Published in final edited form as:

Neurobiol Aging. 2012 February ; 33(2): 234–241. doi:10.1016/j.neurobiolaging.2010.03.001.

Multivariate analysis of differential lymphocyte cell cycle activity in Alzheimer's disease

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Abstract

Mounting evidence suggests cell cycle dysregulation is involved in the pathogenesis of Alzheimer's disease (AD) and that this failure is systemic, affecting not only neurons but also peripheral blood lymphocytes (PBLs). This study analyzed if differences in PBL proliferation activity could be used as a diagnostic biomarker for AD. CD69 and CD28 expressions on PBL T, B, and monocyte cells were measured by flow cytometry with and without mitogenic stimulation in healthy controls (HC), probable AD, and Parkinson's disease dementia (PDD) subjects. Univariate and multivariate scoring models were employed to evaluate the data relative to the clinical diagnoses. Eleven CD expression markers were significantly altered in AD subjects compared with a mixed pool of PDD and HC subjects using univariate models. Using multivariate models, seven CD expression markers were significantly altered in AD subjects compared with PDD subjects. Multivariate scoring demonstrated up to a 91% positive and 92% negative agreement with subject clinical diagnosis and had little correlation with the severity of dementia. Present findings suggest that with further development this analytical and multivariate modeling procedure could aid the current differential diagnosis of Alzheimer's disease.

Keywords

Biomarker; Cell cycle activation; Dementia; Diagnosis; Parkinson's disease dementia; Peripheral blood lymphocytes

The early diagnosis of Alzheimer's disease (AD) is the basis for a successful medical intervention in AD extending or maximizing the patient's quality of life (Cummings et al., 2000; DeKosky, 2003). However, the clinical differential diagnosis of AD, as outlined by the NINCDS-ADRDA work group (McKhann et al., 1984; Tierney et al., 1988), is often

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Disclosure statement

Thomas Arendt and Jens Stieler report holding a patent for the flow cytometry assay applied in this study. F. Randall Grimes, Donald Weber, and William Gartner are employees of Provista Life Sciences, LLC, a company that is developing a commercial test application based on the methodology applied in the present study.

problematic. It is time-consuming, costly, and does not always attain adequate accuracy levels, especially when detecting early stages of AD, differentiating it from other forms of dementia, or when employed at the primary care level (Andreasen and Blennow, 2005; Maccioni et al., 2004; Mayeux et al., 1998; Mendez et al., 1992; Varma et al., 1999). Accordingly, only confirmation by postmortem neuropathology is considered a definitive diagnosis of AD.

To improve the diagnostic process, a wide variety of techniques and specimen types have been the subject of investigation, including brain imaging techniques and analysis of cerebrospinal fluid (Frank et al., 2003), but the associated costs and procedural risk limit their widespread use. In contrast, venous puncture is a widely accepted low risk, low cost specimen collection procedure. Therefore, a blood-based test for AD diagnosis would be favorable, provided such a test complies with conditions for an “ideal” AD biomarker as outlined in the Consensus Report of the Working Group on: “Molecular and Biochemical Markers of Alzheimer’s Disease” (Consensus Report of the Working Group, 1998). In that regard, the collection and analysis of peripheral blood lymphocytes could potentially fulfil this unmet clinical need.

The use of peripheral blood lymphocytes as potential diagnostic tool for AD stems from the cell cycle hypothesis of AD, the known connections between the nervous and immune systems, and prior study data. According to the cell cycle hypothesis, AD pathogenesis is triggered by genomic instability and cell cycle re-entry of postmitotic neurons that leads to apoptotic cell death (Arendt et al., 1996; Busser et al., 1998; Nagy et al., 1997; Vincent et al., 1996).

It appears that this dysregulation is not restricted to the brain, but rather represents a systemic aspect of the disease that might also be relevant to peripheral cells such as lymphocytes and fibroblasts (Emiliani et al., 2003; Hirashima et al., 1996; Ikeda et al., 1991; Inestrosa et al., 1994; Shalit et al., 1995; Stieler et al., 2001; Zhang et al., 2003). The nervous system and the immune system are connected by a complex bidirectional communication (Payan et al., 1986). There exists a close association between the state of the immune system, in lymphocytes particularly, and major psychiatric disorders such as depression, stress, anxiety, schizophrenia, and AD (Gladkevich et al., 2004). Several factors, including neuroinflammatory cytokines, that link the peripheral immune system and nervous system (Reale et al., 2009) can trigger aberrant neuronal cell cycle activation in Alzheimer’s disease (Arendt, 2008). While the clinical phenotype of AD mainly arises from the neuronal manifestation of the disease, there is no doubt that in addition, there exists a peripheral aspect of the disease that, so far, had been ill-defined.

Several AD-related cellular and molecular changes have been observed on lymphocytes and fibroblasts, most notably, altered levels of circulating cytokines or cytokines produced by peripheral blood mononuclear cells following immune stimulation (Bossù et al., 2008; Kassner et al., 2008; Ray et al., 2007; Reale et al., 2008). Further, peripheral lymphocytes show telomere shortening (Honig et al., 2006; Jenkins et al., 2006; Panossian et al., 2003; Zhang et al., 2003) and altered expression of genes involved in apoptosis and DNA-repair (Gatta et al., 2009; Maes et al., 2007). These findings suggest that disease-related alterations

of lymphocytes may explain deficiencies of the immune response of AD patients (Ikeda et al., 1991; Kusdra et al., 2000; Lombardi et al., 1999; Pirttila et al., 1992; Stieler et al., 2001; Tan et al., 2002) which is a constant and very early phenomenon of AD that becomes detectable at preclinical stages of the disease (Bonotis et al., 2008; Larbi et al., 2009; Magaki et al., 2008).

The present cross sectional study was conducted to confirm and expand on a prior study of cell cycle dysregulation that simultaneously measured the cell cycle activation status of PBL subtypes in AD and Control subjects (Stieler et al., 2001). That study found that AD subjects' PBL response to mitogenic stimulation was significantly suppressed compared with that of age matched healthy controls. The present study expands on those findings by including subjects with PDD, correlating univariate and multivariate results to the subjects' clinical diagnosis, determining the multivariate scores' correlation with severity of dementia, and investigating confounding factors that affect immune system response.

1. Methods

1.1. Participants

Written informed consent was obtained from 92 subjects or their legal representatives, with study approval from IRC institutional review board. Four subjects were excluded from the study because they did not meet the eligibility criteria that excluded subjects with autoimmune disease, suspected comorbidity with other dementia, or other significant medical problems. Of the 88 subjects that were included, 32 had a clinical diagnosis of probable AD, 26 were demented and diagnosed with PDD, and 30 were classified as HC. Clinical diagnosis of AD was made according to NINCDS-ADRDA (McKhann et al., 1984; Tierney et al., 1988) or DSM-IV criteria (Diagnostic and Statistical Manual of Mental Disorders, American Psychiatric Association). PDD was defined as meeting Huges criteria and DSM-IV criteria for dementia. All evaluations were conducted without knowledge of PBL's response to a mitogenic stimulation.

1.2. Assay methods

Blood samples were taken by venous puncture into 10-mL Collection Preparation Tubes (Naheparin, BD Biosciences) at five clinical sites across the USA. Samples were shipped overnight at room temperature to BD Biosciences Pharmingen's Custom Technology Laboratory (La Jolla, CA), which performed all blood cell preparation within 24 h after collection, along with all subsequent cell stimulations, staining, and flow cytometry analysis.

Peripheral blood mononuclear cells (PBMC) were isolated as described in the product guidelines for CPT tubes and suspended in complete media (RPMI-10% low IgG FBS, BD Pharmingen) at 2.5×10^6 cells/mL. Isolated cells were stimulated with Phytohaemagglutinin (PHA-L, 12 μ g/mL, Sigma Aldrich) and pokeweed mitogen (PWM, 4 μ g/mL, Sigma Aldrich). The nonstimulated reference sample contained an equal volume of phosphate-buffered saline. Samples were incubated at 37 °C with 7% CO₂ for 4 h, subsequently mixed with 5 mL of FACS-Lysing solution (BD Biosciences), immediately frozen and stored at

–70 °C. Staining of cells was performed in batches after thawing of samples with the following custom antibody-cocktails (custom reagents, BD Biosciences Pharmingen):

- A – IgG1-FITC/IgG1-PE/CD45-PerCP-Cy5.5/IgG1-APC,
- B – CD8-FITC/CD69-PE/CD3-PerCP-Cy5.5/CD4-APC,
- C – CD14-FITC/CD69-PE/CD45-PerCP-Cy5.5/CD19-APC,
- D – CD8-FITC/CD28-PE/CD45-PerCP-Cy5.5/CD4-APC,
- E – CD45RA-FITC/CD45RO-PE/CD3-PerCP-Cy5.5/CD4-APC.

This antibody cocktail built upon that used in the prior study but included CD28 and was reformulated to provide maximum sensitivity to markers of activation (e.g., CD69 and CD28 on PE channel). Reference samples (nonstimulated) were prepared with all antibody cocktails whereas the stimulated samples were probed with antibody-cocktails B, C, and D only. The samples were stored at 2–8 °C for a maximum time of 8 h before the flow cytometer analysis. Samples were analyzed using a calibrated Becton Dickinson FACS Calibur flow cytometer and CellQuest Pro™ software for data acquisition. A total of 1×10^4 gated events were collected for each data file. Blinded raw data analysis was also performed using CellQuest Pro. All sample preparations and flow cytometry were done without knowledge of the subject's cognitive status.

1.3. Data analysis and statistical evaluation

All flow cytometry data were first self normalized, where each subject's mitogenic response data were adjusted to account for his or her basal mitogenic activity as determined by their corresponding nonstimulated reference sample. This process involved the calculation of two different Stimulation Indices (SI) that accounted for the nonstimulated reference samples' basal activity in slightly differing manners. SI 1 was defined by the ratio of the percentages of either CD69 or CD28 positive cells with and without mitogenic stimulation. SI 2 was defined by the ratio of the mean CD69 or CD28 expression of the analyzed cell population with and without mitogenic stimulation.

$$SI1 = \frac{(CDx + /CDxt)_{stim}}{(CDx + /CDxt)_{unstim}} \text{ and } SI2 = \frac{(CDx)_{stim}}{(CDx)_{unstim}}$$

Student's *t*-test and Mann–Whitney *U* tests were employed to determine if the continuous or discrete markers were significantly different between AD subjects and PDD subjects, HC subjects, or between AD subject and all subjects in the mixed non-AD group, respectively. Markers with a *p*-value < 0.05 were considered statistically significant. A multi-iterative regression analysis was performed using the clinical diagnosis as the dependent variable. Statistically significant markers were used as continuous independent variables and markers with an identified cut-point were used as discrete independent variables. This regression was continued until the adjusted *R*² was maximized and all remaining variables had *p*-value < 0.05. Several alternate scoring models were generated using datasets limited to the data resulting from PHA- or PWM-stimulation only. As with the full dataset modeling, the same multi-iterative regression analyses and statistically significant criteria were applied.

The impact that individual subject demographic and life-style characteristics had on measures of mitogenic response was also investigated. Regressions were conducted using the simulation index as the dependant variable and all characteristic information as either continuous (such as age, BMI, etc.) or discrete (such as asthma, allergies, or smoking) independent variables. This regression was continued until the adjusted R^2 was maximized and all remaining variables had p -value < 0.05 .

2. Results

2.1. Characterization of the subjects

All subjects were well-matched for age, gender, severity of dementia, and most other characteristics. Subjects had a mean age of 73.4 (8.9 SD) for AD subjects, 75.3 (10.4 SD) for PDD subjects, and 69.9 (13.4 SD) for healthy controls. The severity of dementia was broad and well-matched between AD and PDD subjects with mean MMSE scores of 18.0 (6.1 SD) and 17.3 (7.1 SD) for AD and PDD subjects, respectively. AD subjects had a range of MMSE scores from 7–26, with 26%, 58%, and 16% of subjects being characterized as mild, moderate, or severe dementia, while PDD subjects had a range of MMSE scores from 10–25, with 11%, 89%, and 5% of subjects being characterized as mild, moderate, or severe dementia, respectively. Several statistically significant differences were found among the cohorts. Most significant were higher rates of depression ($p = 0.004$) and arthritis ($p = 5 \times 10^{-5}$) among PDD subjects, and alcohol use among HC ($p = 0.008$), as shown in Table 1.

2.2. Statistically significant markers

The self-normalized mitogenic response, as measured by CD69 expression, of helper T Cells (CD4), cytotoxic T cells (CD8), myelomonocytic cells (CD14), and B cells (CD19) were found to be statistically different ($p < 0.05$) in AD patients compared with either PDD subjects (Table 2) or a pool of mixed non-AD subjects (Table 3). Compared with either PDD or HC subjects, the mitogenic response of AD subjects was depressed.

2.3. Univariate differentiation

Figure 1 shows a box-and-whiskers plot of self normalized B cell mitogenic responses indicating both the differentiation and overlap in the AD, PDD, and HC datasets. This is also observed in Figure 2, the associated cumulative frequency histogram. Based on cumulative frequencies, single marker differentiation between AD and either PDD or HC ranged from 19.05% to 33.8% in statistically significant markers.

2.4. Multivariate model differentiation

Multivariate models based on the self normalized data were able to differentiate between AD subjects and PDD subjects with a 91% positive agreement and a 92% negative agreement with the clinical diagnosis, as shown in Figure 3, the corresponding scoring model histogram. Multivariate models were also able to distinguish between AD subjects and mixed non-AD subjects with a 78% positive agreement and 91% negative agreement with the clinical diagnosis (not shown). Single stimulant scoring models based on either PHA- or PWM-stimulation data achieved comparable results.

Receiver Operator Characteristic (ROC) curve for the multivariate model was constructed relative to the clinical determinations of AD and PDD (Fig. 4) yielding an area under the ROC curve (AUROC) of 0.965. Other derivations of scoring models, such as those constructed with a single mitogenic stimulant or those for differentiating AD from a mixed pool of subjects yielded AUROC values from 0.988 to 0.905 (not shown).

2.5. Multivariate score correlation with MMSE

Each AD and PDD subject's multivariate scores were plotted against their MMSE scores and fit using the least squares method. As can be seen in Figure 5, the AD subjects' multivariate scores were only weakly correlated with the severity of dementia ($r^2 = 0.12$), and the PDD subjects' multivariate scores had essentially no correlation ($r^2 = 0.03$) with the severity of dementia.

2.6. Confounding factors

Several subject-specific factors were found to have statistically significant correlations with the measures of mitogenic response. The broadest impact was found with age and gender differences, as shown in Table 4.

Given these relationships, models were constructed with a normalized dataset that removed attributable intrasubject variability. This model was statistical equivalent to the self normalized models, producing an AUROC of 0.954.

3. Discussion

The present study replicated and expanded upon work (Stieler et al., 2001) that suggested that a profile of PBLs stimulated with mitogenic compounds will correlate with the clinical diagnosis of AD. As in the previous study, the expression of cell cycle activity antigen CD69 was measured after mitogenic stimulation on CD3, CD4, CD8, CD14, CD19, and CD45 positive cells and then "self normalized" against a matching nonstimulated reference sample. As previously found, the self normalized CD69 expression was significantly higher on CD3, CD4, CD8, and CD19 in control subjects than in AD subjects. In the present study, this finding was replicated and extended to PDD subjects, finding that expression was likewise significantly higher than in AD subjects. The examination of CD28 expression on CD4, CD8, and CD45 positive cells was somewhat less successful, only finding one parameter that was significantly different between AD and PDD subjects. Univariate scoring of significant markers showed a moderate discrimination between AD and either PDD or HC subjects ranging from 19.05% to 33.8%. In contrast, multivariate scoring models of these markers achieved over 90% discrimination, with a 91% copositive agreement with AD diagnosis and a 92% conegative agreement with PDD diagnosis with little or no correlation with the subjects MMSE scores. Combined, these findings move a multivariate peripheral blood lymphocyte AD diagnostic test several steps closer to use in clinical practice.

This study's results largely replicated the original findings (Stieler et al., 2001). That is, AD subjects' peripheral blood lymphocytes were found to have significantly less CD69 expression following mitogenic stimulation than age matched cognitively intact controls. In both studies, the AD subjects' suppressed response was most evident in CD4 and CD19

positive cells. Some differences in the absolute value were noted, however. The current study yielded mean CD4 stimulation index values of 8.82 and 12.13 for AD and control subjects, respectively, following PHA stimulation compared with 2.57 and 3.55 for AD and control subjects found in the previous study. While these differences are significant, they appear to be due to changes in flow cytometry gating protocol.

The findings for PDD subjects largely mirrored those found in age matched healthy controls, with PDD subjects showing slightly better differentiation from AD. In univariate models, the observed differentiation between AD subjects and PDD or HC was moderate (19–33.8%) with limited clinical utility as individual markers. In contrast, multivariate models of AD vs. PDD achieved very high correlations with the clinical diagnosis and yielded an AUROC exceeding 95%. Multivariate models of AD vs. mixed non-AD subjects performed nearly as well with an AUROC of 0.905. Efforts to rationalize the test to a single stimulant produced equivocal results. There was variation in markers of significance, with PHA achieving slightly better differentiation within the T Cell population and PWM achieving slightly better results in the monocyte population. However, in aggregate, both PWM and PHA stimulants produced useful results with statistically equivalent discrimination power. With either PHA or PWM stimulation, nearly all of this differentiation was attributable to measurements of CD69 expression, as shown on Tables 2 and 3. The addition of CD28 expression profiles had only a minor impact on study results, with only one CD28 measurement achieving statistical significance.

The lack of correlation between the multivariate scores and the subjects' MMSE scores found here is highly encouraging for two reasons. First, should the cell cycle theory hold, measures of cell cycle dysfunction should be reflective of the disease process and not of the accumulated loss of neuronal networks. As such, it should not be correlated with measures such as the MMSE. Second, it begins to address the potential for use early in the diagnostic process. As seen in Figure 5, the trend lines for the mean AD and PDD scores maintain considerable separation even when they are extended to nondemented MMSE scores. While not conclusive, the lack of correlation and continued separation are suggestive of potential in MCI or asymptomatic subjects.

The finding that a subject's characteristics affect individual response to mitogenic stimulation is not surprising, as age, gender, and other conditions are well documented in the literature as affecting immune system response. However, it was somewhat surprising that removing this attributable intrasubject variability did little to improve the correlation with the disease state. Models that corrected for these factors produced nearly identical AUROC as unadjusted models. This could be due to inaccurate estimates of normalization coefficients that are inherent in small sample set.

While the confirmation of prior results and new findings are significant, they also highlight that further studies are needed to address remaining technical and clinical uncertainties. From a technical point of view, the fact that 15% of potential markers achieved statistical significance (or 3-fold the expected Type I error), and that two demonstrated p -values of 10^{-5} , indicates that over fitting is unlikely to explain most of these findings. Still, the lack of a cross validation data due to the study's moderate size ($n = 92$) indicates the need for a

second validation study with a larger population to confirm the results. Similarly, the shift in absolute values found between studies indicates the need for consistent flow cytometry protocol so that results can be compared across studies, equipment, and operators.

In terms of potential clinical use, the differentiation observed between PDD from AD is also encouraging, but highlights the need to broaden the scope of other neurological disorders. It also indicates the need to clearly define the intended use population as either differentiating AD from other chronic progressive dementias or screening for AD in an asymptomatic population. Additionally, the finding that subject characteristics, other than the disease state, are correlated with mitogenic response indicates the need for continued investigation, as such characteristics may predictably increase intrasubject variability and thus reduce AD differentiation.

While the multivariate model's high level of agreement with the clinical diagnosis (~92%) and high AUROC (~0.95) indicate that a test based on this model could become an additional AD diagnostic tool, caution must be used as the accuracy of these results are limited by clinical diagnostic uncertainty, even when subjects are enrolled by specialists in neurodegenerative diseases. Assuming that a 90% accurate clinical diagnosis was achieved at these specialty sites, the multivariate model's true accuracy has been estimated to be between 82% and 98% by considering the 8% discrepant results between the model and clinical diagnosis (i.e., $90 \pm 8\%$ disagreement). While this is only an estimate, it is interesting to note that at the low end of estimated performance, 82%, the model appears to be performing with an accuracy that is substantially equivalent to general neurological sites and at the high end (98%) substantially equivalent to neuropathology. Because these are only estimates, further studies are needed that compare the multivariate model's results to neuropathological findings.

Acknowledgements

The authors are indebted to the patients and their families for their participation. This study was supported by the NIH grant (1R43AG024719-01). Founding was also provided for this study by Provista Life Sciences, LLC (GW Medical Technologies). Provista Life Sciences, LLC (GW Medical Technologies) was involved in study design, analysis, and interpretation of data and writing of the manuscript.

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Fig. 1. Box-and-Whiskers Plot of self normalized CD69 expression of B cells (CD19) in response to PWM mitogenic stimulation. Response to mitogenic stimulation was suppressed in AD subjects compared with either PDD or nondemented subjects.

Fig. 2.

Cumulative Frequency diagram of self normalized B-Cell response (CD19) to PWM mitogenic stimulation with maximum differentiation of 29.8% at a stimulation index (SI 2) 4.05. Also note that the AD cumulative frequency line shift to the left indicates reduced mitogenic response in the AD population compared with either the PDD or nondemented subjects.

Fig. 3. Multivariate Scoring Model for differentiating AD and PDD. Mean scores of 0.16 and 0.84 were obtained for PDD and AD subjects, respectively.

Fig. 4. ROC curve for Multivariate Model for differentiating AD from PDD. Area under the ROC curve was 0.96 with peak co-AD agreement of 91% and PDD agreement of 92%.

Fig. 5.

Dot plot showing individual subject multivariate scores and corresponding MMSE scores.

Trend line shows least squares fit. Little correlation exists between AD subject's MMSEs

and multivariate scores ($r^2 = 0.117$) and almost none exists between PDD subjects' MMSEs

and multivariate scores ($r^2 = 0.029$). Group mean scores are marked by arrows.

Table 1

Selected subject demographic characteristics

	AD	PDD	HC	<i>p</i> -value
n, total	32	26	30	NA
n, male	16	13	17	0.84
n, female	16	13	13	0.84
Age, mean (SD)	73.4 (8.9)	75.3	69.9 (13.4)	0.18
BMI, mean (SD)	25.92 (6.3)	26.9 (8.2)	27.18 (4.7)	0.73
MMSE, mean (SD)	18.1 (6.1)	17.3 (7.1)	29.2 (0.6)	0.62
Depression	13%	38%	7%	0.004
Seasonal allergies	16%	12%	23%	0.49
Alcohol use	22%	12%	47%	0.008
Arthritis	31%	85%	40%	5 E-05

Subjects are well-matched for age, gender, and other demographic characteristics with the notable exception of higher alcohol use among nondemented subjects and higher prevalence of arthritis in PDD subjects. AD and PDD subjects were well-matched for severity and broadly distributed among mild, moderate and severe dementia (ranging from MMSE of 7–25).

Table 2

Significant markers differentiating AD from PDD

Cell marker	Activation marker	Stimulant	Measure	<i>p</i> -value
CD4	CD69	PWM	SI 2	0.028
CD8	CD69	PWM	SI 1	0.005
	CD28	None	Ratio	0.041
CD14	CD69	PWM	SI 2	0.016
CD19	CD69	PWM	SI 1	0.005
		PWM	SI 2	0.009
		PHA	SI 2	0.025

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. The additional measurements of CD28 markers only yielded a single marker of significance.

Table 3

Significant markers differentiating AD from mixed subject pool

Cell marker	Activation marker	Stimulant	Measure	p-value
CD4	CD69	PHA	SI 1	0.023
CD8	CD69	PWM	SI 1	0.018
		PHA	SI 2	0.009
CD14	CD69	PWM	SI 1	0.03
		PWM	SI 2	0.004
		PHA	SI 1	0.013
		PHA	SI 2	0.018
CD19	CD69	PWM	SI 1	0.002
		PWM	SI 2	0.007
		PHA	SI 1	0.009
		PHA	SI 2	0.008

Markers differentiating AD from Mixed Non-AD at $p < 0.05$ significance (two-tailed). All markers that proved statistically significant in differentiating AD from a mixed pool of non AD subjects measured CD69 expression.

Table 4

Sources of intrasubject variability

Cell	Activation marker	Stimulant	Significant factor	<i>p</i> -value
CD4	CD69	PWM	Gender	0.027
			Depression	0.047
		PHA	Gender	0.008
			Age	0.009
	CD28	None	Seasonal allergies	0.007
		PHA	BMI	0.044
CD8	CD28	None	Age	0.022
		PHA	Age	0.007
		PWM	Age	0.03
CD14	CD69	PHA	Age	0.037
CD19	CD69	PHA	Light alcohol	0.042

Potential sources of intrasubject variability in mitogenic response based on two-tailed *t*-test $p < 0.05$. Age and gender were the most common differences in response within a clinical cohort.