Am J Neurodegener Dis 2013;2(1):40-45
www.AJND.us /ISSN:2165-591X/AJND1301003

Original Article

Multiplex assessment of a panel of 16 serum molecules for the differential diagnosis of Alzheimer's disease

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Received January 15, 2013; Accepted February 5, 2013; Epub March 8, 2013; Published March 18, 2013

Abstract: One of the current challenge in Alzheimer's disease (AD) is the identification of reliable biomarkers that might improve diagnostic accuracy, possibly correlating with the disease progression and patient's response to therapy. As the clinically validated AD biomarkers evaluate cerebrospinal fluid (CSF) parameters, the need for less invasive diagnostic markers is well evident. To this respect, blood circulating cytokines or growth factors have provided some encouraging results, even though no clinically validated to date. In 2007 Ray et al suggested a panel of 18 circulating molecules that might increase AD diagnostic accuracy. In an attempt of replicating their data, we designed a multiplex fluorimetric assay comprising 16 independent analytes and covering 15 out of the 18 described proteins. We collected serum samples from three diagnostic groups: probable AD (*n*=33), matched healthy controls (CNT, *n*=23) and non AD demented (NAD, *n*=14). After correction for age, we found an increased level of EGF-1 in AD in comparison to CNT and NAD, while an increase of TRAIL-R4 was found in NAD. However, evaluation of specificity/sensitivity by ROC curve analysis gave weak evidence of diagnostic accuracy (area under the curve = 0.63 and 0.66 for EGF and TRAIL-R4, respectively). Finally, we tried to find a diagnostic classifier by a multivariate algorithm. We found indication of diagnostic evidence for AD only, while NAD samples did not show a diagnostic pattern.

Keywords: Alzheimer's disease, diagnosis, peripheral biomarkers, multiplex analysis, EGF-1, multivariate classifier, machine learning, artificial neural networks

Introduction

Alzheimer's disease (AD) is a prevalent type of dementia whose social and sanitary burden is expected to raise in the next future due to general population aging [1]. No disease-modifying therapy is currently available, and a second important limitation is the lack of reliable and non invasive biomarkers of the pathology [2]. Currently, AD is diagnosed by a multitasking process involving exclusion of other possible cause of dementia, neuropsychological tests, imaging and cerebrospinal fluid (CSF) assessment of the proteins amyloid-beta 42 (Aβ42), MAPT (tau) and phospho-MAPT [2-4]. However, the latter biomarkers, though clinically validated, are not routinely used and are not ideal in a perspective of mass-screening to find early AD alteration in the general population. To this respect, blood-derived circulating biomarkers would be a better solution, but to date, despite extensive research in the field and some promising data on plasma Aβ42, no clinical validation has been proposed [5-7]. One of the possible explanation of the failure of single marker approach is that at peripheral level the clinical picture is much more variable and concurrent pathologies or medications might act as confounding factors. Also for this reason, a multiplexing approach evaluating at the same time a

panel of different circulating molecules has the potential to aid in finding an AD diagnostic signature [8-10]. This was done with promising results by Ray et al, who starting from the assessment of a panel of 18 plasma circulating molecules developed an algorithm discriminating AD from controls and even mild cognitive impairment (MCI) subjects that had converted to AD from MCI without conversion [11]. In an attempt of replicating this panel and shifting the analysis to serum as biologic material, we measured with a multiplex technology 16 circulating factors covering 15 of the 18 already described molecules in probable AD patients, non AD demented and cognitively healthy controls.

Materials and methods

Patients recruitment

Patients were consecutively enrolled by the Neurology Unit at the clinical center "Multimedica" (Castellanza, Varese, Italy). They were classified as probable AD (*n*=33, mean age at inclusion ± standard deviation: 76.8 ± 7.5 years; female percentage: 0.69) according to the NINCS-ADRA international criteria [12]. Non AD demented (*n*=13, mean age at inclusion ± standard deviation: 78.9 ± 4.4 years; female percentage: 0.69) were diagnosed with a different type of dementia [vascular dementia (*n*=4), frontotemporal dementia (*n*=4), corticobasal degeneration (*n*=1), Lewy bodies disease (*n*=1), progressive supranuclear palsy (*n*=1), unspecified non AD dementia (*n*=2)]. Healthy controls (*n*=24, mean age at inclusion ± standard deviation: 74.7± 8.6 years; female percentage: 0.55) were not cognitively affected as clinically ascertained at recruitment. Experiments involving human subjects were in accordance to Declaration of Helsinki and approved by local ethical committees.

Biologic material preparation and assay description

Fasting blood was collected by venipuncture, divided into 5 aliquots of 1.5 mL and stored at room temperature for 15 minutes to allow coagulation. Each aliquot was then centrifuged for 10 minutes at 4000 rpm and the supernatant (serum fraction) was transferred into a new 1.5 mL tube and immediately frozen. Serum was stored at -80°C with constant temperature monitoring until required.

One aliquot of serum (25 μL) was used for a custom-made slide-based multiplex assay (Ray Biotech Inc, Norcross, GA, USA) of a panel of molecules including: Angiopoietin-2 (ANG2); epidermal growth factor (EGF); granulocyte colony stimulating factor (G-CSF); growth regulated oncogene-alpha (GRO); intercellular adhesion molecule-1 (ICAM-1); insulin-like growth factor binding protein-6 (IGFBP-6); interleukin-1 alpha (IL-1α); interleukin-8 (IL-8); interleukin-11 (IL-11); monocyte chemotactic protein-3 (MCP-3); macrophage colony stimulating factor (M-CSF); macrophage inflammatory protein 1delta (MIP-1δ); pulmonary-activation-regulated-chemokine (PARC); platelet-derived growth factor-BB (PDGF-BB), regulated and normal T cell expressed (RANTES); TNF-related apoptosis-inducing ligand receptor 4 (TRAIL R4).

Briefly, using a multiplexed sandwich ELISA-based technology, samples (arrayed in quadruplicate), after a room temperature blocking step were incubated overnight to get the target cytokine trapped on the glass surface. The array was washed on an orbital shaker, incubated with a primary antibodies cocktail for 2 hours and subsequently with a fluorogenic secondary mix for one hour. Finally, it was gently centrifuged in a 50 mL conical tube to completely dry the surface. Then, the slide was placed in a high resolution scanner (Agilent Technologies, USA) for fluorescence quantification. Raw fluorescence spots were quantified using a macro implemented in ImageJ (www.nih.gov/ImageJ). Fluorescence values were also converted into a graphical representation by association of the corrected fluorescence level to an arbitrary colorimetric scale. Fluorescence values corrected for background were converted to molar concentrations thanks to standard curve (linear or logaritmic depending on the best fit for each molecule). Only standard curves with r^2>0.9 were considered.

Classifier development

A multilayer feed-forward artificial neural network (ANN) and a naïve bayes (NB) classifier were employed for the classification of the cases [13, 14]. For training and validation purposes, the available data was divided into two subsets; the 80% of the data was used for

Table 1. Single variable assay for the panel of circulating molecules assessed in serum of patients

PROTEIN (alias)	CONTROL (*n=23*) mean ± SE (pg/mL)	NAD (*n=14*) mean ± SE (pg/mL)	AD (*n=33*) mean ± SE (pg/mL)
ANG-2	1433.8 ± 284.5	2243.9 ± 622.3	1612.5 ± 112.5
EGF	52.7 ± 13.3	28.5 ± 7.9	75.9 ± 13.2*
G-CSF	98.9 ± 45.6	161.4 ± 44.8	92.4 ± 16.1
GRO	3340.9 ± 543.8	2984.5 ± 827.7	3314.2 ± 576.9
ICAM-1	78215.7 ± 14139.9	125879.5 ± 34912.7	135021.8 ± 23504.3
IGFBP-6	27234.0 ± 2751.1	27026.0 ± 7495.9	25773.2 ± 4486.5
IL-1A	7.2 ± 4.3	7.9 ± 2.2	22.4 ± 3.9
CXCL8 (IL-8)	81.7 ± 13.8	88.3 ± 24.5	96.7 ± 16.8
IL-11	1000.3 ± 265.3	1175.2 ± 325.9	683.4 ± 118.9
CCL7 (MCP-3)	1505.1 ± 541.4	3748.5 ± 1039.6	1650.9 ± 287.4
M-CSF	143.7 ± 55.6	245.3 ± 68.0	114.1 ± 19.9
CCL15 (MIP-1delta)	2366.0 ± 157.3	2283.3 ± 633.3	2076.5 ± 361.5
CCL18 (PAR-C)	23324.3 ± 2715.1	23885.9 ± 6624.8	17197.1 ± 2993.6
PDGF-BB	4108.4 ± 143.3	4126.6 ± 1144.5	4048.3 ± 704.7
CCL5 (RANTES)	27178.5 ± 1857.3	24695.9 ± 6849.4	24582.3 ± 4279.2
TRAIL R4	136.9 ± 57.1	1248.4 ± 37.9**	217.7 ± 37.9

*ANCOVA p-value: 0.013; Kruskal-Wallis p-value: 0.0067. **ANCOVA p-value: 0.02; Kruskal-Wallis p-value: 0.045. CCL, chemokine that contains a C-C motif; CXCL, chemokine that contains a C-X-C motif; SE: standard error; AD: Alzheimer's disease; NAD: non AD dementia.

training (training set, including 27 AD, 11 NAD and 18 CNT) and the 20% of the data was used for validation (testing set, including 6 AD, 3 NAD and 5 CNT).

As far as the ANN is concerned, the Back Propagation (BP) algorithm was used for the learning process, while the mean squared normalized error (mse) was used as the network performance function. The input layer of the ANN consists of 16 nodes, one for each of the variables. The output layer contains 3 neurons, one for each class of the dataset. In order to identify the optimal topology of the network, we trained and tested 289 ANNs with two hidden layers and hidden neurons from 5 to 22 for each layer. All neurons in the hidden and the output layers of the networks use a sigmoidal activation function. During this procedure, the learning rate and the momentum of the BP algorithm were set equal to 0.01 and 0.9, respectively.

Statistics

Univariate analysis was conducted by ANOVA and multivariate with ANCOVA considering age as covariate. In case of non normal distribution, associations were tested with non parametric Kruskall-Wallis test. ROC curve analysis was performed by a free online resource (http://vassarstats.net/).

Results

Multiplex assessment of serum molecules level

The quantification of the panel of 16 serum circulating molecules in the three different groups is summarized in **Table 1**. After performing ANCOVA analysis correcting for age, almost all the assessed analytes did not change among groups, with the exception of EGF and TRAIL-R4. EGF serum level resulted higher in AD than CNT and NAD, the latter having the lowest level of the growth factor. As for TRAIL-R4, its level was considerably higher in NAD in comparison to the other groups, where the assessed mean level was comparable.

A color-based representation of the single variable measured in each subject is shown in **Figure 1A**. Fluorescence signal intensity, corrected for background, was transformed into a colored square, according to a reference scale.

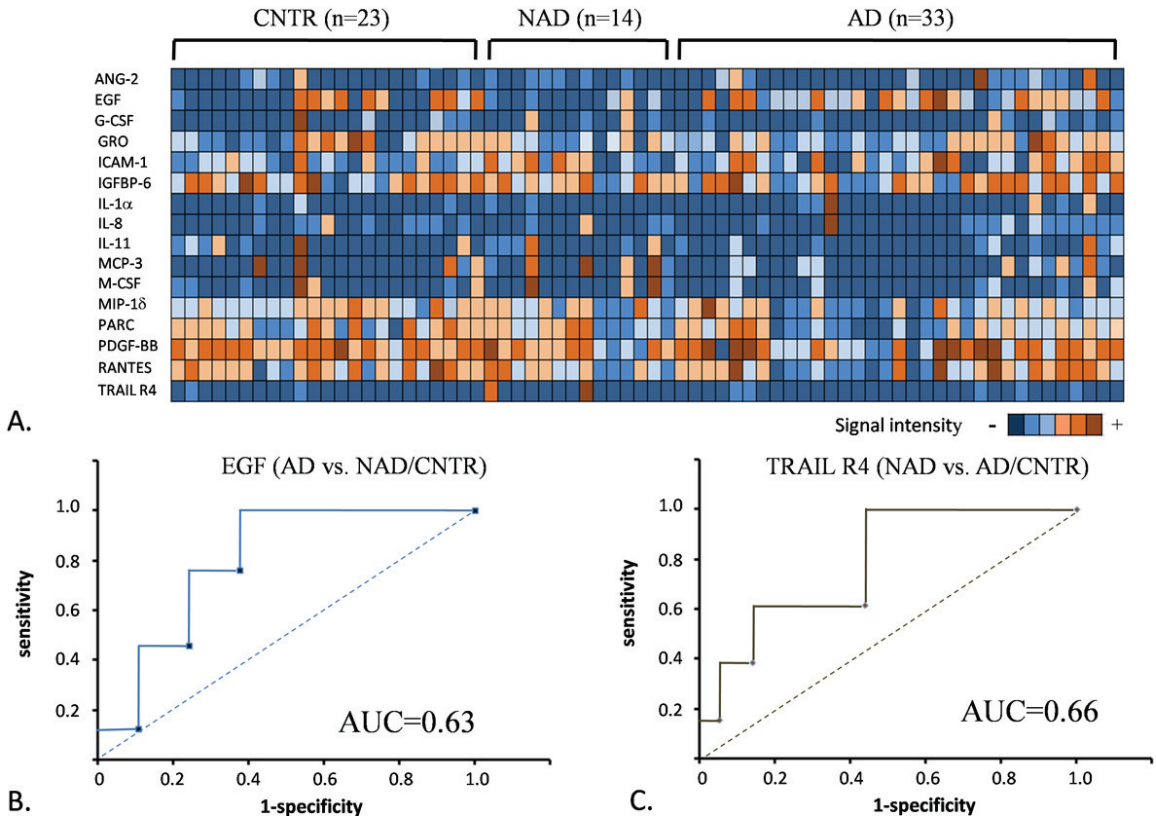

Figure 1. Multiparametric assessment of serum proteins for the differential diagnosis of Alzheimer's disease (AD). A. The circulating proteins listed in Table 1 were simultaneously measured using a glass slide-based protein array as described in Methods. The resulting fluorescence signals were converted to a color code reflecting signal intensity after background subtraction. Vertical columns are single patients, horizontal rows are panel proteins as identified by the labels on the left. B, C. Receiver operating characteristic (ROC) analysis for EGF and TRAIL-R4. The plotted curves were calculated hypothesizing a diagnostic value for Alzheimer's disease (AD) in the case of EGF and for non-AD dementia (NAD) for TRAIL-R4. The reported area-under-the-curve (AUC) value is suggestive of modest diagnostic usefulness (for the null hypothesis: AUC =0.50). AD: Alzheimer's disease; NAD: non AD dementia; CNTR: cognitively healthy control.

This representation highlights the increased level of EGF in AD subjects, while TRAIL-R4 mean increase in NAD was mainly due to the contribution of a limited number of cases.

We have then plotted the specificity/sensitivity graph and run a ROC analysis for EGF and TRAIL-R4 [**Figure 1B** and **1C**]. EGF ROC analysis was conducted for discriminating AD from NAD/CNT, while for TRAIL-R4 the hypothesis was to differentiate NAD from the other groups. The resulting curves were similar for the calculated area under the curve (AUC), that was <0.70 in both cases.

Multivariate analysis and classifier elaboration

Based on the experimental results, the optimal artificial neural network (ANN) was found to be a network with 15 neurons on the first hidden layer and 13 neurons on the second.

The performance of the classifiers was computed on the testing set (holdout validation). The confusion matrices obtained through holdout validations are given in **Table 2**.

Performance rates of the classifier ANN in predicting AD dementia regarding the patients of the testing set were as follows: sensitivity 83.3%, specificity 62.5% and accuracy 71.4%. The same values for NB were: 83.3% sensitivity, 87.5% specificity and 85.7% accuracy.

Discussion

Differential diagnosis of AD is still complex and in some cases not confirmed at autopsy [15].

Table 2. Confusion matrix of the ANN and NB classifiers based on holdout validation

1) ANN classifier				
		ANN predictions		
		Negative	NAD	AD
Diagnose	Control (*n*=5)	4	0	1
	NAD (*n*=3)	1	0	2
	AD (*n*=6)	1	0	5
2) NB classifier				
		NB predictions		
		Negative	NAD	AD
Diagnose	Control (*n*=5)	5	0	0
	NAD (*n*=3)	2	0	1
	AD (*n*=6)	1	0	5

ANN: artificial neural network classifier; NB: naïve bayes classifier; NAD: non Alzheimer's disease type dementia; AD: Alzheimer's disease.

Beside the need of a reliable diagnosis for therapeutic purpose, the availability of minimally invasive AD biomarkers is an urgent unmet issue. In this pilot study we have included three different groups of patients having a clinical classification and we have performed a multiplex assay aimed at finding a possible diagnostic profile. Our approach was not original as for the selection of assayed molecules, that were almost entirely listed by Ray et al [11]. For technical reasons linked to cross-reactivity, we were able to include in a single array only 15 out of 18 of the molecules measured in the just cited paper. We also assessed the protein GRO that was not listed by Ray et al [11] but that was recently considered as possible interesting biomarker for AD diagnosis [16]. However, this should not have been a major limitation in replicating diagnostic accuracy, as according to the published data the classification error using >10 predictors up to 120 was not remarkably increased. In fact, others reported a diagnostic classification starting from the same 18 protein panel but using 16 predictors and a bioinformatic elaboration reduced the number of predictors down to 5 without affecting diagnostic accuracy [17, 18]. At difference from Ray et al, we used serum as biological material instead of plasma. Our purpose was to test the diagnostic panel in a clinically easy handling type of sample, without the need of anticoagulant agents, and to add knowledge about this option, as to date only plasma was used for replication of Ray et al. In agreement with other studies that attempted a replication [17, 19], our results at single predictor level were mostly negative, with no difference among groups for 13 out of 15 molecules. The additional protein tested, GRO, did not differ among groups. However, it is worth to notice that difficulties in replicating the original data might also depend on the complex algorithm used by Ray et al [11]. Others have tried to differently re-analyze their data from the bioinformatic point of view and found evidence for the differential diagnosis of AD [20]. Our positive finding on EGF deserves attention, as our AD group was sufficiently sized (*n*=33) and an increase of this growing factor in AD was independently reported also by [17] and [19], so it is possible that further research is warranted on this point, even though EGF alone was not a good diagnostic predictors in our hands (AUC= 0.63). The multiparametric analysis gave some preliminary suggestions of a possible improvement of diagnostic accuracy using the 15 protein panel as a whole, as the development of a naïve bayes (NB) classifier allowed specificity, sensitivity and accuracy estimation above 83% for the discrimination of AD from healthy controls. However, this analysis suffers from reduced sample size, particularly in the validation step and independent replication in larger datasets is mandatory. In conclusion, our proposed diagnostic panel gave mostly negative findings in agreement with the available literature, but confirmed in serum the evidence for EGF increase in AD patients.

Acknowledgements

This work was supported by the grant 2008-2359 from Fondazione CARIPLO (Milan, Italy) awarded to DA and by "Fondazione Italo Monzino" (Milan, Italy).

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