Lymphocyte Proliferation as a Means to Assess Cell Cycle Dysregulation in Alzheimer's Disease Subjects: Clinical Performance of the LymPro Assay

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ABSTRACT

A blood based test for Alzheimer's disease would be advantageous for early identification of Alzheimer's disease (AD). Multiple lines of evidence have identified Cell Cycle Dysfunction (CCD) as a key pathology in Alzheimer's disease. Furthermore, it appears likely that this dysfunction is systemic, affecting Peripheral Blood Lymphocytes (PBLs) as well as neurons. Here we summarize the clinical data of the Lymphocyte Proliferation (LymPro®) test as a means of measuring CCD. The original findings [1] have been replicated and extended [2] with the technique subsequently enhanced ("Version 2") in unpublished pilot research presented here. The LymPro test consists of measuring cell surface expression of CD69 in subsets of mitogenically stimulated PBLs. Enhanced assay methods demonstrated up to a 2.5 fold increase in expression of CD69 on multiple cell types. Using the enhanced assay methods in a small pilot trial and employing multiple regression techniques, up to a 91% positive and 92% negative agreement with subject clinical diagnosis. With further development, LymPro may become a useful blood based biomarker.

CELL CYCLE DYSFUNCTION IN AD

See Poster 45398 for More Detail on CCD in AD Neurons

In AD, terminally differentiated neurons express aberrant re-entry into the cell cycle progressing through the S phase and arresting at the G2 phase. This increases the risk of neurodegeneration. AD brains express cytokine dependent kinases (CDKs, 1, 2, 4, 5) and cyclins (A, B, D E, G1) associated with the cell cycle. In addition proteins associated with G1-S checkpoint regulation are also altered. AD neurons express duplicated DNA and tetraploid neurons show increased risk of cell death. Furthermore, the CDKs may be involved in phosphorylation of tau. Some authors have related CCD to amyloid-β (Aβ) with evidence of Aβ triggering cell cycle re-entry and with increased production of amyloid. CCD has been documented to be an early phenomenon in subjects with mild cognitive impairment (MCI) and in mouse models expressing CCD prior to much of the subsequent development of AB and ptau.

CELL CYCLE DYSFUNCTION IN PBLs

See Poster 45398 for More Detail on CCD in AD PBLs

AD is likely a systemic disorder with evidence of CCD in peripheral blood lymphocytes. Up-regulation of P53 mutant-like conformation, Calmodulin, Cyclin E and CDK2 are all observed in AD lymphocytes. G1/S Checkpoint inhibitor Rapamycin blocks the G1-S transition in lymphocytes from healthy donors but not AD subjects. Reduced expression of CD69, an indicator of lymphocyte activation, is observed in AD but not cognitively intact (CI) subjects [1,2]. PBLs, being a much more accessible tissue, represents an opportunity for a blood based biomarker of CCD.

METHODS

Clinical subjects:

The Stieler 2001 study [1]: N_{TOT}=72; N=27 with probable AD and N=45 age-matched cognitively intact (CI) controls.

The Stieler 2012 study [2]: N= 88; 32 with probable AD, 26 with Parkinson's disease dementia (PDD) (active control) and 30 age matched cognitively intact controls.

Enhanced assay study: N_{TOT} = 44; N=15 with probable AD, N=18 with other dementia (OD) and N=11 cognitively intact (CI) controls.

Assay methods:

Sample preparation: Sample preparation for [1] and [2] were as published and similar. In brief, peripheral blood mononuclear cells (PBMC) were isolated from whole heparinized blood samples as described previously [1]. Aliquots of each sample were stimulated with either phytohemaglutinin (PHA, 12 µg/mL), pokeweed mitogen (PWM, 4 µg/mL) or none (Unstimulated) and each cell culture incubated at 37°C with 7% CO2 for four hours [1] and [2] (Version 1 = V1).

The enhanced version (Version 2 = V2) included 8 µg/mL PWM with up to 20 hours incubation. Samples were then diluted and frozen prior to staining for flow cytometry.

Assay measurement: Antibody cocktails for flow cytometry used in [1] included antibodies against the following: CD45, CD3, CD4, CD8, CD19 and CD69. Subsequent studies included antibodies against CD14 and CD28 in addition to those used in [1]. Studies [1], [2] and Enhanced Assay analyzed samples using a Becton Dickinson FACSCalibur flow cytometer.

RESULTS

Univariate scoring:

Stieler 2001: Demonstrated differential PBL proliferative response between Alzheimer's disease cases and age matched controls. Univariate results were replicated in Stieler 2012.

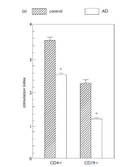


Figure 1: Impairment of mitogenic activation of CD4 and CD19 lymphocytes after PHA stimulation in AD. P=0.001; Student's t-test [1].

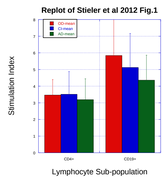


Figure 2: Re-plot of the Box-and-Whiskers Plot of self normalized CD69 expression of B cells (CD19) in response to PWM mitogenic stimulation representing the mean & standard deviation. (Stieler 2012 [2] – re-plot by Amarantus 2014).

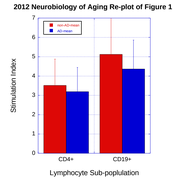


Figure 3: Re-plot of Figure 2 combining the OD and CI groups into an AD- group compared to an AD+. (Stieler 2012 [2] – re-plot by Amarantus 2014).

Cell Marker	Activation Marker	Stimulant	Index	p-Value
CD4	CD69	PWM	SI 2	0.028
CD8	CD69	PWM	SI 1	0.005
CD14	CD69	PWM	SI 2	0.016
CD19	CD69	PWM	SI 1	0.005
	CD69	PWM	SI 2	0.009
	CD69	PHA	SI 2	0.025

Table 1: Markers differentiating AD from PDD at p <0.05 significance (two- tailed). It should be noted six of the seven statistically significant markers were measures of CD69 expression, which was the basis of the original study. (Stieler 2012)

Multivariate Scoring Model

Integrated differential PBL subpopulations with IVDMIA algorithm to produce a singular score that differentiates Alzheimer's subjects from either PDD or control subjects, as shown in Figure 4.

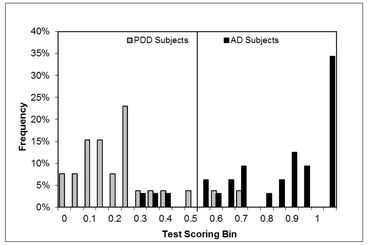
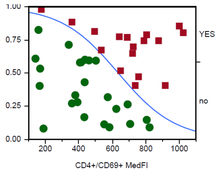


Figure 4: Multivariate Scoring Model for differentiating AD and PDD. Mean scores of 0.16 and 0.84 were obtained for PDD and AD subjects, respectively.

RESULTS



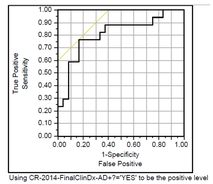


Figure 5: A preliminary predictive model of each factor was conduced in a univariate manner in JMP PRO v11. The left figure (above) shows the logistic regression of the median fluorescence intensity of the CD69+ cells measured within the CD4+ subpopulation (non AD = AD- = green circles, AD positive = AD+ = red squares). On the right is a Receiver Operator Characteristic (ROC) curve which shows an Area Under the Curve (AUC) of 81% in a univariate model that was not adjusted with internal k-fold cross validation.

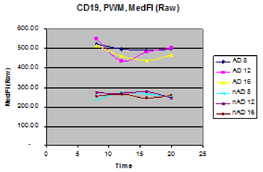


Figure 6: Time / Dose Studies examined CD69 expression using three concentrations of the mitogenic stimulants PWM or PHA at four different incubations times. Median fluorescence intensity {MedFI (raw)} was observed significantly larger in AD subjects versus non-AD subjects (3 of each class plotted above).

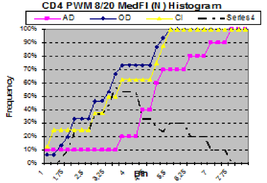


Figure 7: Increased CD69 expression observed with higher concentrations of mitogenic stimulants and longer incubation times translated to greater univariate differentiation between Alzheimer's subjects and those with other chronic progressive dementias. As shown here, the median fluorescence intensity of CD69 expression on CD3 Cells is increased on OD and CI cells relative to AD (cumulative probability curves). Difference between CI and AD shown in black dashed trace.

FUTURE WORK

The ongoing work includes:

Pilot trial:

- Replicate the original data set.
- Further explore the enhanced stimulation techniques presented here to maximize differentiation.

Top line results should be ready by July 31 2014 and presented at the C4CT Concussion Awareness Conference at the United Nations

Analyte performance package: Full Analyte Performance/Validation Package will be completed in Q3

Continued development: Will launch much larger trial incorporating biomarker qualified AD and cognitively intact subjects as well as multiple types of other dementias

Collaboration: Plans to work with academia and industry to support their research activities

CONCLUSIONS

Stieler 2001

- A moderate degree of differentiation is seen in the PBL proliferative response between Alzheimer's disease subjects and cognitively intact controls.
- Statistically significant differences in the stimulation index were reported for T-helper-/inducer lymphocytes (CD3+/CD4+) and B-lymphocytes (CD19).
- These findings support the hypothesis that dysfunction of cellular proliferation control in AD is not restricted to neurons but also affects immune cells outside the CNS.

Stieler 2012

- Using methods similar to 2001, a similar degree of differentiation is seen in the PBL proliferative response between Alzheimer's disease subjects and subjects with Parkinson's disease dementia or cognitively intact controls.
- Statistically significant differences in the stimulation index were reported for T-helper-/inducer lymphocytes (CD3+/CD4+) and B-lymphocytes (CD19) as well as for T-suppressor-/cytotoxic lymphocytes (CD3, CD8) and monocytes (CD14).
- Linear and logistic regression models were created that could differentiate Alzheimer's disease subjects from age matched Parkinson's disease dementia subjects with a 91% positive agreement and a 92% negative agreement with the clinical diagnosis.

Enhanced Assay

- Increasing concentration of mitogenic stimulants and longer incubation times significantly increase the cell cycle activation marker CD69.
- Increased CD69 expression appears to increase univariate differentiation.
- Results demonstrate that multiple parameters can be tuned to optimize assay performance, signal to noise, and maximize differentiation between Alzheimer's disease and other chronic progressive dementia that confound diagnosis.

Key References

1. Stieler, JT, and Arendt, T.; et al. Neuroreport 2001; **12(18):**3969-39722.
2. Stieler, J et al, 2012. Neurobio Aging **33:**234-341

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For References: