

# Longitudinal Analysis of Cerebrospinal Fluid Visinin-like protein-1, chitinase-3 like-1, synaptosomal-associated protein 25 and neurogranin

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## Summary

Recent preclinical biomarker studies in AD have shown that development of the pathological hallmarks of AD begins as early as 10-15 years before the development of cognitive decline<sup>1-5</sup>. Clinicians, researchers, and pharmaceutical companies have begun to appreciate possible intervention in the preclinical stages of AD as a patient's best option for the prevention of dementia and, in the shorter term, as the best opportunity to test proposed disease-modifying drugs currently in development. Cerebrospinal fluid (CSF) Visinin-like protein-1 (VILIP-1) and chitinase-3 like-1 show utility in distinguishing AD from cognitively normal (CN) controls and in some cases from other dementias<sup>6-8</sup>. Importantly, they appear useful as prognostic markers, particularly when used in combination with A $\beta$ 42. The synaptosomal-associated protein 25 (SNAP-25) and neurogranin (NRGN) proteins have also received recent press for their utility in distinguishing individuals with Alzheimer Disease from Cognitively Normal individuals and as prognostic markers<sup>9-11</sup>. Further work, including in-depth cross-sectional studies across multiple cohorts and especially longitudinal follow-up of CN subjects, is needed to improve the ability of biomarkers to contribute to AD research and clinical trials. Adding additional, non-pathogenic, novel biomarkers to a panel including A $\beta$ 42, tau, and ptau181 may improve our diagnostic and prognostic capabilities. Better knowing the order of changes in biomarkers as preclinical AD progresses to symptomatic AD will enable clinical trials to select those participants who are most likely to benefit from drug intervention, as well as to reduce the number of participants needed to attain statistical relevance.

## Method

### VILIP-1

Mouse anti-human VILIP-1, clone 3A8.1 and sheep anti-human VILIP-1 were provided to Singulex, Inc. (Alameda, CA) for development and manufacture of an Erenna® immunoassay assay kit (#03-0008-02) according to an agreement with Washington University. The proprietary custom order kit reagents included preparation of the monoclonal capture antibody for binding to



Invitrogen (Carlsbad, CA) MyOne magnetic micro particles and Invitrogen Alexa fluor dye labeling of the sheep polyclonal detection antibody and all assay buffers.

A sandwich ELISA was developed using the Erenna® immunoassay system to measure VILIP-1 in CSF. Prior to the assay all samples were centrifuged (11,000 g x 3 minutes) to remove particulates. All assay steps were performed at room temperature unless otherwise indicated. A calibration curve was prepared using dilutions of recombinant human VILIP-1 ranging from 3.9 to 3000pg/mL and , with each concentration assayed in triplicate. 15µL standards or CSF were combined with 135ul assay buffer and 50µL antibody coated micro particles for measurement of VILIP-1 in CSF. The assay plate was incubated for two hours on a plate shaker set to 525 revolutions per minute. Micro particles were then magnetically separated and washed one time using an Agilent (Santa Clara, CA) Bravo Automated Liquid Handling Platform using Singulex wash buffer. Fluorescent dye labeled detection antibody prepared by Singulex (20µL per well) was added and incubated for one hour. After washing the magnetic micro particles 5 times, 20µL per well of Singulex elution buffer was added for 10 minutes to separate detection antibody from the micro particles. Eluted antibodies were then transferred with the Bravo instrument to a clean 384 well plate for reading in the Erenna® immunoassay system.

#### YKL-40

YKL-40 was measured using the MicroVue YKL-40 ELISA assay (Quidel Corp.). Prior to the assay, all samples were lightly vortexed for 5 seconds. All assay steps were performed at room temperature unless otherwise noted. The complete standard curve of YKL-40 purified from osteosarcoma MG-63 cells is provided with the assay kit, each standard and sample was assayed in duplicate. All CSF samples were diluted 1:2 in Standard A (0 ng/ml) on an ice cold preplate before transferring 20µl to the coated ELISA plate. After adding 100µl capture solution, the plate was incubated for 60 minutes followed by washing 4 times with 250µl wash buffer. Enzyme conjugate, 100µl, which was prepared prior to beginning the assay, was added to the assay plate followed by a 60 minute incubation. The substrate solution was prepared during this step to ensure dissolution of the substrate tablet. After another wash, 4 times with 250µl was buffer, 100µl substrate was added to the assay plate followed by a 60 minute incubation. Finally, 100µl stop solution was added to the assay plate and the samples were read at an Optical Density of 405nm and analyzed with a linear regression curve-fit.

#### SNAP-25

Mouse anti-human SNAP-25 antibodies were used for development of an Erenna® immunoassay assay according to an agreement between Singulex, Inc (Alameda, CA) and Washington University. Assay reagents included preparation of the monoclonal capture antibody 6H07-2C12 for binding to Invitrogen (Carlsbad, CA) MyOne magnetic micro particles and Invitrogen Alexa fluor dye labeling of monoclonal antibody 9E11, using Singulex labeling kits (capture antibody labeling kit 03-0077-xx and detection antibody labeling kit 03-0076-02).

A sandwich ELISA was developed using the Erenna® immunoassay system to measure SNAP25 in CSF. Prior to the assay all samples were centrifuged (11,000 g x 3 minutes) to remove particulates. All assay steps were performed at room temperature unless otherwise indicated. A calibration curve was prepared using dilutions of recombinant human SNAP25 [CSI15602] from Cell Science, Inc (Seattle, WA) ranging from 0.078 to 90pg/mL in Thermo Scientific, Inc [Rockford IL] Blocker Casein in TBS plus 0.1% Tween-20 from Sigma-Aldrich, Inc [St Louis MO] and , with each concentration assayed in triplicate. 100µL standards or CSF diluted 4-fold were combined with 100µL antibody coated micro particles diluted in Blocker Casein in TBS plus 1% Tween-20, for measurement of SNAP25 in CSF. The assay plate was incubated for two hours on a plate shaker set to 525 revolutions per minute. Micro particles were then magnetically separated and washed one time using an Agilent (Santa Clara, CA) Bravo Automated Liquid Handling Platform using Singulex wash buffer. Fluorescent dye labeled detection antibody diluted in Blocker Casein in TBS plus 1% Tween-20 (20µL per well) was added and incubated for one hour. After washing the magnetic micro particles 5 times, 20µL per well of Singulex elution buffer was added for 10 minutes to separate detection antibody from the micro particles. Eluted antibodies were then transferred with the Bravo instrument to a clean 384 well plate for reading in the Erenna® immunoassay system.

## NRGN

Two epitope-specific rabbit anti-human NGRN antibodies were used for development of an Erenna® immunoassay assay according to an agreement between Singulex, Inc (Alameda, CA) and Washington University. Assay reagents included preparation of C-terminal specific antibody (P-4793) for binding to Invitrogen (Carlsbad, CA) MyOne magnetic micro particles and Invitrogen Alexa fluor dye labeling of N-terminal specific antibody (P-4794), using Singulex labeling kits (capture antibody labeling kit 03-0077-xx and detection antibody labeling kit 03-0076-02).

A sandwich ELISA was developed using the Erenna® immunoassay system to measure NGRN in CSF. Prior to the assay all samples were centrifuged (11,000 g x 3 minutes) to remove particulates. All assay steps were performed at room temperature unless otherwise indicated. A calibration curve was prepared using dilutions of recombinant human GST-NGRN produced at Washington University, ranging from 1.75 to 3000pg/mL in standard diluent (TBS, 2 mg/ml rabbit IgG from Equitech-Bio [Kerrville TX] plus 0.1% Tween-20) with each concentration assayed in triplicate. 50µL standards or CSF diluted 10-fold were combined with 100µL antibody coated micro particles diluted in assay buffer (TBS, rabbit IgG plus 1% Tween-20), for measurement of NGRN in CSF. The assay plate was incubated for two hours on a plate shaker set to 525 revolutions per minute. Micro particles were then magnetically separated and washed one time using an Agilent (Santa Clara, CA) Bravo Automated Liquid Handling Platform using Singulex wash buffer. Fluorescent dye labeled detection antibody diluted in assay buffer (20µL per well) was added and incubated for one hour. After washing the magnetic micro particles 5 times, 20µL per well of Singulex custom elution buffer (PN 02-0002-03) was added for 30 minutes to separate detection antibody from the micro particles. Eluted antibodies were then transferred with the Bravo instrument to a clean 384 well plate for reading in the Erenna® immunoassay system.

## All Assays

All samples (each on the same freeze/thaw cycle) were run in triplicate on a single lot number for VILIP-1, SNAP-25 and NRG1 and duplicate for YKL-40. Within-person longitudinal samples were run on the same assay plate to reduce inter- and intra-plate variability. Quality control (QC) for VILIP-1, SNAP-25 and NRG1 included analysis of three internal standard CSF pools run on each plate. For YKL-40, two internal standard CSF pools were run on each plate. QC mean and tolerance limits for VILIP-1, SNAP-25 and NRG1 were established by computing the average of at least 15 values collected over at least 4 runs prior to running ADNI samples. Tolerance limits are defined at +/- 2 standard deviations (2SD) and +/- 3 standard deviations (3SD). QC mean and tolerance limits for YKL-40 were determined by the kit manufacturer. Plates with 2 or more QC sample values greater than 2SD from the mean were reanalyzed (VILIP-1, SNAP-25 and NRG1) for YKL-40, plates with two or more internal pooled controls and/or kit provided controls were reanalyzed. Further, any individual sample with a Coefficient of Variation (% CV) greater than 25% was reanalyzed. Samples that failed quality control (QC) were refrozen and stored at -80°C for at least 48 hours before being reanalyzed. When samples were reanalyzed due to QC failure, all within-person longitudinal samples were reanalyzed as well, on the same freeze/thaw cycle. The proteins VILIP-1, SNAP-25 and NRG1 were run on the first freeze/thaw cycle, YKL-40 was run on the second freeze/thaw cycle as were any VILIP-1 samples that failed QC. Any samples that required repeat SNAP-25 or NRG1 were performed with third freeze/thaw cycle samples. Due to protein loss from multiple freeze/thaw cycles, SNAP-25 repeats did not pass QC – therefore any samples that failed QC from the first freeze-thaw were removed from the SNAP-25 data completely.

## Dataset Information

This methods document applies to the following dataset(s) available from the ADNI repository:

| <b>Dataset Name</b>                           | <b>Date Submitted</b> |
|---|-----------------------|
| Fagan Lab – Longitudinal VILIP-1 CSF Analysis | July 15, 2015         |

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