chicago, who was not involved in the study.

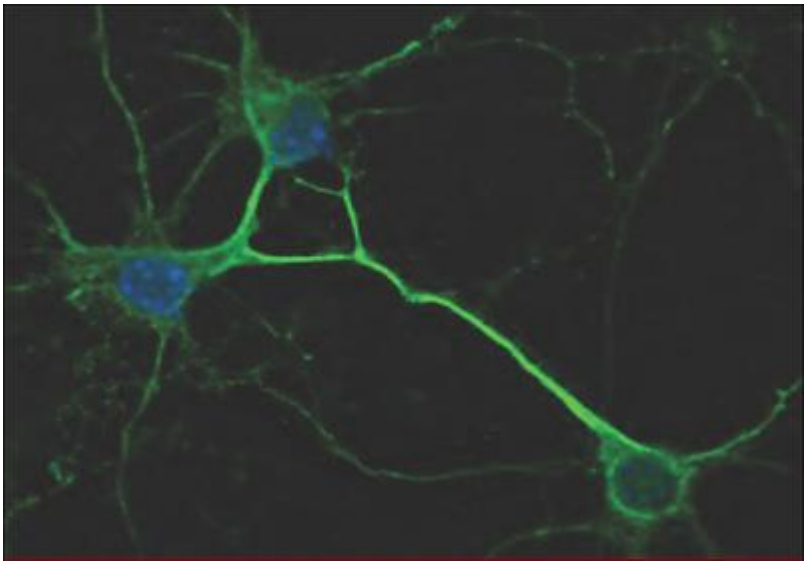
SOD1 Carriers

How might the snarled SOD1 travel from one cell to the next? The team investigated this question in the mouse motor neuron NSC-34 cell line. Other work suggests that recipient cells can swallow mSOD1 aggregates via macropinocytosis, in which the cell engulfs some of the liquid around it (Münch et al., 2011). The Cashman group examined how donor cells might release those aggregates, hypothesizing that the misfolded SOD1 inclusions escape dying cells. Collaborator Justin Yerbury of the University of Wollongong, Australia, observed via light microscopy that transiently transfected neurons, carrying either wild-type or mutant SOD1, released micron-sized aggregates as they expired.

While aggregates released by a dying cell could certainly infect neighbors, Cashman suspects that is not the main mechanism for the misfolding propagation, because ALS pathology seems to spread faster than neurons die. He looked for a mechanism that would explain transmission from living donor cells. Because prions have been shown to propagate via exosomes (Février et al., 2005), Cashman collaborated with exosome expert Bradley Turner of the University of Melbourne, Australia, to see if SOD1 spreads the same way. On electron micrographs, Turner observed that misfolded SOD1 colocalized with exosome markers on small extracellular vesicles.

Cashman suspects misfolded SOD1 factors in more than familial ALS cases caused by SOD1 mutations. That is because the wild-type protein, too, can infect neighboring cells if it adopts the snarled conformation. In fact, Cashman and other researchers have observed misfolded, wild-type SOD1 aggregates in sporadic ALS (see ARF related news story on Bosco et al., 2010). What would cause normal SOD1 to fold in the absence of SOD1 mutations? In Chicago, Cashman presented evidence that the pathological mislocalization of ALS-linked proteins TDP-43 and FUS tips normal SOD1 toward the misfolded conformation (see photo below). SOD1 aggregates showed up in spinal cord tissue from people who died of ALS caused by mutant FUS or the C9ORF72 expansion, or from sporadic ALS linked to TDP-43 inclusions. Transfecting human neuroblastoma SH-SY5Y

cultures with mutant FUS, or mutant or wild-type TDP-43, has also produced SOD1 misfolding (Pokrishevsky et al., 2012). The work suggests that TDP-43, FUS, SOD1, and other mutations do not fall into distinct boxes, but are all "players in the same game," Ozdinler said. The Cashman group has not worked out the pathway between FUS/TDP-43 and SOD1 misfolding, but it is likely indirect, because aggregated SOD1 did not colocalize with either.



The presence of mutant TDP-43 in mouse primary spinal cord neurons causes wild-type human SOD1 (green) to misfold. *Image courtesy of Edward Pokrishevsky, Neil Cashman, University of British Columbia*

"I think that SOD1 is the cause of most, if not all, types of ALS," Cashman proposed. "The propagated misfolding of SOD1 is the fundamental mechanism." If his model is correct, then blocking intercellular transfer could halt the spread of neurodegeneration. Biogen Idec of Cambridge, Massachusetts, plans therapeutic development of misfolded SOD1 antibodies generated in the labs of Cashman and Jean-Pierre Julien of Laval University in Québec City, Canada.

ALS Clinical Trials: New Hope After Phase 3 Setbacks

Researchers, therapists, patients, and caretakers shared a common goal of finding a cure. "We look forward to hearing the word 'survivor,'" said **Wendy Abrams**, executive director of the Les Turner ALS Foundation in Skokie, Illinois, which hosted the symposium. Toward that end, early results from Phase 1 and 2 trials for several treatments offered a glimmer of hope, but more immediately, two large studies disappointed (see below).

Researchers also puzzled over one bit of good news for patients, namely that placebo controls are performing better than expected in trials. In the unsuccessful Phase 2/3 study for olesoxime (see ARF related news story), presented by **Timothée Lenglet** of Pitié-Salpêtrière Hospital in Paris, France, placebo survival over 18 months approached 70 percent—compared to 50 percent over a median of 18 months in a 1996 study (Lacomblez et al., 1996). In another trial, one-tenth of untreated controls showed no change on the ALS Functional Rating Scale

(ALSFRS), a common trial outcome measure, over six months. Lenglet suggested this might be because their symptoms are being better managed nowadays. In addition, simple alterations to diet or activities might have a noticeable impact over time, suggested **Angela Genge** of the Montréal Neurological Institute and Hospital, Canada, in an interview with Alzforum. The findings echo a current trend in Alzheimer's trials, where placebo groups decline more gradually than has been seen in the past (see ARF related news story).

The slower decline in patients is not just academic. Modern clinical care and increased survival may underlie the recent failures of trials for ceftriaxone and olesoxime, suggested Benjamin Brooks of the University of North Carolina School of Medicine, Charlotte, in an e-mail to Alzforum. Careful analysis may show that the disease trajectory researchers predict at the start of a trial is too steep, he suggested. "If that is true, then we are at a point where longer trial times are necessary, similar to the early days of breast cancer treatment in the past," Brooks wrote.

In addition, changing placebo numbers could affect the use of historical controls in trials. That practice was approved for the first time by the Food and Drug Administration in 2011 to amplify the power of an ALS trial for sodium chlorite, a mineral formulation also known as NP001. **Robert Miller** of the Forbes Norris MDA/ALS Research Center in San Francisco, California, who presented the trial results at the meeting (see below), hoped the use of historical controls would make recruitment easier. Since amyotrophic lateral sclerosis progresses quickly, some patients are reluctant to join a trial when they risk being assigned a placebo. Coupled with the rarity of ALS, this can make it difficult to fill a trial. By tapping old records for control data, researchers performing Phase 1 or 2 studies should be able to reduce the control populations they need, the theory goes, boosting participants' chances of receiving active drug and improving recruitment (see ARF related news story).

Given the lengthening survival of control subjects, Genge considers use of historical controls a mistake. Historical data may not reflect the current state of ALS progression, Genge said. Miller agreed in an e-mail to Alzforum that people with ALS are living longer, but countered that rates of decline in the ALSFRS have remained steady (Miller et al., 2011). He noted the historical approach has received support from the FDA as well as other scientists (Donofrio and Bedlack, 2011).

Large Phase 3 Studies Fall Flat

Long-term hopeful signs notwithstanding, ALS researchers in Chicago had to deal with two major setbacks immediately before them. They discussed disappointing results from two recent Phase 3 studies. In addition to the olesoxime report, **Merit Cudkowicz** described final results of a Phase 3 study of ceftriaxone. This drug upregulates the astrocyte excitatory amino acid transporter 2, which recycles synaptic glutamate to counteract excitotoxicity in ALS (Rao and Weiss, 2004). This trial used an adaptive design, meant to move seamlessly from Phase 1 through 3 as long as the data indicated it should continue (see ARF related news story). The study had reached Phase 3, with 513 subjects enrolled, when the organizers at the Northeast ALS Consortium in Charlestown, Massachusetts,

decided to call it quits because it did not look as if the drug was working. There was no improvement in survival, ALSFRS score, or lung capacity, Cudkowicz reported.

Since the ALS/MND meeting, Biogen Idec of Weston, Massachusetts, announced on 3 January 2013 that dexpramipexole, a mitochondrial modulator similar to olesoxime, also failed to meet its endpoints in a Phase 3 study. The company released top-line results showing that none of the outcome measures—primary and secondary endpoints and subgroup analyses—of the 943-patient trial in 11 countries showed efficacy (see press release). The news was particularly disappointing because the trial was well designed and Phase 2 results seemed promising, said **Steven Perrin** of the ALS Therapy Development Institute in Cambridge, Massachusetts, who was not involved in the study. Phase 2 results indicated that dexpramipexole slowed decline on the ALSFRS by 31 percent (see ARF related news story and Cudkowicz et al., 2011). Perrin noted that the ceftriaxone trial also had encouraging Phase 2 data. The lesson here, he said, is that Phase 2 studies should enroll more than 100 patients in order to give clear results that can support a go or no-go decision on a Phase 3 trial.

Phase 2 Trials Offer Hope for Treating Disease, Symptoms

NP001, as Miller described, presented encouraging Phase 2 results that reached statistical significance once he added the historical data. The rationale for using this drug is based on the link between ALS and neuroinflammation (see ARF related news story; ARF news story; ARF news story), which suggests that dampening the immune response could slow disease. NP001 has been reported to reorient toxic inflammatory macrophages that invade the central nervous system into normal, protective ones (Zhang et al., 2005). The 12-month, multisite study of 136 participants with early-stage ALS, sponsored by Neuraltus Pharmaceuticals, Inc., of Palo Alto, California, wound down two months ago. In Chicago, Miller shared data from the first six months.

In a double-blind trial, patients were randomly assigned to groups that received placebo, or 1 or 2 milligrams of NP001 per kilogram of body weight. They received intravenous infusions in short series: five days in a row for initial treatment, followed by three days in a row once a month for five months thereafter. This treatment was safe and well tolerated, said Miller, though the drug caused pain at the infusion site. This was a concern, as it may have unblinded some participants.

The researchers tracked rate of decline on the ALSFRS compared to a person's rate of decline before starting treatment. Those on the higher dose of NP001 deteriorated 13 percent more slowly than did controls. This was not statistically significant, Miller said. Looking at the data a different way, and adding in historical controls, Miller found that the drug crossed the significance threshold for some patients. While 27 and 19 percent of those on the high- and low-dose treatment, respectively, remained stable during the trial, 11 percent of those in the placebo group retained baseline function.

Neuraltus plans to start a Phase 3 study this year. Despite the experimental nature of NP001, some people with ALS have started taking sodium chlorite outside the

trial (see Wall Street Journal article). Physicians do not recommend this. Miller noted that swallowing the medication would not work; it must be injected to be absorbed properly.

Scientists are considering other immunomodulators besides NP001. In Chicago, Perrin discussed his hopes for Gilenya®, the trade name for fingolimod. Made by Novartis, this fungal derivative locks up T cells in the lymph nodes and prevents them from circulating. Already approved for multiple sclerosis, Gilenya increases survival in ALS mice by a week—a meaningful amount given the severity of the model, Perrin said. ALS-TDI has been planning a Phase 2a safety trial since early 2012 (see ARF related news story). Filing paperwork with the FDA took longer than planned, Perrin told Alzforum, but he expects to hear back from the FDA later in January and to enroll patients before April. **Neil Cashman** of the University of British Columbia in Vancouver, who attended the Chicago meeting, told Alzforum he was excited about the Gilenya studies because the role of the immune system in ALS has spent a long time on the back burner. "Finally, this orphan hypothesis is getting some play," he said.

Although the immune-system drugs aim to extend survival, that is not the only outcome people with ALS are seeking, noted Genge in a satellite session on clinical trial design. They also want to maintain muscle strength and function. **Jeremy Shefner** of the State University of New York Upstate Medical University in Syracuse reported on a drug aiming to amp up muscle power. Tirasemtiv has been through three Phase 2a studies so far, each asking a specific question as researchers fine-tuned the dosage. Developed by Cytokinetics, Inc., of South San Francisco, tirasemtiv, aka CK-2017357, was designed to counter the effects of muscle atrophy that occurs as input from damaged motor neurons wanes. It works by tightening the interaction between troponin and its inhibitor, calcium; the ion keeps the contraction-blocking troponin away from actin and myosin fibers (Russell et al., 2012).

Three Phase 2a studies, examining a single dose, daily doses, or escalating doses, indicated that tirasemtiv enhanced muscle strength and endurance. Although it caused dizziness when people started taking the medication, that went away with time and the medicine was generally well tolerated (see ARF related news story and Shefner et al., 2012). At the symposium, Shefner focused on the second trial. In addition to testing three doses of tirasemtiv, that trial examined interactions between the muscle enhancer and riluzole, the only FDA-approved drug for ALS. Riluzole is metabolized by cytochrome P450 1A2, but tirasemtiv inhibits that breakdown pathway, leaving researchers concerned that the new treatment would raise concentrations of riluzole.

Shefner described that, of the 49 people with ALS who participated in the Phase 2a study, 24 did not use riluzole while the others took 50 milligrams daily—half the normal dose. The goal was to achieve the appropriate plasma level of riluzole despite blocking its metabolism. Each subject group was then split into those who received tirasemtiv or a placebo for two weeks. Patients on the drug received escalating doses, starting at 250 mg daily and going up to 500 mg a day. All doubled riluzole levels in plasma. Shefner concluded that their approach, halving

the riluzole, worked to maintain the desired concentration for people taking tirasemtiv.

Shefner told Alzforum in an e-mail that tirasemtiv development progresses apace. A multicenter Phase 2b trial now underway will enroll approximately 400 people, half to receive placebo and half to receive tirasemtiv for three months. People who are on riluzole at the start of the trial will take the reduced dose during the study.

Phase 1 Candidates Prove Safe

On separate drugs, two Phase 1 studies offered safety results. **Tim Miller** of Washington University in St. Louis, Missouri, reported work from Isis Pharmaceuticals, Inc., of Carlsbad, California. Isis is developing an antisense oligonucleotide therapy to dampen expression of the mutant superoxide dismutase 1, which is responsible for 13 percent of familial ALS cases, Miller said.

A multisite trial of ISIS 333611 took place in four stages. Each included two people on placebo, plus six receiving the oligonucleotide via intrathecal infusion. The researchers started with a dose of 0.15 milligrams and raised it with each stage, to 0.5, 1.5, and 3 mg. The infusion was safe and tolerable; in fact, some subjects returned to complete two or even three stages of the study. Most adverse events were related to the intrathecal delivery; the worst were headaches from leaking cerebrospinal fluid, suffered by one-third of participants. Researchers are now redesigning the oligonucleotide to make it more potent and safer, Miller said.

Jonathan Glass of Emory University in Atlanta described the latest findings from a stem cell trial. Glass reported on three new patients who have been treated, adding to the 12 previously described (Riley et al., 2012; see also ARF related news story). Surgeons injected spinal cords of the recipients with half a million or more neural stem cells, derived from fetal spinal cord by Neuralstem Inc., in Rockville, Maryland. Previously, the team injected cells into the lumbar region; in Chicago, Glass reported on some of the most recent patients who received cervical injections.

In this Phase 1 trial, the treatment appeared safe. Since the surgeries began in 2010, six patients have died—five from ALS and one from heart problems. The deaths were unrelated to the treatment, Glass said. Autopsies revealed that the stem cell injection sites had healed, and donor DNA was found in the autopsied spinal cords, Glass reported.

Most patients in Neuralstem's trial continued their downward trajectory, but there was one exception. Glass reported on a patient "who needed a walker and a cane before surgery, and was dancing with his wife at a wedding after it" (see article in Crain's Detroit Business). The researchers do not understand why this man improved so much, and his result is a topic of intense discussion, Glass said. The team is awaiting FDA approval to double the number of stem cells implanted. Genge expressed doubt about stem cell therapies, suggesting that any benefit might have resulted from the immunosuppressant drugs the participants received, that is, their ability to quell neuroinflammatory pathology.

For its part, preclinical research offers reason to hope neural stem cell transplants could eventually work (see Teng et al., 2012). Researchers led by joint first authors Yang Teng of Brigham and Women's Hospital, and Susanna Benn of Massachusetts General Hospital, both in Boston, meta-analyzed data from 11 independent, double-blinded experiments. In each, researchers implanted neural stem cells from the same cell bank into mice expressing mutant human superoxide dismutase 1. The transplanted cells slowed disease onset and progression, and prolonged survival, concluded the researchers. The work was supervised by senior authors Robert Brown of the University of Massachusetts Medical School in Worcester; Evan Snyder of the Sanford-Burnham Medical Research Institute in La Jolla, California; and Yang Teng. More stem cells yielded longer survival. Some animals passed the one-year mark—a significant effect in an animal model that lives but a few months. The implants did not replace degenerating neurons, but supported endogenous ones by making trophic factors and stifling inflammation, suggested the authors. Stem cells were particularly effective when they settled across a broad area, or in regions that control survival functions such as breathing.

Overall, scientists in Chicago were optimistic about future ALS trials. "Some may look at recent failures and be discouraged," wrote Richard Bedlack of the Duke ALS Clinic in Durham, North Carolina, in an e-mail to Alzforum. Thanks to advances in genetics and biomarkers, and a list of potential target pathways, "I think this is a time of unprecedented hope and excitement in ALS research" (see full comment, below).

"People's expectations have to be reasonable," added Genge. If any of the current crop of experimental drugs manages to extend life by even a year, then that would indicate which pathways are worthy targets for further drug development, she noted. And if more than one adds even months to survival, then researchers can start building cocktails to look for additive effects.

Devilish Duo: Two Mutations Add Up to Familial ALS

As if the genetic basis for amyotrophic lateral sclerosis was not complicated enough, attendees at the symposium heard another wrinkle to the story. Michael van Es, of the University Medical Center in Utrecht, the Netherlands, proposed that in certain familial cases, not one but two major ALS risk genes contribute to disease. After screening families for known mutations, Van Es and his colleagues discovered far more double mutants than they would expect by chance alone. Mutations in one gene, a second, or both might explain why carriers in the same family often present with different phenotypes, or escape disease altogether, suggested van Es. The mechanism behind the dual-gene pathology remains unclear, and some scientists even doubt that mutant pairs work together.

Geneticists have typically explained the variable age at onset, speed of progression, and symptoms of people with the same ALS mutation by hypothesizing that other factors in their genomes, perhaps many variants, influence the disease. The Utrecht group's "oligogenic" hypothesis is not that. It blames ALS phenotypes on two genetic co-stars, instead of one headliner and a supporting cast of bit players (Van Blitterswijk et al., 2012).

Van Es' colleague Marka van Blitterswijk headed the study before moving to Rosa Rademakers' lab at the Mayo Clinic in Jacksonville, Florida. In an interview with Alzforum, she said the geneticists happened upon people carrying two ALS risk alleles when they set out to perform exome and whole genome sequencing. They checked their samples for known ALS mutations first, and discovered that five of their 97 families carried pairs of mutations. The angiogenin variant K17I showed up with FUS-R521C or TARDBP-N352S, a variant of the TDP-43 gene. C9ORF72 expansions paired with TARDBP-N352S, SOD1-D90A, or FUS-Q210H. In a further study published last month, Van Blitterswijk reported on a C9ORF72 expansion/VAPB-V234I duo (Van Blitterswijk et al., 2012).

In healthy control genomes, variants of known ALS genes only appeared in 0.5 percent of samples, and never more than one in a given person. The researchers calculated that the frequency of double mutations in families with ALS was well above the rate that should occur by chance.

However, the oligogenic interpretation is complicated by the fact that not all of the implicated variants are confirmed as pathogenic mutations. For example, FUS-Q210H and angiogenin-K17I also occurred individually in control subjects. Thus, they might not be causative mutations, but could still represent risk factors or disease modifiers, Van Blitterswijk suggested in an e-mail to Alzforum. Researchers debate the role of angiogenin as an ALS risk factor (see ARF related news story on Greenway et al., 2006; Corrado et al., 2007; Kirby et al., 2012), but several of the other mutations crop up in previously reported ALS cases. This includes FUS-R521C (Sproviero et al., 2011) and TARDBP-N352S, which was fairly common among Van Blitterswijk's pedigrees (Kühnlein et al., 2008; Kamada et al., 2009). C9ORF72 expansions, recently discovered to be a long-sought risk factor, are prevalent in cases as well (see ARF related news story on Renton et al., 2011, and Dejesus-Hernandez et al., 2011). And SOD1-D90A is among the most frequent disease-causing SOD1 mutations globally (Rabe et al., 2010; Giannini et al., 2010; Eisen et al., 2008).

Other researchers have also reported cases with double mutations (Luigetti et al., 2011; Chiò et al., 2012; Lattante et al., 2012; Millecamps et al., 2010). Van Es said he is unsure how common pathogenic mutation pairs are overall. He hypothesized that the additive effects of two genetic risk alleles cause full-blown ALS in some families, while a single mutant allele may lead to different ALS or FTLD phenotypes, which may manifest as later onset of disease or slower progression. "It is more the sum of mutations that determines what the phenotype looks like, rather than individual mutations causing different phenotypes," Van Es said. Importantly, that does not mean all inherited ALS cases result from a genetic double whammy, just that it is possible in some families, Van Blitterswijk noted.

Van Blitterswijk has taken the work a step further in her new position. Rademakers' group studies frontotemporal dementia (FTD), which shares several genes and features with ALS. As outlined in a poster at the Chicago symposium, Van Blitterswijk examined DNA from people with known mutations in ALS and FTD genes, such as tau and progranulin, for the presence of the newly discovered C9ORF72 expansion. Again, she observed an unexpectedly high rate of double mutants—out of 218 families, three had a C9ORF72 expansion plus progranulin

variants, and one had the C9ORF72 variant plus a tau mutation. The progranulin mutations included two frameshifts and an R493X substitution; the tau was P301L. All of these are linked to FTD beyond a doubt, Van Blitterswijk wrote (Huey et al., 2006; Spina et al., 2007; Pickering-Brown et al., 2008; Nasreddine et al., 1999). "This confirms the oligogenic pathogenesis and shows that it is not just specific for ALS; it can be detected in FTD patients as well," Van Blitterswijk said.

Reaction from scientists has been mixed. Not all have embraced the hypothesis. Rademakers told Alzforum that she was initially skeptical, but now that she has seen how frequently mutations co-occur, she agrees that the pairs constitute a real phenomenon.

Guy Rouleau, of McGill University in Montréal, Canada, sees it differently. He argued that one of the two mutations is not a pathogenic mutation, but simply a benign variant. Just because the polymorphisms are present does not mean they contribute to disease, he told Alzforum, noting that every person's genome is littered with variants that do not cause problems.

The presence of two mutations in so many ALS and FTD families has major implications for both researchers and the families, the presenters said. Scientists often eliminate carriers of known genes from new genetic analyses, but that would be a mistake if those carriers have more important mutations lurking in their genomes that researchers want to find. "I would advise we include [samples with known mutations] in any future screening to find evidence for double mutations," Van Es said at the meeting.

Double mutations will complicate genetic counseling and risk prediction for families that harbor risk alleles. "I really do not know how we are going to work around this in clinical practice," Van Es told Alzforum. "We do not know what to tell them anymore." Already, Van Blitterswijk said she knows of one family that was seeing a genetic counselor, thinking they had only the angiogenin K17I variant to contend with (Van Es et al., 2009). Further testing showed that they also carried the TARDBP-N352S mutation.

Dynamic Repeats: C9ORF72 Expands and Shrinks in ALS

For years, scientists hunted for the mutation on chromosome 9 that causes amyotrophic lateral sclerosis, and the subsequent discovery of the mysterious C9ORF72 was hardly the end of the challenge. At the symposium, researchers discussed having to contend with a nucleotide expansion that varies in size not only from one person to the next, but also between tissues within the same person. Presented by **Mariely Dejesus-Hernandez** of the Mayo Clinic in Jacksonville, Florida, this finding could complicate diagnosis and some cellular disease models. Those based on lymphocytes, for example, may not exhibit the full extent of the mutation in the nervous system. This phenomenon is a familiar theme in repeat disorders, commented **John Wilson** of Baylor College of Medicine in Houston, Texas; for example, variable repeat size also occur in myotonic dystrophy type 1 and Fragile X syndrome.

Healthy people tote up to 30 hexanucleotide repeats in C9ORF72. People with ALS or frontotemporal dementia typically have hundreds or thousands. Since its discovery in 2011 (see ARF related news story on Renton et al., 2011, and Dejesus-Hernandez et al., 2011), there have been hints of different repeat lengths in different tissues in the same person (see ARF related news story). In Chicago, Dejesus-Hernandez described data from three people who came to autopsy. Two had died of classical ALS, and one had frontotemporal lobar degeneration and lower motor neuron disease. She examined DNA from blood, spleen, heart, muscle, liver, spinal cord, and brain tissue, the last including the frontal, temporal, parietal, and occipital cortices as well as the cerebellum. Within one person, the size of C9ORF72 repeat ranged from as little as three kilobases to 10 Kb. The person with mixed brain and spinal disease had 80-100 C9ORF72 repeats in the blood, and more than 1,000 in the cerebellum.

Even within individual tissues, there is mosaicism. Southern blots for C9ORF72 revealed smudges and smears rather than the crisp bands that would indicate a gene of fixed size. The central nervous system tends to have the longest repeats. The same is true in other repeat disorders, Wilson said. **Johnathan Cooper-Knock** of the University of Sheffield in the U.K. told Alzforum he has seen a similar pattern in his own C9ORF72 studies. In approximately one in 15 patients, he said, the repeat length in blood cells comes out 10-fold smaller than in brain.

"That is going to hugely complicate things, because we tend to [rely on] drawn blood [for many analyses]," commented **Michael van Es** of the University Medical Center Utrecht in the Netherlands. He called the finding "intriguing and horrible at the same time." **Rosa Rademakers**, in whose laboratory Dejesus-Hernandez works, speculated that scientists and clinicians could miss people with C9ORF72 expansions if their blood does not reflect the lengthy repeats in the nervous system. Emphasizing that no evidence yet exists for this kind of false negative test result, Rademakers told Alzforum that it would be hard to determine if such a misdiagnosis occurred, since that would require brain biopsies. Nevertheless, missing people with C9ORF72 expansions in the brain would not only confuse genetic diagnosis in the clinic. It could also confound studies that attempt to characterize the symptoms of C9ORF72-based disease, and/or that correlate phenotype with repeat length. Cooper-Knock recommended that scientists making patient-based cell models not use blood cells from people who have only a hundred or so repeats in their blood, because that does not guarantee a sufficient number of repeats in the brain to cause pathology.

C9ORF72 is hardly the first repeat disorder to show this kind of instability, noted Wilson and **Tom Cooper**, also at the Baylor College of Medicine. In myotonic dystrophy type 1, which is due to a repeat expansion in dystrophia myotonica-protein kinase 1 (DMPK1), repeat shrinkage and expansion both occur in cell culture experiments, Wilson said. In animals the repeats tend toward growth. In the case of C9ORF72, Rademakers said it is unknown if people start out with one shortish repeat that grows over time, or if contraction occurs as well.

John Hardy of University College London, U.K., commented in an e-mail to Alzforum that if people with C9ORF72 expansions have different lengths in different tissues, that might explain why they have different disease phenotypes.

People with the C9ORF72 repeat can exhibit symptoms of ALS, frontotemporal dementia, or both. Rademakers speculated that people with longer repeats might suffer earlier disease onset.

Murky Mechanism

How might the expansions grow and contract? Scientists agree that the repeat sequences could cause replication or repair enzymes to stutter, adding or deleting sections as they go; however, the precise mechanism remains controversial. Some researchers who spoke with Alzforum believe the size change must occur early in development, while others argue that the gene could keep stretching and contracting in adults.

The resizing likely happens during DNA replication, reasoned **Michael Baughn** of the University of California, San Diego (UCSD). "The polymerases that copy DNA have a hard time with repetitive sequences," he said. Since neurons do not divide, neuronal repeats must have arisen during early development, when precursors are still reproducing. Why, then, would non-dividing cells—the neurons—have longer repeats than regularly mitotic types such as blood cells?

At what point in development the expansions occur might explain their tissue distribution. Dejesus-Hernandez presented preliminary evidence that the length of repeats correlated, loosely, with the embryonic origin of each tissue. In one patient, repeats were shortest in mesoderm-derived cells such as spleen and blood, medium length in endoderm-based tissues such as liver, and longest in nerve tissues derived from the ectoderm. One would expect this pattern if the C9ORF72 size was set early in embryonic development, Baughn said. "It is just an observation at this point," Rademakers cautioned. Her laboratory will gather as many tissue types from as many subjects as possible to better characterize the repeats' size, range, and the cutoff between health and disease.

Cooper suggested an alternative explanation to when expansions might change. "While DNA replication does not happen in neurons, what does happen in all cells is DNA repair," he said. Repeats could confuse repair enzymes, making them add to the expansion. In myotonic dystrophy type 1, Cooper noted, muscle biopsies in adults show that the DMPK expansion grows over time. Similarly, huntingtin repeats grow in Huntington's disease model mice as they age (Kennedy et al., 2003).

Two cellular processes recruit repair enzymes to repeat sequences, Wilson said. One is transcription, which exposes and separates DNA strands. This allows the repeats to form structures such as hairpins, which the DNA repair machinery recognizes as abnormal and tries to repair (reviewed in Lin and Wilson, 2011). The formation of hairpins by CAG repeats, such as found in Huntington's disease, and CGG repeats as in Fragile X, has long been considered a factor in expansion (Gacy et al., 1995). Supporting Wilson's theory, knocking out some DNA repair proteins stabilizes the length of trinucleotide repeats (reviewed in Lin et al., 2009). The second possibility Wilson noted is that the high oxygen concentration in the brain damages DNA. That, too, would recruit repair enzymes and prime the system to alter the number of repeats (see ARF related news story on Kovtun et

al., 2007). Rademakers suggested that multiple mechanisms might destabilize repeats.

A final answer on the process is still a way off. “One of the most fascinating aspects of all these repeat diseases is the pattern of expansions in different tissues. But it is incredibly complicated,” Wilson said. For now, the heterogeneity of the repeats is an important factor for researchers studying C9ORF72 to keep in mind, Dejesus-Hernandez concluded.

RNA Inclusions Offer Therapeutic Target in ALS

No one knows what the amyotrophic lateral sclerosis (ALS) gene C9ORF72 does, or how exactly it causes motor neuron disease (MND). No matter—some researchers are speeding ahead to develop and test a potential therapy. ALS researchers, still on a high over the gene’s discovery in 2011 (see ARF related news story), were excited to hear progress reports from two separate collaborations pursuing antisense oligonucleotide therapy at the symposium. The treatment is based on a theory that mRNAs from mutant C9ORF72 alleles form toxic inclusions, and the antisense oligos should dissolve those poisonous nucleic clots. **Michael Baughn** of the University of California, San Diego, and **Jeffrey Rothstein** of Johns Hopkins University in Baltimore, Maryland, described their respective efforts in a poster and oral presentation. They claimed that early results look promising, with the oligos destroying the inclusions and righting abnormal gene expression caused by a hexanucleotide repeat expansion in the C9ORF72 DNA.

Inclusions Confirmed...

The first task, Baughn told Alzforum, was to confirm that C9ORF72 mRNA inclusions exist. Researchers who discovered the genetic association with ALS reported the presence of C9ORF72 mRNA in nuclear foci (Dejesus-Hernandez et al., 2011), but other researchers struggled to replicate the finding. The challenge lay in the repeat's GGGGCC sequence; GC-rich regions are tricky to label because probes pick up other, nonspecific GC-containing strands as well.

Baughn, who works in the laboratory of **John Ravits** at UCSD, nailed a method to specifically label C9ORF72 foci in collaboration with **Clotilde Lagier-Tourenne** in the laboratory of **Don Cleveland**, also at that institution. The trick was to use a locked nucleic acid (LNA) probe. LNA probes are made of DNA with methylene bridges to stabilize the structure in just the right conformation to hook up, specifically, with the C9ORF72 GGGGCC sequence. “It is basically a braced DNA base,” Baughn said. That bracing increases affinity and specificity for the desired target. Researchers aiming to silence the repeat expansion in Huntington’s disease are using a similar technique (see ARF related news story).

With an LNA probe consisting of three hexanucleotide repeats, Baughn and Lagier-Tourenne detected C9ORF72 inclusions. The foci dissolved in the presence of RNase but were resistant to DNase. The inclusions appeared in primary cell lines, including fibroblasts, lymphoblasts, neurons, and glia, isolated from people who had ALS. They were absent in control cells from healthy tissues or from people who had sporadic ALS, familial ALS due to non-C9ORF72 mutations, or Parkinson’s disease.

The foci were mainly nuclear, typically one or two per cell, although occasional cells were jam-packed with inclusions, Baughn said. He and Lagier-Tourenne have not finished characterizing how frequent focus-positive cells are, but Baughn told Alzforum that 9-45 percent of fibroblasts and 22-33 percent of neurons exhibit foci, depending on the cell line they examined.

Simply confirming the presence of C9ORF72 RNA foci was an exciting finding, commented **Johnathan Cooper-Knock** of the University of Sheffield in the U.K., because their presence only in C9ORF72 repeat-toting cells lends support to the theory that the inclusions are indeed toxic.

"Not only do these foci exist, not only are they composed of RNA, but they are actually pretty common," Baughn concluded. In spite of the C9ORF72 expansion and foci, the cell lines are fairly healthy, although some grow slowly, he told Alzforum. He was pleased to see the RNA foci were present in fibroblasts and lymphoblasts, because those cell types are easy to grow and would make good models in which to test treatments. Curiously, other researchers have suggested that in approximately one in 15 C9ORF72 patients, the repeat region is shorter in blood than in brain, perhaps making blood cells less than ideal for modeling (see ARF related news story). Baughn used the patient fibroblast lines with the highest rates of RNA inclusions to test an antisense therapy.

…And Denied

For his part, Rothstein, who coauthored one of the C9ORF72 discovery papers (Renton et al., 2011), told Alzforum he and his collaborators started their antisense project the day that manuscript was submitted. They based their idea, in part, on early results with antisense oligonucleotides in fibroblasts and myoblasts from people with myotonic dystrophy type 1, another repeat expansion disorder (reviewed in Mulders et al., 2010).

Rothstein's group benefited from a ready-made model to test the treatment. He and other researchers have been building a collection of induced pluripotent stem (iPS) cell lines from people with ALS (see ARF related news story), and luckily discovered that one of their lines, from an apparently sporadic case, harbored a C9ORF72 expansion. The researchers made motor neurons and astrocytes from the iPS lines. Like Baughn and Lagier-Tourenne, Rothstein found that RNA inclusions were present, but hardly affected the cells' health. By profiling the transcriptomes of their lines, the researchers identified some 20 genes encoding secreted proteins that were reliably over- or underexpressed in the C9ORF72 cultures. This signature could eventually make a biomarker for a C9ORF72-targeted therapy, Rothstein said.

Both the Cleveland-Ravits and Rothstein groups teamed up with Isis Pharmaceuticals of Carlsbad, California, which provided candidate antisense oligonucleotides for potentially treating C9ORF72-based ALS. Isis pursues antisense therapies for several neurodegenerative conditions (see ARF related news story on Kordasiewicz et al., 2012, and ARF related news story on Passini et al., 2011). The company is already working on another antisense treatment for ALS, aimed at the ALS gene superoxide dismutase 1 (see ARF related news story). The researchers tried oligonucleotides that bind all C9ORF72 splice forms.

In addition, Baughn's group also targeted sequences that appear only in the expanded isoform. Several of the oligonucleotides reduced the frequency of RNA foci, and apparently stabilized gene expression as well.

This evidence from foci-labeling and antisense studies supports the toxic aggregate model. It does not eliminate other possible mechanisms for C9ORF72-based disease. Cells carrying the repeat might suffer haploinsufficiency. For that reason, Baughn and Cooper-Knock are particularly interested in those antisense oligonucleotides that would knock down the abnormal, expanded transcript but leave C9ORF72 mRNA of the proper length alone. "You do not want to create another disease by getting rid of all [of the protein]," Cooper-Knock said.

In an e-mail to Alzforum, **John Hardy** of University College London, U.K., noted, "This is a hard road. It is a rational approach, but, as with all those antisense [therapies], getting effective delivery will be the challenge."

Reference:

Teng YD, Benn SC, Kalkanis SN, Shefner JM, Onario RC, Cheng B, Lachyankar MB, Marconi M, Li J, Yu D, Han I, Maragakis NJ, Lládo J, Erkmen K, Redmond DE Jr., Sidman RL, Przedborski S, Rothstein JD, Brown RH Jr., Snyder EY. Multimodal actions of neural stem cells in a mouse model of ALS: a meta-analysis. Sci Transl Med. 2012 Dec 19;4(165):165ra164. Abstract