Lymphocyte Proliferation as a Means to Assess Cell Cycle Dysregulation in Alzheimer's Disease Subjects: Clinical Performance of the LymPro Assay With 7-Year Longitudinal Diagnosis Refinement

Marwan Sabbagh, MD[1] F. Randall Grimes, MBA[2],Gerald Commissiong, MBA[2] Paul Jorgensen, MS[2], Louis Kirby, MD[2]
[1]Banner Sun Health Research Institute [2]Amarantus Bioscience Holdings Inc.

Abstract

Cell Cycle Dysfunction (CCD) has increasingly been identified as a key pathology in Alzheimer's disease (AD). This dysfunction can be identified from whole blood by purifying Peripheral Blood Lymphocytes (PBL) and stimulating them for a period of time at certain concentrations (LymPro Test ®, LymPro), reported twice by Stieler et al [1,2], . In 2008, Provista Life Sciences, Inc. (Provista) completed a preliminary set of assay optimization experiments to understand whether modifying assay stimulation parameters of time and dose (Time Dose Studies) could improve the univariate and multivariate accuracy of the assay. A total of 44 subjects were enrolled and donated whole blood (Alzheimer's, Other Dementia and Cognitively Intact Controls) for the Time Dose Studies that were performed in-house at Provista using a pre-defined matrix of parameters. Amarantus Bioscience Holdings, Inc. (Amarantus) licensed the Assay from Provista in 2012 and acquired the data underlying the Time Dose Studies in 2014. As biomarker accuracy is only as good as the original subject diagnosis, Amarantus revisited the subjects' current diagnosis through a review of updated patient chart diagnoses (Chart Review) of the subjects, yielding up to 6 years of additional clinical diagnosis data, certain post-mortem autopsy confirmation data, and clinical diagnosis change over time. Using the algorithms based upon the longitudinally more accurate Chart Review diagnosis as inputs, Amarantus re-evaluated univariate and multivariate models to assess the ability of the LymPro assay to predict AD from non-AD. Based on the results of the Time Dose Studies, Amarantus concluded that univariate and multivariate analyses, LymPro appears to have a strong potential as a biomarker for Alzheimer's.

Introduction

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. The LymPro Test ® (Lymphocyte Proliferation) is a measure of the cell surface expression of CD69 in defined subpopulations of lymphocytes as a means of quantifying the extent to which lymphocytes have entered the cell division cycle in response to a mitogenic stimulus. Previous research has shown that cell cycle abnormalities are characteristic and important in AD pathogenesis [1] and measurement of the cell cycle dysfunction was able to differentiate Alzheimer's from Cognitively intact controls (CI) in previous studies [1, 2] and may, once further characterized, be a useful means for identifying the presence or absence of Alzheimer's disease in patients.

Post mortem studies have indicated that the clinical diagnosis of Alzheimer's disease has up to 15% false diagnosis rate [3,4]. The purpose of this study is to compare the original clinical diagnosis (from 2008) with the current clinical diagnosis based on a chart review and then to compare the original (2005) LymPro Test results in light of any changes in diagnosis, disease progression, and cognitive decline as refined through the lens of time.

CELL CYCLE DYSFUNCTION IN AD

See Poster 45398 for More Detail on CCD in AD Neurons

In AD, terminally differentiated neurons aberrantly re-enter 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-b (Ab) with evidence of Ab triggering cell cycle re-entry and with increased production of amyloid. CCD has been documented to be an early phenomenon in subjects with MCI and in mouse models expressing CCD prior to much of the subsequent development of AB and ptau. (references available through QR code)

CELL CYCLE DYSFUNCTION IN PBLS

See Poster 45398 for More Detail on CCD in AD PBLs

AD likely has systemic manifestations with evidence of CCD in peripheral blood lymphocytes. Upregulation of P53 mutant-like conformation, calmodulin, cyclin E and CDK2 are all observed in AD lymphocytes. Rapamycin blocks G1-S transition in healthy but not AD lymphocytes. Reduced expression of CD69 in response to a mitogen, an indicator of lymphocyte activation, is significantly reduced in in AD compared to controls [1,2]. PBLs, being a much more accessible tissue, represents an opportunity for a blood based biomarker of a key AD pathology.

All unpublished data presented on this poster was generated by Provista Life Sciences and prior to Amarantus acquiring the assets of Provista Life Sciences

Methodology

Subjects Three original subject cohorts (AD), Other Dementia (OD) and Cognitively Intact (CI)) were recruited from Banner Sun Health Research Institute with a total n of 44: AD=15; CI=11; OD=18. The other dementia breakdown included Parkinson's disease dementia (11), Vascular dementia (5) and mild cognitive impairment (MCI) (2). For the retrospective chart review, the investigator (MS) performed the review under an IRB waiver to generate a de-identified limited dataset that included the original cohort, the 2007-8 diagnosis, and the most recent diagnosis available. Where there were biomarker or pathology data available these were used to inform the final diagnosis.

Patient inclusion rules: The objective was to select as clearly defined subjects in each of the categories as possible. Where the diagnosis was clear, the subject was assigned a diagnosis and included in the analysis. When the diagnosis was not clear, they were excluded. For example, three subjects had a designation of mild cognitive impairment and were excluded from the model. If they had pathologically determined AD plus another diagnosis (Lewy Body or vascular dementia) they were included in the AD category. The reasoning here was that the subject with AD would have the cell cycle dysfunction related to AD regardless of the concurrent pathology. See Table 1. These revised diagnoses were used to generate a scoring model.

Sample preparation: Peripheral blood mononuclear cells (PBMC) were isolated from whole heparinized blood samples as described previously [1]. Aliquots of each sample were stimulated with up to 8 µg/mL PWM and up to 20 hours incubation. Samples were then diluted and frozen prior to staining for flow cytometry.

Assay measurement: Antibody cocktails for flow cytometry included antibodies against the following: CD45, CD3, CD4, CD8, CD19, CD69, CD14 and CD28. Assay samples were analyzed using a Becton Dickinson FACSCalibur flow cytometer.

Statistical approach: The OD and CI cohorts were analyzed separately and combined into a non-AD cohort and AD formed the second cohort. Univariate statistics were run on each cohort for each lymphocyte subtype and analytes with p values below 0.05% were included in the multivariate analysis. Multivariate models were constructed by first randomly selecting subjects in each cohort for a training set and the subsequent results compared to the remaining subjects as a validation set.

Results

Of the original 44 subjects, 3 were excluded because of Mild Cognitive Impairment. Two original AD subjects were determined on post not to have AD pathology and 2 PDD subjects had pathologic evidence of AD.

Excluded from anaylysis		
Original 2007 DX	**Current 2014 DX**	**Comment**
Normal	MCI	Cognitive decline
MCI	MCI	Erroneously recruited
MCI	MCI	Erroneously recruited
Changed Cohort		
Original 2007 DX	**Current 2014 DX**	**Comment**
PDD	AD	Pathlology
PDD	AD	Pathlology
AD	VaD	Pathlology
Normal	VaD	Pathlology

MCI – Mild Cognitive Impairment; PDD – Parkinson's disease dementia; AD – Alzheimer's disease; VaD – Vascular Dementia.

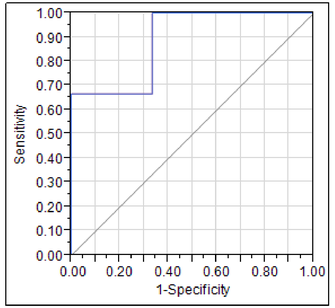
Table 1: Change in category of the listed subjects plus their reasons for change.

Change in diagnosis: 7 of 44 subjects (16%) had a diagnosis that upon chart review caused an exclusion or a change in cohort. Several subjects had additional diagnoses (AD = VaD) however, they would still be considered to have AD and therefore would stay in the AD cohort. This presumes, however, that the VaD does not cause abnormalities in CCD.

RESULTS

Summary of changes		
Group	**All Subjects**	**After Clinical Review**
AD	15	15
N	11	9
OD	18	17
Total	44	41

NON-OPTIMIZED MULTIVARIATE DATA



		Predicted			
		no	YES		
Truth	no	21	5	0.81	Spec
	YES	4	11	0.73	Sens
		0.84	0.69	78%	
		NPV	PPV		Accuracy

Figure 1. A predictive model built from the 6 Lymphocyte subgroups (CD45+, CD3+, CD4+, CD8+, CD14+, CD19+) using the CD69+ Median Fluorescence Intensity (MedFI) of each sub population in a Random Forest (built from 100 Trees) adjusted using 5-fold internal cross validation. Representative clinical performance can be seen in the truth table below.

FUTURE WORK

Ongoing work (as of July 15th, 2014) includes:

1) **Bridging trial:** A 72 subject study is underway at Becton Dickinson employing the original and enhanced assay stimulation parameters. Objectives include

a) Replicate the original data sets of Stieler et al, 2001 and Stieler et al, 2012
b) Further explore the enhanced stimulation techniques presented here to maximize differentiation in stable assay environment at Amarantus' contract laboratory, Becton Dickinson Biosciences, Inc. (BD Biosciences, www.bd.com).

2) Top line results are expected to be presented on July 31, 2014 at the #C4CT Concussion Awareness Conference at the United Nations.

3) **Analytical performance:** Analyte Performance/Validation Package suitable to support pharmaceutical clinical trials will be completed in second half of 2014

4) **Continued development:** Amarantus intends to conduct multiple robust clinical performance trials incorporating biomarker qualified AD and cognitively intact subjects, as well as multiple types of other dementias, clinically validate LymPro.

5) Collaboration: Plans to work with academia and industry to support research activities.

Summary

Because a clinical diagnosis of dementia is not uncommonly made in error, we sought to take the data from a 2008 pilot study of LymPro and increase the accuracy of the clinical diagnosis by undertaking a chart review to attempt to clarify the diagnosis in 2014.

- The chart review clarified 7 original cohort designations, resulting in excluding three and a change in cohort of 4, a change in 16%
- The increased precision of diagnosis yielded univariate accuracy less than 80% in select subpopulations of lymphocytes
- ROC Area Under the Curve (AUC) range around 80% with multivariate models
- Sufficient evidence exists to support the further evaluation of LymPro across original and optimized assay conditions
- An ongoing Bridging study will yield initial specificity and sensitity data for LymPro in original and optimized assay conditions that will be presented at the #C4CT Concussion Awareness Summit at the United Nations on July 31st, 2014 (www.c4ctsummit.com)

CONCLUSION

Optimization conditions hypothesized to improve the original LymPro assay conditions improved performance in a consistent manner across all univariate and multivariate analyses and models attempted, based on clinical diagnosis data confirmed based on chart review data retrospectively evaluated for up to 6 years, to support the further evaluation of both assay conditions in the Amarantus Bridging Study in order to determine optimal assay conditions for research and commercial development, and develop robust multivariate algorithms required for clinical diagnosis of Alzheimer's disease.

Key References

1. Stieler, JT, and Arendt, T.; et al. Neuroreport 2001; 12(18):3969-39722.
2. Stieler, J et al,. Neurobio Aging 2012 33:234-341.
3. Joachim, C. L. et al Ann Neurol. 1988, 24: 50–56. July
4. Mirra S. S.et al. Neurology April vol. 41 no. 4 479 1991

For additional information:

Mr. Kerry Segal
VP of Business Development
Amarantus Bioscience Holdings
kerry.segal@amarantus.com

PDF of this Poster:

For References: