

**Title (82/100 characters):**

**Neurogranin as cerebrospinal fluid biomarker for dementia - assay  
comparison study**

Eline A.J. Willemse<sup>1,2,3</sup>, Ann De Vos<sup>4</sup>, Elizabeth M. Herries<sup>5</sup>, Ulf Andreasson<sup>6</sup>, Sebastiaan Engelborghs<sup>3</sup>, Eugeen Vanmechelen<sup>4</sup>, Wiesje M. van der Flier<sup>2,7</sup>, Philip Scheltens<sup>2</sup>, Dan Crimmins<sup>5</sup>, Jack H. Ladenson<sup>5</sup>, Henrik Zetterberg<sup>6,8,9</sup>, Anne M. Fagan<sup>5</sup>, Kaj Blennow<sup>6</sup>, Maria Bjerke<sup>3</sup>, Charlotte E. Teunissen<sup>1</sup>.

<sup>1</sup> Neurochemistry laboratory, Department of Clinical Chemistry, Amsterdam Neuroscience, VU University Medical Center Amsterdam, The Netherlands

<sup>2</sup> Alzheimer Center, Department of Neurology, Amsterdam Neuroscience, VU University Medical Center Amsterdam, The Netherlands

<sup>3</sup> Reference Center for Biological Markers of Dementia (BIODEM), Department of Biomedical Sciences, Institute Born-Bunge, University of Antwerp, Antwerp, Belgium

<sup>4</sup> ADx NeuroSciences, Ghent, Belgium

<sup>5</sup> Washington University School of Medicine, The Knight Alzheimer's Disease Research Center and Department of Neurology and Hope Center for Neurological Disorders, St. Louis, USA

<sup>6</sup> Clinical Neurochemistry Laboratory, Institute of Neuroscience and Physiology, the Sahlgrenska Academy at the University of Gothenburg, Mölndal, Sweden

<sup>7</sup> VU University Medical Center, Epidemiology & Biostatistics, Amsterdam, The Netherlands

<sup>8</sup> UCL Institute of Neurology, Department of Molecular Neuroscience, Queen Square, London, United Kingdom

<sup>9</sup> UK Dementia Research Institute, London, United Kingdom

**Corresponding author:**

Eline AJ Willemse

Room PK2Br129

Laboratory of Neurochemistry

Dept. of Clinical Chemistry and Alzheimer center

VU University Medical Center

De Boelelaan 1117

1081 HV Amsterdam

The Netherlands

Phone: +31204443029

E-mail: e.willemse@vumc.nl

**Running title:** CSF biomarker Neurogranin assay comparison

## **Abstract (399/400 words)**

CSF neurogranin levels are elevated in Alzheimer's disease patients and have been related to cognitive decline. As such, neurogranin holds promise as a monitoring biomarker for disease progression in Alzheimer's disease. Three commonly used neurogranin assays, measuring slightly different forms of the analyte, report divergent concentrations ranges, which hampers direct comparison of results. Here, we perform an in depth analysis of the analytical and diagnostic performance of these assays in the same cohort of patients to increase the interpretability of CSF neurogranin test results.

First, protein quantity of the calibrators of the neurogranin Singulex assay from WashU (St. Louis, MO), the neurogranin ELISA from ADx (Ghent, Belgium), and the neurogranin ELISA from UGot (Mölndal, Sweden) were compared using silver staining after gel electrophoresis. Affinity of all capture and detection antibodies for the different calibrators, CSF, and brain homogenates was studied using western blot. Second, intra- and inter-assay variation and analytical sensitivities were defined. We used Passing-Bablok regressions in clinical CSF samples (n=108). Lastly, we compared clinical performance of the three assays in samples from subjects diagnosed with subjective cognitive decline (n =22), and in patients with Alzheimer's disease (n=22), frontotemporal dementia (n=22), dementia with Lewy Bodies (n=22), or vascular dementia (n=20), adjusted for gender and age.

The calibrator of the UGot assay contained about 7-fold more protein than the WashU and ADx calibrators. Capture and detection antibodies recognised their own calibrator best. The assays detected different peptides of neurogranin: the WashU assay the N-terminal part of neurogranin (S10-D23), the ADx assay neurogranin truncated at P75, and the UGot assay neurogranin at the C-terminus (D78). All assays had good analytical performance, i.e. intra-/inter-assay variability of 5-8%. Absolute neurogranin levels ranged from (median+range)

1881(330–8320)pg/mL for the WashU assay, 372(71–1191)pg/mL for the ADx assay, and 416(115–1481)pg/mL for the UGot assay. The results for clinical CSF samples correlated well (Spearman's rho 0.95, 0.87, 0.81 for WashU–ADx, WashU–Goth, and ADx–GothU, respectively). Passing-Bablok regression demonstrated proportional differences amongst all assays and a systematic difference between WashU and ADx only. ANCOVA showed group differences for ranked neurogranin levels in each assay (all  $p < 0.05$ ), with specific elevation in Alzheimer's disease.

The three assays targeted different epitopes of neurogranin. Together with difference in value assignment, this could explain the divergent concentration ranges of the assays. Profiles of neurogranin concentrations across diagnostic groups were comparable amongst all three assays. The targeting of different epitopes by these assays enables in-depth studies into neurogranin's role in Alzheimer's disease pathology.

**Keywords (max. 5):** cerebrospinal fluid, biomarker, Alzheimer's disease, assay comparison, Neurogranin

**Abbreviations:**

WashU= Washington University

ADx= ADx Neurosciences

UGot=Gothenburg University

ELISA= enzyme-linked immunosorbent assay

LLOD= lower limit of detection

CV= coefficient of variation

CI= confidence interval

## Background

Neurogranin is a post-synaptic protein involved in synaptic plasticity and long-term potentiation (Gerendasy and Sutcliffe, 1997; Kaleka and Gerges, 2016). Post-mortem analyses suggested a relation of neurogranin with Alzheimer's disease pathology, as neurogranin was found to be reduced in Alzheimer's disease brain tissue in western blot analysis (Davidsson and Blennow, 1998; Reddy *et al.*, 2005). In cerebrospinal fluid (CSF), neurogranin levels were increased in Alzheimer's disease patients compared to controls, which led to growing interest in neurogranin as a novel biomarker candidate for Alzheimer's disease (Thorsell *et al.*, 2010). Subsequent studies confirmed that CSF neurogranin levels are increased in Alzheimer's disease as well as in mild cognitive impairment, compared to controls (Kvartsberg *et al.*, 2014, 2015; De Vos *et al.*, 2015; Kester *et al.*, 2015; Portelius *et al.*, 2015; Mattsson *et al.*, 2016; Remnestål *et al.*, 2016; Sanfilippo *et al.*, 2016; Tarawneh *et al.*, 2016), and compared to other dementias (Hellwig *et al.*, 2015; Janelidze *et al.*, 2015; Wellington *et al.*, 2016; Lista *et al.*, 2017), suggesting that it could be an Alzheimer's disease specific biomarker. Even though neurogranin levels did not correlate with cognitive scores at baseline, they could predict cognitive decline in Alzheimer's disease patients (Portelius *et al.*, 2015; Mattsson *et al.*, 2016; Tarawneh *et al.*, 2016). Neurogranin could even be a pre-symptomatic marker, as in a former study, neurogranin levels increased over two years in controls, while no further increase was observed in levels in mild cognitive impairment and Alzheimer's disease (Kester *et al.*, 2015). Thus, neurogranin could serve as a predictive and possibly monitoring marker for Alzheimer's disease specific cognitive decline, which is highly needed for treatment intervention studies.

The measurements of CSF neurogranin levels in the aforementioned publications have been performed using three independent assays (Kvartsberg *et al.*, 2014; De Vos *et al.*, 2015;

Kester *et al.*, 2015), which all target different epitopes of the protein (figure 1). Yet, the discriminative power to distinguish patients from controls are strikingly comparable for all assays, even while the absolute neurogranin concentration ranges vary widely among the assays, e.g. ~5-100 pg/mL (De Vos *et al.*, 2015) compared to ~1000-4000 pg/mL (Kester *et al.*, 2015) and ~100-340 pg/mL (Kvartsberg *et al.*, 2014). These differences could be explained by differences in cohorts, but also by differences in assays, e.g. the use of various types of calibrator. To allow direct comparison of results across studies, it is important to understand the assay specifics and to directly compare the outcomes within one cohort of patients with different dementia diagnosis.

In this study, we aimed to link the three commonly used neurogranin assays, developed at Washington University (WashU), ADx NeuroSciences (ADx), and Gothenburg University (UGot). First, we characterised the three assays through analysis of their calibrators and antibodies. Next, we compared CSF neurogranin concentrations measured in the same clinical dementia cohort. To compare the discriminative potentials of the assays, we analysed neurogranin levels in CSF of patients with dementia with Lewy Bodies (n=22), vascular dementia (n=20), frontotemporal dementia (n=22), dementia due to Alzheimer's disease (n=22) and controls (n=22). Bridging these CSF neurogranin assays will help translate neurogranin in multicentre comparisons and improve our understanding of the use of neurogranin as a biomarker.

## **Methods**

### Samples

#### Brain homogenate preparation

Two different brain homogenate samples were used, the first to characterise the three assays using Silver stain and Western blot: 100-150 mg of frozen frontal cortex tissue (Biobank Institute Born-Bunge, University of Antwerp) was homogenized using RIPA buffer containing protease inhibitor cocktails PhosSTOP (Roche, Basel, Germany) and complete Tablets (Roche, Basel, Germany). The homogenate was centrifuged at 10,500g for 30 min at 4°C and the supernatant was stored at -80°C until further use.

The second brain homogenate was used in the immunoassay measurements, to compare the affinity of the three assays for neurogranin peptides/protein present in brain tissue: frozen hippocampal tissue (Brain bank, dept. of Pathology, VU University Medical Center) was homogenized with Mammalian Protein Extraction Reagent (M-PER, 0.1 g/ml, Thermo Scientific, Waltham, USA) containing EDTA-free Protease Inhibitor Cocktail (1:25, Roche, Basel, Germany). The homogenate was centrifuged at 10,500g for 30 min at 4°C. The protein content in the supernatant was 2.06 mg/ml, quantified using bovine serum albumin (BSA) standards (Thermo Scientific, Waltham, USA) and the Bio-Rad Protein Assay (Bio-Rad, Hercules, USA). Brain lysate was kept at -80°C until further analysis (Del Campo *et al.*, 2014). Before neurogranin measurement by immunoassays, brain lysate was initially diluted 1:400 followed by serial dilutions in sample diluent resulting in the following concentrations: 5150, 1030, 515, 103, and 51.5 pg total protein/ $\mu$ l.

### Clinical samples

108 CSF samples from patients from the Amsterdam Dementia Cohort (van der Flier *et al.*, 2014) were collected and processed according to the international consensus guidelines (Teunissen *et al.*, 2009, 2013). Twenty-two patients with a diagnosis of dementia due to Alzheimer's disease were matched for age and sex to 22 controls (individuals with subjective cognitive decline), as well as patients with a diagnosis of frontotemporal dementia (n=22),

dementia with Lewy bodies (n=22), or vascular dementia (n=20) (all diagnosed according to consensus criteria (Román *et al.*, 1993; Neary *et al.*, 1998; McKeith *et al.*, 2005; McKhann *et al.*, 2011)(table 1)). The dementia due to Alzheimer’s disease patients and controls were additionally selected based on their CSF biomarker profiles: CSF A $\beta$ 1-42 (<640 pg/ml for Alzheimer’s disease), t-Tau (>375 pg/ml for Alzheimer’s disease), p-Tau181 (>52 pg/ml for Alzheimer’s disease) as measured by Innostest (Fujirebio, Belgium). All patients signed informed consent, and the study was approved by the local ethical committee.

Table 1 Patient sample characteristics

	Controls	Dementia due to Alzheimer’s disease	Dementia with Lewy bodies	Fronto-temporal dementia	Vascular dementia	p-value
<b>N</b>	22	22	22	22	20	
<b>Sex = m</b>	13 (59%)	13 (59%)	21 (96%)	15 (68%)	13 (65%)	0.026
<b>Age</b>	64 (6)	65 (8)	68 (6)	63 (5)	68 (6)	0.023
<b>APOE <math>\epsilon</math>4 carriers</b>	4 (18)	15 (68)	12 (55)	8 (36)	13 (65)	0.004
<b>MMSE</b>	28 [27,29]	18 [16,23]	25 [21,27]	24 [18,26]	24 [22,26]	<0.001
<b>A<math>\beta</math>42 (pg/ml)</b>	891 (268)	511 (160)	730 (299)	914 (245)	586 (241)	<0.001
<b>t-Tau (pg/ml)</b>	311 (155)	800 (378)	366 (256)	434 (249)	404 (188)	<0.001
<b>p-Tau (pg/ml)</b>	49 (21)	98 (45)	54 (33)	55 (26)	53 (22)	<0.001

Legend: Data are represented as n(%), mean(sd) or median[range]. Groups were compared using Fisher Exact Test for sex and APOE  $\epsilon$ 4 carriership, Kruskal-Wallis rank sum test for MMSE, and ANOVA for age, A $\beta$ 42, t-Tau, and p-Tau. Abbreviations: A $\beta$ 42= Amyloid- $\beta$  1-

42, IQR= inter-quartile range, m= male, MMSE= Mini Mental State Examination, p-Tau= phosphorylated Tau at P181, sd= standard deviation, t-Tau= total Tau.

#### A priori power calculation

Sample size was based on two power calculations using G\*Power version 3.1.9.2, the first to discriminate between Alzheimer's disease and controls (power > 0.90 with effect size Cohen's  $d = 1.06$  (De Vos *et al.*, 2015) and  $\alpha$ -error = 0.05) and the second to discriminate between Alzheimer's disease and controls, frontotemporal dementia, dementia with Lewy Bodies, and vascular dementia (power > 0.82 with effect size estimated at  $f = 0.35$  and  $\alpha$ -error = 0.05).

#### Sample distribution

Calibrators and detection and capture antibodies of the three assays were sent to the BIODEM laboratory at the Institute of Born-Bunge (University of Antwerp) to perform the Silver stain and Western Blot experiments. For the immunoassays measurements, the same set of blinded clinical samples, the same brain lysate stock, and calibrators and controls of the assays were distributed by VUmc and transported frozen to St. Louis (USA), Gothenburg (Sweden), and Ghent (Belgium). Neurogranin measurements were performed on-site according to each assay's protocol. The blinded clinical samples were measured in random order. Raw data were reported to the VUmc Amsterdam and statistically analysed there.

#### Neurogranin immunoassays

See figure 1 for the composition of the neurogranin calibrators, antibody epitopes, protein, and peptides.

#### Washington University in-house neurogranin Singulex assay

A sandwich immunoassay was developed for CSF on a Singulex Erenna system using the 2 epitope-specific rabbit antibodies (recognising N-terminal epitope S10-D23 and C-terminal epitope G49-G60) (for development of antibodies see (Kester *et al.*, 2015)). The C-terminal-specific antibody (P-4793) was coupled to magnetic beads and used as the capture antibody, and the N-terminal-specific antibody (P-4794) was labelled with a fluorescent dye and used as the capping/detection antibody. Synthetic 78-mer human neurogranin was prepared and characterised by AAPPTec, LLC (Louisville, KY, USA) using C18-reversed phase-high performance liquid chromatography and electrospray ionization-mass spectrometry, and this material was used as the immunoassay standard. CSF samples were measured in triplicate. Curve fitting was done by weighted regression of the three signal types (detected events, event photons, and total photons) using an algorithm (SMDCurve Fit, Singulex software SGX Link) resulting in a 5-parameter logistic equation for interpolation.

#### ADx NeuroSciences neurogranin ELISA

This sandwich ELISA combines two monoclonal mouse antibodies, as previously reported (De Vos *et al.*, 2016): one (ADx403 (clone ADxNGCI2)) directed against the R53-A64 sequence of neurogranin, the other (ADx451 (clone ADxNGCT1)) directed against the C-terminus of the protein, truncated at P75 specifically, i.e. G62-P75. CSF samples were analysed undiluted (15 $\mu$ L) in duplicate. Final concentrations of neurogranin were intrapolated ( $\log(X)$ ; 4-parameter logistic) based on a synthetic calibrator, custom-made by Proteogenix (France), covering the C-terminal sequence truncated at P75.

#### Gothenburg University in-house neurogranin ELISA

A sandwich-immunoassay was developed using a monoclonal antibody against the G52-G65 epitope of human neurogranin as capturing antibody and a polyclonal antibody against the C-terminus (V66-D78), specifically designed against the D78 terminus (ab23570; Upstate

Biotechnology). Full-length recombinant human neurogranin calibrators with concentrations ranging between 78 – 10,000 pg/mL, blanks, and CSF samples were incubated in duplicate together with the detector antibody. A fitted 4-parameter logistic model was used as the calibration curve and the blank was included as zero concentration of neurogranin (Kvartsberg *et al.*, 2014).

### Analytical performance

Analytical validation parameters for each assay were evaluated: the lower limit of detection (LLOD) had been previously determined in each assay by the mean concentration of 16 blanks plus 10 times the standard deviation. Clinical samples with undetermined values <LLOD were assigned the LLOD-value for inclusion in the statistical analyses. Intra-assay coefficients of variation (CV) was defined as the mean of the duplo CVs of all patient CSF samples used in this study. Inter-assay CV was defined as the mean of 4 – 6 in-house prepared quality control CSF pools with high and low neurogranin concentrations.

### Characterisation of assays - gel electrophoresis

Calibrators, two control CSF samples from the BIODERM laboratory (Institute Born-Bunge, University of Antwerp), and brain homogenate were separated based on their molecular weights using a 12% Bolt Bis-Tris gel (ThermoFisher Scientific) with 1x MES SDS running buffer (ThermoFisher Scientific). Samples were prepared in 1x loading buffer with 0.05 M Dithiothreitol and the gel was run at 200V.

### Characterisation of assays - Silver stain

Protein quantities in calibrator solutions were determined using the Pierce Silver stain kit (ThermoFisher Scientific) according to manufacturer's protocol. In short, after gel electrophoresis the gel was fixed in 30% ethanol and 10% acetic acid, sensitized, stained

overnight, and the colour was developed for 2-3 minutes and stopped with 5% acetic acid.

Bands were quantified in ImageJ (Schneider *et al.*, 2012).

### Characterisation of assays - Western Blot

After gel electrophoresis, gels stayed in transfer buffer (20% methanol Tris Glycine) for 10 minutes before assembling the blotting sandwich using the iBlot Transfer Pack containing a 0.2 µm nitrocellulose membrane according to manufacturer's instructions. Transfer was done for 6 minutes at 20V. Next, the membrane was blocked for 1 hour using 1:5 Odyssey blocking buffer in PBS (Li-Cor). Primary antibody incubation was performed overnight at 4°C, using the capture and detection antibodies of the assays: polyclonal rabbit P-4794 1:1000 (WashU) and polyclonal rabbit P-4793 1:1000 (WashU), monoclonal mouse ADx403 1:2000 (ADx) and monoclonal mouse ADx451 1:1000 (ADx), monoclonal mouse Ng7 1:2000 (UGot) and polyclonal rabbit 07-425 1:2000 (UGot), all in 1:5 Odyssey blocking buffer with 0.1% Tween-20. Secondary antibody incubation was done with either or both IRDye 680RD Goat Anti-Mouse (Li-Cor) and IRDye 800CW Goat Anti-Rabbit (Li-Cor) diluted 1:20000 in 1:5 Odyssey buffer (Li-Cor) with 0.01% SDS for 1 hour at room temperature and blots were kept in the dark. Blots were read using channel 700 and 800 of the Odyssey imaging system (Li-Cor).

### Data analysis

Passing-Bablok regression analyses were performed to compare the WashU, ADx, and UGot assays on proportional and systematic differences based on the 108 clinical samples.

For clinical performance validation, rank-transformed neurogranin levels were used since assumptions for normal distribution were not met. To compare neurogranin levels amongst diagnostic groups, an ANCOVA corrected for age and gender was performed per assay,

followed by Bonferroni adjusted post-hoc comparisons. The effect size of every assay was defined as the partial  $\eta^2$  of these ANCOVA models. Spearman correlations were used to correlate neurogranin concentrations with MMSE scores and Alzheimer's disease biomarker values for each assay. Analyses were done in R version 3.4.0 (R Core Team, 2017).

## Results

### Calibrator quantification using silver stain after SDS-PAGE gel electrophoresis

All calibrators were separated by gel electrophoresis and visualised by silver stain (figure 2). The silver stain showed a single sharp band at 14 kDa for the WashU calibrator only for the highest calibrator concentration (100 ng). For the ADx calibrator, a clean band at 6 kDa was observed when 100 or 20 ng was loaded. The UGot calibrator showed a large smear at higher molecular weights and lower clean bands at 14 kDa and at 6 kDa, although the 6 kDa band was only observed in the highest calibrator concentration (100 ng). The UGot calibrator contains by far the largest amount of protein, around 7-fold more than the WashU and the ADx calibrators. The WashU and ADx calibrators both had similar levels of staining, indicating that the same relative amounts of calibrator were present in the samples.

### Neurogranin epitope recognition by the three assays

To better understand what forms of neurogranin are recognised by the three different assays, the calibrators of each assay were measured in the other two assays (table 2). The WashU neurogranin assay did not detect the P75-truncated ADx calibrator. The WashU assay fully recognised the UGot calibrator, and the neurogranin values obtained by the WashU assay were approximately 30% higher than the concentrations obtained by the UGot assay itself. The ADx assay, designed to detect neurogranin truncated at P75, did not detect the calibrators of WashU nor the calibrators of UGot, although for the latter very low concentrations at the

border of the LLOD were detected for all calibrator dilutions. The UGot assay could not detect the P75-truncated ADx calibrator since the signal was too weak, whereas the signal of the WashU calibrator was detected above the upper limit of detection in all dilutions (12-399 pg/ml).

Additionally, neurogranin was measured in brain lysate to compare the affinity of the three assays for neurogranin peptides in brain (figure 3). All assays recognised neurogranin in this control brain lysate sample. The WashU and UGot assay quantified neurogranin in brain lysate at similar concentrations, whereas approximately 70-fold lower neurogranin concentrations were detected by the ADx assay compared to the WashU and UGot assays.

Quantitative and qualitative comparisons of neurogranin epitope recognition by capture and detection antibodies of immunoassays

After separation by gel electrophoresis, the calibrators of the three assays, as well as CSF and brain homogenate samples were immunoblotted with the antibodies of all three assays (figure 4). All antibodies showed the strongest and cleanest bands when exposed to their own assay calibrator. The N-terminal WashU antibody (S10–D23) recognised both its own calibrator and the UGot calibrator at 14 kDa, where the latter gave the strongest signal. The ADx calibrator was not recognised (figure 4A). The G49–G60 WashU antibody recognised its own and the UGot calibrator at 14 kDa, again the UGot calibrator gave the strongest signal. The ADx calibrator was detected at 6 kDa (figure 4B). The combination of WashU antibodies strongly detected the UGot calibrator, but did not recognise the ADx calibrator, which is in line with the fact that this calibrator lacks the N-terminal part of neurogranin. The R53–V64 ADx antibody recognised its own calibrator at 6 kDa and the UGot calibrator at 14 kDa. The WashU calibrator showed a very weak band at 14 kDa (figure 4C). The G62–P75 antibody, on the contrary, only recognised its own calibrator at 6 kDa (figure 4D). The combination of

ADx antibodies did not recognise the WashU nor the UGot calibrator, due to the specific targeting of neurogranin truncated at P75. The G52–G65 UGot antibody recognised its own calibrator and the WashU calibrator at 14 kDa, and weakly stains the ADx calibrator at 6 kDa (figure 4E and G). The WashU calibrator in figure 4E had been stored at 4°C for 2 days and was degraded, therefore the experiment was repeated with freshly prepared WashU calibrator in figure 4G. The V66–D78 UGot antibody detected its own and the WashU calibrator at 14 and at 6 kDa, where the ADx calibrator gave no signal (figure 4F). The combination of UGot antibodies – specifically targeted at neurogranin ending at P78 – recognised the WashU calibrator, but did not recognise the P75-truncated calibrator of ADx. The multiple bands at higher molecular weights in the UGot calibrator were recognized by all neurogranin antibodies, except for the ADx451 antibody that only recognised the neo-epitope at neurogranin truncated at P75.

Next, we assessed the affinity of the neurogranin antibodies to CSF and brain lysate samples (figure 4). Neurogranin was recognised in CSF by the WashU and UGot antibodies, bands were shown around 60 or 70 kDa, but not by the ADx antibodies. Neurogranin in brain lysate was recognised as a single band at 14 kDa by most antibodies, where the polyclonal WashU antibodies additionally recognised bands at multiple, mainly higher molecular weights, and the ADx451 antibody recognised a low intensity band around 27 kDa.

#### Analytical performance of the assays

Neurogranin levels could be determined in all samples using the WashU and ADx assays, while 9 out of 108 samples were below the LLOD using the UGot assay. All duplo neurogranin measurements had CVs <20% in the WashU and ADx assays, where 20% of the samples measured with the Gothenburg assay had CVs >20%, mainly in samples with concentrations near the LLOD. Intra-assay/ inter-assay CVs were 5%/6% (WashU); 7%/7%

(ADx) and 6%/8% (UGot). Neurogranin levels ranged from (median (range)) 1881 (330-8320) pg/mL for WashU, 372 (71-1191) pg/mL for ADx, and 416 (115-1481) pg/mL for UGot. The strongest correlation for neurogranin in clinical samples was found between the ADx and WashU assays, with a Spearman's Rho of 0.95, while for UGot versus WashU and UGot versus ADx, Spearman's Rho was 0.87 and 0.81, respectively. Passing-Bablok regression analysis demonstrated proportional differences among all three assays (figure 5), where the WashU assay showed about five times higher neurogranin concentrations than the ADx and UGot assays. Slopes of regression lines were 5.78 (95% CI= 5.36; 6.09) for ADx versus WashU, 5.17 (95% CI= 4.38; 5.83) for UGot versus WashU, and 0.82 (95% CI= 0.71; 0.94) for UGot versus ADx. A systematic difference was observed between the ADx versus WashU assays (intercept -169.72 (95% CI= -266.41; -56.46), but not between ADx and UGot, or WashU and UGot.

#### Neurogranin results differ between diagnostic groups

ANCOVA's showed a specific increase in neurogranin levels in Alzheimer's disease patients compared to all other clinical groups in each assay ( $p < 0.05$ ; figure 6). Effect size was highest in the UGot assay:  $\eta^2$  for the WashU, ADx, and UGot assay was 0.14, 0.11, and 0.21, respectively. Note that effect sizes are relatively small due to the rank-transformed neurogranin levels that were used in these analyses instead of the absolute concentrations. Post hoc Bonferroni tests showed a specific increase for in the Alzheimer's disease group compared to the Lewy body dementia ( $p < 0.05$  for WashU and ADx,  $p < 0.01$  for UGot) and vascular dementia groups ( $p < 0.01$  for WashU,  $p < 0.05$  for ADx,  $p < 0.001$  for UGot), while the differences between Alzheimer's disease patients and controls were only significant for the WashU ( $p < 0.05$ ) and UGot assay ( $p < 0.01$ ). Gender and age did not significantly influence neurogranin levels (supplementary materials 1-2). *APOE*  $\epsilon 4$  carriers had slightly

higher neurogranin levels than *APOE*  $\epsilon$ 4 non-carriers but this difference reached significance in the UGot assay only (supplementary material 3).

Relation of neurogranin with MMSE scores and other Alzheimer's disease biomarkers in the three assays

A modest negative correlation was found in the UGot assay ( $\rho = -0.23$ ,  $p < 0.05$ ), but not in the WashU or ADx assay (supplementary material 4). None of the assays showed a correlation with A $\beta$ 1-42, but all had positive correlations with t-Tau and p-Tau (supplementary material 5).

## **Discussion**

In this study, we compared three commonly used neurogranin assays on their calibrators, their epitope affinities, and their clinical performances. The WashU and UGot assays could mutually recognise their calibrators, on western blot as well as in the immunoassays, while the ADx assay detects a specific form of truncated neurogranin which was not detected by the others nor could it detect the other calibrators. All assays detected different neurogranin peptides, since the WashU assay targets the N-terminal part of the protein and UGot specifically targets the C-terminal end. Results from our clinical cohort showed that CSF neurogranin levels measured by the different assays correlated well amongst each other, but showed large differences in absolute values. Neurogranin levels were elevated in Alzheimer's disease patients compared to controls (although not significantly in the ADx assay), dementia with Lewy bodies, and vascular dementia, but not compared to frontotemporal dementia.

We characterized and quantified the calibrators of the three assays using silver stain to better understand the large differences in absolute values observed in previous studies. Where the WashU and ADx calibrators showed clean bands at respectively 14 and 6 kDa, the UGot

calibrator showed many protein bands at different molecular weights on Silver stain, which were recognised by neurogranin antibodies on western blot. The recombinant origin of the UGot calibrator might explain the presence of multiple larger protein complexes that comprise neurogranin, possibly due to post-translational modifications such as citrullination of neurogranin at its C-terminal part (Liu *et al.*, 2013). The differences in absolute neurogranin concentrations were, however, not explained by the amount of protein found in the calibrators, since the UGot calibrator contained the most protein, while the WashU assays produced the highest absolute values. A potential explanation could be differences in value assignment of the calibrators, especially since the WashU assay is developed on an Erenna system. Another cause could be different antibody affinities amongst the antibodies in the assays to their calibrator.

The neurogranin levels in CSF and brain homogenate detected by the immunoassays were not reflected in high staining intensities in the western blot experiments. To illustrate, CSF samples on western blot did not show the typical 14 kDa neurogranin band, except for a weak signal detected by the N-terminal WashU antibody only, while neurogranin in CSF is abundantly detected by all immunoassays. This is probably due to altered conformational states of the protein in the different experiments, since samples were denatured and reduced for the SDS-PAGE gel and western blot compared to native conditions used in the immunoassays.

Our results confirmed that CSF neurogranin concentrations were specifically elevated in Alzheimer's disease patients compared to controls and other types of dementia, although levels in frontotemporal dementia were slightly elevated as well. Also, we observed an overlap amongst the different disease groups which had as consequence that neurogranin levels were often not significantly changed amongst the groups. These findings are consistent with findings from previous studies (Hellwig *et al.*, 2015; Janelidze *et al.*, 2015; Tarawneh *et*

*al.*, 2016; Lista *et al.*, 2017), indicating that neurogranin has limited value as biomarker for differential diagnosis of dementia.

Importantly, neurogranin concentrations amongst all three assays correlated well in all clinical dementia groups. Nevertheless, we do not support use of a conversion factor between the neurogranin levels of the different assays, since we showed that the assays have affinity for different neurogranin peptides. This was not reflected in the clinical neurogranin levels, although it could explain our finding that neurogranin levels measured by UGot correlated with MMSE score and related to APOE $\epsilon$ 4 carriership, where the levels measured by WashU and ADx did not. Clinical performance of the immunoassays compared through their effect sizes of ANCOVA, was highest in the UGot assay, followed by WashU and ADx. A limitation of the UGot assay was, however, that it measured 8% of the samples at the lower limit of detection value, which artificially reduced the variance in statistical comparisons.

The major strength of this study is the thorough approach in which calibrators of the immunoassays were directly compared on Silver stain and western blot, and, that the assays were directly compared using a similar set of clinical CSF samples.

A limitation of this study was the large difference in assay calibrator composition and quantities for comparison on western blot. High abundant proteins required other transfer conditions than did low abundant proteins, likewise did the high molecular weight complexes compared to the small 14 kDa neurogranin peptide. This hampered semi-quantification of the bands observed in western blot, limiting the quantification to the calibrator bands that were stained in the gel by Silver staining.

The remarkable fact that the different neurogranin peptides detected by the three assays are not differentially expressed between dementia subtypes, suggests alternative hypotheses regarding neurogranin's role in dementia pathology. Potentially, synaptic loss, which is

mainly described as hallmark of Alzheimer's disease (Terry *et al.*, 1991; Blennow *et al.*, 1996), also plays a prominent role in other types of dementia (Clare *et al.*, 2010; Herms and Dorostkar, 2016). Studies using ratios of biomarkers, for example neurogranin together with the pre-synaptic protein BACE1 (De Vos *et al.*, 2016), the amyloid pathology marker A $\beta$ 42 (Tarawneh *et al.*, 2016), or the neurodegeneration marker t-Tau (Mattsson *et al.*, 2016) could yield better discriminatory power amongst differential diagnoses of dementia. Moreover, the different neurogranin peptides were not differentially expressed in this cross-sectional design, but could have increased value in longitudinal designs focused on disease progression.

In conclusion, our research showed that different peptides of neurogranin in CSF can be measured using the three assays described here, WashU targets full-length neurogranin, ADx targets P75-truncated neurogranin and UGot targets neurogranin ending at D78. Through directly comparing these three commonly used assays, we are one step closer to implementation of neurogranin as an additional CSF biomarker for dementia. The next challenge would be the development of reference material for neurogranin assays. Also, insight in the relative performance of the assays compared to each other provides new opportunities for studying neurogranin as a pathological player in the progression of dementia, since the assays measure relatively similar neurogranin values but different neurogranin peptides.

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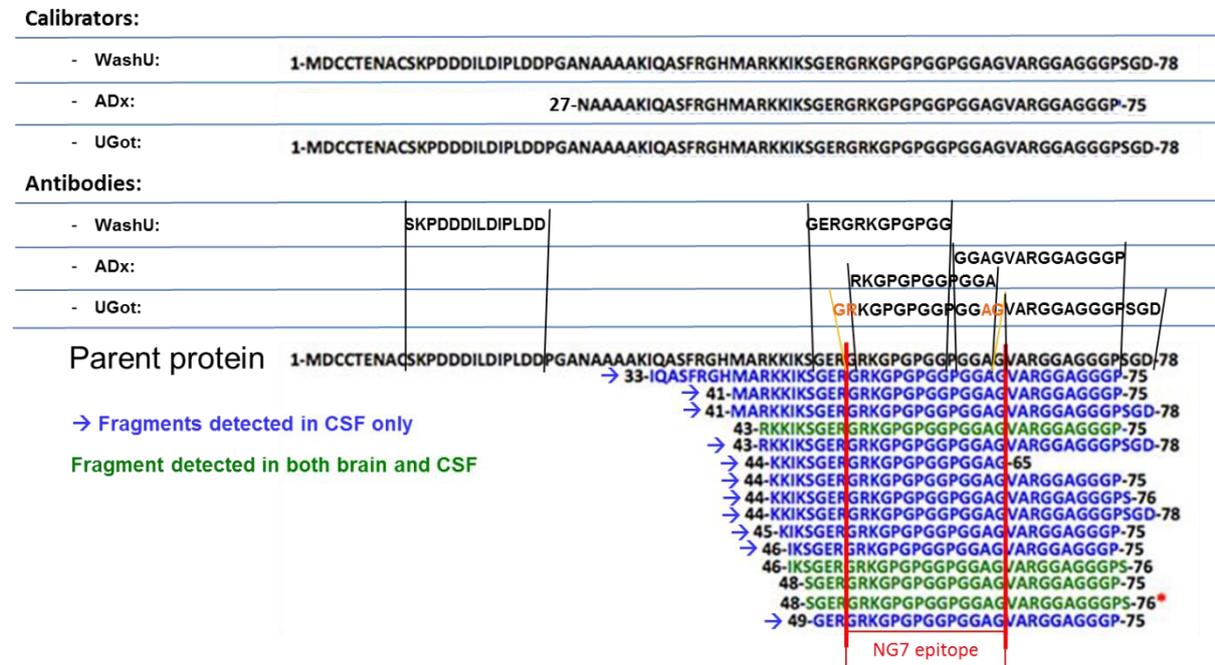
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Figure 1. Amino acid sequences of full-length human neurogranin, its physiological peptides found in CSF and brain tissue, and the calibrators and antibody epitopes of the neurogranin assays.



Legend: Amino acid sequence of full-length human neurogranin and its epitopes found in CSF and brain tissue. The upper three rows indicate what epitopes are tagged by the capture and detection antibodies used in the WashU assay (upper row: S10-D23 and G49-G60), ADx assay (middle row: R53-A64 and G62-P75), and UGot assay (bottom row: G52-G65 and V66-D78). The red asterisk indicates the fragment found as the most abundant one in brain and brain-specific (Kvartsberg *et al.*, 2015). Adapted from (Kvartsberg *et al.*, 2015).

Figure 2. Silver staining of the WashU, ADx, and UGot calibrators.

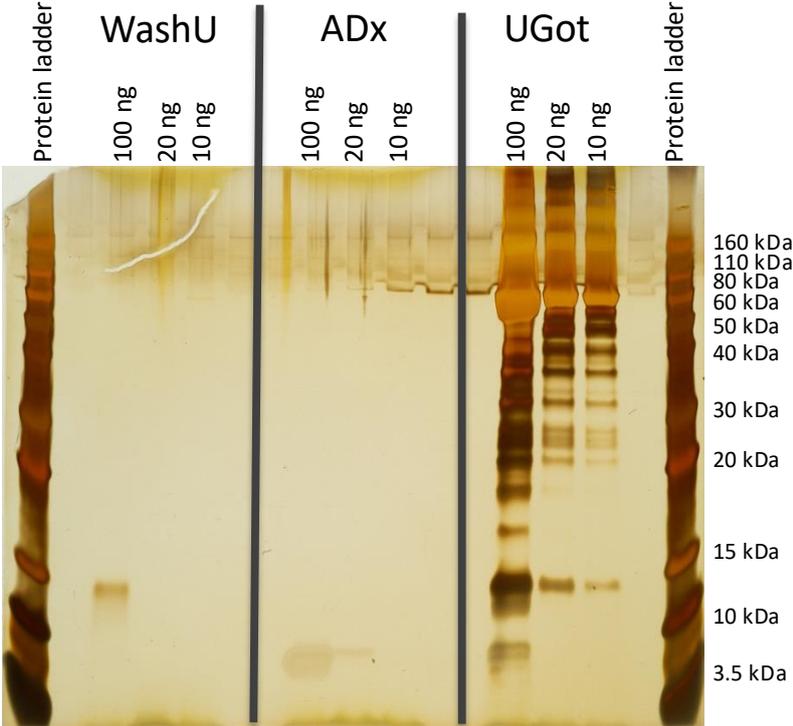


Table 2. Recognition of neurogranin assay calibrators and neurogranin in brain lysate by the WashU assay, ADx assay, and UGot assay.

Recognise calibrator of:	WashU assay	ADx assay	UGot assay
<b>WashU (synthetic, full-length)?</b> Calibrator concentrations:	399 pg Ng/ml 61 pg Ng/ml 12 pg Ng/ml	- - <LLOD	+ + >ULOD
<b>ADx (synthetic, truncated at P75)?</b> Calibrator concentrations:	- 3.3 pg Ng/ml 1.8 pg Ng/ml 0.8 pg Ng/ml -	500 pg Ng/ml 300 pg Ng/ml 150 pg Ng/ml (75,30,15,5,0 pg Ng/ml)	- - <LLOD
<b>UGot (recombinant, full-length)?</b> Calibrator concentrations:	+ + 5703 pg Ng/ml 1772 pg Ng/ml 506 pg Ng/ml	- 18 pg Ng/ml 16 pg Ng/ml 22 pg Ng/ml	4458 pg Ng/ml 1114 pg Ng/ml 279 pg Ng/ml

Degree of detection of a calibrator by another assay is scored from + + (very good) to - - (very poor).

Figure 3. Neurogranin concentrations in dilutions of one brain lysate sample measured by the three immunoassays.

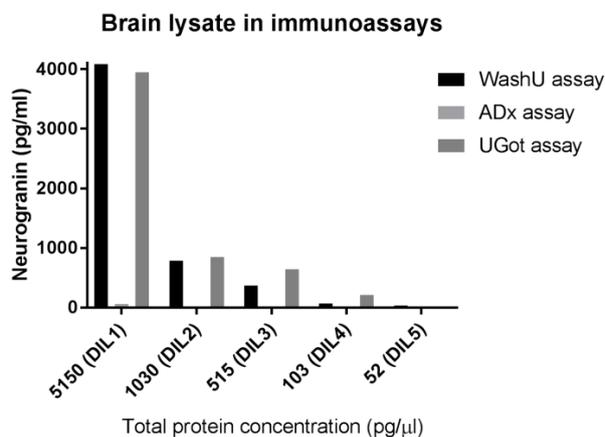
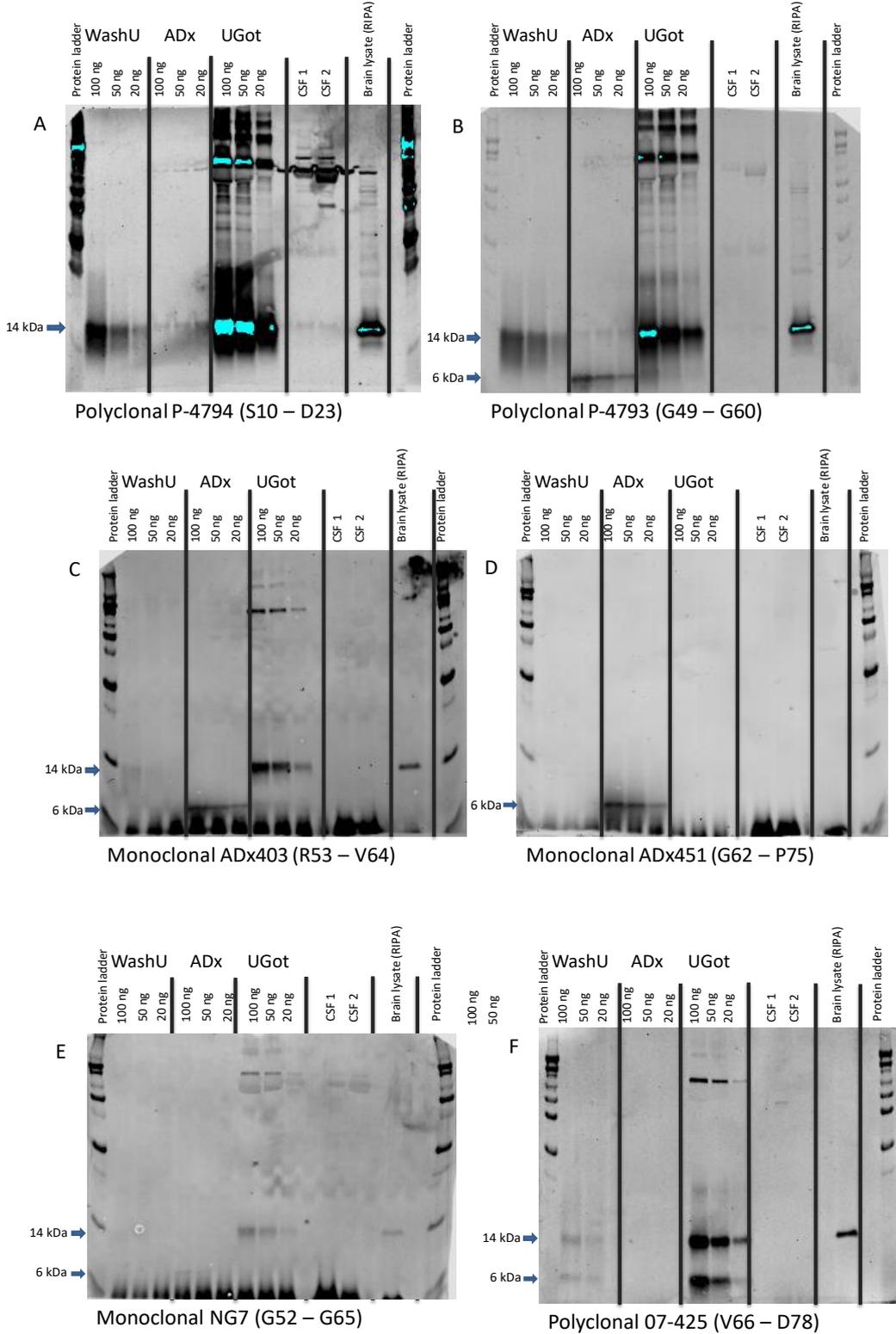


Figure 4. Western blot analysis of neurogranin calibrators, CSF, and brain homogenate stained by the antibodies of the three immunoassays.



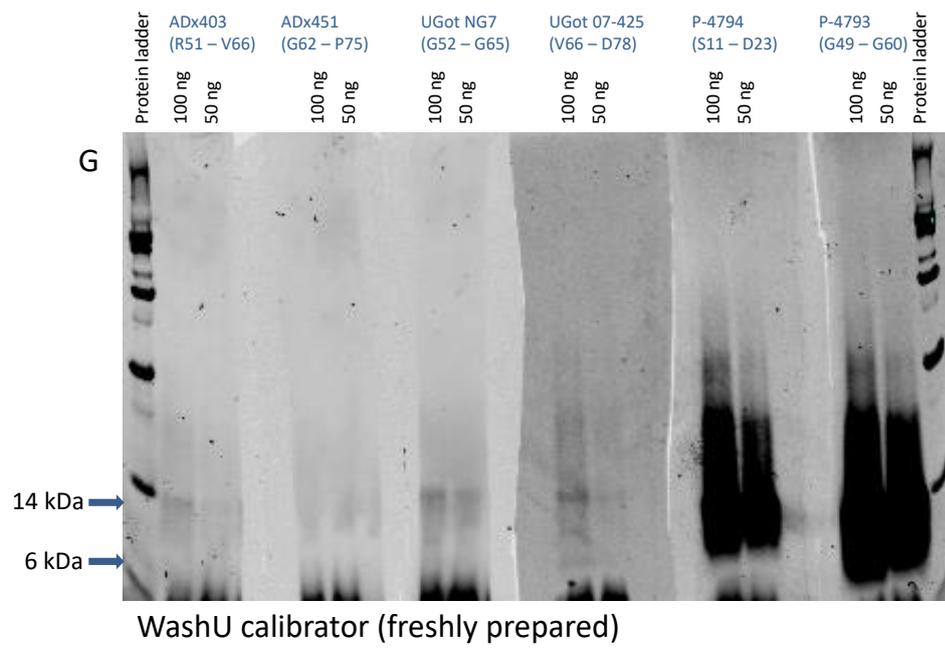


Figure 5

Legend: Passing-Bablok regression analysis of the neurogranin assays. a) The ADx vs. the WashU assays exhibit a proportional difference (slope (95% CI) of regression line: 5.78 (5.36; 6.09)), as well as a systematic difference (intercept (95% CI): -169.72 (-266.41; -56.46)).

b) The UGot vs. the WashU assays exhibit a proportional difference (slope (95% CI) of regression line: 5.17 (4.38; 5.83)) but not a systematic difference (intercept (95% CI): -177.48 (-448.19; 45.18)). c) The UGot vs. the ADx assays exhibit a proportional difference (slope (95% CI) of regression line: (0.82 (0.71; 0.94)) but not a systematic difference (intercept (95% CI): 10.36 (-29.37; 57.93)).

Orange dots indicate individual CSF samples (n=108), the dotted lines represent the equation  $x=y$  (identity line), and the blue areas show the 95% confidence intervals of the regression lines.

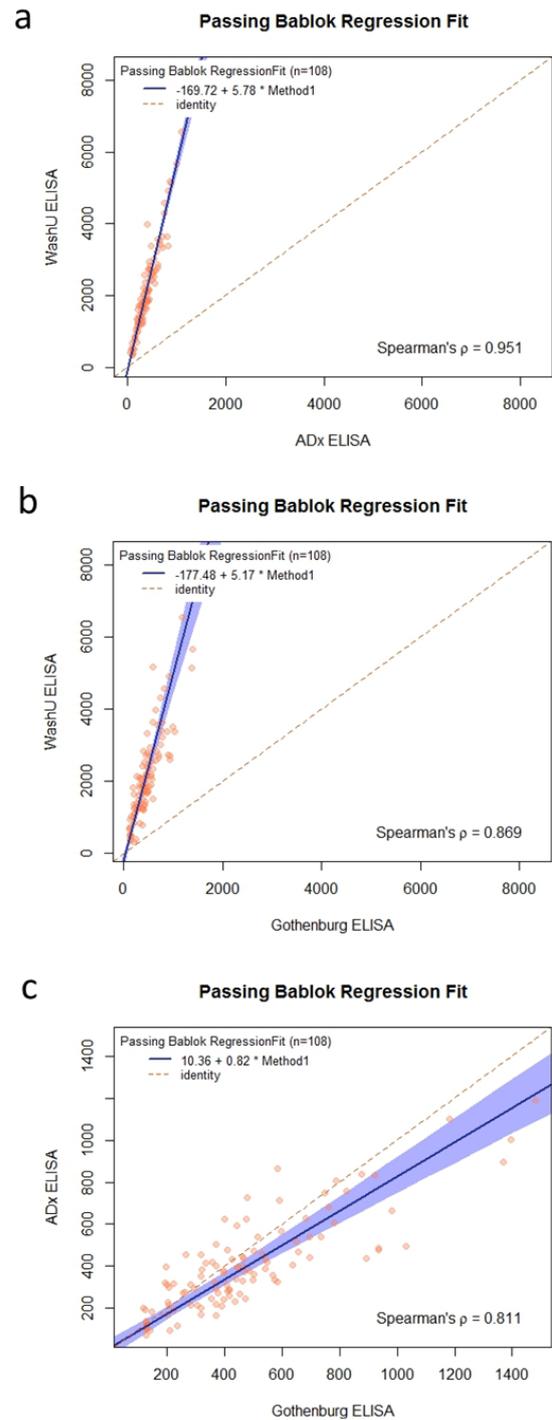
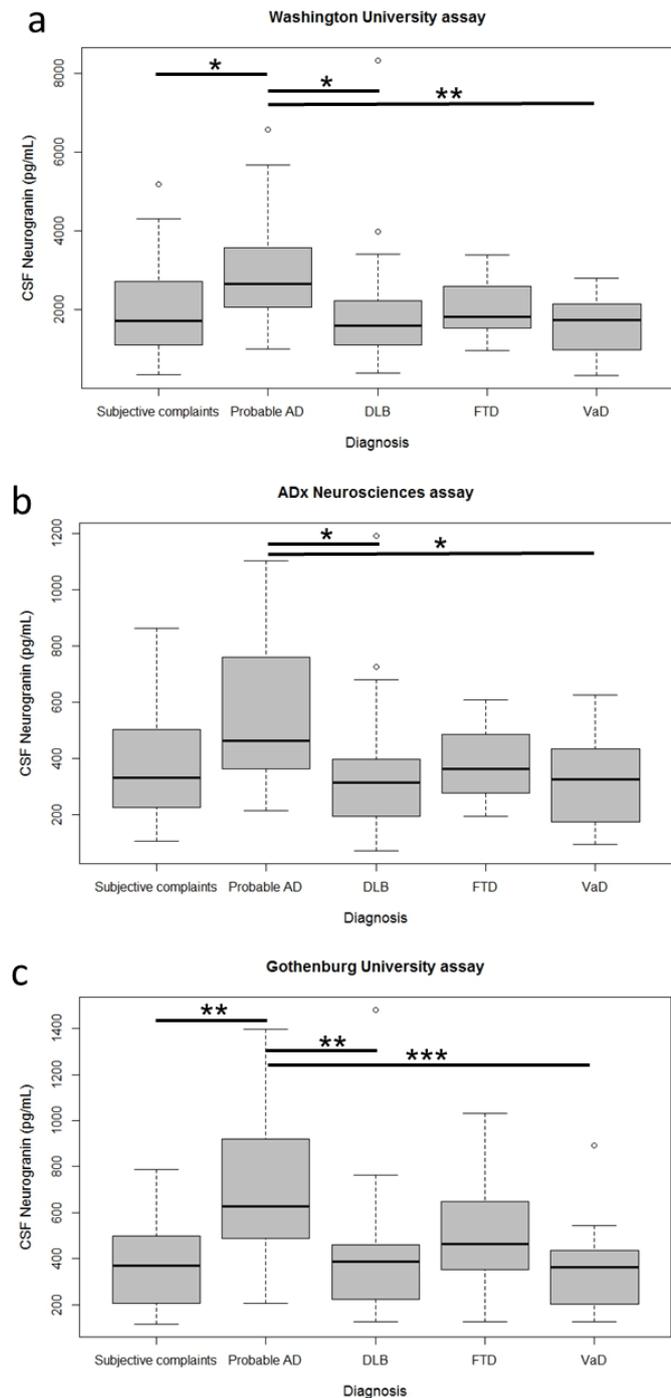


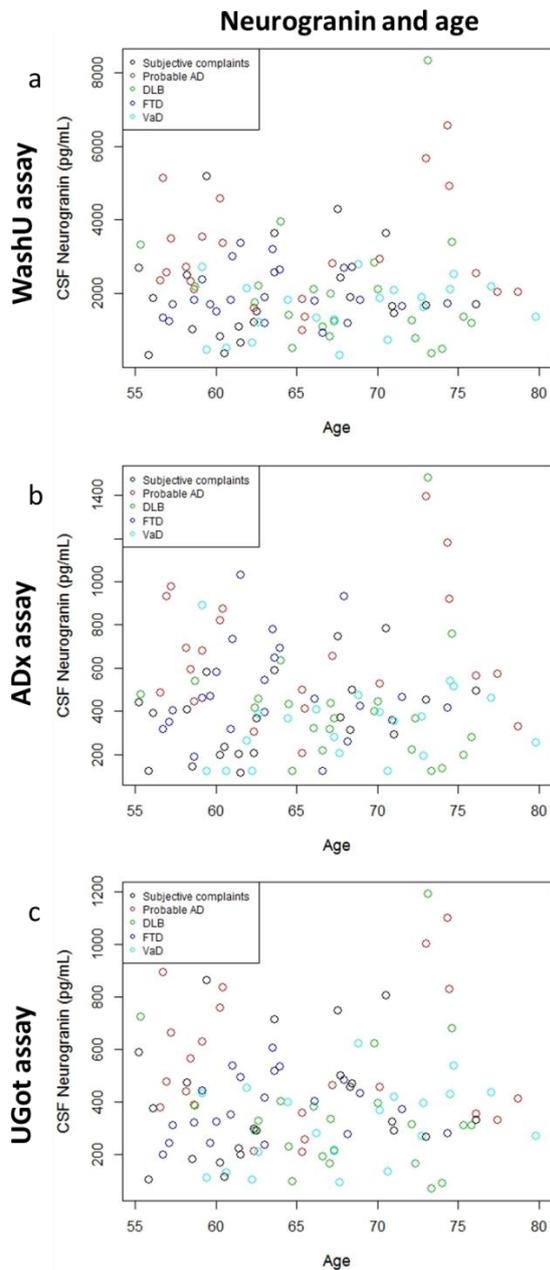
Figure 6.

Legend: Boxplots of CSF neurogranin concentrations (pg/mL) in dementia differential diagnosis groups measured with the a) WashU assay, b) ADx assay, c) UGot assay. Absolute neurogranin ranges vary among the three assays, but the neurogranin levels within the clinical groups show the same pattern amongst the three assays. DLB= dementia with Lewy Bodies, FTD= frontotemporal dementia, AD= Alzheimer's disease, VaD= vascular dementia. Asterisks indicate significance of \*=  $p < 0.05$ , \*\*=  $p < 0.01$ , \*\*\*= $p < 0.001$  in Bonferroni post-hoc comparisons following ANCOVA on the ranked neurogranin levels corrected for age and gender.



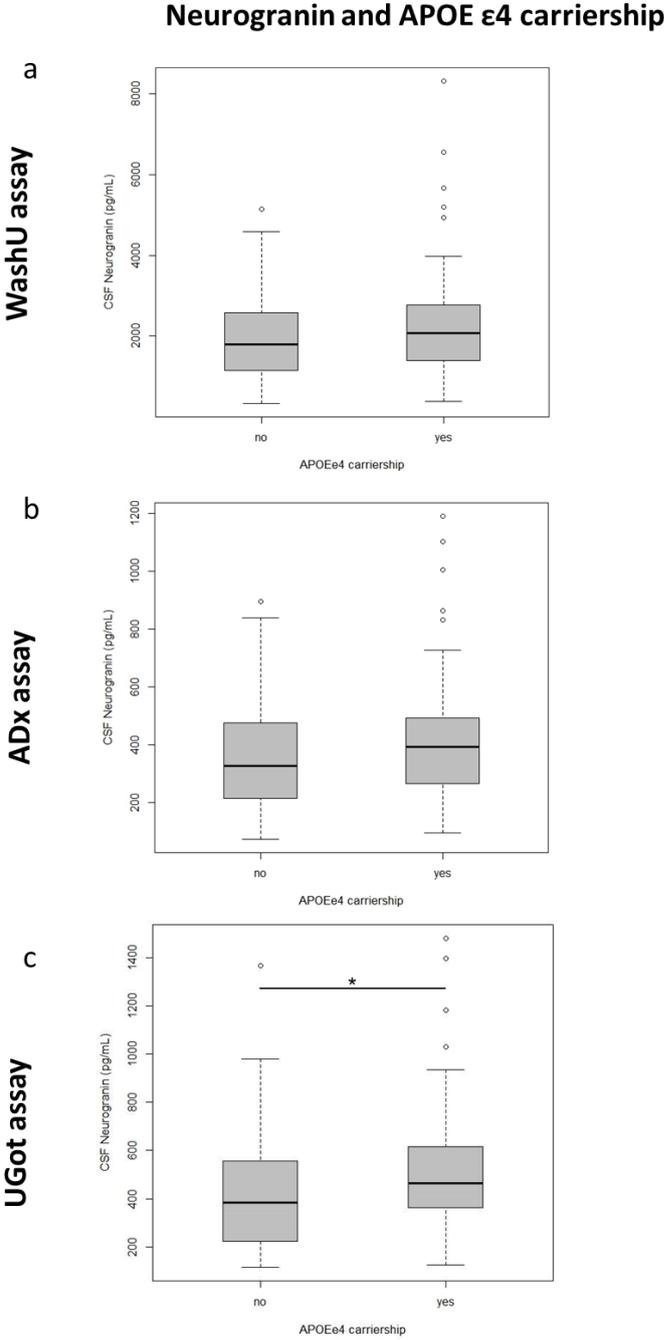
## Supplemental material

Supplemental figure 1



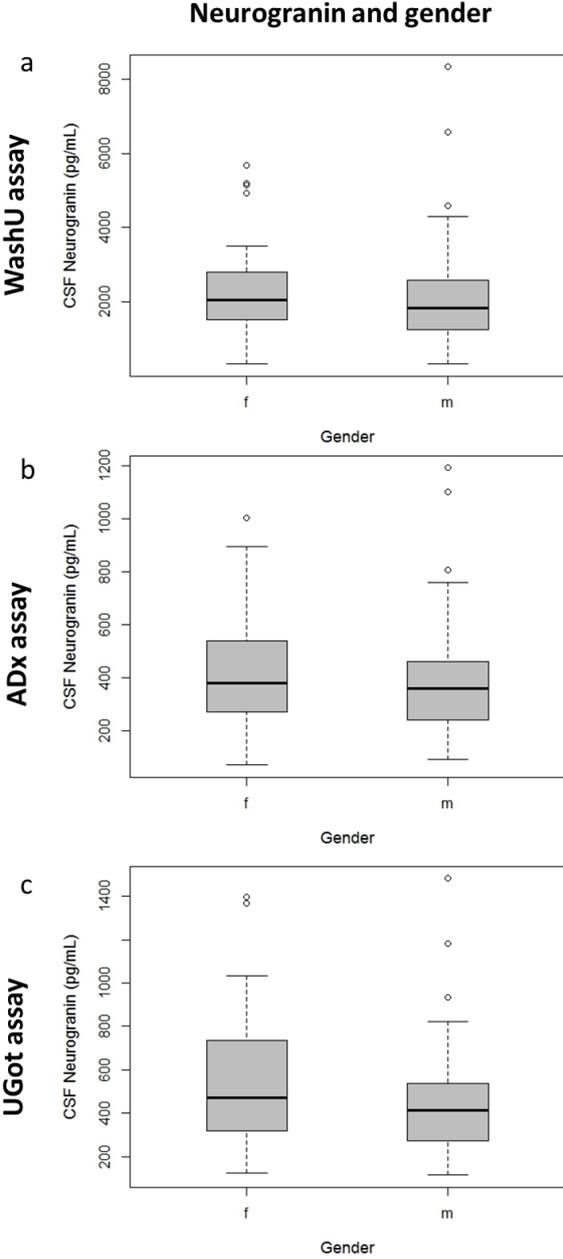
Legend: Association between age and neurogranin levels in the three assays. Correlation plots of neurogranin levels with age and neurogranin levels measured with the WashU assay (a), ADx assay (b), and UGot assay (c). Colours indicate different clinical groups. Spearman correlations showed no correlation with neurogranin levels and age in any of the assays.

Supplemental figure 2



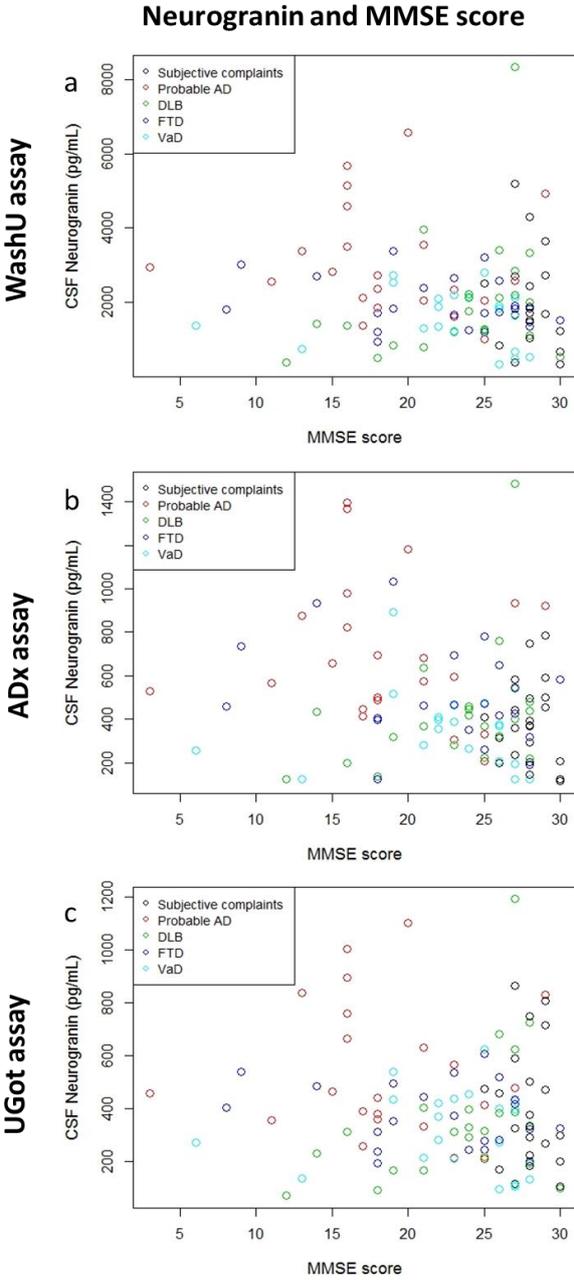
Legend: Neurogranin levels are slightly higher in APOEε4 carriers compared to non-carriers and in the three assays, although this differences reached statistical significance in the UGot assay only (c), but not in the WashU assay (a) and ADx assay (b). T-tests on rank-transformed neurogranin levels were performed.

Supplemental figure 3



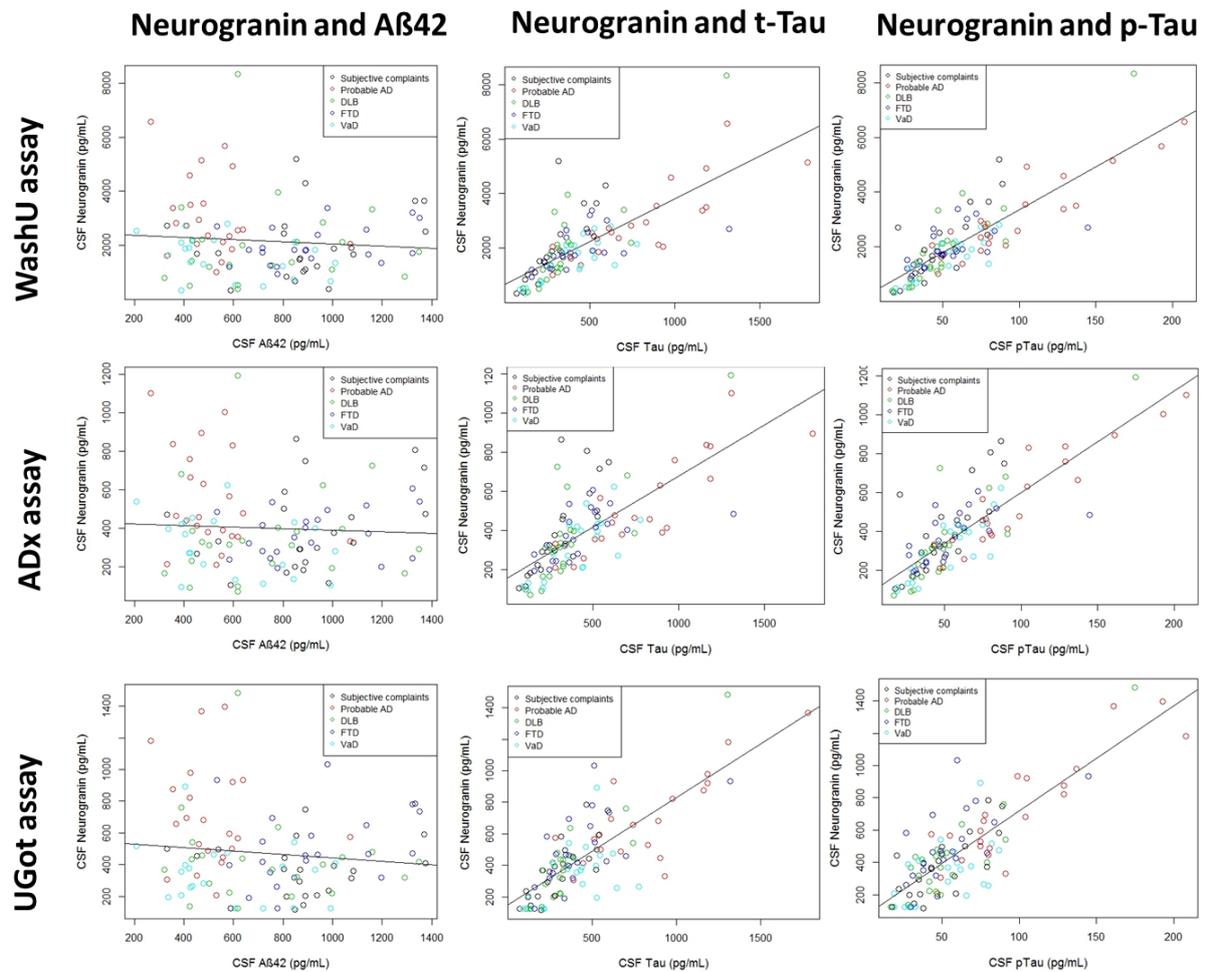
Legend: Neurogranin levels were not different between females and males. Mean neurogranin levels were f = 2344 pg/mL and m= 2074 pg/mL for WashU (a), f= 433 pg/mL and m= 389 pg/mL for ADx (b), and f= 551 pg/mL and m= 436 pg/mL for UGot (c). T-tests on rank-transformed neurogranin levels were performed.

Supplemental figure 4



Legend: Correlation between neurogranin levels and MMSE in the WashU (a), ADx NeuroSciences (b), and UGot assay (c). A significant (negative) correlation was found between Neurogranin level and MMSE score only for the Gothenberg assay score (Spearman correlation,  $\rho=-0.23$ ,  $p<0.05$ ). MMSE scores can range from 0-30, with 30 being perfect performance.

Supplemental figure 5



Legend: Correlation between Alzheimer’s disease biomarkers and neurogranin levels.

Neurogranin did not significantly correlate with Aβ42 in any of the assays. In all three assays, neurogranin correlated with t-Tau (Spearman’s  $\rho = 0.78$  ( $p < 0.0001$ ),  $0.76$  ( $p < 0.0001$ ), and  $0.73$  ( $p < 0.0001$ ) for the WashU, ADx, and UGot neurogranin assay, respectively) and p-Tau (Spearman’s  $\rho = 0.81$  ( $p < 0.0001$ ),  $0.80$  ( $p < 0.0001$ ), and  $0.71$  ( $p < 0.0001$ ) for the WashU, ADx, and UGot neurogranin assay, respectively).