Supporting Information

Immunochemical Detection of α-Synuclein Autoantibodies in Parkinson's Disease: Correlation between Plasma and Cerebrospinal Fluid Levels

Istvan Horvath †,#, Igor A. Iashchishyn †,‡,#, Lars Forsgren §, and Ludmilla A. Morozova-Roche†,*

†Department of Medical Biochemistry and Biophysics, Umeå University, Umeå, Sweden.

‡Department of General Chemistry, Sumy State University, Sumy, Ukraine.

§Department of Pharmacology and Clinical Neuroscience, Umeå University, Umeå, Sweden.

#Authors with equal contribution

*Corresponding author: Ludmilla A. Morozova-Roche (ludmilla.morozova-roche@umu.se)

METHODS

ELISA The content of α-synuclein autoantibodies in the CSF and blood plasma samples of PD and controls were measured by indirect ELISA. Initially 100 µl/well of 1 µg/ml α-synuclein in phosphate buffered saline (PBS) buffer was incubated overnight in 96 well plates (Maxisorp, Nunc), followed by 1 hour blocking with 200 µl/well of 5% PBS. After washing with 200 µl PBS, 100 µl of body fluid samples were applied on the plates, each sample was taken in triplicate. The initial dilution for serum samples was 20-fold in PBS and for CSF samples was 7.5-fold, respectively. This followed by 2-fold serial dilution steps in PBS, keeping the volumes of all samples at 100 µl. Finally, we reached 2560-fold dilution for serum and 480-fold dilution for CSF samples, respectively. After 3 hour incubation the plates were washed 3 times with 200 µl PBS-Tween (0,1%). Following that the plates were covered with horse radish peroxidase labelled anti-human IgG antibodies in PBS-Tween (20000x dilution, A8792, Sigma) and incubated for 1 hour. After washing away unbound secondary antibody the detection was performed by using an EC-Blue Enhanced TMB substrate (10-9405, Medicago). The reaction was stopped by adding 1 M sulphuric acid and absorbance was measured in each well at 450 nm on a Tecan M200 plate reader. Each sample was assessed at least in triplicates. In order to determine the titers of α-synuclein antibodies in plasma and CSF samples their serial dilutions *I(c)* were fitted by 4-parametric logistic model function developed previously for ELISA titrations:[1]

$$I(c) = \beta_1 + \frac{\beta_2 - \beta_1}{1 + e^{\beta_4(\ln(c) - \beta_3)}}, \qquad (1)$$

where c – dilution steps and β_1 to β_4 – plate-specific model parameters.

Each experimental data point was weighted by a reverse of its standard deviation, i.e. the points with lower standard deviation had a higher weight. 95% confidence bands were calculated for each titration curves using a *NonlinearModelFit* function built in *Mathematica-11*. To account for non-specific binding the measurements were performed in wells without immobilized α-synuclein and the fitted curve with corresponding confidence bands was used as a background. The antibody titers in the patient samples were determined by selecting the lowest of three following values (Fig. 1): *i*) intersection of the sample low confidence band with the background high confidence band; *ii*) intersection of the sample high confidence band with the background low confidence band or *iii*) intersection of the sample fitted curve with the background curve. This approach was used to

account for two possible scenarios when the sample titration parameters were either higher or comparable with background.

Human subjects. A cross-sectional cohort of 60 participants, who donated CSF (60) and blood plasma (40, subgroup of the 60) samples, was recruited from the Department of Neurology, Umeå University Hospital, Sweden. The cohort consisted of 30/30 patients/controls for CSF analysis and 20/20 patients/controls for blood plasma evaluation. Individuals were in an age range of 39 – 80 y.o., with the majority above 60 y.o. Patients were neurologically examined at the Department of Neurology on several occasions and diagnosed as having clinically definite PD according to the UK Parkinson's Disease Society Brain Bank clinical diagnostic criteria.[2] All PD patients have sporadic but not familial PD. The mean disease duration from the diagnosis of PD to the CSF and blood sampling was 3 months. Severity was assessed by the Hoehn and Yahr (H–Y) score.[3] Patients were divided into 2 subgroups according to the severity of disease: the mild PD subgroup included 24 patients, who were in a less advanced stage with 1.5 to 2 H–Y scores, and the moderate PD subgroup included 6 patients in a more advanced stage with 2.5 to 3 H–Y scores. Patients with concomitant neurological or psychiatric diseases, cancer and other severe diseases were excluded. The control subjects were patients, whose CSF and blood plasma were collected for analysis of disorders others than neurodegenerative diseases, including headache, polyneuropathy, neurogenic pain, dizziness, diplopia and fatigue.

Sample treatment. CSF and blood plasma samples were collected on the same patient visit according to the standard operating procedure implemented at the Department of Neurology, Umeå University. Samples were collected in the morning, without overnight fasting, in polypropylene tubes (including EDTA as anticoagulant for plasma), gently mixed to avoid gradient effects (for CSF from lumbar puncture) and stored in 1 ml aliquots at –80 ºC. Prior to measurements the samples were thawed on ice and thereafter gently shaken. Undiluted CSF samples were used for further analysis.

Data analysis. Statistical data analysis was performed by using a *Wolfram Mathematica* 11 package. Binary logarithms of titers were used since all dilutions were factor of 2. The normality of the α-synuclein antibody titer distributions both in plasma and CSF was examined by plotting

their quantiles *versus* quintiles of a normal distribution. Since there were significant deviations from the normal distribution in all data sets, non-parametric methods such as Mann-Whitney test and bootstrap were applied as major statistical tools.[4, 5] We used the combination of the standardized effect size and associated confidence intervals to assess (1) the magnitude of an effect of interest, i.e. the difference in the α-synuclein antibody levels between PD and controls and (2) the precision of the estimate of the effect magnitude. Such approach enables us to conclude on the biological importance of the observed effects, rather than merely statistical significance defined by *p* values.[6]

Cliff's delta and Spearman's rho were used as measures of the effect size due to non-normality of the data distributions.[7] Their confidence intervals were calculated by a bootstrap corrected and accelerated (BCa) nonparametric method with 10000 replications.[4, 5] Binary logarithm transformation did not affect confidence intervals values since BCa method is transformation-respecting.[5] Efficiency of the diagnostic tests based on titers of α-synuclein autoantibodies in plasma and CSF was estimated by using nonparametric receiver operating characteristic (ROC)[8].

REFERENCES

[1] Higgins, K. M., Davidian, M., Chew, G., and Burge, H. (1998) The effect of serial dilution error on calibration inference in immunoassay, *Biometrics 54*, 19–32.

[2] Hughes, A. J., Daniel, S. E., Kilford, L., and Lees, A. J. (1992) Accuracy of clinical diagnosis of idiopathic Parkinson's disease: a clinico-pathological study of 100 cases, *J. Neurol. Neurosurg. Psychiatry 55*, 181–184

[3] Hoehn, M. M., and Yahr, M. D. (1967) Parkinsonism: onset, progression and mortality, *Neurology 17*, 427–442.

[4] DiCiccio, T. J., and Efron, B. (1996) Bootstrap confidence intervals, *Stat. Sci. 11*, 189–228.

[5] Efron, B., and Tibshirani, R. J. (1993) *An introduction to the bootstrap*, Chapman & Hall, New York.

[6] Nakagawa, S., and Cuthill, I. C. (2007) Effect size, confidence interval and statistical significance: a practical guide for biologists, *Biol Rev Camb Philos Soc 82*, 591–605.

[7] Cliff, N. (1993) Dominance statistics: Ordinal analyses to answer ordinal questions, *Psychological Bulletin 114*, 494.

[8] Goddard, M. J., and Hinberg, I. (1990) Receiver operator characteristic (ROC) curves and non-normal data: an empirical study, *Stat. Med. 9*, 325–337.