HTRF analysis of soluble huntingtin in PHAROS PBMCs

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ABSTRACT

Objective: We measured the levels of mutant huntingtin (mtHtt) and total huntingtin (tHtt) in blood leukocytes from Prospective Huntington At-Risk Observational Study (PHAROS) subjects at 50% risk of carrying the Huntington disease mutation using a homogeneous time-resolved fluorescence (HTRF) assay to assess its potential as a biomarker.

Methods: Peripheral blood mononuclear cells from consenting PHAROS subjects were analyzed by HTRF using antibodies that simultaneously measured mtHtt and tHtt. mtHtt levels were normalized to tHtt, double-stranded DNA, or protein and analyzed according to cytosine-adenine-guanine repeat length (CAGn), demographics, predicted time to clinical onset or known time since clinical onset, and available clinical measures.

Results: From 363 assayed samples, 342 met quality control standards. Levels of mtHtt and mt/tHtt were higher in 114 subjects with expanded CAG repeats (CAG ≥37) compared with 228 subjects with nonexpanded CAG repeats (CAG <37) ($p < 0.0001$). Analysis of relationships to predicted time to onset or to phenoconversion suggested that the HTRF signal could mark changes during the Huntington disease prodrome or after clinical onset.

Conclusions: The HTRF assay can effectively measure mtHtt in multicenter sample sets and may be useful in trials of therapies targeting huntingtin. *Neurology*® 2013;81:1134–1140

GLOSSARY

CAG = cytosine-adenine-guanine; **CAGn** = cytosine-adenine-guanine repeat length; **dsDNA** = double-stranded DNA; **GLP** = Good Laboratory Practice; **HD** = Huntington disease; **HTRF** = homogeneous time-resolved fluorescence; **Htt** = huntingtin; **MGH** = Massachusetts General Hospital; **mtHtt** = mutant huntingtin; **PBMC** = peripheral blood mononuclear cell; **PHAROS** = Prospective Huntington At-Risk Observational Study; **QC** = quality control; **Tb** = terbium; **tHtt** = total huntingtin; **UHDRS** = Unified Huntington's Disease Rating Scale.

Huntington disease (HD) is caused by the expression of the toxic mutant huntingtin (mtHtt) protein, which contains an expanded polyglutamine repeat sequence near its N-terminus.[1] mtHtt misfolds, undergoes posttranslational modifications, fragments, and forms soluble oligomers and insoluble intracellular aggregates,[2–4] which are differentially toxic.[5,6] Huntingtin (Htt) is the most salient target for neuroprotective therapies[7–9] and it is both essential and challenging to reliably measure it[1,2,10] to enable the development of therapies. We adapted a semiquantitative cell-based immunoassay that measures soluble mtHtt and total Htt (tHtt) using homogeneous time-resolved fluorescence (HTRF) Förster resonance energy transfer.[11,12] This HTRF assay is sensitive, reliable, and specific for soluble mtHtt in tissues and blood from HD mouse models,[11] in postmortem tissue, and in single-site studies using human peripheral blood mononuclear cells (PBMCs) from subjects with premanifest and manifest HD.[11–13] We optimized and technically validated the HTRF assay according to Good Laboratory Practice (GLP) standards for analyzing mtHtt and tHtt in clinical PBMC samples.[12] To validate the HTRF assay in the context of a blinded multicenter study encompassing subjects with and without the HD mutation, to assess normalization methods for Htt values, and to examine whether the HD prodrome or the development of clinical

Supplemental data at www.neurology.org

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symptoms might be associated with alterations in PBMC Htt, we assessed the relative levels of mtHtt and tHtt in a sample set from the Prospective Huntington At-Risk Observational Study (PHAROS)[14] of clinically unaffected adults nominally at 50% genetic risk for developing HD.

METHODS

Data source and human subjects. PHAROS,[14] an NIH-sponsored multicenter observational study of 1,001 individuals at 50% risk of carrying the HD mutation by virtue of a diagnosed first-degree relative, enrolled subjects from 1999 until 2004 and concluded follow-up in 2009. Blood for DNA was collected at enrollment for double-blinded analyses of genotype and cytosine-adenine-guanine (CAG) length. The PHAROS biostatistics team at the University of Rochester is solely able to perform genetically unblinded analyses. An exploratory biomarker aim was added late to PHAROS and consisted of a single collection of blood and urine samples from 433 subjects still participating in the study.

Standard protocol approvals, registrations, and patient consents. Use of the leukocyte fractions from a 5-mL tube for the HTRF assay was approved as an ancillary study by the PHAROS steering committee and by the Partners Institutional Review Board. The ClinicalTrials.gov registration for PHAROS is NCT00052143. Blood samples for prespecified, as well as future unspecified, biomarker studies were collected from 433 consenting PHAROS subjects with the approval of the institutional review boards at the participating clinical centers.

PHAROS blood samples. The collection, processing, and storage methods for the blood samples were previously optimized and standardized, and all site staff received training before implementation to reduce variability and improve sample quality. The blood specimens used for HTRF were from 5-mL ethylenediaminetetraacetic acid tubes that were held on wet ice for a maximum of 30 minutes and centrifuged at a gravitational force and duration based on the available equipment (from 6,000*g* to 8,000*g* for 35 to 20 minutes, respectively, at 4°C). The tubes with blood were carefully flash frozen on dry ice or by insertion into tube-caliber holes in an aluminum block prefrozen (−80°C) so as not to disturb the in situ layering within the blood tubes. The frozen blood was shipped to the Matson lab (Veterans Administration Hospital, Bedford, MA), where it was expelled from the collection vial and dissected manually, while frozen, into plasma, PBMC (leukocytes), and red blood cell fractions and archived at −80°C. Coded PBMC samples were transferred to the Hersch lab at Massachusetts General Hospital (MGH) for HTRF analyses. PBMC samples from 3 additional manifest HD subjects were collected at MGH, processed using the PHAROS protocol, and used to evaluate the effect of red blood cell contamination on the leukocyte HTRF signal.

Brain tissue samples. Postmortem frontal cortex samples from patients with HD and controls obtained from the Brain Bank of the Alzheimer's Disease Research Center at MGH were prepared as previously described.[12] Lysates were prepared, their protein concentrations were determined, and stocks were aliquoted and stored at −80°C for use as quality control (QC) samples on each HTRF assay plate.

Antibodies. The monoclonal antibodies used in this cell-based HTRF assay are specific for selected epitopes on the Htt molecule.[12] The antibodies include the following: 2B7 monoclonal antibody (Novartis, Basel, Switzerland) specific for the first N-17 amino acids of normal and mtHtt[11]; MW1 monoclonal antibody[15] (Developmental Studies Hybridoma Bank, University of Iowa) binds preferentially to expanded polyglutamine sequences (polyQ), hence binds to mtHtt and to a lesser extent to normal Htt[11]; and 2166 monoclonal antibody (MAB2166; Millipore Corp., Billerica, MA), which binds to the Htt epitope starting at amino acids 443–457 and recognizes both normal Htt and mtHtt, hence tHtt.[16] The 2B7 was conjugated by Cisbio to the lanthanide terbium (Tb) that served as the donor for HTRF.[11,17] The Tb was excited at 340 nm and its peak emission captured at 615 nm. MW1 was conjugated in-house to AlexaFluor 488 (Invitrogen, catalog no. A20181) per the kit instructions and 2166 was conjugated to d2 by Cisbio.[12] AlexaFluor 488 and d2 peak emissions were captured at 510 nm and 665 nm, respectively, and they served simultaneously as acceptors of Tb emission, enabling multiplexing in each well in HTRF (table e-1 on the *Neurology*® Web site at www.neurology.org).[12,13]

HTRF Htt assay. The assay was performed and recorded according to a GLP-compliant standard operating procedure[12] and met the Health Insurance Portability and Accountability Act of 1996 requirements. The assay conditions for the detection of mtHtt, tHtt, and double-stranded DNA (dsDNA) content from an individual sample, using PBMCs derived from a single 5-mL blood sample, were as previously described.[12] The ratio of signals at 665/615 nm and 510/615 nm, respectively, were multiplied by 10^4 and represent a specific, artifact-corrected determination of the signals from 2166-d2/2B7-Tb and MW1-AF/2B7-Tb, simultaneously bound to Htt, revealing information about the relative tHtt/Max and mtHtt/Max signals, respectively. The assay run for any plate was accepted if the brain lysates used as QC samples yielded $Z' > 0.5$, as previously described.[12] Mt/tHtt designates the ratio of mtHtt and tHtt and represents a normalization of mtHtt to tHtt.

dsDNA content. As an approximation for normalizing the HTRF results to the concentration of PBMCs in the buffy coat fractions, we quantified the dsDNA content of each sample. The Quant-iT PicoGreen dsDNA Quantitation Kit (Invitrogen, catalog no. P11496) was used to measure dsDNA content in the HTRF assay wells (excitation at 485 nm and emission of 535 nm), as previously described.[12] A standard curve for dsDNA was generated on every plate to ensure that the measurements were within the dynamic range of the assay.[12] We also evaluated the effect of red blood cell contamination on the leukocyte HTRF signal by controlled mixing of the 2 cell types, which enabled us to define dsDNA >1 ng/mL (data not shown) as a QC threshold corresponding to a sufficient quantity of leukocytes for statistical analyses.

Protein concentrations. To normalize HTRF results to the protein content of the samples, we used DC Bio-Rad Protein Assay Reagents, per the kit instructions to measure protein in the human brain lysates and leukocyte samples.

Statistical analysis. The complete HTRF data set was transferred to the PHAROS biostatistical team for unblinded analyses. Medians and 25th and 75th percentiles were chosen to represent the demographic and experimental variables. Subjects were assigned to 2 or 3 CAG groups based on the longer of the 2 Htt alleles: either 1) CAG <37 (nonexpanded) vs CAG ≥37 (expanded), or 2) CAG <27, CAG 27–39 (intermediate and reduced penetrance), or CAG >39[18–20] based on the longest of the 2 Htt alleles.[14] Kruskal-Wallis tests (nonparametric), χ^2 tests, and Jonckheere-Terpstra trend tests (nonparametric tests for

ordered differences among groups) were used to compare groups. For subjects in the expanded CAG group, Spearman correlation (based on ranks) was used to calculate the correlation of the mtHtt signal with CAG repeat length, the Unified Huntington's Disease Rating Scale (UHDRS)[21] motor score, and disease burden, calculated as age × (CAG − 35.5).[22] Clinical onset of HD (phenoconversion) was based on the diagnostic certainty item from the UHDRS and was defined as the first occurrence of a diagnostic value of 4 by the independent rater, indicating that the rater was ≥99% confident that the subject had motor abnormalities that are unequivocal signs of HD.[21] Langbehn formula, which is based on age and CAG repeat length, was used to calculate the probability of onset in the expanded group.[23] The STROBE (Strengthening the Reporting of Observational studies in Epidemiology) recommendations[24] for reporting on observational studies were followed.

RESULTS **Demographic and experimental characteristics.** Of the 433 samples collected, 70 samples were not analyzed because of missing or illegible barcodes. The data from another 21 samples with dsDNA <1 ng/mL were excluded as described above. The subjects providing the 91 samples that were not included in this report did not differ significantly by CAG, age at blood draw, or sex from the subjects providing the 342 samples from 35 clinical centers included in the final analyses. Demographic characteristics and experimental assay results are shown in table 1, in which, as prespecified in PHAROS, nonexpanded subjects were defined as having CAG <37 (n = 228) and expanded subjects as those with CAG ≥37 (n = 114). The cohort includes 32 subjects with repeat lengths of CAG 27–39,[18–20] which could potentially be associated with intermediate mtHtt signals (table 1), including 14 subjects with reduced disease penetrance (CAG 36–39)[19] and 18 subjects with meiotic instability who can transmit a pathogenic CAG length to their offspring (CAG 27–35).[20] Subjects in the nonexpanded CAG group were slightly older than subjects in the expanded group, but the groups did not differ by sex.

Comparison of expanded and nonexpanded groups. Expanded subjects had a significantly higher HTRF signal for mtHtt ($p < 0.0001$) compared with subjects with CAG <37 or CAG <27 (excluding the CAG 27–39 repeats subjects). HTRF signals for tHtt did not differ significantly between groups. Significant differences between groups were also present upon normalizing the mtHtt signals to tHtt ($p < 0.0001$), to leukocyte concentrations (dsDNA, $p = 0.0097$ for the CAG <37 vs CAG ≥37 and $p = 0.0112$ for CAG <27 vs CAG >39), or to protein concentrations ($p = 0.0533$ for the CAG <37 vs CAG ≥37 and $p = 0.0195$ for CAG <27 vs CAG >39) (table 1). These results suggest that the CAG 27–39 subgroup does not significantly affect the group analyses. The signals for mtHtt were not significantly correlated with CAG repeat length, disease burden, or the UHDRS motor

Table 1 Demographic and experimental characteristics

	CAG <37 (n = 228)	CAG ≥37 (n = 114)	p Values, CAG <37 vs CAG ≥37	CAG <27 (n = 209)	CAG 27–39 (n = 32)	CAG >39 (n = 101)	p Values, CAG <27 vs CAG >39
CAGn	19 (17–22)	42 (40–43)	—	18 (17–21)	34 (31–38)	42 (41–43)	—
Age, y	51 (45–57)	49 (44–55)	0.0476	51 (44–57)	52 (45–57)	48 (43–55)	0.0360
Males, n (%)	77 (34)	37 (32)	NS	70 (33)	13 (41)	31 (31)	NS
mtHtt	1,183 (1,006–1,377)	1,423 (1,213–1,644)	<0.0001	1,162 (1,001–1,372)	1,271 (1,107–1,510)	1,432 (1,234–1,644)	<0.0001
tHtt	1,432 (1,302–1,654)	1,403 (1,291–1,527)	NS	1,431 (1,313–1,658)	1,432 (1,267–1,648)	1,401 (1,297–1,504)	NS
mt/tHtt	0.79 (0.66–0.96)	0.98 (0.82–1.13)	<0.0001	0.78 (0.64–0.96)	0.86 (0.75–0.96)	1.00 (0.84–1.16)	<0.0001
mtHtt/dsDNA	28 (18–44)	35 (21–58)	0.0097	28 (17–44)	33 (19–69)	34 (22–54)	0.0112
mtHtt/protein	17 (12–24)	18 (14–29)	0.0533	16 (12–23)	19 (14–24)	18 (14–30)	0.0195

Abbreviations: CAGn = cytosine-adenine-guanine repeat length; dsDNA = double-stranded DNA; mtHtt = mutant huntingtin; NS = not significant; tHtt = total huntingtin.
Data are medians (25th–75th percentiles) unless otherwise indicated. Comparisons of 1) CAG <37 vs CAG ≥37 groups, and 2) CAG <27, CAG 27–39, and CAG >39 groups for age, sex, mtHtt, tHtt, and mtHtt normalized to tHtt (mt/tHtt), to dsDNA (mtHtt/dsDNA), and to protein concentration (mtHtt/protein). The data were analyzed using Kruskal-Wallis tests or χ^2 tests, as appropriate. For the analysis comparing the CAG <27 with the CAG >39 group, the intermediate and reduced penetrance group (CAG 27–39) was omitted.

scores in the expanded group, and there were no sites with extreme data that might skew the results (data not shown).

Relation of Htt values to disease onset. To assess potential relationships between HTRF signals and disease progression, data from subjects with CAG ≥37 were ordered into 5 groups, based on predicted time to onset and the time since phenoconversion. Premanifest subjects were divided into 3 groups based on their calculated 2- and 5-year probabilities of HD onset using Langbehn formula: low risk of onset = 5-year probability of onset <0.2; moderate risk of onset = 5-year probability of onset ≥0.2 and 2-year probability of onset <0.2; high risk of onset = 2-year probability of onset ≥0.2. Symptomatic subjects were divided into 2 groups based on the time from their clinical diagnosis to the blood draw: <2 years and ≥2 years (range 2–8.8 years). Median (25th–75th percentile) values for subjects with CAG <37 and the 5 groups ordered by their relationship to disease onset are shown in table 2.

Tests for trends in mtHtt and mtHtt normalized to tHtt ($p < 0.0001$), dsDNA concentration (mtHtt/dsDNA, $p = 0.0295$), and protein concentration (mtHtt/protein, $p = 0.0263$) across the 6 groupings were significant, but this is largely reflective of the difference between the expanded and nonexpanded groups. In the expanded group, when normalized to tHtt (mtHtt/tHtt), the HTRF signal tended to increase somewhat across the onset groupings; when normalized to dsDNA concentration (mtHtt/dsDNA), the HTRF signal tended to increase with diminishing time to onset and to decrease after clinical diagnosis, perhaps marking phenoconversion. When normalized to protein (mtHtt/protein), the HTRF signal was stable through the HD prodrome and increased after clinical diagnosis. Although complex, these changes in the HTRF signal and its normalized values may be capturing information about molecular alterations to mtHtt in PBMCs, such as oligomerization and aggregation.

Table 2 mtHtt and mtHtt values normalized to tHtt (mtHtt/tHtt), dsDNA (mtHtt/dsDNA), and protein concentration (mtHtt/protein) by onset group

		Premanifest, CAG ≥37, risk of onset			Phenoconverters, CAG ≥37, time since onset		
	CAG <37	Low	Moderate	High	<2 y	≥2 y	J-T trend test
No.	228	31	37	20	10	16	342
mtHtt	1,183 (1,006–1,377)	1,249 (1,121–1,530)	1,430 (1,242–1,674)	1,438 (1,159–1,632)	1,457 (1,384–1,507)	1,505 (1,263–1,775)	<0.0001
mtHtt/tHtt	0.79 (0.66–0.96)	0.89 (0.78–1.03)	1.01 (0.88–1.17)	1.06 (0.80–1.22)	1.00 (0.98–1.11)	1.02 (0.76–1.15)	<0.0001
mtHtt/dsDNA	28 (18–44)	36 (23–73)	37 (23–48)	37 (23–63)	29 (21–39)	27 (17–47)	0.0295
mtHtt/protein	17 (12–24)	18 (14–21)	18 (14–23)	18 (13–31)	18 (11–33)	31 (15–34)	0.0263

Abbreviations: CAG = cytosine-adenine-guanine; dsDNA = double-stranded DNA; J-T = Jonckheere-Terpstra; mtHtt = mutant huntingtin; tHtt = total huntingtin.
Data are medians (25th–75th percentiles) unless otherwise indicated. Using Langbehn formula, premanifest subjects (CAG ≥37) were divided into 3 groups (low, moderate, and high risk of onset) based on their calculated probabilities of Huntington disease; manifest subjects (phenoconverters) were divided into 2 groups based on time since onset. The CAG <37 group, the 3 onset groups, and the 2 symptomatic groups were compared using J-T tests for trend.

DISCUSSION We analyzed soluble mtHtt and tHtt in PBMCs from subjects with expanded CAG repeat lengths (CAG ≥37) compared with nonexpanded subjects (CAG <37) participating in PHAROS, utilizing simultaneous detection in each sample of mtHtt and tHtt in a GLP HTRF assay. Analyzed samples originated from 35 clinical sites trained to follow uniform collection and processing procedures. From the 433 samples that were collected, 342 could be analyzed statistically, after losses caused by unreadable labels and sample processing or QC criteria. The demographic and genotype characteristics of the analyzed and unanalyzed groups were not significantly

different. Because PHAROS is an observational study of individuals with a 50% risk of carrying the HD genetic mutation, the majority of subjects included in our analyses (92%) were subjects with CAG <37 or premanifest individuals destined to develop HD in the future, whereas 8% of subjects were prospectively diagnosed as having symptomatic HD during the course of the study, based on UHDRS clinical criteria. The expanded subjects (table 2) had a median CAGn of 42 (range 37–48), which is typical of the premanifest and manifest adult subjects who participate in clinical research. There were no subjects with extreme CAG lengths (CAG >48) that might have skewed the results[25] and indeed there was no correlation between CAGn and the assay results in expanded subjects in the PHAROS sample set (data not shown). Previous results in mice and humans, which included subjects with much higher repeat lengths than 48, have demonstrated increased mtHtt signals using HTRF assays with increased CAG length.[11,13,26]

Our goal in this study was to assess the potential for the HTRF assay to be a useful outcome measure for multicenter HD clinical research studies. The PHAROS sample set provided an opportunity to examine the sensitivity and specificity of the HTRF assay in a multicenter sample set under blinded Good Clinical Practice and GLP conditions, using routinely available equipment, simple preparatory techniques within the capabilities of typical Huntington Study Group sites, blood from a single 5-mL ethylenediaminetetraacetic acid tube, and no special PBMC processing. The large number of blood samples from premanifest individuals with a range of predicted times to disease onset, as well as from subjects with observed phenoconversion, provided an opportunity to examine whether PBMC Htt levels undergo any changes that occur as premanifest individuals progress through the HD prodrome and become symptomatic. Our results demonstrate that the assay can effectively measure mtHtt and tHtt (tables 1 and 2) in multicenter sample sets without undue site or analytic laboratory burden. The assay has limited sensitivity in distinguishing expanded from nonexpanded subjects (data not shown), because immunoassays are inherently variable and because the MW1 antibody has relative but not absolute selectivity for mtHtt and so signal/noise is limited. The genetic test is far more sensitive and specific for determining the presence of the HD mutation.[26] However, the primary usefulness of this assay is not as a diagnostic assay but for detecting changes in mtHtt caused by treatments affecting it or by clinical covariates, and the current results suggest that it could serve as a pharmacodynamic marker for treatments affecting Htt levels, much as we have used it in HD mouse models.[27]

When normalized to leukocyte concentrations, levels of mtHtt tended to diminish with progression through the HD prodrome and phenoconversion, suggesting reduced detection of mtHtt, either because of disproportionate loss or lost HTRF signal caused by Htt aggregation. A shift from soluble Htt to insoluble Htt aggregates, which are not detected by HTRF, with time and progression has been observed with HTRF studies of mtHtt in cell and mouse models.[28,29] When normalized to protein concentrations, mtHtt levels tended to remain stable through the HD prodrome and increase after phenoconversion suggesting impaired proteolysis of Htt. We speculate that mtHtt turnover may be slowed in leukocytes or more specifically in monocytes[25] perhaps because of impaired proteolysis or autophagy, leading to accumulation of soluble holoprotein and fragments.[25,29]

Although this is a cross-sectional study, these data suggest that the Htt HTRF assay could possibly mark progression in prodromal HD or phenoconversion, depending on the normalization used. Prospective longitudinal studies in premanifest and manifest subjects will be necessary to examine these possibilities. It is also interesting to consider why neurologic symptoms based on brain pathology might coincide with mtHtt signals in PBMCs. One possibility is that systemic influences, such as oxidative stress or inflammation, could concordantly modulate brain pathology and cause peripheral changes such as Htt oligomerization and aggregation.[30] The increase in plasma levels of the DNA damage marker 8-OH2dG that occurs as clinical symptoms develop is consistent with this possibility.[31,32] We have also described gene expression changes in leukocytes that indicate progressive influences on them from HD.[33] We hypothesize that PBMCs may act as sensors for HD with alterations in Htt, measured by HTRF, being one of many markers.

AUTHOR CONTRIBUTIONS

Miriam Moscovitch-Lopatin, PhD, PMP: study concept and design, drafting/revising the manuscript, analysis and interpretation of data, study supervision, acquired funds (National Institute of Neurological Disorders and Stroke). Rachel E. Goodman, BS: revising the manuscript, technical assistance, and analyzing raw data. Shirley Eberly, MS: drafting/revising the manuscript, statistical analysis, and analysis and interpretation of data. James J. Ritch, BS: revising the manuscript, technical assistance, and analyzing raw data. H. Diana Rosas, MD, MSc: revising the manuscript. Samantha Matson, BS: revising the manuscript, technical assistance. Wayne Matson, DSc: revising the manuscript. David Oakes, PhD: revising the manuscript, statistical analysis, and analysis and interpretation of data. Anne Buckingham Young, MD, PhD, and Ira Shoulson, MD: study design integration with PHAROS, revising the manuscript. Steven M. Hersch, MD, PhD: study concept and design, drafting/revising the manuscript, analysis and interpretation of data, study supervision, acquired funds (NIH, National Institute of Neurological Disorders and Stroke).

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