Pharmacodynamic Biomarkers for HTT-Lowering Therapies

A White Paper

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EXECUTIVE SUMMARY

Audience and objective

The purpose of this white paper is to inform the Huntington's disease (HD) community, including key stakeholders involved in the development of huntingtin protein (HTT) lowering therapeutics, about the strategy, timelines and current study status of the CHDI Foundation HTT-Lowering Biomarker Initiative.

Background

HTT-lowering is a key therapeutic strategy for HD. Reducing the amount of the disease-causing expanded HTT protein in HD-affected brains is predicted to reduce signs and symptoms and slow the progression of the disease.[1,2] Several approaches are being developed to lower HTT, including antisense oligonucleotides and siRNAs, and gene therapy using viral delivery of miRNAs, shRNAs, and zinc-finger repressor proteins.[2-10] To advance these therapeutics to the clinic, translatable HTT-lowering pharmacodynamic biomarkers are being studied in both preclinical HD models and observational clinical studies.

Our aim is to develop and validate outcome assessment measures that indicate that the delivery of an HTT-lowering therapy to the CNS does indeed lower the amount of HTT protein in HD patients.

Overview

Pharmacodynamic biomarkers are considered vital to confirm that a therapeutic agent will reach its intended target and have a biological effect. CHDI has committed to developing pharmacodynamic biomarkers of HTT-lowering to support HTT-lowering clinical development programs. We began this process by establishing a Task Force and holding a series of several workshops with internal and external experts to assess the landscape of potential biomarkers that could be developed in a timeframe consistent with the anticipated timing of clinical trials. We then prioritized several classes (referred to as 'domains') of biomarkers by both their scientific rationale and their likely timeline to the clinic, and began evaluating these in preclinical HD models.

Methodology

We are currently focusing on three domains

1) Static and dynamic CSF markers, in particular HTT protein quantification
2) Neuroimaging, including PET and MRS
3) Physiological measures, including qEEG

We are evaluating these domains using a consistent methodology that includes evaluation for clinical suitability (in observational studies) and preclinical proof-of-principle (PoP) validation.

While the scope of work under the auspices of the CHDI HTT-lowering Biomarker Task Force is focused exclusively on identifying pharmacodynamic biomarkers of HTT lowering, it should be noted that the Foundation has parallel efforts committed to identifying biomarkers of disease progression and disease state. Those biomarkers will be critical for PoP and efficacy trials. Many of the same domains of biomarkers that we have been evaluating as potential disease progression or state biomarkers—such as MRS, PET, and qEEG—are now also being evaluated as potential pharmacodynamic markers. While it is conceivable that a given domain of biomarker could have dual applications, it is important to evaluate each biomarker specifically for its intended use and context. Accordingly, the focus of this white paper will be on pharmacodynamic biomarkers of HTT lowering, but relevant information already obtained regarding the profile of some biomarkers in the course of the disease may also be highlighted.

Summary Results

	HTT Protein in CSF	PET Imaging	HTT PET Imaging	CSF Proteomics: Static	CSF Proteomics: Kinetic	qEEG	MRS
Biological plausibility	HIGH	MODERATE	HIGH	MODERATE	MODERATE	MODERATE	MODERATE
Measurable in humans	YES	YES	UNKNOWN	YES	YES	YES	YES
Technological feasibility	YES	YES	STUDIES ONGOING	YES	YES	YES	YES
Repeatable within subjects	YES	YES	YES	YES	YES	YES	YES
Reliably measured in HDGECs	STUDIES ONGOING	STUDIES ONGOING	TBD	STUDIES ONGOING	STUDIES PLANNED	YES	YES
Signal metrics: dynamic range, variance	STUDIES ONGOING	STUDIES ONGOING	STUDIES ONGOING	STUDIES ONGOING	STUDIES PLANNED	YES	YES
Measurable in HD animal models	YES	YES	STUDIES ONGOING	STUDIES ONGOING	YES	YES	YES
PoP: Changes in response to central lowering of HTT in animal models	STUDIES ONGOING	YES	STUDIES PLANNED	STUDIES PLANNED	STUDIES ONGOING	STUDIES PLANNED	STUDIES ONGOING
Changes in response to an HTT-lowering intervention in HDGECs	TBD	TBD	TBD	TBD	TBD	TBD	TBD

Legend: This table assesses the seven prioritized biomarker domains according to the nine established criteria. TBD = to be determined.

ABBREVIATIONS

ASOs	Antisense oligonucleotides
BAC rat	BAC HD rat model
BDNF	Brain-derived neurotrophic factor
BP_{ND}	Binding potential
CSF	Cerebrospinal fluid
CNS	Central nervous system
GP	Globus pallidus
HD	Huntington's disease
HDGEC	Huntington's disease gene expansion carrier
HTT (*HTT*)	Huntingtin protein (*gene*)
ICV	Intracerebroventricular (administration)
mHTT	Mutant huntingtin protein
miRNAs	MicroRNAs
MRM	Multiple reaction monitoring
MRS	Magnetic resonance spectroscopy
PET	Positron emission tomography
PoP	Proof-of-principle
Q175 HET	Q175 heterozygote knock-in mouse model of HD
Q175 HOM	Q175 homozygote knock-in mouse model of HD
qEEG	Quantitative electroencephalography
RNAi	RNA interference
RISC	RNA-induced silencing complex
SRM	Selected reaction monitoring
SNPs	Single-nucleotide polymorphisms
shRNAs	Short hairpin RNAs
siRNAs	Small interfering RNAs
TFC	Total functional capacity score
WT	Wild type
ZFPs	Zinc-finger repressor proteins

1. INTRODUCTION

1.1 Huntington's disease and huntingtin

HD is an autosomal dominant, progressive, neurodegenerative disorder caused by an expanded trinucleotide CAG sequence in exon 1 of *HTT*, which encodes a stretch of glutamines in HTT.[11] HTT plays a role in a variety of cellular processes, and is expressed ubiquitously with the greatest expression found in the CNS.[12] The mutant polyglutamine expanded form of HTT is cytotoxic leading to the hallmark pathology of HD, including pronounced atrophy of the striatum and other brain regions.[13] The temporal effect of mHTT is dependent on the number of CAG repeats in the gene, resulting in an inverse relationship between the age of motoric symptom onset and the CAG repeat size.[14,15]

There are no disease-modifying therapies for this neurodegenerative disease that is characterized by the development of progressive motor dysfunction, cognitive decline, and psychiatric disturbances.[16,17] Available treatments for HD are currently limited to the management of symptoms using drugs developed for other indications. To date, most of the potential therapeutic candidates evaluated specifically for HD have had limited success in clinical studies. At best these candidates have targeted individual pathways or mechanisms hypothesized to be downstream of the expression of mHTT, and the success of such approaches may require further elucidation of the mechanism by which mHTT causes disease. On the other hand, mHTT is the most proximal therapeutic target and may affect multiple mechanisms and downstream cellular pathways.[14,18] It is therefore believed that preventing or reducing the expression of mHTT (HTT lowering) is one of the most promising approaches for the treatment of HD.[1,2,19] At the present time, it is suggested that decreasing the levels of mHTT may be less likely to have unwanted side-effects and more likely to have efficacy on all aspects of the HD phenotype than therapies directed at downstream targets.[20]

1.2 Overview of HTT-lowering interventions

Several approaches are being developed to lower HTT, including antisense oligonucleotides and siRNAs, as well as gene therapy using viral delivery of miRNAs, shRNAs, and ZFPs.[2-9] Modulation of HTT levels at the transcriptional level has been achieved in experimental models by the use of ZFPs,[5,21] while at the translational level ASOs, siRNAs and miRNAs have all been used to lower HTT.[3,4,8-10] Approaches to increase the clearance of mHTT protein have also been addressed by several groups.[22-24]

These approaches can be separated into 'chemical' therapeutics, which includes ASOs, siRNAs and other small molecules, and gene therapy approaches that deliver a therapeutic by gene expression, such as ZFPs, miRNAs or shRNAs. If the particular agent targets a sequence common to the normal and mutant HTT allele, it is termed a pan-lowering agent. Alternatively, one can design allele-selective lowering agents that preferentially lower expression of the mutant rather than the normal protein by directing them towards either the expanded CAG repeat domain of the mutant allele or specific SNPs present in the mutant allele.[10,25-28]

ASOs are modified single-stranded DNA molecules that are designed to be complementary to the target mRNA and form a DNA/RNA hybrid complex that is degraded by the endogenous enzyme RNase H. siRNAs are double-stranded RNA molecules that are processed by the cytoplasmic endoribonuclease enzyme Dicer and assembled into a RISC. Activated RISC binds the target mRNA, resulting in mRNA cleavage and a decrease in gene expression. Current HTT-directed ASOs and siRNAs are unable to cross the blood brain barrier and must be directly delivered into the CNS, which has been shown to have discrete distribution limited to the specific targeted areas of the brain.[19,29] Therefore, the main challenge is the delivery of the potential RNAi agent to the brain and, consequently, the ongoing preclinical studies described in this report are necessarily based on direct infusion into the CNS, either to the brain parenchyma or into the CSF, similar to the expected administration in the clinic.

Gene therapy is the use of nucleic acids to deliver and express proteins or interfere with the expression of proteins. Example cargo constructs include miRNAs, shRNAs, and larger proteins including ZFPs.[30] For HTT lowering, several such agents have been developed for delivery by viral transduction into the brain parenchyma.

These novel approaches are rapidly progressing towards the clinical phases of development; however, a key barrier is the lack of appropriate pharmacodynamic biomarkers that adequately demonstrate target engagement and PoP for HTT-lowering effects. Rapid identification of appropriate biomarkers will decrease the risk of having clinical trial results that are not interpretable due to uncertainty regarding the potential clinical outcome.

1.3 Pharmacodynamic biomarkers

Biomarkers are now a cornerstone of the drug development process, and may combine physiological and molecular measures of disease.[19,31] Biomarkers are considered essential for early Phase I/II clinical trials and can be categorized as *trait* (biomarkers which are stable over time), *state* (biomarkers which change with disease progression or treatment), and *pharmacodynamic* (sometimes referred to as mechanism of action markers).[19]

Biomarkers allow drug developers to make good and timely decisions regarding the clinical development of an investigational drug. If a therapeutic candidate does not affect a relevant biomarker, ideally in a dose-dependent manner, this will call into question whether to continue the development of the compound. The earlier this happens, the better, as this will save the financial and human expense of late phase clinical trials and encourage more effort in the design of alternative candidates or dosing strategies.

In the absence of evidence that an investigational treatment actually reaches its intended target and has a mechanistic effect, it is impossible to fully interpret trial results. Consider, for example, a Phase I trial of a potential HTT-lowering therapy that ends without signs of undue toxicity and allows a conclusion of good tolerability. This result is only trustworthy regarding potential intervention-specific effects if the therapeutic agent actually reached its intended target and lowered HTT in the brain. If it does not lower HTT levels in the brain, or it cannot be demonstrated that it did, no conclusions can be made about the safety of 'on-target' HTT reduction. Such a trial can still be informative for issues related to the technique of administration but not beyond.

In a typical clinical development plan, safety and tolerability are first assessed in healthy volunteers in Phase I studies. Maximum tolerated doses can be determined by escalating dosage until adverse effects are seen. Demonstration of target engagement and PoP then follow in Phase II trials in patients. However, as with many target-specific oncology interventions,[32-34] HTT-lowering trials in humans are expected to conduct Phase I trials in patients rather than in healthy volunteers. In such trials, the key Phase I question the trial is addressing is the on-mechanism safety of target engagement rather than systemic toxicity.[32-34] We consider it very important that Phase I trials of HTT-lowering interventions attempt to demonstrate target engagement whenever possible. As biomarkers with appropriate supporting data become available, Phase I trials should be designed to achieve at least a percentage of HTT reduction monitored by the biomarker response instead of basing dosing on drug concentrations.

This white paper focuses on evaluating potential pharmacodynamic biomarkers of HTT lowering that can be used to confirm that a therapeutic agent reaches its intended target and has a biological effect. Current biomarker approaches under preclinical and clinical evaluation include the use of molecular, imaging, and electrophysiological outcomes. One or more of these pharmacodynamic markers will be essential tools for demonstrating that the delivery of a HTT-lowering therapy does, in fact, lower the amount of HTT in the brains of HD patients.

2. TASK FORCE METHODOLOGY

2.1 CHDI Huntingtin-Lowering Biomarkers Task Force

The CHDI Huntingtin-Lowering Biomarkers Task Force was initiated to accelerate the process of identifying pharmacodynamic biomarkers of HTT lowering by conducting a systematic and pragmatic approach to biomarker development. To achieve this, the Task Force brings together expert members from CHDI and collaborators from the academic, pharmaceutical, and biotech sectors.

A first step for the Task Force was to take an overview of current biomarker development, and define the essential criteria for an HTT-lowering pharmacodynamic biomarker. Next, a thorough evaluation and prioritization was made of the potential domains of biomarkers that could be explored in animal models and humans. We then developed a coordinated and streamlined process for evaluating each of these prioritized domains. This white paper provides an overview of each of the identified domains and their current status.

2.2 HTT-Lowering Biomarker Task Force objective

The main objective of the Task Force is the identification and/or development of one or more pharmacodynamic biomarkers that can be used to measure changes in response to HTT-lowering interventions in HD patients. With the rapid advancement of such therapeutic approaches, it is critical that this biomarker development advance within a short time frame so that it can be integrated into the design of clinical trials now being planned. As such, the goal is to have reliable pharmacodynamic biomarkers ready for use in trials of HTT-lowering therapies due to start in the near future. We acknowledge that not all biomarkers will be applicable to all HTT-lowering agents, such as an agent directed to the striatum versus the cortex; nevertheless, we have focused on the identification of up to four potential strategies or candidates that can be actively pursued.

2.3 Criteria for an appropriate biomarker

The Task Force has used the following set of criteria to define pharmacodynamic biomarkers that would be useful in the context of clinical trials. Considerations for criteria included both the merits of the technique itself as well as the informative value of the biomarker.

Biomarker criteria

1. ***Biological plausibility:*** *The connection between a putative reduction in the levels of HTT and the outcome measurement (biomarker readout) should be biologically plausible.* Biological plausibility is a requirement in the causality relationship. It is a matter of judgment, taking into account the knowledge that exists in the literature regarding the disease pathophysiology and the conceptual basis of the measurement method. For pharmacodynamic biomarkers, it is important to consider the degree of distance from the original disease insult.
2. ***Technological feasibility:*** *The technology should be feasible in humans and animal models.* The technology should already exist or be easily adaptable to measure the biomarker in both HD animal models and HD patients.
3. ***Measurable in humans:*** *It must be possible to measure the biomarker in humans.* This informed our decision to focus on non-invasive approaches.
4. ***Repeatable within subjects:*** *The biomarker should be amenable to repeated measurements.* Repeated biomarker measurements will most likely need to be made over weeks or months in a clinical trial, both before and after treatment, so it is important to consider biomarkers that can be repeatedly measured within individuals. Biomarkers should also demonstrate good test–retest results, giving comparable measures when administered to the same individual multiple times over days, weeks, or months.
5. ***Reliably measured in HDGECs:*** *The biomarker should be measurable in a reliable fashion in HD patients at stages of the disease likely to be targeted in upcoming clinical trials.* Clinical studies should demonstrate that the biomarker can be reliably measured in late pre-manifest, and early- to mid-stage manifest HD.
6. ***Signal metrics:*** *dynamic range, variance, etc.* The biomarker should be robust enough to detect any treatment-induced change. It should have a good signal to noise ratio, good dynamic range, and acceptable variance to be able to distinguish effects with relatively small sample sizes, and have strong inter- and intra-subject and site reliability.
7. ***Measurable in preclinical HD models*** The biomarker must be quantifiable in HD animal models in order to conduct the critical PoP studies.

8.	***PoP - Changes in response to central lowering of HTT in preclinical models*** The biomarker must be shown to respond to HTT lowering in a relevant HD model (i.e. confirming PoP). The Task Force specified that an appropriate biomarker should reflect HTT lowering and not simply the delivery of the therapeutic itself (e.g. an inflammatory marker might be elevated by the therapeutic delivery). Although qualitative measurements may initially suffice, quantitative measurements that demonstrate responsiveness are preferred. When possible, there should be a demonstrated correlation of the biomarker with lowering of HTT levels.
9.	***Changes in response to a HTT-lowering intervention in HDGECs*** The ultimate validation of the biomarker will occur only after successful, positive clinical trials of HTT-lowering agents have been completed.

2.3.1 Peripheral versus central biomarkers

The Task Force noted that by lowering HTT (either WT, mutant, or both) in the CNS there may be peripheral consequences that can be detected in blood, such as a downstream signaling molecule. This possibility will be of interest if the opportunity arises (e.g. if a likely candidate is available for development) but it was agreed that the majority of efforts would be concentrated on CNS biomarkers because a change in a central marker, as compared to a peripheral marker, is more likely to reflect a change in central HTT levels.

The Task Force also noted that differences in HTT-lowering therapeutic approaches (e.g. target brain regions, modes of administration, *etc*.) might require different biomarkers. Some HTT-lowering agents may only be delivered selectively to modulate HTT levels in the striatum while others might selectively target the cortex. It is therefore important to develop specific biomarkers to those brain regions. For example, phosphodiesterase (PDE) 10A expression, which is localized to the basal ganglia, is quantified using the PET ligand ^{18}F-MNI-659, which is currently under study as a striatal-specific marker of neuronal changes. Other examples are detailed in sections below.

3. METHODOLOGY FOR EVALUATING PHARMACODYNAMIC BIOMARKER DOMAINS

The validation of each potential biomarker includes a variety of key steps ranging from validation of the analytic methodology to preclinical PoP studies and clinical studies to evaluate the reliability, reproducibility and repeatability of the biomarker in humans. The main steps are described below.

3.1 Prioritization of biomarker domains for study

To streamline the process, the Task Force has prioritized seven biomarker domains and designated CHDI Science Directors to lead each of the clinical and preclinical efforts:

Biomarker Domain	Project Leaders
HTT Protein in CSF	*Douglas Macdonald & Beth Borowsky*
PET Imaging	*Ladislav Mrzljak & Andrew Wood*
PET Imaging HTT	*Jonathan Bard, John Wityak & Andrew Wood*
CSF Proteomics: Static	*Jonathan Bard & Beth Borowsky*
CSF Proteomics: Kinetic	*Jonathan Bard & Valentina Dilda*
qEEG	*Roger Cachope*
MR Spectroscopy	*Larry Park, Beth Borowsky &Valentina Dilda*

While we are taking a parallel approach to evaluate these biomarker domains, they are prioritized based on the available methodologies, preliminary data, and potential utility in the upcoming clinical trials. Initially, we are focusing on HTT protein measurements in CSF, existing PET tracers (PDE10, D1, D2, and CB1 ligands), and kinetic proteomic measurements in our preclinical model studies. The second tier of studies includes other existing PET tracers for targets of interest as well as a novel HTT tracer, MRS, qEEG, and static proteomics.

The domains selected reflect the criteria listed as relevant for a pharmacodynamic biomarker and the readiness for the immediate use of the technologies. All of the biomarkers listed have available technologies with the exception of the PET imaging of HTT itself, which is still in development. Some of the biomarkers are specific to a given brain region; for example, qEEG is mainly relevant for the cortex. In other cases, it remains unclear whether the biomarker reflects HTT lowering other than in the compartment measured (as is the case for measuring HTT in CSF). Excluding the circumstances where there is a clear-cut specificity of use, several different biomarkers should have convergent responses.

Therefore the concurrent use of different domains is advisable. We strive to find the best combinations of biomarkers that, together, will increase our confidence that there is indeed target engagement and a pharmacodynamic response clearly attributable to the administered intervention. Using several biomarkers simultaneously implies a trade-off between the confidence regarding the outcome measured and the burden that each measurement puts on subjects.

3.2 Analytic methodology

Validation of the analytic methodology will be obtained (if available) from the providers of the methods, or may need to be developed and confirmed experimentally by CHDI (as is the case for HTT quantitation assays). Several of the technologies being employed, such as PET and qEEG, are in common use and are therefore readily available with international standards (and the sites where they are available are regulated and/or certified).

3.3 Clinical studies

A key consideration in the prioritization of biomarker domains for study was the known ability to measure the biomarker in humans. The next steps in this process are to assess the reliability, variability, and potential effect size of the prospective biomarkers in the disease state. Of course, it is not yet possible to clinically test whether a biomarker is sensitive to change induced by the intervention, since none of the interventions have yet been tested in humans. The intention is to conduct preliminary observational studies of the candidate biomarkers in the clinic that will be updated when data from the initial clinical trial can be incorporated into the validation plans.

Current clinical studies aim to answer the following key questions:

1. ***Can the biomarker be measured consistently in HD patients?***
2. ***Is the measurement robust enough (in terms of reliability and variability) that we could expect to see a change, if one occurs, after HTT lowering?***
3. ***Is the biomarker altered by the disease (an indirect indication that the biomarker might change with HTT lowering)?***[1]

[1] *Note that this is not a requirement for a pharmacodynamic biomarker, which by definition are intervention-related and independent of the disease. However, in the case of HTT-lowering interventions this information might help understand whether the biomarker is likely to be responsive to mHTT levels. HD pathogenesis is considered to be at least partially dependent on mHTT levels and, as such, disease-induced alterations in biomarker readouts can be considered an indirect indication that the biomarker might change with mHTT levels.*

3.3.1 Populations under study

Predictive testing allows HDGECs to be identified in their premanifest phase before they develop symptoms.[35] While the first clinical trials of HTT-lowering therapies are expected to be in people with early manifest disease (Stages 1 and 2), we are assessing the reliability and effect size of candidate biomarkers in both premanifest and manifest HDGECs.

3.4 Proof-of-principle studies in animal models

PoP studies evaluate the ability of the potential biomarker to change when HTT protein is lowered in the brain of an animal model of HD. This is an extremely important step in biomarker validation since we cannot yet test the HTT-lowering interventions in HD patients.

Current preclinical PoP studies aim to answer the following key questions:

1. ***Can this biomarker be measured in HD animal models?***
2. ***Does the biomarker change in response to HTT-lowering in a dose-[2] and time-dependent manner?***

3.4.1 Selection of preclinical animal models for use in PoP studies

A large number of preclinical HD models are available that have a variety of mHTT constructs, including fragment transgene models, full-length artificial chromosome models, and genetic expansion knock-in models.[36-40] For the purposes of validating the selected HTT-lowering biomarkers, it was decided that the animal model should:

1. ***Reflect the genetics of human HD.***
   A genocopy heterozygous knock-in model with one expanded and one non-expanded HTT allele.
2. ***Exhibit measurable deficits that reflect appropriate stages of the disease.***
   The primary experimental setup being employed allows the testing of the most relevant time point of intervention in phenotypic 'manifest' animals since the current HTT-lowering therapies will most likely be tested first in stage 1 symptomatic HD patients.

We have selected the Q175 knock-in heterozygote (Q175 HET) HD mouse model[41,42] as the primary model for our HTT-lowering biomarker validation studies. Q175 HET mice exhibit first signs of motor symptoms

[2] *Dose and time dependence are not an absolute requirement as it may be difficult to demonstrate dose-dependency with some modalities (e.g. AAV therapies where only one dose is administered).*

from 3 to 4 months of age and behavioral deficits accompanied by marked brain atrophy and brain metabolite changes by 8 months.[41,42] Depending on the measure, dosing will occur in 4-6 month old Q175 HET mice. We will also examine interventions at earlier stages. However, mouse models can only provide a limited amount of tissue so we have included an additional HD model, the BAC HD transgenic rat model[43] that, for example, can provide a 10-fold volume of CSF compared to a mouse. BAC HD rats have an early onset and progressive HD-like phenotype including motor deficits and anxiety-related symptoms,[43] and this model can provide serial draws of CSF (an advantage over the mouse Q175 knock-in model) allowing for pre- and post-dosing collections.

3.4.2 HTT-lowering agent delivery methods

Another important consideration in the design of preclinical studies is the delivery method of the intervention. To date, all HTT-lowering agents must be delivered directly to the CNS and each agent has a specific mode of delivery that dictates biodistribution;

- *ASOs:* In rodent models, ASOs are being delivered either intraventricularly by bolus or pump/infusion resulting in distribution to the majority of the brain, or intrathecally by bolus resulting in distribution predominantly to the spinal cord and brain cortex. The latter is being employed to best mimic what is expected to be the administration route and distribution of ASOs in the planned upcoming clinical trial. Furthermore, intrathecal delivery can be used in large animals (i.e., non-human primates).
- *siRNAs:* In rodent models, siRNAs are being delivered by intraparenchymal infusion into the striatum by pump resulting in distribution that is mostly limited to that part of the basal ganglia. This somewhat mimics the expected clinical dosing paradigm using convection enhanced delivery infusion systems as demonstrated in non-human primates.
- *Viral vectors (i.e., AAV-ZFPs, AAV-miRNAs):* In rodent models, gene therapy approaches using viral delivery and transduction are being delivered by intraparenchymal bolus infusion into specific brain regions (i.e., striatum) resulting in distribution to the target area. This mimics what is expected in the clinic and is also applicable to large animal models (i.e., non-human primates). It is also worthwhile noting that some of these viral expression systems may be actively transported to more distant structures and affect other brain regions, but this needs to be examined more closely. Recent reports from several groups have demonstrated intravascular peripheral dosing of viral vectors resulting in CNS exposure, and this may become an alternate delivery option for such agents in the future.[44]

4. OVERVIEW OF BIOMARKER STUDIES

4.1 HTT protein measurement in CSF

The first biomarker approach considered by the Task Force is the measurement of HTT protein in the CSF compartment. This proteomic approach uses recent technological advances that enable measurement of proteins at much lower concentrations than were previously achievable. Using a novel, ultra-sensitive, single-molecule counting immunoassay (the Erenna® Immunoassay System by Singulex), CHDI and collaborators have shown in two cohorts of HD patient CSF samples that soluble mHTT can be quantified in the femtomolar range. [45,46] CHDI is also developing HTT assays to detect total, full-length, fragment, and aggregated HTT to enable the profiling of HTT proteins in such biosamples. Other advanced technologies such as the Quanterix Simoa HD-1 Analyzer and the Meso Scale Discovery S-Plex platform may also provide the sensitivity required to detect low-level analytes in CSF and other investigators may be exploring these alternate platforms.

4.1.1 Methodology

Quantifying HTT protein in CSF may be a valuable biomarker to assess HTT-lowering treatment response. The Erenna® Immunoassay System represents an important advance in this technology and, in addition to quantifying mHTT in HD patient CSF samples, has also been used to quantify mHTT in CSF from rodent HD models enabling preclinical PoP experiments. Depending on the HTT expression level in preclinical models, assays already developed on the Meso Scale Discovery platform can also be used to measure HTT proteins in such biosamples.[47]

Summary of HTT assay platforms - quantifying HTT in human and rodent model CSF samples

Assay Platform	HTT Detection	HTT in Human CSF	HTT in HD Model CSF
TR-FRET	Mutant	No	tbd
	Total	No	tbd
	Oligomeric	*ND*	tbd
MSD	Mutant	Prelim	YES
	Total	No	tbd
	Mouse	*ND*	tbd
	Full length	No	tbd
	Aggregated	*ND*	tbd
Singulex	Mutant	YES	YES
	Total	*ND*	tbd
	Full length	*ND*	tbd

Green = assay quantifies indicated HTT species; beige = studies ongoing; red = assay does not quantify indicated HTT species; ND = not done; tbd = to be determined

4.1.2 Clinical evaluation

Using the Erenna® technology described above, Wild and colleagues have reported that mHTT is undetectable in control subjects but quantifiable in nearly all HDGECs.[46] Interestingly, the CSF mHTT concentration was ~3-fold higher in patients with manifest HD as compared to premanifest HD.[46]

While this is a promising start toward developing a pharmacodynamic biomarker for HTT-lowering, there remain several potential limitations of this approach. First, the amount of HTT measured in the CSF was in the femtomolar range, close to the limit of detection of the assay, so it's still unclear whether the assay is sensitive enough to measure modest reductions in CSF mHTT levels in HD patients. Second, while it is assumed that CSF mHTT is brain-derived due to the correlation with brain-specific proteins and the lack of hemoglobin detection in the samples, a peripheral source cannot be completely ruled out, particularly since the concentration of mHTT in blood is several orders of magnitude higher than in CSF. And third, even if CSF mHTT is indeed brain derived, we do not know the relative contribution of newly synthesized mHTT versus older stores of mHTT from dying cells. Short-term exposure to a HTT-lowering treatment is likely to only affect newly synthesized mHTT, and only from regionally discrete parts of the brain, meaning that reduction of newly synthesized HTT in a restricted portion of the brain would need to lead to a

measurable decrease in the already very low levels of mHTT in CSF. With those caveats, CSF mHTT remains a highly attractive potential pharmacodynamic biomarker that needs additional evaluation.

CHDI has also partnered with University College London (UCL) to launch a clinical study called HDClarity, a collection of CSF and blood samples from HDGECs and healthy controls to enable the discovery and development of pharmacodynamic and disease progression biomarkers. Around 10 clinical sites in North America and Europe that participate in Enroll-HD (www.enroll-hd.org) will recruit healthy controls and patients with late premanifest, early, moderate and advanced HD to complete regular clinical assessments and donate CSF and plasma for biomarker discovery and validation. A CSF consortium—consisting of CHDI, UCL, and the clinical site principal investigators—is being established to conduct a large number of CSF biomarker studies, including HDClarity. The CSF consortium will oversee access to these biosamples for various research efforts, taking into account the merits of the research proposal, ranking of priorities and rules of use for limited samples. The identification and characterization of an HTT-lowering pharmacodynamic biomarker is a top priority for the consortium. The CSF consortium is committed to obtaining longitudinal as well as cross-sectional data, and whenever possible intra-subject test-retest data.

To better understand the contribution of newly synthesized HTT to the HTT pool detected in CSF, CHDI will study the dynamics of HTT production and its half-life in HD patients, similar to studies conducted for amyloid in Alzheimer's disease.[48] Such studies can be done by labeling newly synthesized HTT and then following the output to the CSF. This will require a mass-spectrometer assay that is sensitive enough to measure labeled and unlabeled HTT in femtomolar concentrations; current MS assays have a limit of detection of 100pM. We are currently working with contract research organization collaborators to incorporate an immunoprecipitation step to concentrate CSF HTT in order to achieve the required sensitivity. We will then proceed with a flux study to measure appearance of newly synthesized HTT into the CSF.

Progress points:

- ***mHTT protein can be measured in CSF from premanifest and manifest HDGECs; however, intra-subject variability over relatively short time periods (1-3 months) still needs to be evaluated.***
- ***Absolute concentration of mHTT in human CSF is very low and the sensitivity to small changes in mHTT concentration is unknown. Furthermore, we currently have limited understanding of the dynamics of production, half-life and origin of the HTT quantified in CSF.***
- ***Evidence suggests an increase in mHTT with disease burden in cross-sectional samples, but needs replication.***

4.1.3 Preclinical validation

Several studies to evaluate the effects of HTT-lowering interventions in HD preclinical models on mHTT CSF levels are being conducted in order to validate the measure as a pharmacodynamic biomarker. One major challenge is obtaining sufficient volume of CSF for the analyses; CHDI's current assay requires 5µl of rodent HD model CSF for each data point, in triplicate. Pooling of such samples should be avoided due to the risk of blood contamination during the harvesting procedure; therefore, great care must be taken to acquire as much CSF as possible per animal (ideally >20µl per animal). We are using two HD models for these studies, the preferred Q175 HET mouse model as well as the BAC HD rat model because of the greater CSF volume the latter can provide; see section 3.4.1.

It is important that these preclinical studies address whether reducing HTT in specific brain regions will be reflected in the measurable levels of mHTT in the CSF. This is an experimental challenge but the desired area can be selectively treated using specific HTT-lowering agents with well-defined distribution pharmacokinetics. For example, intrastriatal delivery of an AAV-HTT ZFP is being used to lower mHTT in the striatum of Q175 HET mice. Alternatively, intrathecal delivery of an HTT ASO is being used to lower HTT in the spinal cord and cortex of BAC HD rats. We are also delivering the HTT ASO by ICV in both the Q175 HET mouse and HD BAC rat for a broader distribution and HTT-lowering throughout the rodent brain to see whether global lowering is required to observe changes in mHTT levels in the CSF.

Important preliminary results were reported at the 2015 Huntington's Disease Therapeutics Conference. In collaboration with Isis Pharmaceuticals, CHDI analyzed the CSF from HTT ASO-treated HD BAC rats. In the ASO ICV-treated rats, significant decreases in CSF mHTT protein were observed compared to pre-dose

levels (p = 0.001), demonstrating that central lowering of mHTT in the rodent brain can result in quantifiable decreases in CSF levels; follow-up studies are planned.

Progress points:

- ***mHTT protein can be measured in the Q175 HET mouse and the BAC HD rat CSF using the Singulex or MSD assay system.***
- ***Studies are ongoing to evaluate whether CSF levels reflect the amount of mHTT in the HTT-lowering treated region of interest (i.e., striatum, cortex, other) in a dose- and time-dependent manner if possible.***

4.1.4 HTT protein measurement in CSF biomarker domain status

Biological plausibility	**High**
Technological feasibility	**Yes**
Measurable in humans	**Yes**
Repeatable within subjects	**Yes**
Reliably measured in HDGECs	**Studies ongoing** (*partial data available)*
Signal metrics: dynamic range, variance	**Studies ongoing** *partial data available)*
Measurable in HD animal models	**Yes**
PoP: Changes in response to central lowering of HTT in animal models	**Yes, studies ongoing** (*partial data available)*
Changes in response to an HTT-lowering intervention in HDGECs	**TBD**

4.2 PET and microPET imaging

PET imaging is a well-established, readily available technique that provides both structural and kinetic information and, compared to other imaging techniques, provides high sensitivity and high spatial and temporal resolution.[49] Importantly, development of micro-PET instrumentation for small animal imaging has made this technology accessible for the quantitative and repetitive imaging of biological function in preclinical models. This approach was selected as the second highest priority because there are already several PET studies showing clinical markers that are clearly expressed in brain regions of interest and are dysregulated in HD.[49-53]

4.2.1 Methodology

The main PET tracers being evaluated or under consideration are shown in the table below.

PET tracers being evaluated or under consideration

Target	PET Ligand	Localization	Preclinical Evaluation Status	Clinical Evaluation Status
D1 receptor	11C-NNC112	Basal ganglia/cortex	**Studies completed (uPET)**	*Currently no planned studies*
D2 receptor	11C-raclopride	Striatum/cortex	**Studies completed (uPET)**	**Studies completed and ongoing**
PDE10A enzyme	18F-MNI659/ [11C]IMA107	Basal ganglia	**Studies completed (uPET)**	**Studies ongoing and planned**
CB1 receptor	18F-FMPEP-d2/ [11C]MePPEP	Basal ganglia/cortex	**Studies ongoing (ARG)**	**Studies completed** and planned
5HT2a receptor	11C-MDL100097	Basal ganglia/cortex	**Studies completed (uPET)**	Studies planned
H3 receptor	11C-GSK189254/ [11C]MK-8278	Basal ganglia/cortex	**Studies ongoing (ARG)**	Studies planned
Glucose uptake	18F-FDG	Cortex and subcortical	**Limited profiling (uPET)**	**Studies completed** and planned
GABA-A receptor	11C-Flumazenil	Basal ganglia/cortex	Studies planned (ARG)	*Currently no planned studies*
mGluR5 receptor	18F-FPEB	Basal ganglia/cortex	Studies planned (uPET)	*Currently no planned studies*
M1 receptor	11C-GSK1034702	Basal ganglia/cortex	*Currently no planned studies*	*Currently no planned studies*
5HT1a receptor	11C-WAY100635	Cortex	*Currently no planned studies*	*Currently no planned studies*
NK1 receptor	18F-FE-SPA-RQ	Basal ganglia/cortex	*Currently no planned studies*	*Currently no planned studies*

Other novel PET targets are being explored as a second tier priority. Extensive qPCR studies were performed by CHDI in collaboration with Dr. Gillian Bates to explore transcriptional changes that occur in the R6/2 and Q175 HET mouse HD models over different ages. Both striatal and cortical transcriptional

targets were chosen from published data, RNA sequencing data and, in part, corresponding available translational microPET/PET tracers (i.e. for mouse and human imaging). Those PET tracers that have been used in humans and mice, *and* are reflective of corresponding mRNA changes observed in HD mouse models compared to WT controls, have been selected for microPET and PET studies.

The ideal PET ligand would be one that targeted HTT protein itself. CHDI is currently developing such a ligand and preliminary studies in Q175 HET mice show specificity of signal to HTT lowering; non-human primate pharmacokinetic studies are also planned.

4.2.2 Clinical evaluation

There is currently one ongoing study in this domain. PEARL-HD is a cross-sectional adaptive-design PET study (associated with a longitudinal study – LONGPET) evaluating the expression of PDE10A enzyme and D2 receptor levels using [^{18}F]MNI-659 and [^{11}C]raclopride, respectively, in pre-manifest and manifest HDGECs and healthy controls.[54] To date, 10 premanifest and five manifest HDGECs (stage I) and 15 age- and sex-matched control participants have been examined in PET using the aforementioned imaging tracers and the high-resolution research tomograph (a total of 45 subjects will be scanned). The outcome measure was the binding potential (BP_{ND}), using the simplified reference tissue model (D2R) and the Logan graphical analysis (PDE10A) with the cerebellum as reference region. The regions examined were caudate, putamen, and globus pallidus (GP).

In this study, [^{11}C]raclopride and [^{18}F]MNI-659 BP_{ND} were significantly lower in HDGECs compared with controls. In manifest Stage I participants, the mean BP_{ND} reduction vs. controls for D2R and PDE10A availability was 63% and 91% in the caudate, 43% and 69% in the putamen and 25% and 65% in the GP. In premanifest participants, the corresponding BP_{ND} reduction was 32% and 53% in the caudate, 31% and 43% in the putamen, 16% and 41% in the GP. These results show that striatal PDE10A is already more severely reduced than striatal D2R in HD, at the earliest stages of HD examined.

Russell et al., examined PDE10A levels in 11 premanifest and manifest HDGECs and nine healthy controls using [^{18}F]MNI-659. Compared to controls the HDGEC cohort had significantly lower striatal [^{18}F]MNI-659 uptake (mean difference, −48.4%; $P < 0.001$).[55] Striatal [^{18}F]MNI-659 uptake correlated strongly with severity of disease as measured by the clinical scale (UHDRS Motor subscale; $R = 0.903$; $P < 0.001$), burden

of pathology as measured by age × [CAG repeats − 35.5] (BOP; R = 0.908; P < 0.001), and regional atrophy (R = 0.667; P <0.05).

Additional PET studies are planned, one of which will assess PDE10A binding in a larger cohort of HDGECs to better understand the observed variability in binding potential and get a better understanding of the effect size. This study will also be the first to evaluate histamine H3 receptor levels using [18F]FMH3 in HDGECs. H3 therapeutics have shown promise in the treatment of memory and cognitive performance[56,57] and are presumed to be involved in HD. Indeed, the hypothalamic tuberomamillary nucleus (from where all histamine neurons originate[58]) contains the highest density of nuclear and cytoplasmic inclusions of mHTT.[59] Autoradiographic examination of postmortem HD brains has indicated lower H3R in the caudate (-69%), putamen (-77%) and pallidum (-80%) compared to controls, suggesting involvement of the H3 receptor in the pathophysiology of HD.[60] Inclusion of this target will provide additional information on H3 receptor availability and assess the utility of [^{18}F]FMH3 as a potential imaging biomarker.

Another study under discussion, PETMARK-HD, will characterize potential longitudinal progression and pharmacodynamic biomarkers in substantial participant cohorts of early premanifest to early manifest HD and matched healthy controls. Multiple PET targets will be imaged longitudinally over a 4-year period to provide a corticostriatal 'signature' of disease progression. PET targets include PDE10A, H3R, 5HT2AR and CB1R, assessed using [^{11}C]IMA107, [^{11}C]MK-8278, [^{11}C]MDL100907 and [^{11}C]MePPEP ligands respectively. The PETMARK-HD study will establish a series of 6-month PET readouts describing multi-target pathologic change from premanifest to early HD; this temporal feature will facilitate evaluation over intervals relevant to future investigational studies. MRI structural and diffusion data will be combined with PET to perform connectivity-based functional analyses of target expression, and to correlate volumetric and metabolic changes with PET and clinical data, across the different stages of HD.

Progress points:

- ***All ligands currently being evaluated as potential biomarkers in animal models are being used clinically. For some (raclopride and MNI-659) we have acquired detailed data in HDGECs; for others it is not known how the disease affects the expression of the target and therefore whether the PET imaging is consistently measurable in HD patients.***
- ***PET signals are robust and can be assessed in multi-site trials. However, evaluation of required effect size, longitudinal reliability, and inter-subject variability for each ligand is ongoing.***
- ***Cross-sectional differences in PDE10A, CB1, D2, and D1 are established and longitudinal assessment is ongoing to address alterations during disease progression. Work is also ongoing to establish the utility of new targets such as 5HT2A and H3.***

4.2.3 Preclinical validation

4.2.3.1 Longitudinal microPET imaging of the Q175 HET mouse model

Male heterozygous Q175 and WT animals were imaged in a longitudinal fashion to assess D1, D2, 5HT2A and PDE10A ligand binding at 6 and 9 months of age using the nanoScan® PET/MRI and nanoScan® PET/CT scanners (Mediso Ltd, Hungary).

At 6 months of age, the BP_{ND} in the striatum was lower in Q175 mice compared to WT by 40% in the case of D2 receptors ($p<0.0001$), by 52% in the case of PDE10A ($p<0.001$), by 29% in the case of D1 receptors ($p<0.001$) and 12% in the case of 5HT2A receptors ($p<0.01$). In the rostral cortex, D1-receptor binding was 24% lower in Q175 mice compared to WT. In the hippocampus, the BP_{ND} of 5HT2A receptors in Q175 mice was 12% lower compared to WT. At 9 months, there was a slight additional reduction of D1, D2 and 5HT2A in the striatum compared to 6 months (with a 4–7% further decrease in Q175 compared to WT), whereas PDE10A reached a plateau after 6 months (i.e. similar levels at 9 months). Cortical markers D1 and 5HT2A were also slightly further decreased at 9 months in Q175 mice (up to 20%). These results suggest that the proteins measured by microPET may be useful downstream markers to validate the effect of HTT-lowering therapies in HD animal models.

4.2.3.2 Longitudinal effect of lowering mHTT with ZFP on the expression of dopamine D2 receptor and PDE10A enzyme in the striatum of Q175 mice, as measured by microPET

The primary preclinical study to evaluate whether intrastriatal injections of ZFPs affect striatal dopamine (D2 and D1) receptor and PDE10A expression in the Q175 mouse model uses the so-called reversal treatment paradigm of intervention after the appearance of the disease phenotype. In this paradigm, AAV-HTT ZFP repressor of the mutant HTT was unilaterally injected into the striatum of 4-month old Q175 mice (i.e. at the age when loss of D2 and PDE10A has already begun). The contralateral non-injected hemisphere was used as the intra-subject control. In addition, another group of 4-month old Q175 mice received unilateral intrastriatal injections of the control AAV-GFP construct. All animals were longitudinally imaged at 7 and 10 months of age using [^{11}C]-raclopride (D2 receptor) and [^{18}F]-MNI 659 (PDE10A) ligands in the high resolution MRI-microPET. This experiment demonstrated 12% and 15% improvement in PDE10A ligand BP_{ND} in HTT ZFP repressor injected striatum compared to non-injected striatum at 7 and 10 months of age, respectively; this improvement was not observed in a control Q175 cohort. In contrast, no improvement of D2 receptor BP_{ND} was observed. While these results need to be repeated, they do indicate that an increase of [18F]-MNI 659 binding to PDE10A could potentially be used to indicate HTT-lowering.

We are employing another intervention paradigm (prevention) where we treat 2-month old Q175 mice with unilateral intrastriatal injections of AAV-HTT ZFP repressor and plan to analyze D2 and PDE10A ligand binding at 5-months of age.

4.2.3.3 Development of HTT-directed PET tracer

Work is ongoing to develop an HTT-directed PET tracer to measure HTT levels in specific brain regions. Using amyloid-binding ligands from the Alzheimer's disease field as starting points (which themselves were poor binders to HTT aggregates) and a number of *in vitro* assay systems to detect and quantify binding of ligands to HTT aggregates, medicinal chemistry efforts have significantly modified and matured several small-molecule series.

Several lead compounds have displayed significantly greater binding to mHTT and/or aggregates in specific regions of the brain (e.g. striatum, cortex, hippocampus) from a number of mouse HD models and human HD post-mortem samples (e.g. insula, thalamus) compared to analogous regions in WT mice and non-HD controls, respectively, using autographic radioligand binding analyses. We are also using high-

resolution microscopic emulsion autoradiography, combining autographic binding and immunohistochemistry (using the HTT-aggregate recognizing antibody EM48) to follow radioligand binding and HTT aggregate-specific immunoreactivity. Results to date have indicated clustering of silver grains (representing radioligand) selectively co-localizing with EM48 immunoreactive HTT aggregates; no silver grains nor EM48 immunoreactivity were detected in the WT sections.

Also, a lead compound, when tested *in vivo* via tail vein administration of radioligand, exhibited rapid and robust uptake in Q175 HOM HD mice compared to only marginal uptake in WT controls. Additional studies are now ongoing to radiolabel such compounds with ^{11}C to allow micro-PET and PET imaging in HD mice and non-human primates, respectively, to help define regional distribution, specific uptake, kinetics and non-specific binding. Analogous lowering paradigms are being used to test the HTT-directed radioligands (data analysis using ARG is in progress in Q175 mice treated with AAV-ZFPs).

Progress points:

- ***The expression of PDE10A and D1, D2 and 5HT2A receptors has been observed using microPET imaging in the Q175 HET mouse HD model.***
- ***Lowering of HTT in striatum using AAV-HTT ZFP resulted in significant 12–15% increases in PDE10A binding potential in 7- and 10-month old Q175 HET mice 3 and 6 months post-administration as demonstrated by microPET. However, no significant change of the binding potential was observed in the above paradigm for dopamine D2 receptor, suggesting that biomarker changes in response to HTT lowering may be protein-specific under certain paradigms.***

4.2.4 PET imaging (other than HTT) biomarker domain status

Biological plausibility	**Moderate**
Technological feasibility	**Yes**
Measurable in humans	**Yes**
Repeatable within subjects	**Yes**
Reliably measured in HDGECs	**Studies ongoing** (*partial data available)*
Signal metrics: dynamic range, variance	**Studies ongoing** *(uncertain)*
Measurable in HD animal models	**Yes**
PoP: Changes in response to central lowering of HTT in animal models	**Yes**
Changes in response to an HTT-lowering intervention in HDGECs	**TBD**

4.2.5 HTT PET imaging biomarker domain status

Biological plausibility	**High**
Technological feasibility	**Unknown**
Measurable in humans	**Studies ongoing** *(uncertain)*
Repeatable within subjects	**Yes**
Reliably measured in HDGECs	**TBD**
Signal metrics: dynamic range, variance	**Studies ongoing** *(uncertain)*
Measurable in HD animal models	**Studies ongoing** *(uncertain)*
PoP: Changes in response to central lowering of HTT in animal models	**Studies planned**
Changes in response to an HTT-lowering intervention in HDGECs	**TBD**

4.3 Quantitative EEG

EEG measurements are a reflection of brain electrical activity with millisecond temporal resolution, and are the most direct and non-invasive correlate of brain processing. As a technique, qEEG has the advantages of being relatively affordable and easily obtainable, although the technique's reliability depends heavily on well-trained technicians. It can also be performed in both small and large animal models.

EEG signals result from the functioning of the cortex in its full complexity. Many types of insults alter EEG readouts, which are also sensitive to a number of medications and mind-altering states. Importantly, there is evidence supporting EEG abnormalities in HD patients such as decreases in absolute alpha power in both manifest and premanifest HDGECs.[61] Differences in oscillatory synchrony – both spatially across the brain as well as across the frequency spectrum – have also been shown to correlate with disease severity, cognitive dysfunction, total functional capacity, and CAG length.[62]

Due to the large number of background conditions that must be standardized in order to detect a sub-acute or chronic drug effect, the technique may have a lower degree of reliability. As such EEG is a second tier priority, but we are committed to assessing its potential because it is currently one of the few windows available to evaluate effects at the level of the cortex.

4.3.1 Methodology

EEG is the measurement, using digital technology, of electrical patterns at the surface of the scalp, which primarily reflect cortical electrical activity. qEEG is the mathematical processing of digitally recorded EEG to highlight specific waveform components, transform the EEG into a format or domain that elucidates relevant information, or associate numerical results with the EEG data for subsequent review and comparison. These techniques provide a neurophysiological approach to viewing the dynamic changes taking place throughout the brain (e.g. while performing a cognitive task). Subjects are always tested in the awake resting state (the clinical norm). Outcomes of interest include global spectra power, inter-hemispheric coherence and power spectra per hemisphere.

4.3.2 Clinical evaluation

Two clinical studies of this domain are in planning and aim to characterize qEEG alterations in the context of premanifest and manifest HDGECs. The first planned study is designed to characterize the patterns of

qEEG in HDGECs as compared to controls. The acquisition of data is standardized to allow the prospective meta-analysis of the two datasets. One pilot study evaluating whether a change in the qEEG signature can be identified in premanifest and early-manifest HDGECs has already completed.[62] In this study, all HDGEC groups showed an increase in global delta power, and loss of normal anterior-posterior gradient of relative alpha and delta power. Relative alpha power gradient loss correlated with lower TFC scores, greater cognitive dysfunction and increased CAG.[62] The second study is more complex and aims to study the potential of several imaging modalities including FDG-PET, fMRI, and ASL to identify signature networks in the connectome that can be specifically linked to HD to confirm and expand previous work; [63-65] qEEG is an optional measurement in this protocol and it is expected that a sufficient number of participants will consent to undergo qEEG and, by doing so, will contribute to further characterization of the metrics of this measurement. Additionally, the co-registration of imaging and qEEG will allow a better understanding of the qEEG patterns.

Progress points:

- ***qEEG can be measured in HD patients.***
- ***qEEG is sensitive to change, acutely to the action of different psychotropic drugs. Signals are generally robust and reliable; however, it is currently unknown whether lowering HTT will alter the qEEG signal.***
- ***Studies have shown an increase in global delta power, loss of normal anterior-posterior gradient of relative alpha and delta power in HDGECs vs. control subjects.***

4.3.3 Preclinical validation

There are currently two preclinical studies planned to use the Q175 HET HD mouse model to evaluate the effect of HTT-lowering interventions on qEEG readings, both designed to test the effect of an intervention after the appearance of a disease phenotype. Specifically, HTT ASOs are being used to treat the brain more globally by ICV bolus dosing and HTT ZFPs are being used to selectively treat the striatum.

Progress points:

- ***qEEG can be used in both mouse and rat preclinical HD models.***
- ***PoP studies to assess whether qEEG signals change in response to HTT-lowering in a dose- and time-dependent manner are planned to start in Q1 2015.***

4.3.4 qEEG biomarker domain status

Biological plausibility	**Moderate**
Technological feasibility	**Yes**
Measurable in humans	**Yes**
Repeatable within subjects	**Yes**
Reliably measured in HDGECs	**Yes**
Signal metrics: dynamic range, variance	**Yes**
Measurable in HD animal models	**Yes**
PoP: Changes in response to central lowering of HTT in animal models	**Studies planned**
Changes in response to an HTT-lowering intervention in HDGECs	**TBD**

4.4 Proteomics: static and kinetic

This biomarker domain focuses on proteins other than HTT in the CSF and is also considered a second tier priority. Measurement of protein levels and/or protein flux in the CSF may allow us to detect changes resulting from an HTT-lowering treatment. There are two ways to measure such proteins, either at the steady state levels (static) or in a time-dependent manner (dynamic). For the former, changes in the absolute concentration of one or more proteins in the CSF can be readily measured using either established immunoassays or newer proteomic techniques such as SRM or MRM. These techniques are now routinely used to identify proteins that change under pathologic or treatment conditions.

4.4.1 Methodology

4.4.1.1 Static

There is now an SRM assay available for every protein in the human proteome. This approach has attained relative success in other therapeutic areas such as Alzheimer's disease[66] and multiple sclerosis[67] where SRM and MRM have been used to assess 10s of proteins simultaneously and to identify those that are quantifiably distinguishable between patients and healthy controls (i.e., diagnostic biomarkers). For the SRM/MRM approach to work in biomarker development, it will be important to focus on proteins that have human and rodent orthologs to enable the relevant preclinical studies. To identify more accessible biomarkers studies should be carried out in both CSF and plasma, while brain tissue can also be examined in initial preclinical studies to identify protein changes. A critical consideration in identifying and validating proteins that are altered by HTT-lowering treatment is the quality of the tissue source. For CSF, samples should be acquired and processed in a state-of-the-art, standardized way, minimizing blood contamination as well as food and drug effects.

4.4.1.2 Dynamic

Another approach is the analysis of dynamic proteomics by measuring molecular fluxes within cellular processes and pathways. The method proposed involves the use of stable isotope labeling of vesicle cargo molecules (following a period of administration of heavy water, $^{2}H_2O$), and observation of the appearance and disappearance of labeled cargo molecules in CSF as a kinetic marker. This is a proprietary technology developed by Kinemed.[68] Specifically, the method involves measuring the time course of labeled cargo protein release into the CSF following synthesis in the cell body. After *in vivo* labeling of protein cargo molecules, a time course of appearance and disappearance of these labeled cargo molecules in the CSF

(as a fraction of total, unlabeled protein) is calculated. In conditions characterized by impaired kinetics, the time to appearance of labeled cargo proteins in CSF is delayed compared to healthy controls.[69]

4.4.2 Clinical evaluation

4.4.2.1 Static

Previous proteomic discovery studies in blood and CSF samples from HD patients at various disease stages have identified a potential hot list of molecules that were differentially expressed either between controls and HD patients, between patients at different stages of the disease, or over time within subjects. To identify which of these proteins might serve as a pharmacodynamic biomarker to detect the effects of an HTT-lowering therapy, we refined this list with data from mouse models treated with HTT-lowering agents. Since those animal studies assessed changes to the transcriptome, rather than the proteome, and used brain tissue rather than CSF, we limited the search to transcripts that coded for secreted proteins, which are more likely to be released into the CSF. We further refined the list by prioritizing proteins that either already have a readout/assay (i.e. immunoassay) or are considered amenable to assay development. Other proteins under consideration include those expressed at high levels in the brain (particularly the cortex, striatum and cerebellum) and secretome-enriched proteins, such as brain-derived neurotrophic factor (BDNF) and encephalin. Using this approach we have identified a prioritized set of candidate proteins that could be assessed in preclinical or clinical samples for their utility as pharmacodynamic biomarkers following HTT-lowering interventions.

Among the prioritized candidate proteins on the hot list for initial study are proENK, complement (C1qb; C1qc; C4b), BDNF, apoE, cathepsin D, clusterin, and neurofilament light chain. The expression of these proteins will be assessed in the new HD CSF collection, HDClarity. In addition, a new hypothesis-free proteomic discovery approach will also be conducted on this new collection.

4.4.2.2 Dynamic

In collaboration with Kinemed, CHDI is planning a clinical study to monitor kinetic flux of CSF biomarkers in HDGECs compared to healthy controls; the time profile of the appearance and disappearance in CSF of pulsed deuterium-labeled cargo proteins utilizing LC-MS/MS will be analyzed (consistent with the Kinemed methodology for preclinical studies, see below). Cargo proteins to be assessed for kinetics in CSF will be: sAPPα amyloid precursor protein, chromogranin B, neuregulin-1, α-synuclein, proenkephalin A, neurosecretory protein VGF, neuroendocrine protein 7B2, chromogranin A, clusterin, major prion protein

precursor, neurexin-3, galanin, BDNF, semaphorins (Sema3A and 3D), neuroserpin and neurexophilin 1. An initial cohort of manifest HD Stage 2 patients will be compared to healthy controls in the first phase of the study. Depending on results, additional HDGEC cohorts will be recruited in subsequent phases to obtain a preliminary overview of potential biomarkers in kinetic flux across the HD disease spectrum.

Progress points:

- ***All proteins being explored for static measures have been measured in human CSF, and most in HD CSF.***
- ***For the kinetic flux studies, all of the cargo proteins have been measured in human CSF, but no studies in HD CSF have been conducted to date.***
- ***Studies are planned to determine how robust the readouts are in HD CSF.***
- ***Many of the proteins being explored in the static measures have shown some trend of cross-sectional difference in mass-spectrometry-based assays.***

4.4.3 Preclinical validation

Several studies are currently evaluating HTT-lowering interventions on static and dynamic protein expression. These mainly employ the Q175 HET mouse model, but earlier studies have also used the R6/2 transgenic line. The main interventions under study are HTT ASOs given by ICV bolus dosing after the appearance of a disease phenotype to mimic a manifest intervention.

4.4.3.1 Static

Plans are underway to evaluate changes in the concentrations of a prioritized list of proteins including proENK, complement (C1qb; C1qc; C4b), BDNF, apoE, cathepsin D, clusterin, neurofilament light chain, among others, in the CSF of Q175 HET mice. A longer list of proteins is available based on proteomic discovery studies using human HD CSF samples, HTT biology, and transcriptomic changes in HD animal models following HTT-lowering treatments, and could be the basis for a broader SRM/MRM approach to extend the earlier findings.

4.4.3.2 Dynamic

Dynamic proteomics has largely relied upon a pulse-chase paradigm using deuterated water, which has proven safe for both animal and human applications. Kinemed has developed CSF kinetic biomarkers of

axonal transport that have correlated with neurodegenerative disease progression. To date, dynamic proteomic studies have revealed: (1) Altered CSF kinetics (i.e. appearance and disappearance) in R6/2 mice of the neuronal cargo proteins, neuregulin-1 and sAPPα, but not for chromogranin B; (2) altered microtubule dynamics (microtubule hyperdynamicity) in the R6/2 and Q175 HET HD models that was age-dependent and region-specific (found in striatum and cortex but not cerebellum) suggesting a mechanistic link for the observed altered CSF kinetics; and (3) dose-dependent HTT ASO-mediated HTT knockdown amelioration of microtubule hyperdynamicity in striata of Q175 HET (3 months of age), 8 weeks but not 16 weeks post-treatment (in collaboration with Isis Pharmaceuticals and Kinemed). Results are imminent from the CSF study exploring whether neuronal cargo protein kinetics are ameliorated by HTT ASO-mediated HTT knockdown. Additional studies are ongoing to broaden the kinetic proteomic signature to include ~20 additional proteins, exploring two different ages of Q175 HET as well as including an HTT ASO-mediated HTT knockdown arm to the study.

Progress points:

- ***Some of the proteins have already been measured in both static and dynamic studies.***
- ***So far, microtubule hyperdynamicity that is displayed in HD mouse model is normalized in brain after ASO-mediated HTT-lowering; results are imminent for the CSF neuronal cargo protein kinetic outcome measures.***

4.4.4 Proteomics static biomarker domain status

Biological plausibility	**Moderate**
Technological feasibility	**Yes**
Measurable in humans	**Yes**
Repeatable within subjects	**Yes**
Reliably measured in HDGECs	**Studies ongoing** (*partial data available)*
Signal metrics: dynamic range, variance	**Studies ongoing** (*partial data available)*
Measurable in HD animal models	**Studies ongoing** *(partial data available)*
PoP: Changes in response to central lowering of HTT in animal models	**Studies planned**
Changes in response to an HTT-lowering intervention in HDGECs	**TBD**

4.4.5 Proteomics dynamic biomarker domain status

Biological plausibility	**Moderate**
Technological feasibility	**Yes**
Measurable in humans	**Yes**
Repeatable within subjects	**Yes**
Reliably measured in HDGECs	**Studies planned**
Signal metrics: dynamic range, variance	**Studies planned**
Measurable in HD animal models	**Yes**
PoP: Changes in response to central lowering of HTT in animal models	**Studies ongoing** *(partial data available)*
Changes in response to an HTT-lowering intervention in HDGECs	**TBD**

4.5 MRS imaging

MRS is a non-invasive analytical method that enables the quantification of metabolites in samples. Compared with other modalities it is inexpensive, rapidly conducted, widely accessible (it uses clinical MRI equipment), and can be used in both clinical and preclinical settings. It can also be used to evaluate multiple tissue types simultaneously (e.g. grey and white matter). In our program MRS is under study as a second tier priority.

4.5.1 Methodology

MRS allows the detection of relatively small molecules, typically in concentrations of 0.5–10 mM, within cells or in extracellular spaces. The determined MR spectra provides information on metabolic pathways and changes therein. Attention has focused on proton (^{1}H) and phosphorus (^{31}P) MRS, and studies have been conducted using either single or many voxels simultaneously. The main brain regions of interest include the striatum and cortex. In clinical studies the typical field strength of the MRS systems is 3T, but in very particular and circumscribed paradigms, MR systems with field strength higher that 3T (including 7.0 T systems) are used for optimum signal-to-noise ratios. However this option very much limits the feasibility of the technique and it is therefore a research option rather than an implementable solution in multi-site clinical trials.

Suitable brain metabolites for clinical HD studies include:

- NAA: N-acetyl-aspartate, contained only in neurons, serves as healthy neuronal marker. Measurements of total NAA include N-acetyl-aspartyl-glutamate (NAAG).
- GLU: Glutamate, also GLX (glutamate + glutamine); Glu is a neurotransmitter and Gln is involved in neurotransmitter synthesis.
- CR: Creatine/phosphocreatine buffer ATP concentrations.
- CHO: Choline groups are found in acetylcholine and in membrane breakdown products.
- MI: Myo-inositol is a glial cell marker.

The MRS technique may be particularly attractive for studying the cortex since a cortical-focused voxel can be utilized.

4.5.2 Clinical evaluation

MRS was evaluated in a subset of control, premanifest and early manifest HD subjects taking part in the TRACK-HD and Track-On clinical studies.[70] [71] MRS measures with a putaminal voxel were conducted annually for 4 years at a single clinical site. An additional clinical site and the inclusion of a second voxel, in the cortex, were incorporated in the last 3 years.

Analysis of the final year is still ongoing, but the data clearly show that the MRS signal in HD is robust and reliable, both over time and across sites, at least when similar scanners are used. The data also demonstrate that the MRS signal for NAA was lower in both premanifest and manifest HDGECs than controls while the signal for myo-inositol was higher in manifest HDGECs than premanifest HDGECs and controls.[70] So while the ability of the MRS signal to serve as an HTT-lowering biomarker remains to be demonstrated, this measure could be employed in HD clinical trials using already established methodology. Because of the ability to selectively focus on the cortex or putamen, this may be particularly attractive for interventions expected to have limited brain coverage.

CHDI has two additional MRS clinical studies that are mechanistically investigating the energetics pathways and the potential compromise of mitochondrial energy fluxes in HD. These studies were not planned as biomarker studies but might provide some insight to the clinical utility of MRS as a biomarker in HD.

Progress points:

- ***^{1}H-MRS can be performed in HD patients, focusing on cortex and/or putamen.***
- ***The MRS signal is robust and reliable. The use of MRS in multi-site studies is still challenging since combining data from different scanner types is not standardized; developing this standardization is planned. However, it is currently unknown whether HTT-lowering interventions will alter the signal.***
- ***Studies have shown NAA to be lower in premanifest and manifest HDGECs than controls and myo-inositol higher in manifest HDGECs than premanifest HDGECs and controls.***

4.5.3 Preclinical validation

There are currently several ongoing and planned studies to evaluate HTT-lowering interventions on MRS measures. Interventional studies are based on the Q175 HET mouse model, but the R6/2 transgenic line and the BAC HD rat model have also been employed in non-interventional MRS optimization studies. Treatment paradigms include ICV bolus HTT ASO as well as intrastriatal administration of HTT ZFPs and HTT siRNAs to lower HTT. The main intervention being employed is after the appearance of a disease phenotype in the rodent model to mimic manifest intervention.

Additionally, based on recent results in Q175 HET mice showing a progressive decrease in the glutamatergic and GABAergic TCA cycle in neurons, another preclinical study is being planned using HTT-lowering agents to see whether brain function and energy metabolism using MRS measures of the glutamate/glutamine cycle, and neuronal and astrocytic mitochondrial TCA cycle fluxes, can be restored.

Progress points:

- ***1H-MRS can be measured in the Q175 HET preclinical HD models.***
- ***Studies are ongoing to see whether ^{1}H-MRS signals change in response to HTT-lowering in a dose and time-dependent manner.***

4.5.4 MRS biomarker domain status

Biological plausibility	**Moderate**
Technological feasibility	**Yes**
Measurable in humans	**Yes**
Repeatable within subjects	**Yes**
Reliably measured in HDGECs	**Yes**
Signal metrics: dynamic range, variance	**Yes**
Measurable in HD animal models	**Yes**
PoP: Changes in response to central lowering of HTT in animal models	**Studies ongoing** (*partial data available)*
Changes in response to an HTT-lowering intervention in HDGECs	**TBD**

5. CLOSING COMMENTS

Identification of easily measured, reliable, and robust pharmacodynamic biomarkers of dynamic changes in HTT protein levels in the brain are essential for the development and evaluation of the various HTT-lowering interventions currently under development. CHDI is committed to its ongoing biomarker development program to support upcoming HTT-lowering clinical trials. The program is based on a series of pragmatic and scientific decisions ranging from the pre-defined criteria required for a suitable biomarker, to prioritization of candidate biomarkers, to the choice of animal models used for preclinical validation. These decisions have been made to not only ensure that the program is at the forefront of technology but also that the goals are realistic and can be met within a reasonable timeframe. By working in close collaboration with a large number of commercial and academic colleagues, the program has brought together new ideas and methods from other therapeutic areas and applied them specifically to HD.

The program has already achieved much; for each of the biomarker candidates we have a better understanding of how they behave in HD and the robustness of signal. However, we are humbly aware of the challenge of identifying and fully characterizing biomarkers that can successfully and accurately track the lowering of HTT in the human brain. The CHDI HTT-Lowering Biomarker Task Force is an ongoing effort, and we are committed to providing regular and timely updates on the progress made.

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