# Milton Wexler Interdisciplinary Workshop:

Visions of Victory – HD in Our Grasp Twenty Years and Counting!

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Twenty years ago, The Huntington's Disease Collaborative Research Group reported their discovery of IT15, a new gene containing an expanded trinucleotide repeat in individuals suffering from Huntington's disease (HD). This seminal discovery, the identification of the huntingtin gene, was made possible by the drive and creativity of a group of international scientists brought together and supported by the Hereditary Disease Foundation (HDF).

Since its inception in 1968, the HDF has not only provided funding for researchers with bold new ideas to tackle HD—like the proposal to map and identify the HD gene—but, perhaps more importantly, it has fostered the creative process that has fueled many major advances in the field. Nancy Wexler explained that her father, Milton Wexler, had the vision of creating a unique organization that would bring together top scientists from around the world in an environment optimized for the generation and exchange of novel ideas.

M. Wexler's vision crystallized after attending his first meeting of the Society for Neuroscience. At this large, bustling conference, M. Wexler noticed that during the participants' talks, the lights went off, the audience fell silent and, with the exception of the speaker, everyone became a passive listener. Some scientists even fell asleep. During the coffee breaks, however, scientists talked to each other, exchanged ideas for experiments, and established collaborations. Seeing this creative process unfold, M. Wexler decided to set up conditions that would reproduce and nurture it. And so the HDF workshops were born—meetings that eschew formal presentations of data in favor of free-wheeling discussions amongst scientists with different areas of expertise.

True to M. Wexler's vision, this year's January workshop served as a forum to creatively explore new ideas for tackling HD. Participants discussed novel findings suggesting that aberrant splicing of the mutant huntingtin mRNA generates a particularly toxic protein fragment. The implications of these observations for understanding the neuropathology of HD, as well as for optimizing gene silencing therapies currently under development were examined. New findings about the role of oxidative stress in the disease process were also discussed, as were suggestions for future experiments to clarify their role and assess their value as therapeutic targets. Participants also stressed the importance of finding robust, sensitive biomarkers to monitor disease progression and evaluate the effectiveness of candidate treatments. In particular, they discussed the advantages and limitations of a new marker that could help track huntingtin mRNA using brain imaging techniques.

***Courage and commitment***

An inspiring realization that emerged from the workshop was the level of commitment of many of those involved in the fight against HD. Several researchers who have been working on HD for many years expressed their heartfelt dedication to solving HD. David Housman, Diana Rosas, Anne Young and Nancy Wexler, for example, all described their strong desire to continue the hard work involved in understanding HD and, ultimately, curing it. Moreover, the generous participation of Paula Bailey, an intelligent and courageous woman suffering from HD, highlighted the invaluable contributions of people who are afflicted with the disorder. Bailey inspired participants with her remarkable resourcefulness and optimism, and her commitment to help researchers further their understanding of HD.

Sharing her story with participants, Bailey explained that her mother had suffered from depression, alcoholism and HD. The complex interplay of symptoms delayed her HD diagnosis, but as soon as it was established, Bailey realized the possibility of succumbing to the disease herself. She immediately signed up for a genetic test, and when she received the positive result, contacted physicians and specialists to help her manage the HD symptoms which she knew would inevitably ensue. Bailey also enrolled in a medical insurance plan, a private-pay option which was her only choice.

Bailey's willingness to confront the possibility of having HD and planning for her future is unusual. In addition to overcoming the enormous psychological challenge of facing a positive result from an HD genetic test, Bailey risked discrimination once her genetic status was established. Nancy Wexler explained that there is legislation in the U.S. intended to protect individuals against discrimination based on their genetic information, but it often

fails to achieve its goal. As a result, Wexler recommends that individuals pay for HD testing out-of-pocket and keep their results private.

Roughly five years after receiving the results from her genetic test, Bailey began to notice she was having difficulties focusing at work. Bailey was a therapist using highly interactive methods to treat her patients—a situation that required a high level of focused attention every day. To counter her increasing tendency to get distracted, she made a conscious effort to stay present and attentive. She also began taking extensive notes during and after her therapy sessions, which helped for a time. Ultimately, however, her lack of focus became incompatible with her work. She realized she had to shut down her practice, giving up the work she described as both her passion and hobby.

Bailey has been living with HD symptoms for almost a decade now. She has some mild choreic movements and uses a walker to stabilize her stride. She is unable to drive, despite having been a skilled driver since she was very young. Bailey also said she has muscle spasms in her throat and sometimes has trouble swallowing. In addition, she suffers from incontinence and digestive problems. Diana Rosas asked Bailey to perform a few tasks which revealed that Bailey's overall motor abilities, however, are surprisingly intact. Bailey's gait appeared solid, although she had some trouble turning around, and she performed very well on an eye-tracking test and mimicking simple movement sequences. Indeed, Rosas had to repeatedly increase the difficulty of several tasks to reveal some of Bailey's deficiencies.

Bailey was articulate and thoughtful at the meeting, but described having several cognitive deficiencies, stressing that these were far more burdensome than her motor problems. Bailey suffers from anxiety, perseveration, and memory problems. She often gets apprehensive about getting to her appointments on time, for example, and needs help keeping track of everyday chores. Because her handwriting is sometimes illegible, she rarely makes lists (although the act of writing a list sometimes helps her remember things better). Bailey also has a disrupted sleep pattern and feels fatigued throughout most of the day. Her most challenging problem, however, is staying focused. Bailey said she has to make a conscious effort to remain on task even while brushing her teeth. This continuous struggle is exhausting and Bailey deeply grieves the loss of this cognitive ability.

Nevertheless, Bailey has maintained a positive, even cheerful, attitude. A nurse who visits Bailey to help out four times a week, describes Bailey as an intelligent and charming person with an irrepressible zest for life. The nurse said Bailey loves children and makes friends readily. Moreover, Bailey seizes all opportunities to keep her mind sharp and her body fit. She plays video games and reads a lot. She also goes on long walks, sometimes practicing word games as she strolls. In addition, Bailey keeps up with her medications—Paxil (anti-depressant), Zyprexa (to control movements at night), clonazepam (for chorea, as needed), medications for digestive problems, and melatonin (for sleep). She also takes Coenzyme Q10 (CoQ10; 200 mg/day) and creatine (5 g) supplements, the latter of which she described as helpful.

Participants were impressed with how well Bailey is doing ten years into the symptomatic phase of HD and emphasized the importance of environmental factors in determining disease progression. Bailey began experiencing HD symptoms relatively late in life, and late onset is usually associated with a milder phenotype. However, Bailey's attitude and commitment to enhancing her quality of life have undoubtedly been instrumental in ameliorating the progression of her disease.

**Treatments in clinical trials**

Inspired by Bailey's courage and optimism, participants reviewed the status of candidate treatments for HD that are currently in clinical trials. As noted by Carl Johnson, the compounds that are most advanced in the pipeline are Co-Q10 and creatine, two agents that are predicted to be neuroprotective based on their ability to act as antioxidants and improve mitochondrial function and cellular bioenergetics.

Diana Rosas noted that there are currently two international, multi-center phase III clinical trials to test the effects of high doses of Co-Q10 (2400 mg/day). The treatment has been well tolerated so far, but results on its efficacy won't be available until four years from now. Rosas also described ongoing trials for creatine, including the CREST-E and PRECREST trials. Enrollment in the CREST-E study is ongoing, with 420 participants recruited so far. The dosage is the maximum that individuals can tolerate (40 g/day) and the study is expected to be completed in 2016. The primary outcome measures for these studies are total functional capacity and its rate of change, with neuroprotection as the endpoint. Other parameters being monitored include 8-OH2'dG (a marker of oxidative stress) levels, imaging markers, and metabolomics and transcriptomics profiles.

The PRECREST study, a two-phase clinical trial focusing on presymptomatic HD, is of particular interest because its open-label phase is yielding very encouraging results which Rosas said she is now preparing to publish. Rosas reported at the HD2012 meeting in August that premanifest individuals have significant and widespread brain pathology that appears to be countered by creatine treatment, as assessed by MRI. In particular, creatine seems to prevent cortical thinning, preserve white matter integrity and possibly reduce striatal atrophy.

The study is also of interest because it uses a novel strategy for recruiting subjects, which includes at-risk individuals who have chosen not to be tested for the HD mutation. As noted by Rosas at the HD2012 meeting, this strategy avoids genetic testing coercion and automatically provides an unselected, healthy control group. The strategy also greatly increases the pool of potential participants, given that the majority of at-risk individuals choose not to be genetically tested. However, as noted by Rosas at the workshop, the strategy is still controversial and some researchers are opposed to having subjects enroll without genetic testing.

Participants also mentioned a few other candidate neuroprotective agents that are in the early stages of clinical testing. For example, Gill Bates noted that Novartis is testing the safety and tolerability of a Sirt1 regulator. Previous studies have shown that mutant huntingtin interacts with Sirt1, inhibiting its deacetylase activity which results in hyperacetylation of substrates that facilitate cell survival, such as the transcription factor forkhead box O3A. In addition, Jang-Ho Cha noted that Raptor Pharmaceutical Corp in France has an ongoing Phase 2/3 clinical trial to test cysteamine, a compound that has been reported to have neuroprotective effects in HD mice by increasing the levels of brain-derived neurotrophic factor (BDNF).

A few compounds that are in clinical trials to ameliorate HD symptoms were also discussed. For example, Carl Johnson noted that Auspex Pharmaceuticals is currently testing a new version of tetrabenazine—an anti-choreic medication that reduces dopamine levels—in which hydrogen atoms are substituted for deuterium. This "deuteration" of tetrabenazine changes its pharmacokinetic properties, slowing its rate of oxidation and thus extending its half-life by approximately two-fold. The expectation is that lower and less frequent doses of deuterated tetrabenazine will be able to achieve the same therapeutic effects of the unmodified compound and result in fewer side-effects. However, Marc Diamond and Roger Albin expressed concerns about the increased time that will be required to eliminate the medication. Although fewer side-effects are expected with the new compound, if it causes other, unsuspected secondary effects, its longer half-life will be problematic. Also, current treatments with tetrabenazine allow for scheduling of frequent "breaks" between doses. Reducing these breaks may result in more, rather than less, side-effects.

Another compound being tested in clinical trials is a second derivative of chloroquine. Diana Rosas noted that a safety and tolerability study to test this agent is currently underway (scheduled to end in June). The derivative has been postulated as a potential candidate to treat Alzheimer's disease (AD) and may also help ameliorate HD cognitive symptoms by reducing the aberrant deposition of copper and iron observed in HD brains. By affecting lysosomes function, chloroquine may alter the intracellular trafficking and processing of amyloid precursor protein in AD and may affect the release of metals from cellular proteins, such as ferritin, in HD. However, David Housman questioned how well these mechanisms of action are currently understood.

As noted by Carl Johnson, the number of candidate treatments in the pipeline is modest so it will be important to keep focused on the development of gene silencing therapies to directly knock down huntingtin expression, as well as on the identification and validation of new targets for intervention. Bolstering previous findings in yeast and *Drosophila*, a recent article by Christian Neri reported 662 modifiers of HD in a *Caenorhabditis elegans* model of the disease. This wealth of potential targets is encouraging, noted Johnson, as is the abundance of ongoing research in HD. In an online search, Johnson found over 700 articles on HD published in 2012 alone.

**Mechanisms of Pathology**

***Aberrant splicing of mutant huntingtin mRNA***

A deeper understanding of the molecular and cellular mechanisms of HD promises to reveal new targets, as well as provide new insights to improve current candidate therapies. A striking example of this was presented by Gill Bates and David Housman who presented findings indicating that the expanded CAG repeat results in the aberrant splicing of mutant huntingtin mRNA. The observations suggest a novel mechanism of huntingtin fragment generation which may importantly contribute to the molecular pathogenesis of HD and help explain some disparate findings in the field. In addition, the findings could help improve the design of gene silencing agents and suggest new targets for HD therapies.

*A new look at huntingtin fragment formation*

The formation of huntingtin fragments has long been implicated in HD pathology. Several proteases have been identified that cleave huntingtin, but the precise mechanism by which small, toxic N-terminal fragments are generated has remained elusive. Bates said she became interested in huntingtin fragments about a decade ago when she noticed that a knock-in mouse model of HD (Detloff, Q150) had a remarkably similar phenotype to her R6/2 mouse model, a transgenic carrying a genomic fragment spanning the 5' end of the huntingtin gene, exon 1 with approximately 150 CAG repeats, and a portion of intron 1. Bates observed that the knock-in mice developed the HD phenotype more slowly than the R6/2 mice, but the changes in heat shock response pathways, as well as the peripheral pathologies were nearly identical in the two models.

Based on these observations, Bates spent several years studying huntingtin protein fragments. She used various antibodies to map huntingtin polypeptides and assess their sizes. Antibody cross-reactivities and the difficulty of separating some of the fragments on Western blots made the goal of dissecting the fragments' identities and differential effects very challenging, but the team persevered. As various groups identified proteases capable of cleaving huntingtin, Bates's team and others focused on proteolysis as the source of huntingtin fragments.

Taking advantage of advances in high throughput RNA sequencing technologies, however, Bates recently decided to look beyond proteases and investigate the unexamined role of splicing in huntingtin fragment formation. Thus, Bates and Housman set up a collaboration to analyze huntingtin mRNA species. Because of the known importance of N-terminal fragments in HD pathology, the researchers focused on the 5' end of the mRNA. They performed RT-PCR assays to compare the levels of huntingtin cDNAs containing exon 2, exon1-exon2 junctions, exon1-intron1 boundaries, and intron 1 sequences in homozygous and heterozygous knock-in mice, as well as in their wildtype littermates.

Their results revealed the presence of differentially spliced species of mutant huntingtin mRNA. Increased levels of PCR products containing intron 1 and exon1-intron1 boundary sequences were observed in the mutant mice, with higher levels in homozygotes than heterozygotes. Examination of the intron 1 sequence revealed a cryptic poly-adenylation signal. This signal appears to be used to generate an unspliced exon1-intron1 transcript in mutant knock-in mice, as indicated by the presence of short polyadenylated mRNAs that can be detected by 3' rapid amplification of cDNA ends (RACE). The researchers then used total RNA sequencing (RNAseq) to independently verify the presence of the exon1-intron1 transcript in knock-in mouse tissues. Bates explained that, initially, the technique failed to provide clear results because regions of high GC content in the short

transcript interfered with the RNA amplification step. Once this problem was addressed, however, the results were consistent with the 3' RACE data.

The team has now confirmed the presence of the aberrant transcript in all HD knock-in mouse models and shown by quantitative PCR that transcripts containing early intron 1 sequences are increased in a CAG dose-dependent manner. To analyze human transcripts, the reseachers first performed bioinformatics analyses which revealed nine predicted polyadenylation signals in intron 1. Using 3'RACE to assess the transcripts in YAC128 and BACHD mice, both of which carry human huntingtin constructs, the team found a polyadenylated transcript that corresponded to the poly-A signal with the highest predictive score, as well as a second transcript only found in the YAC128 mice. Mice carrying mixed CAA/CAG repeats also express the aberrant transcript. Searching for the mutant transcript in human tissues has been challenging, however. As explained by Bates, the quality of mRNA in post-mortem tissues is often poor, making it impossible to conduct reliable 3' RACE assays. So far, the team has succeeded in clearly identifying the transcript only in human HD fibroblast lines.

To determine if the aberrant transcripts are translated into protein, Bates and colleagues isolated polysomes and found that, indeed, the transcripts are associated with ribosomes and translated to produce an exon 1 huntingtin protein, as assessed by immunoprecipitation and Western blots. Bates explained that the exon 1 protein terminates in a proline residue as expected given the presence of an evolutionarily conserved stop codon at the 3' end of unspliced exon 1. This protein corresponds to the smallest N-terminal fragment generated in the Q150 knock-in mice, providing a compelling possible explanation for Bates's initial observation of the similarities in phenotypes between these mice and the R6/2 exon 1 model of HD.

To investigate the underlying mechanism of the aberrant splicing, Bates and co-workers used bioinformatics to examine exon 1 and identified a CAG or CAGCAA repeat as a binding site for the splicing factor SRSF6. RNA co-immunoprecipitation experiments using an antibody against SRSF6 pulled down mutant huntintin 5' UTR and early intron 1 sequences, but not transcripts containing exon 2 sequences. SRFS6 regulates splicing and facilitates translation of partially spliced transcripts. In addition, SR proteins can displace the U1 snRNP, promoting polyadenylation from cryptic poly-A signals within introns. Thus, the increased association of SRSF6 with expanded CAG repeats could account for the production of the unspliced exon 1 transcript in a CAG repeat-dependent manner.

*General implications for HD pathology*

The observations are exciting because they point to a potential source of a key toxic fragment that may be responsible for many aspects of HD pathology. All exon 1 protein appears to derive from aberrant splicing (there are no proteases known to generate this particular fragment), and decades of research using the R6 lines—which express this fragment—indicate it results in a phenotype that mirrors many facets of HD in humans. Furthermore, Bates noted she observes some degree of correlation between the levels of the aberrant transcript in the different mouse models and their phenotype severity. And as previously mentioned, aberrant transcript levels vary in a CAG length-dependent manner.

The findings also help explain why the R6/2 model of HD expresses a phenotype that is very similar to other HD models, but develops at an accelerated pace. R6/2 mice start life with a high expression of the exon 1 fragment, while in other models, the fragment is only a fraction (5-20%, see below) of total mutant huntingtin protein expression that accumulates gradually over time.

Another interesting implication of the new findings is the existence of an allele-specific fragment in HD that may be of pathological importance. As noted by Marian DiFiglia, all huntingtin fragments identified to date are derived, via proteolysis, from both the wildtype and mutant huntingtin proteins. The exon 1 fragment described by Bates, however, is only produced from mutant huntingtin mRNA.

*Open questions: What is the relative contribution of aberrant huntingtin splicing to HD pathology?*
Participants considered that determining the extent to which the newly identified transcript contributes to HD pathology will be of particular importance. Housman estimated that 5-10% (and potentially up to 20%) of mutant huntingtin transcripts in the Q150 knock-in mouse are aberrantly spliced. However, he noted that quantifying these transcripts carefully will be challenging. Beverly Davidson suggested using junction probes. In addition, several participants proposed strategies to measure exon 1 versus full-length protein levels. Bates noted, however, that exon 1 protein levels are fairly low and difficult to detect by Western blot. An alternative suggested by Anne Young is to use mass spectrometry which Bates said they are beginning to do.

Participants also proposed using double-labeled constructs. A Förster resonance energy transfer (FRET) assay, for example, could be used to distinguish the presence of molecules with labels on their N- and C-termini (full-length) versus molecules with a single label on their N-terminus (exon 1). However, as noted by Bates, this type of approach is complicated by aggregate formation which would allow for inter-molecular, as well as intra-molecular, energy transfer. Another double-label approach, proposed by William Yang, involves making constructs with labels that are differentially available for translation in the full-length versus truncated transcripts. A label encoded at the 3' end of huntingtin RNA would be translated only from full-length transcripts, and a label encoded within the 5' end of intron 1 would be translated only from truncated transcripts. To allow translation of the latter tag, the stop codon for the exon 1 fragment would be modified so that it is distal to the inserted label. Yang added that he could use his BAC model system to generate animals expressing this double-tag construct. Bates noted that a similar idea had occurred to her team and could also be used for developing screening assays to identify molecules that specifically target the exon 1 protein. As noted by Kurt Fischbeck, however, it will be important to use small tags to minimize the potential interference of the tags on the molecules' functions and dynamics.

Another aspect of assessing the pathological relevance of the newly identified mRNA involves dissecting the relative contribution of the exon 1 fragment compared to previously identified proteolytic fragments. Given the large number of identified fragments and the many facets of HD pathology, this will not be an easy task, however. Moreover, much remains to be understood about the role of proteolysis in HD. For example, Bates noted that her team recently published a paper showing that the loss of caspase-6—a protease previously reported to generate a toxic huntingtin fragment key to HD pathology—had no effect on the proteolysis of huntingtin in HD knock-in mice.

The contributions of some fragments to pathology may vary depending on context. For example, Carl Johnson noted that a mouse model of HD with a cDNA construct of mutant huntingtin suffers from HD pathology, and yet aberrant splicing cannot be contributing to its phenotype (Marian DiFiglia suggested that the CpA fragment derived from cleavage by an aspartyl protease is probably responsible for this model's phenotype). On the other hand, Bates noted that the Q150 knock-in mice don't seem to accumulate any proteolytic fragments (unless there's a proteolytic fragment that migrates exactly to the same spot on Western blots as the product of the aberrantly spliced transcript). Thus, mutant huntingtin fragments produced by either proteolysis or aberrant splicing may be able to independently generate HD phenotypes. Participants agreed it is likely that, in most instances, both proteolysis and aberrant splicing contribute to pathology.

To address this question experimentally, participants suggested selectively silencing the exon 1 transcript and/or the full-length transcript. Many different huntingtin silencers have already been tested as part of the efforts to design gene silencing therapies for HD, so previously collected data and frozen tissues might shed light on the contribution of the exon 1 transcript to HD pathology. With this in mind, Marie Françoise Chesselet offered Bates tissue samples of animals treated with a variety of HD antisense oligonucleotides (ASOs). Moreover, Beverly Davidson suggested designing silencers specific to the unspliced exon 1 transcript and assessing if their effects and those of silencers currently being tested for therapeutic purposes are additive (most therapeutic silencers under development target downstream sequences present only in full-length transcripts –see *Therapeutic silencing implications* below).

Davidson added that it might be valuable to examine results from David Corey's lab in which the team silenced mutant huntingtin by targeting the CAG expansion. Corey's approach using anti-CAG duplex RNAs and, more recently single-stranded RNAs, is one of the few that is predicted to unequivocally target both full-length and exon 1 unspliced transcripts. Blocking or altering the aberrant splicing event, as noted by Davidson, is yet another strategy that may help assess the contribution of the exon 1 transcript to pathology.

*Open questions: The where, how and why of aberrant huntingtin splicing*
Basic questions about where, how and why the aberrant transcript is produced were also discussed at length. For example, Leslie Thompson wondered about the age- and tissue-dependence of the process. Bates explained that, so far, her team has only examined 2-month old mice, and found the transcript in all tissues. Furthermore, William Yang asked about the nuclear localization of the exon 1 protein. Bates noted that it is difficult to assess this because huntingtin protein in the nucleus is insoluble. However, she has seen the exon 1 fragment disappear from the cytoplasm with age. As noted by Kurt Fischbeck, it will also be of interest to examine whether aberrant splicing occurs in other CAG repeat disorders in addition to HD.

Anne Young suggested assessing the presence of the aberrant transcript in different HD populations, including individuals with adult-onset versus juvenile HD. She also proposed comparing transcript levels in people with similar CAG repeat lengths but different disease severity and/or age of onset. David Housman said he would like to perform RNAseq analyses on tissues from the Venezuelan population, but as stated previously, working with post-mortem tissues has proven challenging. Bates added that they are currently extending their analyses of human fibroblasts, as well as examining induced pluripotent stem cells. Studies of the RNA species in polysomes and ribosome protection assays are currently underway.

Participants also discussed ways to gain a deeper understanding of the molecular mechanisms underlying the aberrant splicing event. Bates pointed out that there are several hypotheses that can be experimentally tested. One possibility is that SRSF6 is recruiting the spliceosome at an incorrect location, interfering with the normal splicing process. Alternatively, SRSF6 may be sequestering or displacing the U1 snRNP, which in turn reveals and activates one or more cryptic poly-adenylation signals found in huntingtin intron 1. Yet another possibility suggested by William Yang and Marc Diamond, is that the mutant huntingtin transcript could be altering the splicing machinery, behaving like a toxic mRNA, as seen in myotonic dystrophy. Housman pointed out, however, that the splicing alteration in HD must be fundamentally different from what occurs in myotonic dystrophy because the huntingtin mutation appears to act only in cis, not trans.

Diamond said it will also be important to consider non-traditional ways in which the huntingtin mutation may be affecting splicing and translation. For example, Laura Ranum recently reported that CAG and CUG expansions can mediate translation without an AUG-initiation codon, depending on RNA structure and repeat length, and that this occurs in vivo in at least two repeat disorders (DM1 and SCA8). Jeff Kelley added that the translation of proteins from non-traditional transcripts is not constrained to diseased states, but appears to be widespread in normal mammalian cells. Alan Saghatelian's group recently found in normal human cells large numbers of polypeptides encoded by short open reading frames in noncoding RNAs and multicistronic mRNAs that initiate with non-AUG start codons. By Saghatelian's estimation, the abundance of these polypeptides is in the same range as those of known proteins. The role of the antisense strand should also be kept in mind. As noted by Neil Aronin, Russ Margolis recently reported that huntingtin antisense inhibits huntingtin gene expression and its expression is reduced in HD brains.

It will also be important to keep an open mind about the mechanisms by which the aberrant splicing event may affect cellular function. For example, Marie Françoise Chesselet pointed out that generation of the exon 1 fragment may not only result in gain-of-function toxicity, but also contribute to loss-of-function effects by causing less full-length huntingtin protein to be produced. Jang-Ho Cha added that there could be competition for glutamine tRNAs which might affect the levels of the full-length transcript. If glutamine tRNAs are indeed

rate-limiting, ribosomes might stall during huntingtin translation, resulting in the degradation of huntingtin mRNA by no-go decay. Considering this possibility, Cha and Neil Aronin pointed out that limiting glutamine might be a way to decrease all forms of mutant huntingtin translation.

As a first step in exploring the molecular mechanisms involved in producing the exon 1 transcript, Bates said her team will be collaborating with another lab to investigate the effects of knocking down SRSF6. Diane Merry suggested also examining the roles of other SR proteins since bioinformatics analysis, which led to the focus on SRSF6, won't necessarily reveal all the players involved in the process. Bates agreed and added that she plans to use 3' RACE to examine whether there are additional truncated transcripts derived from the use of other cryptic poly-A sites in the huntingtin transcript.

*Implications for gene silencing therapies*

Bates's and colleagues' new findings also have implications for the ongoing development of gene silencing therapies. As noted previously, the majority of silencing agents currently being tested target sequences in the huntingtin message that are downstream of exon 1. As explained by Davidson and Chesselet, exon 1 silencers were previously tested, but because several were toxic, they were not pursued as therapeutic agents. In addition, several allele-specific approaches rely on targeting single-nucleotide polymorphisms (SNPs) in the 3' UTR of huntingtin mRNA. In the context of Bates's new findings, this may be a problem—the silencers will reduce full-length transcripts, but may be incapable of downregulating exon 1 transcripts which are predicted to be more toxic.

A key question, noted Diamond, is the timing of the silencers blocking action. If the agents act upon their targets before splicing and 3' end formation, they may be capable of downregulating all huntingtin transcripts. On the other hand, if they act after the full processing of huntingtin pre-mRNAs has occurred, they will downregulate only full-length messages. As noted by Johnson, understanding the kinetics of transcription, splicing, and 3' end formation, should help differentiate between these possibilities.

Bates explained that splicing is generally co-transcriptional. Moreover, recent data suggest that introns at the 5' end of messages are removed earlier and are thus more likely to be removed co-transcriptionally than those at the 3' end. If splicing and 3' end formation of huntingtin mRNA occur before the message leaves the nucleus, expression-based silencing agents that act in the cytoplasm are predicted to be incapable of blocking translation of the aberrant fragment. ASOs, which can enter the nucleus, have a better chance of targeting exon 1 mRNAs, but their capability will depend on when and how splicing and 3' end formation occur.

The kinetics of splicing, the catalytic activity of splicing factors and the interdependency of transcription, splicing and mRNA 3′ end formation are not well understood, however, and different mRNAs are processed differently. For example, although most mRNAs are spliced in the nucleus, several dendrite-associated pre-mRNAs appear to be exported to dendrites where calcium signaling activates their splicing. It may well be that the processing of huntingtin pre-mRNA is also non-canonical. Its large size, for example, may affect its processing in ways that are difficult to predict. As noted by several participants, the only way to know whether and how current gene silencing efforts are affected by aberrant splicing will be through empirical testing.

As a first step, Davidson and Aronin said they could examine huntingtin mRNA levels, particularly those of the aberrant transcript, in their ongoing experiments. In addition, participants noted that some of the strategies mentioned earlier to dissect the relative contribution of the exon 1 transcript to HD pathology, could be used to re-examine gene silencing approaches. For example, participants proposed generating and testing new silencers that either target both the full-length and exon 1 transcripts or specifically the exon 1 fragment. Jang-Ho Cha added that a novel technique developed by Eric Wickstrom to detect specific mRNA species may prove helpful to this end (see *Tracking huntingtin mRNA using PET imaging*). Analyzing the huntingtin mRNA profiles of frozen cell and tissue samples derived from animals treated with different silencers was also proposed.

Moreover, participants noted that double-tagged constructs, such as the one suggested by Yang above, could help monitor the fates of the two huntingtin transcripts when exposed to different silencing agents.

Participants discussed how to set priorities and move the therapeutic silencers forward, while addressing the questions raised by Bates's work. As previously noted, establishing the relative contribution of the exon 1 fragment to HD pathology will be key. However, this will take time, as noted by Al LaSpada, and participants considered it would not be worthwhile to slow the progress of gene silencing approaches waiting for definitive answers to this question. Several studies in mice and rats have shown that downregulating mutant huntingtin with non-exon1 silencers has dramatic effects on the HD phenotype. Although Johnson suggested re-examining these positive results, it is clear that non-exon 1 silencers have enormous potential and participants agreed they should continue to be pursued as therapeutic candidates.

How can one explain the apparent effectiveness of the non-exon 1 silencers in light of the new splicing findings? As noted by Aronin, it is possible that a partial reduction in full-length mutant huntingtin is sufficient to prevent, or significantly delay, cells from reaching a threshold in which their protective mechanisms are overwhelmed, even when they are still producing exon 1 proteins. Indeed, the fact that the silencing therapies seem to work when reducing mutant huntingtin expression by only 50% indicates that some amount of toxic protein can be effectively handled by cells. Additionally, as previously noted, there are many unknowns associated with mRNA processing. It is thus possible that the non-exon 1 silencers may, in fact, downregulate exon 1 transcripts, in addition to the full-length messages, noted Diane Merry and Davidson.

Bates proposed that researchers simply add a silencing agent that targets the exon 1 transcript to their collection of candidate silencers and continue with their ongoing studies. Davidson agreed and added that, although some of the exon 1 silencers that were originally tested showed toxicity, it is likely that her team and others' can identify alternative sequences that do not have these off-target effects. A reasonable prediction is that targeting exon 1 will result in additional benefits, potentially quite significant, to those already observed with currently used silencers.

***Cellular stress responses***

Directing their attention downstream of mutant huntingtin production, participants subsequently discussed new findings on the links between cellular stress and HD. Multiple lines of evidence point to the involvement of normal huntingtin in cellular stress responses; indeed, huntingtin has been described as a multi-functional stress-response element. At the same time, mutant huntingtin causes cellular pathology that elicits stress responses which appear to be ultimately overwhelmed by the accumulation of toxic protein. Thus, a combination of gain- and loss-of function alterations in cellular stress response systems appears to be a key element of HD pathology. Consistent with this idea, Carl Johnson noted that a new RNAi screen in *C. elegans* performed by Christian Neri's team revealed 662 genetic modifiers of mutant huntingtin toxicity, many of which are genes involved in stress resilience.

*NADPH oxidase and its potential role in HD pathology*

Providing participants with new insights into the nature of oxidative stress in HD, Marian DiFiglia described recent work from her lab showing elevated NADPH oxidase (NOX) activity in HD. DiFiglia explained that her team was looking for reactive oxygen species (ROS) in the brains and primary neurons of HD140Q mice based on previous observations that individuals with HD have oxidative damage in the brain. The researchers found much higher levels of ROS in HD neurons compared to wildtype, even at very early stages of disease (3 months of age in striatum and cortex). Consistent with their findings in brain, the team observed an early increase in ROS in cultures of primary HD neurons. Elevated ROS, as well as oxidized methionine, were also observed in synaptosomes derived from the striatum (6 months of age) and the cortex (12 months of age).

The findings were surprising because previous studies implicated mitochondrial dysfunction as a source of ROS overproduction in HD neurons, but the mitochondrial dysfunction occurs later than the ROS elevations

observed by DiFiglia in both animal tissues and cultured cells. Thus, the researchers looked for other, non-mitochondrial sources of ROS, including NOX, a generator of ROS which is well-known in the infectious disease literature for its role in phagocytic killing of bacteria and viruses. Reviewing the NOX literature, DiFiglia and colleagues found that NOX 2 and NOX 4 are expressed by neurons and microglia. Testing various NOX inhibitors, the team observed the most robust inhibition of ROS production and enhanced cell survival using VAS2870, a small molecule that inhibits NOX 2 activity. The researchers then crossed HD140Q knock-in mice to knock-out mice lacking the gp91-phox subunit of NOX2 and observed lower neuronal NOX activity, normalized ROS levels, and significantly improved survival.

The findings are of interest because they point to an early alteration in HD that could act as a trigger for other, later-stage changes in neuronal function. For example, William Yang said his team has observed activation of ATM, a serine/threonine protein kinase activated by DNA double-strand breaks caused by oxidative stress, in BACHD mouse models. In addition, preliminary findings from his group indicate the presence of several oxidative markers in 12-month-old BACHD mice. Also, DiFiglia observed a normalization of ROS levels in response to treatment with brain derived neurotrophic factor (BDNF), linking her findings to previous studies showing HD is associated with reduced levels of BDNF in the striatum and BDNF treatment can rescue HD phenotypes.

In an effort to tease out the molecular mechanism underlying the elevation in NOX activity, DiFiglia and co-workers examined the activity of Rac 1, a protein required for the assembly of NOX2 components at lipid rafts. Significantly, a rise in Rac1 binding activity in HD neurons preceded the increase in NOX activity and ROS levels, consistent with Rac 1 being involved in the initiation of the process. Interestingly, mutant huntingtin appears to interact with NOX in lipid rafts, but is not pulled down by antibodies to the phox subunit.

Mary Kennedy asked about the potential role of interleukin-6 (IL-6) in the activation of NOX2. According to a study of schizophrenia, the neuronal production of IL-6 is necessary and sufficient for NOX activation mediated by ketamine, an NMDA receptor antagonist that produces psychosis in humans and exacerbates the symptoms of schizophrenia. Scott Zeitlin added that IL-6 upregulation has been reported in the early stages of HD. To investigate the potential connection of DiFiglia's findings with IL-6, Kennedy suggested using IL-6 knockout mice.

Participants also wondered whether NOX activation associated with HD occurs in non-neuronal cells. DiFiglia has yet to examine peripheral tissues, but she said that microglia are not likely to play a significant role because the highest NOX activity her team has detected in brain has been at the pre-symptomatic stage, well before neuroinflammation and concomitant microglial activation are observed. In addition, DiFiglia's studies with primary neurons in vitro do not include microglia.

Looking ahead, DiFiglia said her studies suggest that finding new ways to treat non-mitochondrial oxidative damage could result in novel candidates for therapeutic intervention. Several anti-oxidants have been tested for their ability to treat neurodegenerative disorders, including HD, but have failed to yield robust results. It is possible that agents that specifically target NOX activity may result in better outcomes. DiFiglia's team is now testing the effects of the VAS2870 inhibitor in mice and hoping to test other small molecule inhibitors that might be more specific for NOX2 (VAS2870 has been reported to also affect NOX4 activity). Carl Johnson asked about potential side-effects and wondered if the animals might suffer from an increased susceptibility to infections. So far, DiFiglia noted, the animals appear to be healthy, but the team is still collecting data.

Another approach to addressing the oxidative damage in HD is to make vulnerable molecules more resilient to oxidation. As described by Johnson, deuteration—replacing hydrogen atoms with deuterium—is being examined by some groups as a means to protect key molecules from oxidation associated with neurodegenerative disorders. In particular, researchers are using deuteration to create more robust polyunsaturated fatty acids, which are normally very sensitive to ROS and often associated with

neurodegenerative damage. These deuterated molecules could be provided in the diet as nutritional supplements.

*Targeting proteostatic processes*

Participants also discussed options for the therapeutic targeting of proteostatic processes—cellular functions that coordinately maintain a healthy proteome and are regulated by stress-responsive signaling pathways. For example, Kelly explained that his team is using small molecules to manipulate the stress-associated unfolded protein response (UPR) which may help treat diseases characterized by aggregate formation. The UPR has three branches that regulate the expression of numerous genes that maintain homeostasis in the ER, or induce apoptosis if ER stress persists. Using small molecules to selectively modulate the transcription of genes regulated by a single branch of the UPR, Kelly succeeded in reducing the levels of mutant transthyretin, which causes transthyretin amyloidosis, without affecting the levels of the wildtype protein. Similar strategies, noted Kelly, could be used to treat other aggregate disorders, including HD. Compared to traditional drugs used to target cellular stress responses, such as the heat shock protein inhibitor geldanamycin, Kelly's approach allows for more precise and subtle interventions. Indeed, Kelly envisions the development of a new class of finely-tuned medications that would be taken only once every few weeks.

Another proteostatic mechanism discussed at the workshop was macroautophagy—a lysosomal mechanism involved in the degradation of aggregated proteins which is activated during ER stress. Describing a recent review by Ai Yamamoto and colleagues, Scott Zeitlin noted that an increasing number of studies are focusing on the potential benefits of upregulating macroautophagy to treat neurodegenerative disorders. However, its function and regulation vary across different neuronal populations and different diseases, and enhancing its levels may result in neurotoxicity in some cases. For example, in stroke and amyotrophic lateral sclerosis boosting macroautophagy appears to be detrimental.

In HD, noted Zeitlin, the picture is complicated because of huntingtin playing a role in macroautophagy, as well as being a substrate of macroautophagy. Macroautophagy helps clear mutant huntingtin aggregates, suggesting that increasing its levels would be beneficial. But one must also consider wildtype huntingtin's potential role in cytosolic cargo recognition and how mutant huntingtin interferes with this process, as reported by Ana Maria Cuervo's team. In addition, the pathological landscape of HD is ever-changing, so that a treatment that might be beneficial at one stage, may be harmful at another. For example, Cuervo and colleagues have found that chaperone-mediated autophagy (CMA) appears to be upregulated in HD in response to the dysfunction of macroautophagy. The researchers have suggested that this compensatory mechanism may be helpful in the early stages of HD, but its efficiency may decrease with age and contribute to cellular failure in later stages, when pathology becomes more evident.

Yet another consideration is a recent observation from Al LaSpada's team indicating that HD-associated alterations in transcriptional networks result in reduced activity of a master regulator of macroautophagy, the transcription factor EB (TFEB). Nine years ago, LaSpada's team identified a thermoregulatory defect in HD mice which they traced to the dysregulation of the peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC-1α), a regulator of mitochondrial biogenesis and oxidative stress. LaSpada subsequently showed that overexpressing PGC-1α rescued the motor phenotype of HD mice and virtually eliminated huntingtin aggregates. The researchers further established that the rescue was partially mediated by the reduction of oxidative stress, but it was insufficient to completely explain the results. LaSpada wondered if TFEB, which is activated by PGC-1α, was playing a role. Activation of TFEB promotes the removal of damaged mitochondria, but LaSpada also found that it promotes huntingtin turnover and the elimination of aggregates. Furthermore, TFEB activation alone was able to reduce huntingtin aggregation and neurotoxicity. LaSpada is now exploring TFEB's potential as a therapeutic target for HD, testing the effects of various inhibitors of TFEB phosphorylation.

*Links between metabolism, oxidative stress and proteostasis*
LaSpada emphasized that the links between energy production, oxidative stress, and autophagy may provide additional targets for therapeutic intervention. Leslie Thompson pointed out an important link between autophagy and oxidative stress mediated by p62, a protein that interacts with ubiquitin and triggers autophagy, but also interacts with Keap1 to stabilize Nrf2, a master regulator of a protective pathway against oxidative and electrophilic stress. LaSpada agreed with the potential importance of these interactions and added that the various processes regulated by PGC-1α are interconnected in additional ways that may be amenable to modulation. For example, PGC-1α activates mitochondrial biogenesis, which leads to increased ATP production, as well as increased ROS generation. PGC-1α's concomitant activation of anti-oxidation pathways helps prevent oxidative damage. Increased mitochondrial biogenesis is also linked to greater mitochondrial turnover which is enabled by PGC-1alpha's activation of TFEB.

Because PGC-1α regulates so many cellular processes, LaSpada considered it is unlikely to prove useful as a target, but several downstream effectors, in addition to TFEB, seem promising. For example, LaSpada is looking for modulators of PPAR-δ, a transcription factor activated by PGC-1α that regulates lipid and glucose metabolism and is abundantly expressed in the brain. PPAR-δ appears to interact with huntingtin protein and LaSpada found that a dominant-negative mutation of PPAR-δ results in an HD-like phenotype (although the damage is more widespread).

The pharmaceutical industry is working hard to develop drugs that target PPAR proteins and, although most efforts are focused on PPAR-γ, LaSpada thinks that modulators of PPAR- δ will also emerge from these searches. Bexarotene, for example, is a potent agonist of retinoid X receptors which was recently suggested to act through PPAR-γ, yet LaSpada thinks it could be working through PPAR-δ, instead or in addition to PPAR-γ. The drug is of particular interest because it was recently reported to dramatically reduce the symptoms and pathology of mouse models of Alzheimer's disease (AD) and it has already been approved for use in humans to treat lymphoma. Bexarotene has significant side-effects when used to treat cancer, but LaSpada said lower doses are likely to be effective for treating neurodegenerative disorders. LaSpada is pursuing bexarotene, as well as other PPAR agonists, as candidates for treating HD and other neurodegenerative disorders. Although the mouse study using bexarotene to treat AD is somewhat controversial, PPARs are increasingly being viewed as attractive targets for drug development.

Diana Rosas noted that observations from her team are consistent with PPAR-δ being a potential therapeutic target for HD. She explained that HD is associated with many metabolic alterations— early changes in the hypothalamus, as well as weight loss, appetite changes, hypoglycemia, and mitochondrial dysfunction. And of particular relevance to LaSpada's findings, Rosas noted she has observed small thermoregulatory changes in individuals with HD. Moreover, Rosas pointed out that some brain areas that are very metabolically active, such as the cuneus in the occipital lobe, are damaged very early in HD.

Participants were encouraged by these new insights into HD and the possibilities for developing new treatments. Marc Diamond wondered, however, whether these lines of investigation will help design therapies that are specific for treating HD or generally effective for reducing stress-related neuropathologies. In particular, Diamond noted that both DiFiglia and LaSpada implicate mutant huntingtin in their models of HD pathology, but also suggest that the strategies to normalize the HD-associated alterations could help treat other neurodegenerative disorders.

DiFiglia noted that mutant huntingtin seems to interact with NADPH oxidase in lipid rafts, but the consequences of this interaction are as of yet unknown. Also, Carl Johnson noted that increased NADPH oxidase activity has been implicated in the pathology of other neurodegenerative disorders. Similarly, LaSpada identified a physical interaction between huntingtin and PPAR-δ, but its consequences have yet to be

established. LaSpada noted that alterations in the pathways regulated by PPAR-δ, and more generally PGC-1α, are associated with various neurodegenerative processes, including aging.

Specific therapies are usually sought after to minimize the possibility of detrimental side-effects. On the other hand, if a therapy can help treat several disorders that share features in common, including highly prevalent disorders, the therapy will have more potential applications and its development will be easier to fund. It is possible that some elements of DiFiglia's and LaSpada's investigations will turn out to be generalizable to several disorders, but specific therapies may need to be tailored to individual diseases. Identifying whether the pathologies described above are directly and specifically mediated by mutant huntingtin or not should aid in focusing future directions for therapy development.

***Insights from other diseases and models***

Participants also discussed new ideas that have emerged from the study of other diseases and model systems that could be relevant to HD. For example, Paul Patterson explained that recent studies suggest that manipulating the bacterial population inhabiting the digestive system, the gut microbiome, can help treat many diseases. Patterson noted that probiotics can prevent and cure colitis in mice and Jeff Kelly pointed out that fecal transplants have been successful at treating bowel infections in humans. Even systemic diseases, such as sepsis, have been cured by modifying the gut microbiome, without the use of antibiotics. Equally amazing, some neurological disorders appear to benefit from microbiome manipulations as well. For example, in a recent study using the EAE mouse model of multiple sclerosis (MS), researchers found that alterations in intestinal flora contribute to disease development and normalizing the bacterial population helped ameliorate MS symptoms. Moreover, noted Patterson, a study in mice showed that infection by the bacterium Helicobacter pylori, which causes stomach ulcers, appeared to contribute to the subsequent development of Parkinson's disease-like symptoms.

Working with models of autism and schizophrenia, Patterson has found that these mental disorders may also be amenable to treatment with intestinal bacteria. Patterson and his co-workers had previously found that pregnant women who suffer an infection at a particular stage of pregnancy have a higher risk of having babies who develop autism or schizophrenia. Now the researchers have observed in animal models of autism that, if they treat the offspring at the time of weaning with certain gut bacteria, they can prevent the development of the mental disorder. Patterson's hypothesis is that the offspring have increased intestinal permeability as a result of their *in utero* exposure to the mother's immune response to infection. This leakiness can be corrected, however, by populating the babies' guts with a particular mix of bacteria.

Participants were intrigued by the possibility of manipulating the microbiome to treat or ameliorate HD symptoms. Marie-Françoise Chesselet pointed out that there is some evidence indicating that individuals suffering from HD have a higher incidence of abnormal intestinal permeability. Furthermore, Rosas noted that a metabolite made by the gut flora is reduced in HD and, as HD progresses, intestinal problems become more common and more severe. Rosas said she often advises her patients to take probiotics, which seems to help.

Although only briefly mentioned, another emerging model of HD pathology which is linked to other neurodegenerative disorders is the prion model. As noted by Jeff Kelly, studies in yeast have shown that some proteins with expanded repeats behave as prions, acquiring a misfolded state which they propagate by acting as a template to guide the misfolding of more proteins into the prion form. Some investigators are currently exploring the possibility that HD is a prion-like disease in which mutant huntingtin triggers a chain reaction that produces large amounts of misfolded proteins that form fibrillar aggregates.

Moreover, an unlikely source of insight came from Carl Johnson's description of a new study of the Dictyostelium discoideum genome revealing a very large number of triplet repeats within its 6000 genes. Amazingly, roughly half of the coding sequences are occupied by 3000 triplet repeats, many of which encode polyglutamine (but also polyasparagine, polyserine, and polythreonine) stretches between 25 and 70 repeats in

length. Johnson noted that the Dictyostelium genome has over 70% GC content and an extraordinary degree of protein sequence variation—even among amoebas living in the same geographic region. Furthermore, the variation in repeat loci is similar in coding and non-coding regions. Johnson noted that there is no other known organism like this. The authors of the article suggest the repeats are selfish DNA elements that are well tolerated with little or no selective pressure bearing upon their presence. In the context of HD, and other triplet diseases, understanding the mechanisms that underlie Dictyostelium's lack of vulnerability to polyglutamine repeats might offer valuable insights for therapy development.

**Thinking Ahead: Clinical Trials**

As more candidate therapies are being developed, and a few promising ones are moving into the clinical arena, the need for improving the current tools used in clinical trials and developing new ones is becoming increasingly urgent. As noted by Jang-Ho Cha, even with a well-validated target in hand, without the proper tools to monitor HD in humans, it will be difficult to evaluate its clinical value and interest industry in supporting its development.

Cha noted that candidate treatments for HD fall into two general categories: disease-modifying and symptom-targeting. To test the first category of agents, clinical trials will need to include large numbers of patients and sensitive, robust tools for monitoring disease because of the long, slow progression of HD. The most critical stage to be measured in these cases will be the transition into the stage in which pathology becomes detectable—the time when these therapies will likely be most useful.

***General clinical challenges***

One of the problems with developing these much-needed biomarkers is the variability of HD's symptoms. As noted by Diana Rosas, both motor alterations and cognitive decline are difficult to quantify reliably and their variability is large. The most recognized source of variability is between individuals—the expression of HD, as well as its underlying pathology, varies greatly from one person to another. Some individuals with HD have a predominance of motor symptoms, others mood/cognitive symptoms, and still others a combination of both—a variability that is reflected in brain pathology. The root cause of this variability remains unknown. CAG repeat length is known to affect age of onset to a moderate extent, but there are no other identified modifiers of disease known in humans. As noted by Carl Johnson, a handful of genetic modifiers were reported, but upon further examination, they were withdrawn. Nancy Wexler has identified approximately six genetic loci that appear to harbor genetic modifiers of HD, but the specific genes have yet to be mapped.

Furthermore, there is intra-individual variability. Individuals can present with different symptom severity on different days and at different times of the day. Moreover, individuals' performance on tests is affected by practice. Variability between physicians' assessments of HD is yet another challenge. As noted by Rosas, there is great variability in the scoring of chorea, even amongst experienced physicians. In addition, cognitive tests are also problematic, partly because changes in some aspects of cognition, such as memory, occur slowly over time. It has proven very difficult to design tests to evaluate both motor and cognitive symptoms that generate reliable results.

Yet another complication, noted Rosas, is that the progression of HD is highly non-linear. Even the changes detected by imaging do not follow a smooth evolution. Very rapid changes may occur in one brain region, which may then stay constant for some time—perhaps as compensatory mechanisms come into play—and then change quickly again as the compensation is overwhelmed.

These problems are not unique to HD, however. Neil Aronin and David Housman pointed out that cardiovascular studies have long been encumbered by patient variability. The cholesterol-lowering drugs known as statins, for example, were first thought to help treat and prevent cardiovascular disease in general, but now appear to be beneficial to only a fraction of people. Furthermore, as noted by Aronin, changes in markers for

many diseases follow a biphasic curve. For example, cancer markers often decline at some point during the course of the disease, even though the cancer cells are still growing. As noted by Cha, at least the variability in HD has been identified before major drug trials are underway, so there's an opportunity to address the issues before large amounts of money and effort are invested.

A key strategy to deal with HD variability, noted Rosas, will be to eschew cross-sectional studies in favor of longitudinal ones. Rosas explained that a cross-sectional snapshot of a group provides very little information due to the large degree of variability and non-linearity of disease progression. Paul Patterson added that following subjects individually, comparing the scores of each subject over time, will be particularly useful. Because HD pathology proceeds so gradually, Rosas considered that new algorithms and tools will be needed to detect small changes over time. Aronin further suggested identifying the stages of HD in which pathological or symptom-based changes behave more linearly. Cha said this strategy is being used in Alzheimer's disease trials where the most measurable stage of the disease is used for evaluating candidate drug efficacy. Moreover, Rosas suggested working harder to develop composite scores, which should help reduce the variability and increase the sensitivity of current tests to monitor HD.

Selecting different biomarkers for tracking different stages of the disease will also be important, noted Rosas. For example, the change in striatal volume is a good marker for tracking presymptomatic HD, but ceases to be reliable at later stages of disease. Understanding what aspect of the disease a particular marker reflects is key to deciding when and how to use it, but this principle is not always carefully considered. For example, Rosas noted that the value of using of 8-Oxo-2'-deoxyguanosine (8-OH-dG), a major product of DNA oxidation, as a biomarker for HD has recently been called into question. Rosas explained that its value can only be assessed relative to the means and purpose of its use. As a general marker of HD progression, said Rosas, 8-OH-dG is not very useful because it is an indicator of only one facet of HD pathology, oxidative stress. Furthermore, many individuals suffering from HD take anti-oxidant supplements (sometimes even surreptitiously during clinical trials).

For certain applications, however, 8-OH-dG levels can be very informative. As noted by Rosas, an elevation in 8-OH-dG levels in HD plasma can be detected roughly 10 years before the onset of symptoms (if the individual is not taking anti-oxidants). It has also been shown, by Rosas and colleagues, that creatine decreases 8-OH-dG levels in a dose-dependent manner. Thus, 8-OH-dG is useful as a biomarker for anti-oxidant treatments, provided the subjects are not taking additional anti-oxidant supplements. (Rosas also clarified that various groups have used slightly different assays to quantify 8-OH-dG which have yielded somewhat different results. However, within a single research site, using the same assay system, the results have been consistent.)

Another challenge that needs to be addressed, noted David Housman, is the paucity of quantitative data emerging from pre-clinical studies in animal models of HD. Housman considered that the current means by which the effectiveness of candidate therapies are tested in animals—e.g., using the rotarod test or measuring survival rates—do not yield sufficiently quantitative and informative data to optimally guide clinical tests. Roger Albin noted that, because clinical testing of HD therapies is in its initial stages, it is difficult to know which measures and which model systems will be most predictive of human responses.

***HD clinical strengths***

Despite these challenges, participants noted that HD has several features that make the testing of candidate therapies more accessible than in other diseases. For example, there is a large number of animal models available for studying HD—dozens of models have been generated in mice, in addition to models in rats, sheep and birds. As noted by Marian DiFiglia, each model has its strengths and weaknesses such that, as a collection, they allow for testing of many different facets of HD, as well as helping confirm the value of promising candidates.

Participants discussed a new model of HD, the Q175 knock-in mouse line, illustrating how it brings its own set of advantages and limitations to the HD toolkit. The Q175 model was developed by PsychoGenics and is derived from a spontaneous expansion of a knock-in model developed by Scott Zeitlin. As explained by DiFiglia and Al LaSpada, homozygote, and to a lesser extent heterozygote, Q175 mice develop symptoms and aggregates earlier than other knock-in models, but have a slower progression of the disease than R6/2 transgenics. Several participants found the phenotype to be too aggressive for their applications. For example, Marie Françoise Chesselet noted that the early development of symptoms precluded her from using these mice because her experiments cannot be carried out in very young animals with small brains. In addition, Gill Bates said the repeats in this model are very unstable, with a tendency to increase.
On the other hand, for some applications, Q175 might prove useful as it purportedly provides a longer window to follow disease progression, allowing the study of chronic drug administration, for example. The overall value of this new model will become clearer, noted Bates, as more research groups characterize and test it.

Another feature that helps strengthen HD clinical research, noted by Nancy Wexler, is that there are many people with HD, or at risk for HD, willing to participate in clinical trials. In addition, the root cause of HD is well-defined and unique—the only source of the disease is a single mutation, in a single gene. In addition to this being a diagnostic advantage, it also bodes well for the development of gene silencing therapies. And as noted by Carl Johnson, if the gene silencing approaches currently under development succeed, their effects may be so dramatic that they will be clearly detectable, despite some of the previously mentioned challenges.

Another advantage of the HD clinical pipeline is that several of its candidate therapies might prove useful for the treatment of other neurodegenerative disorders. As previously noted, this means that industry might be more willing to invest effort and money for their development. Ideally for the HD community, HD would be selected as a model disease for pioneering studies of new treatments. Cha noted that companies will often choose to run tests in the "cleanest" disorder, the one in which potential therapeutic effects can be measured most precisely and efficiently. He agreed with other participants that HD has several advantages over other neurodegenerative disorders, but added that better assessment tools are still needed for measuring progression in clinical trials.

## ***The search for new biomarkers***

One of the best assessment tools for HD progression currently available is imaging. In particular, several MRI measurements identified by Rosas and colleagues, such as cortical thinning and white matter alterations, appear to serve as reliable, quantitative markers of HD progression in humans. However, as previously stated, additional markers are sorely needed.

### *Inflammation markers*

Paul Patterson wondered if inflammation markers may prove useful, given that inflammation occurs early in HD. In addition, measuring inflammatory cytokines using assays such as the enzyme-linked immunosorbent assay (ELISA) is relatively straightforward. Beverly Davidson added that there are easy-to-use, inexpensive kits that provide whole cytokine profiles. However, as noted by Gillian Bates, cytokine levels in HD have been examined by some research groups, including Sarah Tabrizi's, and the changes observed have been subtle compared to those that occur during infection.

William Yang and Roger Albin noted that there are new inflammation markers currently under study, including one for microglial activation, that might be more sensitive. Also, recent studies by Oleg Butovsky and colleagues suggest that miRNAs associated with inflammatory monocytes may serve as markers of inflammation, at least in some neurodegenerative disorders such as amyotrophic lateral sclerosis. Key to determining the value of new markers, noted Albin, will be an evaluation of their signal-to-noise ratios.

Stored blood samples could be used to conduct preliminary evaluations of the presence of inflammation markers in HD, noted Carl Johnson, and Patterson added that the new sheep model of HD could be recruited for marker identification. Marian DiFiglia added that monitoring a marker in a constrained brain region—such as

the capsule where activated microglia are abundant, for example—might increase sensitivity and decrease variability. Even if inflammatory markers don't prove useful as general indicators of disease progression, they might be helpful for tracking target engagement of anti-inflammatory compounds, noted Jang-Ho Cha.

*Biomarkers to track gene silencing therapies*

Participants agreed that identifying biomarkers of target engagement should be a top priority in the field. Given that gene silencing agents are quickly advancing to the clinic, a major focus should be finding biomarkers for these agents in particular—i.e., methods to measure mutant huntingtin mRNA and/or protein.

Choosing which huntingtin mRNA or protein species to monitor, and finding sensitive, reliable markers to do so is challenging, however. Attempts to track huntingtin protein levels face the difficulty of detecting huntingtin's many different forms—soluble protein, fragments and various types of aggregates. As explained by David Housman, the detection and efficiency of extraction varies significantly between different aggregate forms. DiFiglia asked if it was possible to measure and visualize soluble huntingtin reliably in cells or tissues. Marie Francoise Chesselet suggested a particular antibody and Scott Zeitlin noted that a triple-label assay can provide reliable visualization, but not in a quantitative manner. Furthermore, the state of huntingtin is dynamic, noted Carl Johnson, and tracking a single protein species will almost certainly yield an incomplete picture of the status of huntingtin protein levels. For example, when inclusions form, soluble huntingtin decreases dramatically.

In addition, the relationships between the levels of mutant huntingtin mRNA, protein, aggregates, and pathology are complex. Housman noted that, although many assume that mRNA levels reflect protein concentrations, there is actually no clear correlation between the two. One way to assess the levels of mRNAs that are actually being translated into protein and obtain a protein production rate, said Housman, is to analyze polysomes and their degree of ribosome loading. DiFiglia added that the relationship between a tissue's levels of huntingtin expression and its degree of pathology is another complex relationship that is not linear. Huntingtin is expressed universally throughout the body and the slight variations in expression levels across tissues do not seem to correlate with tissue susceptibility to HD. For example, DiFiglia noted that huntingtin mRNA levels are relatively high in the globus pallidus, yet inclusions are absent.

Participants agreed that having a technology to monitor mRNA levels directly would be extremely useful to measure target engagement in gene silencing therapies. Housman noted that his team has quantified steady state levels of huntingtin mRNA species using RPKM, a method of quantifying gene expression from RNA sequencing data. In addition, in situ hybridization can be used to both quantify and localize mRNA levels. Although useful for conducting pre-clinical studies, these techniques do not offer a method to monitor mRNAs in human brains non-invasively—a requirement for tracking gene silencing in clinical trials.

A new method, still in its early stages of development, may offer this possibility, however. Eric Wickstrom described work by his team using peptide-nucleic acid (PNA) chimeras to label and track specific mRNAs in living animals using positron emission tomography (PET). PNAs are oligonucleotide analogues in which the sugar phosphate backbone of a nucleic acid is replaced by a synthetic peptide backbone which is resistant to enzymatic cleavage. Using hybridization modeling algorithms, Wickstrom has designed 12-mer PNA probes against various cellular mRNAs, including cyclin D1, c-ras, myc, and monoamine oxidase type A (MAO-A). The hybrid melting temperatures are approximately $65^0$C under physiological conditions, a feature that allows single mismatch differentiation.

Wickstrom has conjugated PNAs to various labels, including fluorophores to monitor the probes in living cells in culture. In addition, he has produced PNA probes labeled with gadolinium, a tracer commonly used in medical imaging, and is now working with a collaborator to label probes with a chelating agent attached to copper-64, another imaging label approved for human use. The kinetics of the PNA probes in vivo are well-defined: they take approximately two days to peak and the washout of unbound probe has a 12.8 hour half-life.

Thus, Wickstrom usually measures signals 24 hours after probe administration. Unbound probe is very stable and comes out intact in the urine.

PNAs don't penetrate cell membranes, so Wickstrom has attached peptide ligands to enable receptor-mediated intracellular delivery. For example, he recently designed a PNA probe to detect MAO-A mRNA which he linked to a μ-opioid receptor targeting peptide. The probe was then successfully delivered, through receptor-mediated endocytosis, to human neuroblastoma cells expressing these opioid receptors. The delivery ligands are engineered to be protease-resistant and bind with high affinity to their receptors ($K_d$ assessed by flow cytometry = 11.6 nM). In vivo tests in animal models with cyclin D1, c-ras and myc probes yielded robust, reproducible results.

To apply this methodology to tracking huntingtin mRNA, Wickstrom is generating PNAs that include the initiator ATG of huntingtin mRNA as their target. He noted that sequences including initiation codons have worked well for other mRNAs, perhaps because they present well to the PNA probes. Johnson suggested making several probes to compare their performance, but Wickstrom said his team usually works with a single probe and its control (the same sequence with a single mismatch). Wickstrom explained that his algorithms to select sequences have so far yielded highly effective probes and, furthermore, it is too laborious and time-consuming to prepare large sets of probes—it takes one month to produce a probe and approximately two months to run the microscopy tests with cells in culture.

Wickstrom also explained that to track huntingtin mRNA in the whole brain, a cell-penetrating peptide could be used to deliver the PNA probes. Participants were concerned about the effectiveness of these peptides, however. Although the detailed mechanisms of how cell-penetrating peptides work are still under investigation, it is now generally thought that endocytosis is usually involved in the translocation mechanism. A potential problem with this mechanism is that endosome entrapment of the peptides' cargo can occur. Wickstrom noted that, so far, his PNA probes have reached the cytosol efficiently when delivered through receptor-mediated endocytosis, so he does not foresee this problem being an issue. Nevertheless, Johnson cautioned that the peptides have sometimes worked in vitro, but failed to live up to their potential when tested in vivo. As reported in a recent review by Asrtid Graslund and colleagues, many factors affect the peptides' uptake pathway, their intracellular distribution and their cytotoxicity, including cargo size, type, and linkage, the presence of fluorescent labels, cell type, temperature, and incubation time.

Another consideration noted by Johnson is that, because PNAs are small charged molecules, intrathecal delivery in humans will probably allow the peptides to reach the cortex efficiently, but not the basal ganglia. Diana Rosas added that variability in the height of individuals will affect the distribution of PNAs. In addition, she noted that the cortical thinning associated with HD might make it difficult to resolve the PNA signal in the cortex. To address this potential problem, she suggested using hybrid PET/MR imaging. The high spatial resolution provided by MRI, together with the sensitivity of PET, could not only improve probe detection in thin cortices, but generally allow a broader range of detection, including the visualization of areas with modest target-to-background ratios. DiFiglia wondered if using receptor peptide ligands to target delivery to cortical cells—and bypass the need for cell-penetrating peptides—might also be worthwhile. To label striatal cells, intraventricular delivery would likely be required, and a different receptor ligand could be used for this purpose.

The major question discussed by participants, however, was whether Wickstrom's technique will be sensitive enough to measure the expected reductions of 25-50% in huntingtin mRNA resulting from gene silencing treatments. Wickstrom considered it feasible, but emphasized that the signal-to-noise ratio will depend greatly on huntingtin mRNA concentrations within cells. If he can detect huntingtin mRNA in cultured cells with at least a three-fold signal over controls, Wickstrom considered it will be worthwhile proceeding with animal testing. Marc Diamond noted, however, that the background signal in vivo is likely to be much greater than in vitro, and worried that three-fold might be too low of a threshold for a go/no-go decision. Neil Aronin added that using a similar technique to study tumors, his group has found it difficult to quantitate their observations

because the signals are non-linear signals and significantly affected by variations in uptake, probe recognition, and probe deterioration.

Addressing these concerns, Wickstrom noted that robust PET signals are usually ten-fold stronger than background levels, but three-fold is considered a reasonable minimum to obtain reliable results. He also noted that the signal-to-noise ratios his team has observed in cultured cells for a particular probe, are similar to those observed in subsequent in vivo studies. Wickstrom also pointed out that PNA probes are very stable, such that probe deterioration is unlikely to be an issue. Once a probe binds, it stays attached to its mRNA for approximately 2 days. In addition, Wickstrom described his technique as highly quantitative, providing results that are generally equivalent to those generated by a scintillation counter, with inter-experiment variability of approximately 10%. Also, he noted that the use of dendromers to attach several labeling molecules to individual probes helps boost sensitivity. For example, Wickstrom has achieved clear visualization of several tumor mRNAs by attaching 2-8 gadolinium molecules to single PNA probes. Wickstrom acknowledged, however, that detecting changes in huntingtin mRNA in the brain will likely be more challenging than his previous work with tumors.

Diamond suggested making a careful comparison of Wickstrom's PNA probes with a well-established PET marker, such as dopamine transporter (DAT) PET ligands which are used to monitor presynaptic neuronal degeneration in Parkinson's disease. Comparing the probes' kinetics, sensitivitities, and signal-to-noise ratios may help predict whether the PNA probes will be clinically useful. Beverly Davidson commented that, even if the sensitivity of the huntingtin PNA probes turns out to be low, they may be sufficient to demonstrate target engagement in gene silencing experiments. Mary Kennedy added that obtaining linear signals for this purpose may be unnecessary.

Participants also discussed the potential toxicity of the PNA probes. Wickstrom noted that the probes are used in extremely small amounts—20 micrograms is a typical human dose. If huntingtin is expressed at very low levels, however, the dose may have to be adjusted to target a large fraction of the huntingtin mRNA population. And because the probes are designed to bind to the transcript initiation site, huntingtin translation could be blocked to a significant extent, noted Johnson. Moreover, Wickstrom noted that intrathecal labeling using PNA probes has yet to be approved by the FDA. However, another type of peptide, similar to PNAs, is close to being approved and Wickstrom suspects that PNA approval will be relatively quick and straightforward.

Kennedy and Davidson opined that, although all these considerations are important, only by running experimental tests will it be possible to practically evaluate PNAs' potential as biomarkers for huntingtin mRNA. With this in mind, participants discussed how best to optimize initial experiments to assess the feasibility of the technique. After considering several options for the in vitro experiments, participants supported Carl Johnson's suggestion of using a commonly used, robust human cell line—the human embryonic kidney line HEK293. Johnson proposed knocking down huntingtin mRNA in these cells with silencing agents, and then assessing mRNA levels with RT-PCR and PNA fluorescent probes. Because the goal of this initial test is to assess whether PNA probes have the sensitivity to detect silencing of huntingtin mRNA, the use of neuronal cells and/or HD cells is not critical. Using human cells is important, however, to assess off-target effects using mismatched probes, noted Diane Merry.

Some participants suggested testing other cells in vitro before moving to animal models. However, Jang-Ho Cha considered it would be more efficient to start working with animals as soon as possible. Wickstrom performs micro-PET experiments routinely, such that the in vivo tests should be relatively straightforward. Several participants suggested using a conditional knockout mouse model of HD developed by Scott Zeitlin for these initial experiments. With the possibility of inactivating huntingtin expression in the forebrain and testis, this model should provide a good control to help assess the PNA probes' signal-to-noise ratio. Marian DiFiglia and Zeitlin added that embryonic stem cells from this knockout model could also be used for in vitro tests, if the HEK293 experiments need to be expanded. DiFiglia noted that Kimberly Kegel has homozygote and

heterozygote embryonic stem cells derived from the Zeitlin knockout mouse that can be differentiated into neurons.

Several other suggestions were put forth for the in vivo experiments. For example, Beverly Davidson proposed injecting the PNA probes into a mouse leg muscle and using the other leg as a control, and/or using one hemisphere of the brain as a control for the other. Diamond added that preclinical studies could be done in sheep to evaluate probe distribution—as noted by Wickstrom, it is impossible to resolve the striatum and cortex in mice using PET. The animal experiments are also expected to help determine how best to perform background subtraction. In his experiments so far, Wickstrom has imaged tissues that do not express the target mRNA to obtain measurements of background noise. Since huntingtin is universally expressed, however, this strategy will need to be modified.

**Concluding Thoughts**

Several challenges were discussed at the workshop, ranging from how to assess the role of aberrant huntingtin splicing in HD pathology to finding additional and better biomarkers of disease progression and target engagement. Participants tackled complex questions about the basic biology of HD, as well as brainstormed solutions to complicated technical problems. New challenges were identified and long-standing ones were re-examined.

Despite the hurdles, participants agreed that, now more than ever, HD is emerging as a tractable disease. Promising candidate therapies are moving forward into clinical and pre-clinical trials, and novel tools and insights are revealing new therapeutic targets. The path to success is coming into focus. As Anne Young declared at the end of the meeting, there is a growing sense in the field that “a cure is within our grasp.”