Exploratory analysis of PENK as an early marker of HD onset and progression.

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Introduction

There are no proven disease-modifying treatments for Huntington's disease (HD), but multiple clinical trials are ongoing, exploring different modalities to stop or slow down disease progression and symptoms.
Several fluid biomarkers have been proposed to support these studies and to complement the already established motor and cognitive assessments. CSF levels of mHTT and Neurofilament light (NfL), a marker for neuronal degeneration, have been shown in several studies to follow disease progression and used in recent clinical trials as surrogate readout. However, the complexity of HD symptoms and the still limited knowledge about the pathophysiology underlining them suggest the need to develop additional biomarkers to support novel therapeutic approaches.

Proenkephalin (PENK) is a pre-pro protein that is highly expressed in some medium spiny neurons of caudate and putamen and is proteolytically processed to produce an array of peptides, including a stable penKid fragment (aa 119-159) and several opioid-like enkephalin peptides. Peptide fragments derived from PENK were recently shown to be significantly reduced in HD patient CSF, using unbiased mass spectrometry approaches highlighting the potential of PENK as a biomarker in HD.
The goal of this study was to independently validate the above findings and furthermore determine if PENK might also serve as an early marker of HD onset/progression. Toward this, we utilized a previously qualified immunoluminometric assay (ILMA) that measures penKid peptide to monitor changes in PENK levels in 90 HDClarity CSF samples.

Materials and methods

Immunoassay:

Sphingotest ® PenKid® is a diagnostic tool for quantitative measurement of Proenkephalin 119-159 (penKid) in human EDTA plasma and is used as an invitro diagnostic for kidney injury[4]. This chemiluminescence sandwich immunoassay was adapted for use in CSF. At the beginning of every experiment, a "Light Inspection Check (LIC)" using sphingotest® Lightning kit was performed using an adapted protocol for measurements on the PHERAstar FSX (BMG LABTECH) to ensure optimal signal response of the luminometer.

The relative luminescence units (RLU) values obtained are used to calculate Tare and Net Dynamics and compared with the expected reference values provided by Sphingotec.

$$\text{Tare Dynamics (TD)} = \frac{\text{Signal (Control Low)}}{\text{NSB}} \qquad \text{Net Dynamics (ND)} = \frac{\text{Signal (Control High) - NSB}}{\text{Signal (Control Low) - NSB}}$$

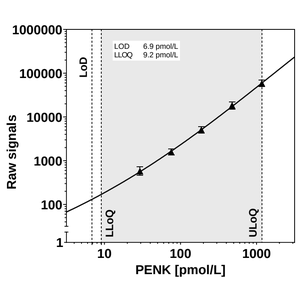
	Measured data	Target value
TD	60	≥ 20
ND	77.6	79.2 ± 13

In pilot-experiments, 3 independent LIC runs confirmed the performance of the reagents and the chosen Pherastar protocol. For the measurements of the HDClarity samples, Tare and Net Dynamics were in acceptance range for all runs.

Assay calibrators and internal controls:
Calibrators and internal controls were included in each assay measurement. The measurements were performed following the protocol provided by the manufacturer with the adaption of the read settings for penKid measurement in human CSF:

Tab. 1. Calibrators and controls

Quality control samples		PENK [pmol/L]	Reagents
CAL1	Calibrator 1	29.3	included in the kit
CAL2	Calibrator 2	75.6	included in the kit
CAL3	Calibrator 3	188	included in the kit
CAL4	Calibrator 4	476	included in the kit
CAL5	Calibrator 5	1174	included in the kit
CONA-Kit	Low control provided by the kit	53	included in the kit
CONB-Kit	High control provided by the kit	301	included in the kit
CONC-Kit	Mid control, set up with kit high control 1:2 diluted	150	generated at Evotec from kit provided material
CONA- synthetic peptide	Low control set up with synthetic peptide	106	generated at Evotec from a synthetic peptide
CONB- synthetic peptide	High control set up with synthetic peptide	565	generated at Evotec from a synthetic peptide



Tab. 2. Acceptance criteria

	within acceptance criteria
Technical duplicate measurements, raw values	CV≤10%
Technical duplicate measurements; back calculated	CV≤15%
Accuracy [%] for calibrator measurements	≤100±25%

Fig. 1. LoD and LoQ estimations. The limit of detection (LoD) is calculated by the mean + 3*SD of the blank samples; Lower limit of quantitation (LLoQ) is calculated by the mean + 9*SD of the blank samples. Mean standard curve from dataset of 12 independent measurements, curve fitting by nonlinear fit (4PL), constrain bottom = 0.

Results

PENK immunoassay (PenKid®) qualification

To detect PENK in human CSF, we optimized and qualified an immunoluminometric assay that was previously validated and used for monitoring PENK in human plasma for kidney injury.
The assay was adapted to be used on the Pherastar instrument and with aCSF + 0.5% tween as standard (and sample) diluent instead of the diluent provided in the kit. In addition to the two penKid controls provided in the kit, a third control (generated from the provided controls) and a synthetic penKid were used to perform the assay qualification (**Tab. 1**).
Assay performance when assessed using Quality Control samples tested over several assay runs showed acceptable accuracy (RE < 20%) and precision (CV < 20%). A summary of 12 runs is shown in **Figure 2** and **Tab. 3**.

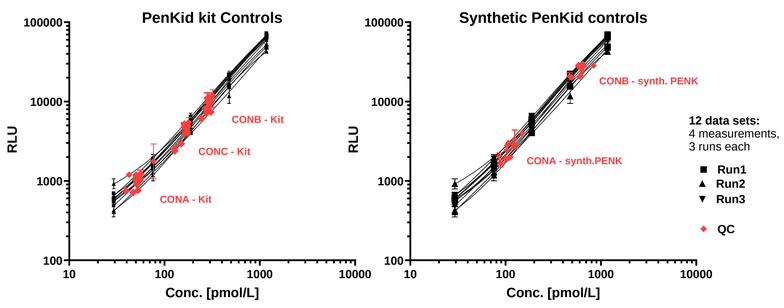


Fig. 2. Evaluation of assay performance. Assay performance was evaluated across 12 assay runs using the penKid kit and synthetic penKid calibrators and QC samples (low, mid and high). The mid QC was generated in-house by 1:2 dilution of high QC from the kit controls. For presentation in the graph, interpolated QC values were plotted on the respective standard curves.

Tab. 3. Inter and intra assay accuracy and precision parameter of quality control samples.

			Variation [%]	
Sample	Conc. [pmol/L]	Accuracy [%]	Intra assay	Inter assay
CAL1	29.3	100	11.60	0.89
CAL2	75.6	101	6.02	1.82
CAL3	188	100	4.39	3.02
CAL4	476	100	5.44	2.91
CAL5	1174	100	5.95	1.08
CONA-Kit	53	99	8.65	18.66
CONB-Kit	301	96	5.68	7.35
CONC-Kit	150	107	6.84	10.71
CONA- synthetic PENK	106	104	6.48	15.69
CONB- synthetic PENK	565	108	4.00	14.97

The matrix effect was evaluated by doping known concentrations of penKid in human CSF from 5 independent donors and performing serial dilutions. A robust recovery (with RE < 25%) was observed at all the dilutions tested indicating no matrix effects (**Figure 3A**). Selectivity of the assay was confirmed by immunodepletion of penKid using a PENK specific antibody (**Figure 3B**).

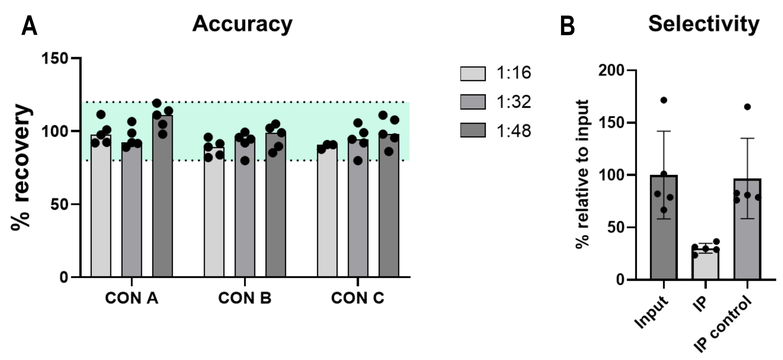


Fig. 3. Matrix effect and selectivity. Matrix effect was evaluated by diluting synthetic PENK in human CSF from five independent donors and calculating the recovery % (A.). Selectivity was evaluated by immunodepletion using an antibody specific for PENK (B.).

The reproducibility of the assay was verified by performing repeated measurement of penKid levels in human CSF from six healthy donors in six independent experiments, using the kit calibrator as reference standard. All CSF samples were obtained from commercial sources. The assay showed excellent reproducibility of the measurements, with CV variation across runs lower than 25% (**Figure 4**).

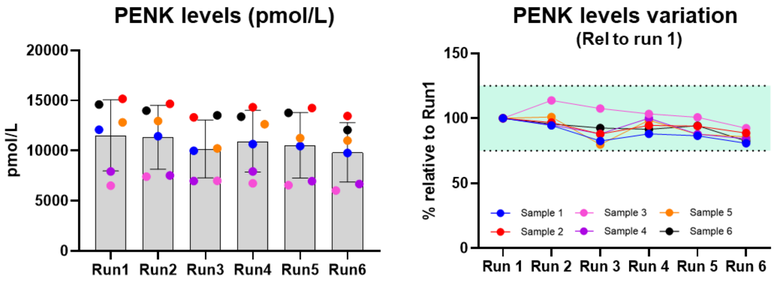


Fig. 4. Assay reproducibility for CSF measurements. PenKid levels in CSF of six healthy human donors obtained from commercial sources were measured in six independent experiments and showed very good assay reproducibility. Calibrator used: Kit calibrator.

PENK measurement in HDClarity CSF samples

90 CSF samples, that included 30 from healthy volunteers (HC samples) were received from the HDClarity collection for PENK evaluation. To re-confirm the minimal required dilution (MRD) and ascertain the optimal dilution for the HDClarity samples, 3 CSF samples each from commercially acquired HC and HDClarity were serially diluted starting from a 1/8 or 1/16 dilution and followed by PENK measurements. Based on the recovery of the PENK signals, a starting dilution of 1/16 was determined to be the MRD and dilution up to 1/64 was acceptable. For further PENK measurements, it was decided to use a 1/32 dilution (**Figure 5A**).
Additionally, PENK measurements were within acceptable range, when HC CSF samples were subjected to up to 4 free-thaw cycles, demonstrating good freeze-thaw stability (**Figure 5B**).

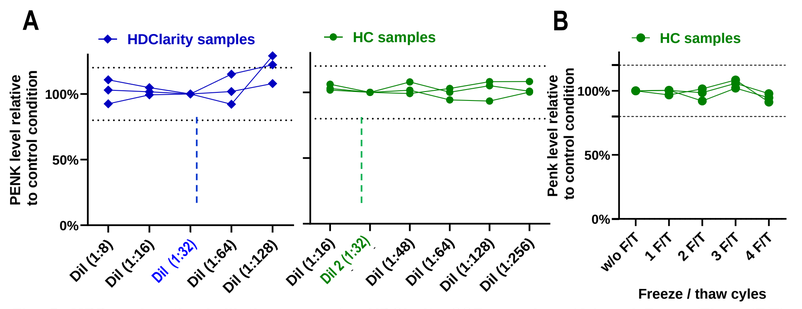


Fig. 5. MRD and optimal dilution range for HDClarity CSF samples (A.) and Freeze/Thaw (F/T) stability of the analyte (B.). Selected samples of the HDClarity cohort (HDClarity samples) and from commercially acquired healthy volunteers (HC samples) were serially diluted and their PENK levels were measured. Dilution corrected PENK levels are plotted relative to the dilution (1/32) used for further PENK measurements. Good recovery could be observed up to 1/64 dilution suggesting a working range between 1/16 – 1/64. Stability of PENK was evaluated for up to 4 F/T cycles with HC samples (B.). PENK levels were plotted relative to the no F/T condition.

PENK levels correlate with HD clinical assessments

PENK measurements on the 90 HDClarity CSF samples were performed in duplicates at three independent times spanning over a month. All the samples showed excellent correlation between the repeat measurements and all, but one sample showed a CV >20%. PENK levels across HD categories (healthy controls, late premanifest, moderate HD, and advanced HD) when analyzed by ANOVA revealed statistically significant differences between healthy controls vs late premanifest HD and late premanifest vs advanced HD (**Figure 6A**), highlighting the potential for PENK as an early HD onset biomarker. A progressive decrease in PENK levels was observed with the advancement of the HD-ISS stages (data not shown), however, a rigorous analysis requires imputation of the missing HD-ISS stageing values in the dataset. To determine if changes in PENK levels can be associated with clinical assessment scores, we performed Spearman correlation analysis. A strong correlation (r = -0.51) could be observed between PENK levels and CAP Score and Total Motor Score (**Figure 6B**). A moderate correlation (r = 0.46) with Total Functional Capacity score was also noted.

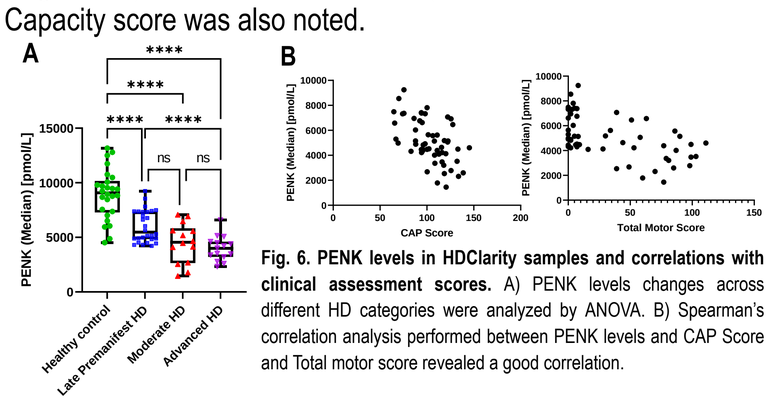


Fig. 6. PENK levels in HDClarity samples and correlations with clinical assessment scores. A) PENK levels changes across different HD categories were analyzed by ANOVA. B) Spearman's correlation analysis performed between PENK levels and CAP Score and Total motor score revealed a good correlation.

Conclusions

- An existing immunoluminometric assay was adapted for the measurement of PENK in human CSF.
- During qualification, the method showed excellent assay performance characteristics with RE and CV lower than 25%.
- Good PENK recovery in dilution studies suggested a MRD of 1/16 for human CSF and a linear range up to 1/64 dilution.
- Measurement of 90 CSF samples from the HDClarity cohort, showed significant differences in PENK levels between HD categories and good correlations with CAP score and total motor score.

References

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