THE LYMPRO® TEST: A BIOMARKER FOR ALZHEIMER'S DISEASE USING BLOOD SAMPLES FROM CLINICALLY DIAGNOSED ALZHEIMER'S DISEASE AND COGNITIVELY INTACT SUBJECTS

Louis Kirby[1], Paul Jorgensen[1], Colin Bier[1], Paula T. Trzepacz[2], Marwan Sabbagh[3]

1. Amarantus Diagnostics 2. Indiana University School of Medicine 3. Banner Sun Health Research Institute

INTRODUCTION

The LymPro® test is a blood assay that has reported differential mitogenic activation in lymphocytes drawn from Alzheimer's disease (AD) subjects compared to healthy controls(HC) (1, 2).

The assay is based on the cell cycle reentry hypothesis for AD (3), which states that post-mitotic neurons in AD have inappropriately reentered the cell cycle with downstream overexpression of cytokines and increased neuronal cell death through apoptosis (4,5). This cell cycle dysregulation (CCD) is likely one of the earliest key pathologies in AD (6) and appears linked to tau hyperphosphorylation (4) and to APP metabolism.

Brain CCD appears to be reflected by systemic manifestations, reported as CCD measured in white blood cells by several research groups. Dr. Thomas Arendt, et al, at Leipzig University developed the specific technique (1, 2) for measuring white blood cells' expression of CD69, a cellular marker related to the cell cycle. When stimulated by a nonspecific mitogen, WBCs normally do not pass the G1/S checkpoint which increases the expression of CD69, whereas in AD WBCs abnormally pass this cell cycle checkpoint (see Fig 1).

We used this *in vitro* assay in lymphocytes and monocytes obtained from AD and HC subjects to further develop a test that might in the future be useful to increase accuracy of clinical diagnoses of AD. Having a peripheral blood-based biomarker of AD would be highly desirable.

METHODS

Subjects were diagnosed as AD by dementia experts using NIA/AA (2011) clinical criteria for the determination of probable Alzheimer's dementia.

Table 1. Demographics

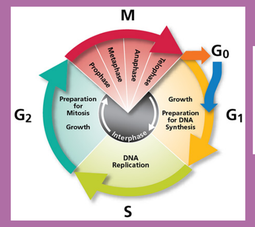
Variable	All	AD	HC	p*
N	125	59	66	
Mean Age	73.1 ± 9.6	77.2 ± 9.0	69.6 ± 7.8	<0.0001
Gender (M/F)	50/75	26/33	24/42	0.02
APOE-4 status (+/-)	44/81	28/31	16/50	0.009

*p values between AD & HC groups

Table 2. MMSE in the AD Cohort (HC was ≥28)

N	59
Mean MMSE ± SD	16.1 ± 5.5
Range	0 - 26

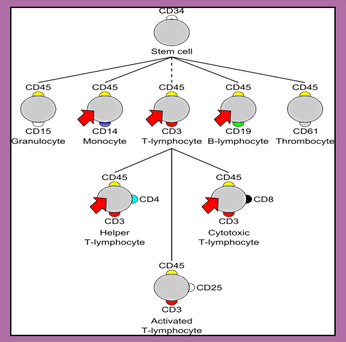
Figure 1: Review of Cell Cycle



G1 – gap phase 1
S – synthesis phase
G2 – gap phase 2
M – mitotic phase
G0 – quiescent state

Mature neurons in G0 should **not** re-enter the cell cycle (go past the G1/S checkpoint, but sometimes they do → Cell cycle dysregulation (CCD)

Figure 2: Five WBC cell subtypes analyzed



LymPro Assay Procedure: 141 whole blood samples were drawn from enrolled study participants in vacutainer tubes designed for lymphocyte culture. Samples were shipped over night, cultured with mitogen (PHA or PWM) or without in separate culture tubes, and then stained with an antibody cocktail to reveal subpopulations of lymphocytes (T, B, and monocytes) as well as expression levels of CD69 (a surface marker of cell cycle activity) cell surface expression. Lymphocyte subpopulation specific biomarkers were measured on an 8 color flow cytometer at a contract lab (Becton Dickinson). After analytical review of the flow cytometry data, N=125 of the samples passed blinded quality control (59 AD and 66 controls). Each subject's WBCs were characterized by 14 measured biomarker features (see Fig 2) in various permutations for statistical analyses (1,2), as well as two stimulation indices that were calculated to produce an additional 8 biomarker variables. Thus, results of 22 variables were analyzed statistically. These 22 were measured for each of three stimulation conditions using mitogens (see Table).

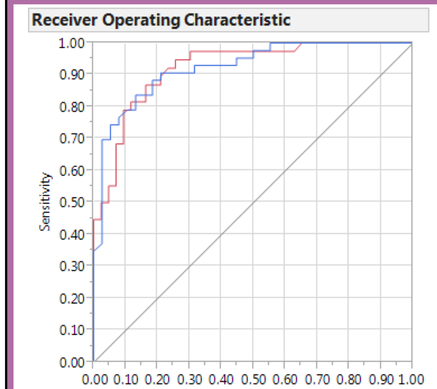
Statistical Analysis: Public domain feature selection algorithms or stepwise methods were used to identify optimal feature sets to maximize diagnostic prediction performance. These included logistic regression, discriminant analysis (linear and quadratic), and decision and random forest methods. Prediction performance was initially assessed with a 65% training set (n=81) and applied to a 35% test set (n=44), All analysis was done in JMP Pro v11.2.1 (SAS, Cary, NC).

MULTIVARIATE RESULTS

- Of the 66 variables, 5 were selected in multivariate analysis as together providing the best differentiation between groups.
- ROC graphs were produced using these 5 candidate features for training and test sets (see Figure 3) where AUC for AD and HC groups were good to very good.
- It is notable that all five candidate features were obtained from the same mitogen stimulation condition.

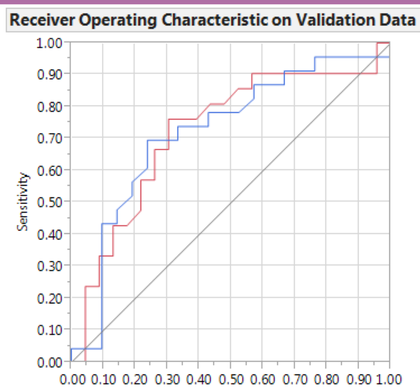
Figure 3: ROC Curves for AD & HC Groups

Training set (n=81); AD=38, HC=43



Cohort of N125-Subset-AMBS-LP202-Join-N125-PerSubject-test-8jul2015	Area
AD	0.9229
HN	0.9229

Test set (n=44); AD=21, HC=23



Cohort of N125-Subset-AMBS-LP202-Join-N125-PerSubject-test-8jul2015	Area
AD	0.7236
HN	0.7236

DISCUSSION

Findings from this expanded analysis of the Lympro test using multivariate analysis are consistent with the two prior published reports using univariate approaches. This lends further support that LymPro test may have utility as a blood biomarker reflective of AD pathology. More in-depth analysis of this cohort is underway. These preliminary findings are encouraging and warrant further studies to demonstrate the utility of this blood biomarker in the differential diagnosis of patients with cognitive impairment.

In addition, the study met its primary and secondary endpoints. Work continues on exploratory objectives.

Limitations:
The study design was predicated upon cohort categorization (AD or HC) on clinical grounds only and there were no biomarkers employed in the confirmation of clinical diagnosis.

Nonetheless, our test sample performed similarly to Beach et al's conclusion about clinical diagnosis (4) that "…when optimizing for sensitivity and specificity, the best [clinical] result was 70.9% sensitivity and 70.8% specificity."

CONCLUSIONS

1. Multivariate analysis using random forest found 5 candidate variables that together generated the best performance results in ROC analysis.
2. This preliminary algorithm holds promise as a step in the development of a bioassay algorithm that can yield both strong sensitivity and specificity.
3. The LymPro test holds promise for use in evaluating patients though further clinical validation in diverse clinical samples, in conjunction with F18-florbetapir PET, is planned.

REFERENCES

1. Stieler, J., Lederer, C., BruĔckner, M., Wolf, H., Holzer, M., Gertzand, H., Arendt, T., 21 December 2001, Impairment of mitogenic activation of peripheral blood lymphocytes in Alzheimer's disease. Neuroreport, 12 :18; 3969-3972
2. Steiler, J., Grimes, R., Weber, D., Gartner, W., Sabbagh, M., Arendt, T., 2012. Multivariate analysis of differential lymphocyte cell cycle activity in Alzheimer' s disease. Neurobiology of Aging 33: 234-341
3. Beach TG, Monsell SE, Phillips LE, et al. Accuracy of the clinical diagnosis of Alzheimer disease at National Institute on Aging Alzheimer Disease Centers, 2005-2010. J Neuropathol Exp Neurol 2012;71:266-73
4. Herrup, K The contributions of unscheduled neuronal cell cycle events to the death of neurons in AD. Front. Biosci.2012; 4:2101-9
5. Yang, Y, Mufson E., Herrup, K. 2003 Neuronal Cell Death Is Preceded by Cell Cycle Events at All Stages of AD. J Neuroscience 23 (7) 2557-2563 1 Apr 2003
6. Graphic by Lokal Profil. - http://commons.wikimedia.org/wiki/File:Cluster_of_differentiation.svg#mediaviewer/File:Cluster_of_differentiation.svg