

Title: Mutant huntingtin and neurofilament light have distinct longitudinal dynamics in Huntington's disease

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One Sentence Summary:

Longitudinal analysis in 80 patients shows that mutant huntingtin and neurofilament light could predict Huntington's disease progression

Abstract: The longitudinal dynamics of the most promising biofluid biomarker candidates for Huntington's disease (HD) – mutant huntingtin (mHTT) and neurofilament light (NfL) – are incompletely defined. Characterizing changes in these candidates during disease progression could increase our understanding of disease pathophysiology and help the identification of effective therapies. In an 80-participant cohort over 24 months, mHTT in cerebrospinal fluid (CSF), and NfL in CSF and blood, had distinct longitudinal trajectories in HD mutation carriers compared with controls. Baseline analyte values predicted clinical disease status, subsequent clinical progression and brain atrophy, better than did the rate of change in analytes. Overall NfL was a stronger monitoring and prognostic biomarker for HD than mHTT. Nonetheless, mHTT possesses prognostic value and might be a valuable pharmacodynamic marker for huntingtin-lowering trials.

Introduction

Despite knowledge of its monogenetic cause, no treatments have been shown to slow neurodegeneration in Huntington's disease (HD)(1, 2). However, multiple approaches aimed at lowering production of the causative mutant huntingtin protein (mHTT) are in human clinical trials(3-5). The ultimate goal – treating mutation carriers early, to prevent disease onset – will require prevention trials in premanifest HD mutation carriers (preHD).

Successful target engagement by the first targeted huntingtin-lowering therapeutic tested in HD patients – the antisense oligonucleotide tominersen (formerly IONIS-HTTRx/RG6042) – was demonstrated by dose-dependent mHTT reduction in cerebrospinal fluid (CSF) in a phase 1/2 trial(3), quantified by ultra-sensitive immunoassay(6). A reliable CSF to brain mHTT relationship has been established in animal studies(7, 8). This notable success led to the first phase 3 trial of such a drug, whose primary outcomes are the Total Functional Capacity (TFC) score of the Unified Huntington's Disease Rating Scale (UHDRS) in the USA, and a composite UHDRS (cUHDRS) measure combining motor, functional, and cognitive scores in the EU(9). Such clinical rating scales quantify overt clinical manifestations, but are less sensitive to detect deterioration, or its therapeutic benefit, in preHD(10-13) making their use as outcomes in prevention trials problematic. Though clinically relevant, they are also far removed from the core disease mechanism: neuronal injury by the HTT gene product. Quantifying biochemical manifestations of neurodegeneration can inform our understanding of pathobiology and the development and testing of novel therapies.

To this end, we recently showed that CSF concentrations of mHTT – the toxic pathogenic protein – and neurofilament light (NfL) – an axonal protein indicative of neuronal injury – are among the earliest detectable changes in HD, and are strongly associated cross-sectionally with

baseline measures of clinical severity and brain volume(14). In the longitudinal Track-HD cohort, we showed that blood NfL concentration independently predicts subsequent onset, clinical progression, and brain atrophy in HD over three years(15).

One unexpected finding from the phase 1/2 ASO trial was a transient elevation of NfL in CSF around five months after first dose(3). In the subsequent open-label extension of this trial(16), NfL rose again then fell in the context of ongoing huntingtin suppression, almost to baseline by month eight. Although it remains unexplained mechanistically, this observation attests to the combined value of mHTT and NfL – as potential pharmacodynamic/response, safety and monitoring biomarkers – to highlight changes of note in the “undiscovered country” of huntingtin-lowering. The relationship between early biomarker changes and subsequent clinical outcomes will of course be critical to follow. It also calls for a more detailed understanding of how these markers change over time throughout the natural history of Huntington’s disease to help the interpretation of their behaviour in clinical trials.

Here we present the mHTT and NfL findings from the two-year prospective longitudinal HD-CSF study, in which an 80-participant cohort of HD mutation carriers and controls underwent clinical assessments, sampling of CSF and plasma, and MR imaging, under strictly standardized conditions. We report here the longitudinal dynamics of CSF mHTT and NfL, studied and compared head-to-head in the natural history of HD. We assessed and compared the ability of the biomarkers at baseline, and their rates of change, to predict longitudinal progression in clinical and neuroimaging measures. We employed advanced methods including random forest methodology and event-based modelling, permitting the exploration of non-linear relationships and multiple variables at once. Finally, we performed computational clinical trial simulations to provide insight into how they could be used and combined in the therapeutic context.

Results

The HD-CSF Cohort

Seventy-four (92.5%) out of the eighty baseline participants returned for the 24-month follow-up assessments. Three (4%) out of the seventy-four opted out of doing the follow-up lumbar puncture but agreed to blood and phenotypic data collection (Fig. 1). No preHD participant progressed to manifest HD during the follow-up period. A more detailed version of the study flow is provided in Fig. S1.

Full cohort characteristics are presented in table S1. Disease groups were well-matched for gender and differed as expected in HD clinical, cognitive and imaging measures. Age differed between groups due to the control group (50.68 years \pm 11.0) being matched to all HD mutation carriers, and manifest HD (56.02 years \pm 9.36) being more advanced in their disease course than preHD (42.38 years \pm 11.04), as previously reported(14).

Longitudinal dynamics of mHTT and NfL

Longitudinal trajectories of each analyte within individuals are shown in Fig. 2A-C. For NfL in CSF and plasma, there was little overlap in the trajectories of HD mutation carriers and healthy controls. Mixed-effects models depict distinct patterns of longitudinal analyte dynamics (Fig. 2D-F). CSF and plasma NfL showed a sigmoidal pattern over time in HD mutation carriers, initially accelerating then later slowing, compared to a slow linear rise with ageing in controls. CSF mHTT, on the other hand, rose linearly with age, albeit with more variability.

To explore the ‘genetic dose-response relationship’ between the causative gene mutation and each biofluid measure we queried our models for the interaction between CAG and age in HD mutation carriers (linear effect for CSF mHTT, $p < 0.0001$; and nonlinear effect for CSF and

plasma NfL, $p=0.006$ and $p<0.001$, respectively; Fig. 2G-I; Fig. S2). Using a change-point analysis, we estimated the disease burden score (a combination of age and CAG) at which each analyte in HD mutation carriers starts to deviate from controls. The DBS change-points for each analyte (CSF mHTT, 188.9; CSF NfL, 248.6; and Plasma NfL, 236.1; Fig. S4) were used to annotate Fig. 2G-I with the age of expected departure from controls, for each CAG length.

Based on simulations of the models in Fig. 2D-F, CSF NfL rose in manifest HD by 98.85 pg/mL/year, in preHD by 79.16 pg/mL/year and in controls by 20.05 pg/mL/year (Fig. 2J).

Similar relative findings were seen for plasma NfL rates of change (controls 0.28 pg/mL/year, preHD 0.84 pg/mL/year, manifest HD 1.04 pg/mL/year; Fig. 2J).

Prognostic value for overall HD progression of baseline analyte versus its rate of change

We assessed the clinical associations of each analyte using the cUHDRS, a composite score derived from large natural history cohorts and combining motor, functional and cognitive symptoms to reflect overall HD clinical severity across clinically important domains(17). All three analytes were associated with cUHDRS cross-sectionally at both baseline and follow-up (Fig. S5). To assess the prognostic value of each analyte for HD progression, we first examined whether their baseline values predicted subsequent change in cUHDRS. All models included age as an independent variable to account for its known confounding effects. Second-tier models also included CAG – a known effect modifier and fundamental driver of HD pathology – as an independent variable. This 2-stepped approach allows the exploration of whether associations, accounting for acquired factors, are driven by the core genetic driver of HD or have predictive and/or prognostic value independent from it. Significant associations with subsequent cUHDRS change were found for all three analytes (CSF mHTT $r=-0.31$, 95%CI -0.57 to -0.03, $p=0.026$; CSF NfL $r=-0.38$, 95%CI -0.52 to -0.18, $p<0.0001$; plasma NfL $r=-0.47$, 95%CI -0.63 to -0.25,

$p < 0.0001$; Fig. 3A-C). When CAG was added to the model, only the association with baseline plasma NfL remained significant (CSF mHTT $r = -0.11$, 95%CI -0.48 to 0.18, $p = 0.513$; CSF NfL $r = -0.21$, 95%CI -0.48 to 0.00, $p = 0.098$; plasma NfL $r = -0.33$, 95%CI -0.58 to -0.08, $p = 0.011$; Fig. 3A-C; Table S2).

Next, we assessed whether the rate of change of each analyte gave any additional prognostic information beyond that given by a single baseline measurement. The rate of change in plasma NfL was associated with the rate of change in cUHDRS whereas CSF NfL showed weaker association and CSF mHTT showed no association (CSF mHTT $r = -0.17$, 95%CI -0.49 to 0.08, $p = 0.253$; CSF NfL $r = -0.20$, 95%CI -0.43 to -0.01, $p = 0.049$; plasma NfL $r = -0.34$, 95%CI -0.65 to -0.12, $p = 0.013$; Fig. 3D-F). These associations for rate of change were weaker than those of the baseline values, and did not survive adjustment for age when also including CAG in the models (CSF mHTT $r = -0.09$, 95%CI -0.46 to 0.15, $p = 0.539$; CSF NfL $r = -0.09$, 95% CI -0.33 to 0.10, $p = 0.401$; plasma NfL $r = -0.22$, 95%CI -0.54 to -0.00, $p = 0.111$; Fig. 3D-F; Table S2).

In our analysis of fast and slow progressors (\geq or $<$ 1.2 decline in cUHDRS respectively), baseline CSF mHTT, CSF NfL and plasma NfL were all significantly higher in faster progressors (mean differences: CSF mHTT 19.27 fM, 95%CI 10.74 to 27.80, $p < 0.0001$; CSF NfL 1066.05 pg/mL, 95%CI 0. 532.62 to 1599.48, $p < 0.0001$; plasma NfL 11.44 pg/mL 95%CI 6.45 to 16.43, $p < 0.0001$; Fig. 3G-I). Only plasma NfL remained associated after adjustment for age and including CAG in the model (mean differences: CSF mHTT 7.38 fM, 95%CI -0.92 to 15.68, $p = 0.081$; CSF NfL 449.75 pg/mL, 95%CI -61.52 to 961.02, $p = 0.087$; plasma NfL 5.59 pg/mL, 95%CI 1.24 to 9.93, $p = 0.032$; Fig. 3G-1).

We repeated this analysis using the rate of change in each analyte. Only the rate of change of plasma NfL was significantly higher in faster progressors (mean differences: CSF mHTT 0.67

fM/year, 95%CI -1.75 to 3.09, $p=0.584$; CSF NfL 230.23 pg/mL/year, 95%CI -29.32 to 489.78, $p=0.082$; plasma NfL 2.83 pg/mL/year, 95%CI 1.03 to 4.62, $p=0.002$; Fig. 3J-L). This did not survive adjustment for age and including CAG in the models (mean differences: CSF mHTT -1.04 fM/year, 95%CI -3.69 to 1.61, $p=0.440$; CSF NfL 27.07 pg/mL/year, 95%CI -253.56 to 307.70, $p=0.849$; plasma NfL 1.22 pg/mL/year 95%CI -0.71 to 3.15, $p=0.210$; Fig. 3J-L).

We next used random forest analysis to compare head-to-head and illustrate the relative importance ranking of the three biofluid analytes – including their baseline value and annualized rate of change – in prediction of HD clinical progression, alongside other established predictors (age, CAG and DBS). Using change in cUHDRS as a continuous outcome, baseline values ranked as stronger predictors than annualized rates of change (Fig. 3M). Similar results were obtained for prediction of fast versus slow progression (Fig. S6A).

Prognostic value of biofluid biomarkers for clinical, imaging and cognitive measures

Next, we compared the prognostic power of each analyte, in terms of both its baseline value and its rate of change, to predict progression in individual clinical and MRI measures (Fig. 4; Fig. S7-8; table S2A-B). Baseline measurements of all analytes had associations with subsequent decline in several clinical and imaging measures (Fig. 4; Fig. S7). Baseline CSF mHTT was associated with worsening in TFC ($r=0.40$, $p=0.001$) and atrophy of whole-brain ($r=0.38$, $p=0.008$), white-matter ($r=0.64$, $p<0.0001$), grey-matter ($r=0.59$, $p<0.0001$) and caudate ($r=0.45$, $p=0.002$), but had no association with TMS and cognitive measures ($r<0.20$, $p>0.242$). Baseline NfL, in both CSF and plasma, was associated with progression in all measures except TMS (CSF NfL: TFC $r=0.39$, $p<0.0001$; SDMT $r=0.20$, $p=0.039$; SWR $r=0.22$, SCN $r=0.26$, $p=0.016$; VFC $r=0.23$, whole-brain $r=0.44$, $p<0.0001$; white-matter $r=0.58$, $p<0.0001$; grey-matter $r=0.37$, $p=0.002$; caudate $r=0.47$, $p<0.0001$; Plasma NfL: TFC $r=0.46$, $p<0.0001$; SDMT $r=0.32$,

p=0.009; SCN $r=0.30$, $p=0.014$; whole-brain $r=0.56$, $p<0.0001$; white-matter $r=0.63$, $p<0.0001$; grey-matter $r=0.47$, $p<0.0001$; caudate $r=0.42$, $p<0.0001$; Fig. 4 and S7, table S2A). After including age and CAG in the models (Fig. S8; table S2B), associations remained significant only between baseline CSF mHTT and subsequent change in white-matter ($r=0.45$ $p<0.0001$) and grey-matter ($r=0.46$ $p=0.0001$); between baseline CSF NfL and subsequent change in TFC ($r=0.25$, $p=0.032$), white-matter ($r=0.37$, $p=0.009$) and caudate ($r=0.32$, $p=0.007$); and between baseline plasma NfL and SDMT ($r=0.29$, $p=0.023$), whole-brain ($r=0.45$, $p<0.0001$), white-matter ($r=0.46$, $p<0.0001$) and grey-matter ($r=0.30$, $p=0.023$).

In contrast, the rate of change in each analyte had no associations with progression in any measure apart from change in TMS (mHTT $r=0.46$, $p<0.0001$; CSF NfL $r=0.32$, $p=0.012$; Fig. 4, table S2A). These associations remained after adjustment for age and including CAG ($r=0.43$, $p=0.001$; $r=0.18$, $p=0.032$ respectively; Fig. S8, table S2B).

To compare the relative prognostic ability of the biofluid and imaging biomarkers within a single model, we repeated the random forest analysis, including only the baseline values for biofluid analytes and imaging biomarkers. Using change in cUHDRS as a continuous outcome, biofluid analytes ranked as stronger predictors than imaging biomarkers (Fig. 5). Similar results were obtained for prediction of fast and slow progression (Fig. S6B).

Using a receiver operating characteristics (ROC) curve analysis, we compared the discriminatory ability of each analyte's baseline value and rate of change, to distinguish between different clinical states: controls versus HD mutation carriers, and between premanifest versus manifest HD. For all three analytes, rate of change had poor ability to distinguish in either comparison; areas under the curves (AUCs) were approximately 0.5 (no better than chance). Baseline concentrations had excellent discriminatory ability with AUCs greater than 0.8 (Fig. 6). In each

condition, and for each analyte, the AUC for the baseline measurement was significantly greater than that for its rate of change (controls versus HD mutation carriers: CSF mhtt $p < 0.0001$; CSF NfL $p < 0.0001$; plasma NfL $p < 0.0001$; premanifest versus manifest HD: CSF mhtt $p = 0.001$; CSF NfL $p < 0.0001$; plasma NfL $p < 0.0001$; Fig. 6).

Simulating clinical trials with biofluid biomarker surrogate endpoints

The data thus far suggests that these analytes indicate current clinical state and might have prognostic value for clinical decline. We used longitudinal data from the HD-CSF cohort to run computationally simulated clinical trials using CSF mHTT, CSF NfL and plasma NfL as possible surrogates for clinical progression. These simulations assume that the intervention-induced change in the analyte emulate the change expected in clinical state by an intervention. Fig. 7 depicts the relationships between statistical power, sample size and trial duration for such trials, using a nominal 20% drug effect per year on the biomarker trajectory. For longitudinal change in NfL, in CSF or plasma, fewer than 100 participants per arm are needed to show an effect over 9 months. More than 10,000 participants per arm would be required to achieve 80% power for a similar trial using the lowering of CSF mHTT as a surrogate outcome over 24 months. Note that this calculation is based on mHTT release and does not apply to any trial in which the intervention reduces its production directly and lowers the protein below baseline values. Comparable simulations for TFC and cUHDRS, commonly used as clinical trial endpoints, are shown in Fig. S9.

Technical validation of cross-sectional baseline results across assays

CSF mHTT, CSF NfL and plasma NfL were re-measured in baseline samples using the same methods used for the follow-up samples, in order to perform the longitudinal analysis.

Comparing the re-measured values with those previously published at baseline, batch, assay or

storage effects did not affect our samples (Fig. 8; Table S3). We used the re-measured data to replicate our previously published cross-sectional findings(14). This included each analytes' inter-group differences (Fig. S10); associations with clinical and imaging measures (Fig. S11-13; Table S4); ROC curves and AUC analysis (Fig. S13A-B); analyte correlations (Fig. S13C-D); and event-based modelling (EBM; Fig. S13E-F). All results for NfL were similar to those previously published(14). For CSF mHTT, the re-measured values had stronger associations with clinical measures and, where it previously lacked statistically significant association with brain volumes, the re-measured mHTT was now associated with grey-matter and caudate volume (Fig. S12; Table S4).

Replication of cross-sectional results in follow-up data

We used the samples and data from the 24-month follow-up to examine whether our cross-sectional findings held true in the same cohort two years later (Fig. S14-17). All results for NfL were similar to those previously published and to the re-measured baseline data. For CSF mHTT, we replicated the stronger associations with clinical measures and found further stronger associations with all brain volumes, similar to those for NfL (Fig. S15-16; Table S5). Our data-driven event-based model for staging participants based on the totality of their baseline data was validated longitudinally, showing that at follow-up nearly all participants had an EBM stage greater than or equal to the baseline EBM (indicating progression; Fig. S18).

Discussion

Here, we present the 24-month results of HD-CSF, a longitudinal study of biofluid biomarkers in HD mutation carriers and matched controls, with longitudinal clinical and MRI data. We have characterized and compared the longitudinal dynamics of mHTT and NfL in CSF in HD for the first time, defining the trajectories of these biofluid biomarkers and the inflection points at which they depart from healthy controls in the natural history of HD. Although rates of change in the analytes had some prognostic value, a single measurement at baseline of each analyte exhibited stronger ability to predict subsequent clinical decline, brain atrophy and disease state. How our prognostic findings add to what was known about these biomarkers is summarised in context in table S6. Using clinical trial simulations, we showed that NfL might be used as an outcome measure of neuronal protection and disease progression, to run trials of feasible duration.

mHTT in CSF, and NfL in CSF and plasma, all rose detectably within participants over 2 years. Over the whole course of the disease, mHTT increases rose linearly with age, whereas NfL rose in a more sigmoidal pattern. The disease-associated rise in NfL was more consistent whereas mHTT was more variable within individuals. The NfL trajectory was distinct from that in healthy controls, with little overlap. This suggests that monitoring these biomarkers against an age-relevant reference range derived from the healthy population might be clinically meaningful. The dynamics of both CSF mHTT and NfL were also CAG-dependent, revealing longitudinally the genetic dose-response relationships that we demonstrated previously for plasma NfL in the TRACK-HD cohort(15). Change-point analysis identified the approximate age and analyte concentration at which HD mutation carriers became detectably different from controls, for a given CAG repeat length. Defining these points of deflection from the trajectories in healthy controls may help us move towards models based on CAG repeat length that could be used to

enrich or stratify clinical trial participants and could eventually be used to personalise treatment approaches(18).

Here, we assessed the clinical prognostic potential of biofluid biomarkers against the cUHDRS – a composite measure derived from large cohort datasets to have high signal-to-noise ratio as a longitudinal measure of disease progression(17). mHTT and NfL concentrations predicted change in cUHDRS, affirming their potential as biomarkers of HD progression. We show that both CSF mHTT and NfL each possess prognostic value for subsequent clinical decline and brain atrophy, as seen previously for plasma NfL in the TRACK-HD cohort.

For rate of change in analytes, there was an association with change in TMS but no other measures. That the rate of change in each analyte had lesser prognostic and discriminatory power than their baseline values may appear surprising. Longitudinal studies of NfL in other genetic neurodegenerative diseases, including dominantly inherited Alzheimer's disease (AD)(19, 20) and frontotemporal dementia (FTD) (21), have revealed that the rate of change in NfL was a stronger predictor of disease progression, with accelerated rate of change in those who converted from presymptomatic to symptomatic. The HD-CSF study was not designed to assess predictors of conversion from premanifest to manifest HD, but the rate of change in plasma NfL did show prognostic value in our comparison of fast versus slow progressors. NfL is an axonal protein but not specific to neuronal sub-populations or to a given disease pathology. It is likely that each disease exhibiting neuronal dysfunction will have a distinct longitudinal NfL profile. It is notable that HD has some of the highest elevated concentrations of CSF NfL compared to other neurological diseases studied to date, greater than concentrations in AD and FTD which have more rapid clinical progression(22). A baseline measure encompasses the totality of a disease's

effects up to the point in a person's life; by comparison, in a slowly progressive disease, even a 2-year change value captures a relatively small further difference.

The relative importance rankings generated from unbiased random forest analyses suggest that a single measurement of plasma NfL has equivalent, if not superior, prognostic value to that of disease burden score – one of the strongest predictors of HD progression. Further, the baseline biofluid biomarkers were stronger predictors of clinical progression than were brain volumes. This is supportive of a single measurement of biofluid biomarkers being closely indicative of the dynamic pathological processes driving disease progression.

Our computational clinical trial simulations offer a means to plan future clinical trials that would use the lowering of these biomarkers as surrogate endpoints. They suggest that a trial using plasma NfL with as few as 100 participants per arm run over six months would have over 90% power to show 20% slowing of the expected longitudinal trajectory of the analyte and, under our assumptions, the slowing of clinical decline. Using CSF NfL, the same trial would need to be run over nine months to achieve the same power. This is promising, when compared to the several hundred participants per arm required to achieve the same effect size over 24 months for both cUHDRS and TFC – current clinical end points of the ongoing phase 3 huntingtin-lowering trial(9). Currently, clinical trials tend to focus on specific subsets of patients, whilst our simulations are based on a broad range of the disease spectrum including from premanifest and manifest HD. In earlier disease stages, such as for prevention trials in premanifest HD, the sample sizes are likely to be higher because longitudinal change is slower. Dedicated study in a large premanifest cohort would be needed to attain the statistical power to model this group independently. Another caveat here is that we assume that the effect size lowering in NfL would be equivalent to the same effect size for improvement in clinical outcome. Until there is a

clinically efficacious intervention for HD, this hypothesis cannot be tested. Because of a slower rate of increase, and more importantly, the intra- and inter-subject variability in the change over time, in our simulations a much larger participant number would be needed to show a deflection in the trajectory of mHTT as a surrogate outcome (not a pharmacodynamic outcome) by slowing disease progression alone. However, given the existing associations of CSF mHTT as a prognostic and pharmacodynamics response marker, it is likely that this measure too will turn out to have some predictive utility in the drug trial context. NfL is less variable and appears to be a better marker of HD progression and prognosis than mHTT. However, it is important to reiterate that mHTT retains its intrinsic value as a direct measure of the causative neurotoxin and as a means of assessing the on-target effects of huntingtin-lowering agents. To achieve this purpose, very small participant numbers are required, as shown by our previous cross-sectional power calculations and findings from the first human trial of such an agent(3, 14). Our finding that lower mHTT concentrations predict slower progression is potentially important in this respect. Importantly, we also show that long-term freezer storage of samples does not adversely impact quantification of mHTT and NfL.

In our previously published baseline analysis(14), CSF mHTT concentration was not cross-sectionally associated with any brain volume measure. However, in our replication of cross-sectional analyses we found associations between CSF mHTT and caudate and grey-matter volume at baseline, and strong associations with all brain volumes with the samples from the 24-month collection. This was likely because the performance of the mHTT assay, which has been enhanced through its use in clinical trial programs, has improved so as to reduce variability in the latest analysis. Further, baseline CSF mHTT values also predicted subsequent brain atrophy, confirming that mHTT concentration in CSF per se has prognostic potential in HD, beyond its

intrinsic appeal as a therapeutic target. It remains important to note that several participants' mHTT values were below the lower limit of quantification of the assay, an indication that more sensitive HTT assays will be needed, especially in the realm of preHD and prevention trials.

Our study is not without limitations. Despite being a large longitudinal natural history cohort CSF collection with matched MRI data in HD, the sample number remains modest. We lack the granularity to make predictions of clinical outcome at the individual level. Larger sample numbers and greater follow-up durations may bring us closer to developing models that could inform clinical prognosis. We are also limited to two longitudinal time points. The lack of associations between rate of change in analyte and the rate of clinical decline was likely driven by both sets of rates being derived from the same time period. Having additional time points would permit the comparison of rates of change in analyte with rates of clinical decline in the subsequent time period. For instance, change in NfL in year 1 may predict brain atrophy in year 2. The range of HD mutation carriers in this study does not cover the whole spectrum of HD. This study was not designed to detect the very earliest disease-related alterations, nor what happens in the later stages of disease. Nevertheless, our range is broader than what is currently recruited for clinical trials. Finally, the sample sizes for TFC and cUHDRS generated from the clinical trial simulations were larger compared to ongoing trials. It is important to note that given the range in HD clinical severity, the variability is larger in our cohort, and we used a conservative effect size, all of which drive larger sample size.

Efforts are well underway to address these issues: HDClarity, a multi-national CSF collection initiative for HD has amassed over 600 CSF and plasma samples across the disease spectrum and is now accumulating longitudinal samples over repeated annual intervals (23). There are also ongoing efforts to build a larger information set with a broader range of age and CAG

distributions encompassing studies such as HD-CSF, HDClarity, Track-HD(24), Predict-HD(25) and HD-YAS(26).

These insights into the longitudinal dynamics of mHTT and NfL shed light on the biology of HD in human mutation carriers and will be of immediate value in the design and conduct of disease-modifying clinical trials, especially as we enter the era of prevention trials where qualified surrogate endpoints will be fundamental(27). The case is far from made that either of the markers is a true surrogate of clinical progression - we will only know this once we have disease modifying intervention. Looking ahead, some centres are already incorporating blood NfL measurement into shared clinical decision-making in neurological disease (18). Continued study may reveal a role for mHTT and NfL in guiding decision-making for individuals living with HD.

Materials and Methods

Study design

HD-CSF was a prospective single-site study with standardised longitudinal collection of CSF, blood and phenotypic data (online protocol: DOI: 10.5522/04/11828448.v1)(14, 28). Eighty participants were recruited. All phenotypic assessment measures were predefined for HD-CSF based on metrics shown to have the largest effect sizes for predicting HD progression(24).

This study was performed in accordance with the principles of the Declaration of Helsinki, and the International Conference on Harmonization Good Clinical Practice standards. Ethical approval was obtained from the London Camberwell St Giles Research Ethics Committee (15/LO/1917). Prior to undertaking study procedures, all participants gave informed consent which was obtained by clinical staff.

Power calculations, inclusion criteria, and clinical and imaging characterization are as previously described (Supplementary methods)(14).

Sample collection and processing

Sample collection and processing were as previously described(14). All collections were standardised for time of day after overnight fasting and processed within 30 minutes of collection using standardised equipment. Blood was collected within 10 minutes of CSF and processed to plasma.

Analyte Quantification

CSF and plasma NfL were quantified in duplicate using the Neurology 4-plex B assay on the SimoaHD-1 Analyzer (Quanterix), per manufacturer's instructions. A 4x dilution for blood samples was performed automatically by the HD-1 Analyser and CSF samples were manually

diluted 100x in the sample diluent provided prior to loading onto the machine. The limit of detection (LoD) was 0.105 pg/mL and lower limit of quantification (LLoQ) 0.500 pg/mL. NfL was over the LLoQ in all samples. The intra-assay coefficient of variance (CV) (calculated as the mean of the CVs for each sample's duplicate measurements) for CSF NfL and plasma NfL was 5.0% and 3.7% respectively. The inter-assay CVs (calculated as the mean of the CVs for analogous spiked positive controls provided by the manufacturer and used in each well plate) for CSF NfL and plasma NfL were 2.7% and 8.4% respectively. We previously quantified NfL in the same baseline samples using an ELISA (NF-Light, UmanDiagnostics) in CSF and 1-plex Simoa kit (NF-Light, Quanterix) in plasma(14). In both biofluids, agreement between assays was good (Fig. 8).

CSF mHTT was quantified in triplicate using the same 2B7-MW1 immunoassay as at baseline (SMC TM Erenna platform, Merck)(6). The LoD was 8fM and LLoQ 25fM. All control samples were below the LoD of the assay except one subject's baseline re-measured sample. These were imputed as 0 fM for analysis purposes. 27 (21%) samples were below the LLoQ and were included in subsequent analyses. One preHD had CSF mHTT below the LoD in their re-measured baseline sample and was not included in the analyses. The intra-assay CV for CSF mHTT was 14.1%. Haemoglobin contamination was quantified using a commercial ELISA (E88-134, Bethyl Laboratories) by Evotec. Only 1 sample (2.186 µg/mL) had haemoglobin just over the 2 µg/mL recommended threshold(29).

Assays were run using same-batch reagents, blinded to clinical data.

Statistical Analysis

As previously observed(14, 15), NfL distributions were right-skewed, therefore log-transformed values were used for analytical purposes. Due to their known effects on HD, models included age and CAG repeat count as covariates.

Cross-sectional analyses: To validate previous findings and compare assays, we replicated the cross-sectional analyses from the study baseline(14), using re-measured baseline data and newly collected 24-month follow-up data. To investigate intergroup differences, we applied generalized linear regression models estimated via ordinary least squares, with analyte concentration as the dependent variable, and group membership, and age as independent variables and then with group membership, age and CAG as independent variables. To study associations in HD mutation carriers between the analytes and clinical or imaging measures we used Pearson's partial correlations adjusted for age and for age and including CAG in the models. Bias-corrected and accelerated bootstrapped 95% confidence intervals (95% CI) were calculated for mean differences and correlation coefficients. To understand the discriminatory power of the studied analytes, we produced receiver operating characteristics (ROC) curves for each analyte to differentiate healthy controls from HD mutation carriers, and premanifest from manifest HD and compared areas under the curves (AUC), formally using the method suggested by DeLong and colleagues(30).

Longitudinal modelling: For modelling analyte trajectories over time generalized mixed effect models were performed, estimated via restricted maximum likelihood, with analyte concentration as the dependent variable. Independent models were developed for healthy controls and HD mutation carriers. Only HD mutation carriers were modelled for mHTT. For CSF mHTT in HD mutation carriers, the model had fixed effects for age and CAG, a random intercept per participant and a random slope for age. A similar model was used for healthy controls for NfL in

CSF and plasma. HD mutation carriers were modelled with fixed effects for age (second-order) and CAG, and random slopes for age were included for both CSF and plasma NfL.

Change-point analysis: We use an offline Bayesian change-point algorithm to estimate the most likely disease time at which a given biomarker changes from a normal to abnormal state. The algorithm was adapted from Zhou et al 2017(31) and estimates the marginal likelihood that the data over a segment of time is generated by a given underlying model. We explicitly model changes in both the mean and covariance and use a minimally informative prior. The change-point is then inferred by a change in likelihood of the underlying model Fig. S4. As we want to estimate the point of change from normality to abnormality, we use data from all groups (control, preHD, and HD) to fit the model over each time segment.

Rates of change simulations: The longitudinal models above were used to estimate rate of change from simulated data. Model parameters, age and CAG distributions, and sample sizes were mimicked from the HD-CSF cohort. Each simulation was repeated 1,000 times and run independently for each analyte for each participant subgroup (healthy controls, premanifest and manifest HD).

Associations of the analytes' baseline values, and of their rates of change, with clinical and imaging changes, were assessed using Pearson's partial correlations adjusted for age, and for age and including CAG in the models. Rates of change were computed as the 24-month follow-up value minus the baseline value divided by the follow-up time in years. Bias-corrected and accelerated bootstrapped 95%CI were calculated for correlation coefficients and mean differences. To further explore clinical prognostic value, we divided mutation carriers into nominally "fast" and "slow" progressors at the previously-described cUHDRS minimal clinically important difference for decline (absolute 1.2-point reduction)(32). Intergroup differences were

investigated with generalized linear regression estimated via ordinary least squares, with analyte concentration or rate of change as dependent variable, and group membership and age, and then group, age and CAG as independent variables.

Receiver operating characteristic (ROC) curves were produced and areas under the curves (AUC) compared for the ability of analytes' baseline values and rates of change to differentiate healthy controls from mutation carriers, and preHD from manifest HD using the method of DeLong(30).

Clinical trial simulation: We used 1,000 repetitions, a parallel design without attrition or placebo effect, a pseudo-control arm emulating the observed longitudinal trajectories, and an intervention arm with constant 20% annualized reduction in the analyte of interest. Synthetic datasets were generated with Monte Carlo simulations using mixed effect models matching the longitudinal models above, including all gene expansion carrier participants. Main effects were estimated as inter-arm mean difference in the mean change from baseline, adjusted for CAG using generalized linear models estimated as above.

Data were analysed using StataMP 16 (StataCorp).

Random forest analyses: To complement our analyses, we used random forest methodology, a supervised ensemble-learning approach based on building multiple independent decision trees from bootstrapped samples(33, 34). This allows the detection of non-linear relationships, and simultaneous ranking of predictors of clinical change in multivariate analyses. We implemented random forests on the following outcomes: annualized rate of change in cUHDRS and cUHDRS as a binary outcome: "fast" and "slow" progressors defined as above. Models were first run with biofluid biomarkers including their baseline value and annualized rate of change, and then with biofluid and imaging biomarkers baseline value as predictor variables. Age, CAG repeat count,

and DBS were included in all models to serve as comparators variables. Each random forest had 1,000 trees and was based on bootstrapped samples with replacement and three randomly sampled predictor variables were considered for splitting each node. Relative importance rankings were based on the mean decrease Gini score across all trees, where a higher mean decrease in Gini indicates greater predictor variable relative importance at predicting outcomes. To explicitly test the stability of our results and generate ranking distributions, we re-ran the model 100 times, each containing 80% of the possible observations (randomly selected). Random forests were implemented using R randomForest package(34).

Supplementary Materials

Fig. S1. Extended HD-CSF study participant disposition.

Fig. S2. Modelling genetic dose-response relationships by individual CAG repeat length.

Fig. S3. Age and CAG repeat count distribution at baseline for HD mutation carriers.

Fig. S4. Change-point analysis for defining the point of deflection from controls in each analyte.

Fig. S5. Cross-sectional associations with cUHDRS at baseline and 24-month follow-up.

Fig. S6. Random forest analysis of fast and slow progressors.

Fig. S7. Scatter plots for associations between biofluids and the annualized change in HD biomarkers

Fig. S8. Correlation coefficients for associations between biofluids and the annualized change in HD biomarkers adjusted for age and including CAG.

Fig. S9. Statistical power, sample size and trial duration of two currently used clinical scales.

Fig. S10. Cross-sectional disease group comparisons in re-measured baseline samples.

Fig. S11. Cross-sectional clinical associations in re-measured baseline samples.

Fig. S12. Cross-sectional associations in re-measured baseline samples between analyte concentrations and imaging measures.

Fig. S13. Receiver Operating Characteristics (ROC) analysis, analyte correlations and Event-Based Modelling (EBM) in re-measured baseline samples.

Fig. S14. Cross-sectional disease group comparisons in 24-month follow-up samples.

Fig. S15. Cross-sectional clinical associations in 24-month follow-up samples.

Fig. S16. Cross-sectional associations in 24-month follow-up baseline samples between analyte concentrations and imaging measures.

Fig. S17. Receiver Operating Characteristics (ROC) analysis, analyte correlations and Event-Based Modelling (EBM) in 24-month follow-up samples.

Fig. S18. Longitudinal validation of EBM. Predicted EBM stages in the HD-CSF cohort at baseline and follow-up.

Table S1. Full cohort characteristics at baseline and 24-month follow-up.

Table S2. Longitudinal correlations between baseline values or annualized rate of change (Δ) of each analyte and the Δ in clinical and imaging measures.

Table S3. Assay comparison and test-retest.

Table S4. Re-measured baseline cross-sectional correlations between analytes and clinical and imaging measures.

Table S5. Follow-up cross-sectional correlations between analytes and clinical and imaging measure.

Table S6. Summary of what this study adds to our previous understanding of the performance of mHTT and NfL as biomarkers for HD.

Supplementary methods

Supplementary references

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Author contributions:

EJW designed the study with the input of HZ, RIS, SS and SJT. FBR and LMB were involved in participant recruitment. Eligibility, clinical examinations and sample collection were performed by FBR, LBM, and RT. Imaging assessments were conceived RIS, EBJ and EDV, data was acquired by EDV, MA, EBJ, and processed by RIS and EBJ. AH and LMB processed and

analysed the patient samples. FBR developed and performed the statistical analysis; PAW and DCA developed and performed the EBM and change-point analyses; NG, HF, RH and SS developed and performed the random forest analysis. FBR, LMB and EJW interpreted the data and wrote the manuscript; and all authors contributed to reviewing the manuscript.

Competing interests:

FBR, LMB, RT, EBJ, PAW, DCA, SJT, RIS, AH, HZ, EJW are University College London employees. MA is a University College London Hospitals NHS Foundation Thrust employee. EDV is a King's College London employee. NG, RH, HF, SS are full-time employees of F. Hoffmann-LaRoche. FBR has provided consultancy services to GLG and F. Hoffmann-La Roche Ltd. LMR has provided consultancy services to GLG, F. Hoffmann-La Roche Ltd, Genentech and Annexon. RIS has undertaken consultancy services for Ixitech Ltd. SJT receives grant funding for her research from the Medical Research Council UK, the Wellcome Trust, the Rosetrees Trust, Takeda Pharmaceuticals Ltd, Vertex Pharmaceuticals, Cantervale Limited, NIHR North Thames Local Clinical Research Network, UK Dementia Research Institute, and the CHDI Foundation. In the past 2 years, S.J.T. has undertaken consultancy services, including advisory boards, with Alnylam Pharmaceuticals Inc., Annexon Inc., DDF Discovery Ltd, F. Hoffmann-La Roche Ltd, Genentech, PTC Bio, Novartis Pharma, Takeda Pharmaceuticals Ltd, Triplet Therapeutics, UCB Pharma S.A., University College Irvine and Vertex Pharmaceuticals Incorporated. All honoraria for these consultancies were paid through the offices of UCL Consultants Ltd., a wholly owned subsidiary of University College London. HZ has served at scientific advisory boards for Roche Diagnostics, Wave, Samumed and CogRx, has given lectures in symposia sponsored by Biogen and Alzecure, and is a co-founder of Brain Biomarker

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Data and materials availability:

The data that support the findings of this study are available at DOI:10.5522/04/12709391 (<https://figshare.com/s/c4b7f3aa731aebf9a9d7>).

Figure legends

Fig. 1. HD-CSF study participant disposition. Baseline visit (n=80) was performed 24-months (± 3 months) before the follow-up visit (n=74). Optional repeat sampling visits occurred 6-8 weeks after baseline. A more detailed version including all study assessments is provided in Fig. S1.

Fig. 2. Longitudinal dynamics of mHTT and NfL over 24 months. (A-C) Individual participant trajectories. Connected dots are measurements within the same participant. Disease groups are colour coded as per (J). (D-F) Modelled biomarker trajectories. Model (solid line), 95% (coloured area) and 99% (dashed line) bias-corrected accelerated confidence intervals were generated from generalized mixed effects models. For CSF mHTT, age was used as first-order fixed effect, whereas for HD mutation carriers for NfL there was a first- and second-order fixed effect for age. All models included CAG repeat count and had a random intercept for participant, and corresponding random slopes for age. Dots represent the observed values. For ease of visual interpretation 2 individual data points ($>6,000$ pg/mL) were included in the model but excluded from the figure. (G-I) Modelling genetic dose-response relationships to show associations between biomarkers, age and CAG repeat count. Solid lines were produced from our observations using the models above; dashed lines are predictions outside the range of our observations. Separate figures with individual data points for each individual CAG repeat count are provided in Fig. S2 and the distribution of age and CAG within the cohort are supplied in Fig. S3. Grey diamonds show the age of predicted onset for each CAG length (as per (35)). Coloured diamonds show the age at which HD mutation carrier trajectories

are most likely to depart from healthy controls trajectories for each CAG repeat count, generated by change-point analysis. (J) Annualized rates of change and 95% confidence intervals. For each biomarker, estimates were computed as the average of the rate of change in 1,000 simulations per group of study participants (healthy controls, premanifest and manifest HD). Note that panels (A), (D) and (G) referring to mHTT do not depict values for controls. For CSF mHTT, shaded areas mark the limits of detection (LoD, 8 fM) and quantification (LoQ, 25 fM) of the assay. CSF, cerebrospinal fluid; mHTT, mutant huntingtin; N/A, not applicable; NfL, neurofilament light.

Fig. 3. Longitudinal associations of mHTT and NfL with disease progression quantified by cUHDRS. Associations between (A-C) baseline values or (D-F) annualized rate of change in each analyte and the annualized rate of change in the cUHDRS. Partial Pearson's correlation coefficient adjusted for age, and p-values are presented. Grey figures were estimated including CAG in the model. Dashed horizontal lines mark no change in cUHDRS, with negative values representing deterioration. Dashed vertical lines mark no change in the biomarker. The baseline values (G-I) and annualized rate of change (J-L) for each biomarker compared between 'fast' (n=24) and 'slow' progressors (n=30), defined as participants with an absolute decrease in cUHDRS greater than or equal to 1.2 over the follow-up period. (M) Random forest plot for prediction of annualized rate of change in cUHDRS including baseline and rate of change of biofluid biomarkers as predictors. Higher relative importance score indicates a greater relative importance of the variable in predicting worsening cUHDRS score. Relative importance scores were based on mean decrease in Gini score. Distributions were generated from re-

running the model 100 times. The boxes show the median, and 25% and 75% percentiles, whereas whiskers are the lower and upper adjacent values (1.5 times the interquartile range minus the 25% percentile or plus the 75% percentile). Dots are values under and above the adjacent values. Violin plots represent ranking distributions. Black diamonds represent the median. CSF, cerebrospinal fluid; cUHDRS, composite Unified Huntington's Disease Rating Scale; mHTT, mutant huntingtin; NfL, neurofilament light; DBS, disease burden score; Δ , annualized rate of change.

Fig. 4. Comparison of prognostic abilities of mHTT and NfL for clinical and imaging

measures. Matrices show the Pearson's partial correlation coefficients adjusted for age only for associations between baseline values or annualized rate of change (Δ) of each analyte and the annualized rate of change in clinical and imaging measures, each expressed such that higher positive values denote clinical worsening. Colour coding displays the magnitude and the direction of the association. Coefficients with corresponding confidence intervals and p-values for each combination are provided in table S2. Scatter plots with individual data points for each association are provided in Fig. S7. CSF, cerebrospinal fluid; cUHDRS, composite Unified Huntington's Disease Rating Scale; mHTT, mutant huntingtin; NfL, neurofilament light; r, Pearson's partial correlation coefficient; SDMT, Symbol Digit Modalities Test; SCN, Stroop Color Naming; SWR, Stroop Word Reading; TFC, UHDRS Total Functional Capacity; TMS, UHDRS Total Motor Score; VFC, Verbal Fluency - Categorical; Δ , annualized rate of change.

Fig. 5. Associations of baseline markers with clinical progression. Random forest plot for prediction of annualized rate of change in cUHDRS including baseline biofluid and imaging biomarkers as predictors. Higher relative importance score indicates a greater relative importance of the variable in predicting worsening cUHDRS score. Note that this figure uses the same technique as Figure 3M but this model examines a different set of variables. Relative importance scores were based on mean decrease in Gini score. Distributions were generated from re-running the model 100 times. Violin plots represent ranking distributions. CSF, cerebrospinal fluid; cUHDRS, composite Unified Huntington's Disease Rating Scale; mHTT, mutant huntingtin; NfL, neurofilament light; Δ , annualized rate of change.

Fig. 6. Discriminatory ability of mHTT and NfL for disease state. ROC curves comparing the discriminatory ability of baseline values for each analyte and its annualized rate of change to distinguish (A) between healthy controls and HD mutation carriers and (B) between premanifest and manifest HD. p-values are for comparison between the baseline AUC and rate of change AUC. AUC, area under the curve; CSF, cerebrospinal fluid; mHTT, mutant huntingtin; HD, manifest Huntington's disease; NfL, neurofilament light; PreHD, premanifest Huntington's disease; Δ , annualized rate of change.

Fig. 7. Statistical power, sample size and trial duration. Monte Carlo simulations predicting the statistical power of using (A) CSF mHTT, (B) CSF NfL and (C) plasma NfL in the clinical trial context, contingent on sample size per arm and trial duration in months. 2-arm parallel design clinical trials with no attrition or placebo effect with a constant effect

size of 20% reduction in each analyte per year were simulated. Each pixel represents 1,000 simulated clinical trials, generated using generalized mixed effects models shaped to estimate the longitudinal trajectories of each biomarker (as in Fig. 2). The main effect in each simulation repetition was calculated as the inter-arm mean difference in the mean change from baseline, using generalized linear models adjusted for CAG. Statistical power was calculated as the proportion of trial simulations with a p-value < 0.05 for the main effect. CSF; cerebrospinal fluid; mHTT; mutant huntingtin; NfL; neurofilament light.

Fig. 8. Time in freezer and comparison of re-measurement of baseline samples. (A-C) Time in freezer and analyte of interest. (D-E) Associations between repeated baseline measurements for CSF mHTT (purple; D, G), CSF NfL (blue; B, E) and plasma NfL (red; C, F) within controls (n=20) and HD mutation carriers (n=60; table S3). (G-I) Bland–Altman plots for re-measurement of baseline samples. The difference between the two measurements is plotted against the mean of the two measurements. Mean difference is represented by the black solid horizontal line (table S3). The upper and lower 95% confidence intervals are designated with dotted horizontal lines. Each point represents one participant. PreHD, premanifest Huntington’s disease; HD, manifest Huntington’s disease; CSF, cerebrospinal fluid; mHTT, mutant huntingtin; NfL, neurofilament light.