DECREASED mRNA EXPRESSION OF CCL5 IN ALZHEIMER’S DISEASE BLOOD SAMPLES
ABSTRACT

A recent study reported that an 18-analyte multiplexed plasma panel of signaling proteins differentiated Alzheimer’s disease (AD) from controls. In this study we measured mRNA expression for nine of these promising biomarkers in 23 AD patients and 23 age-and-sex-matched controls. Total RNA was isolated from PaxGene RNA tubes. Relative mRNA expression levels of CCL5, CSF1, ICAM1, IGFBP6, IL1A, IL3, IL8, PDGFB and TNF were determined by Q-RT-PCR, with GAPDH as housekeeping gene. A panel of 5 markers (CCL5, CSF1, ICAM1, IL8, TNF) with detectable expression levels in all individuals differed between AD patients and controls ($p$ interaction <0.10). Especially, the relative expression level of CCL5 was lower in AD patients than in controls ($p$<0.005). Across groups, levels of both CCL5 and TNF were correlated to CSF levels of tau ($r$=0.39, $r$=0.32), ptau-181 ($r$=0.38, $r$=0.33), and MMSE ($r$=-0.31, $r$=-0.33, all $p$<0.05). In conclusion, the measured panel, and especially CCL5, could aid in the differentiation of AD from controls.

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INTRODUCTION

Alzheimer’s disease (AD) is the most prevalent form of dementia. A clinical diagnosis of AD is based on clinical criteria, but these rely on advanced symptomatology. To develop new treatment strategies, and for individually tailored management of patients, an earlier diagnosis is warranted. AD related brain pathology and subsequent neurodegeneration precede clinical symptoms. Biological markers of this pathology could be of help to identify the onset of disease in vivo. In tertiary referral settings, CSF biomarkers amyloid beta 1-42 (Aβ42), total tau (tau) and tau phosphorylated at threonine 181 (ptau-181) have been shown to perform well for differentiation of AD from healthy controls with sensitivity and specificity of 80-90%. However, assessment of these markers is troubled by lack of standardized procedures, and obtaining CSF by lumbar puncture is considered by many to be invasive. Efforts have been made to develop a plasma test for AD to overcome these problems, with limited success.

A recent study reported that an 18-analyte multiplexed plasma panel could differentiate AD from controls, using plasma signaling proteins. In that study, the abundance of 120 known signaling proteins was measured by filter-based, arrayed sandwich ELISAs. A set of 18 signaling proteins in blood plasma was found to classify blinded samples from AD and controls with close to 90% accuracy. These proteins were involved in pathways of immune response, hematopoiesis, apoptosis and neuronal support. Of these signature proteins, tentatively called the ‘cellular communicome’, nine have been reported to be specifically related to AD. Until now these results have not been independently replicated. Additionally, the study is hampered by methodological issues, e.g. there were age differences between the groups examined.

In the current study, we performed mRNA expression analyses using Q-RT-PCR technique for the nine AD related signaling proteins in 23 AD patients and 23 age-and-sex-matched controls. We used Q-RT-PCR because of the assay performance and the ease to set up a quantitative molecular multimarker analysis of RNA, compared to the filter-based, arrayed sandwich ELISA analysis of proteins. The first aim of the current study was to validate the value of these nine blood markers in the discrimination of AD patients from controls. The second aim was to obtain insight in the biological pathways associated with AD, by analyzing correlations of mRNA expression for the signaling proteins with age, MMSE and levels of CSF biomarkers Aβ42, tau and ptau-181.

METHODS

Study population

We included 23 patients with a diagnosis of probable AD, and 23 patients with subjective complaints, who served as controls, from our memory clinic. Groups were matched for age and sex: AD patients were 64±7 year old and 44% was female, controls were 64±7 years old and 48% was female. All patients underwent a standard dementia screening including physical and neurological examination as well as laboratory tests, EEG and brain MRI. Cognitive screening included a Mini Mental State Examination (MMSE), but usually involved comprehensive neuropsychological testing. The diagnosis of...
probable AD was made according to the NINCDS-ADRDA criteria in a consensus meeting. When the results of all examinations were normal, patients were considered to have subjective complaints (i.e. did not fulfill criteria for MCI). For most patients (n=41) CSF biomarker levels of Aβ42, tau and ptau-181 were determined. If possible patients were additionally selected based on CSF biomarker levels, with abnormal levels for the included AD patients and normal levels of the patients with subjective memory complaints, to obtain a more certain diagnosis. Levels of CSF Aβ42 <550 pg/ml, CSF tau >375 pg/ml and CSF ptau-181 >52 pg/ml were considered abnormal.

**RNA isolation**

Peripheral blood was collected in PAXgene tubes (PreAnalytiX) and left at room temperature for at least 2 hours before freezing at -20°C. Automated RNA isolation was performed on the Biorobot MDX (Qiagen, Benelux b.v., Venlo, the Netherlands) according to the manufacturer’s instructions (PAXgene Blood RNA Mdx kit). Yields and purity of isolated RNA were determined by spectrometric analysis (Nanodrop). Samples were stored at -80°C.

**Quantitative RT-PCR**

The relative expression levels of nine target genes were determined using TaqMan gene expression assays (Applied Biosystems) and detected on an ABI7300/SDS platform (Applied Biosystems, Inc.). Selected target genes were IGBP6, IL8, ICAM1, CCL5, CSF1, TNFA, IL3, IL1A, PDGFB. As calibrator (housekeeping gene), GAPDH was used. The ten genes correspond with the TaqMan assay catalogue numbers GAPDH (hs00266705_g1), IGBP6 (hs00181853_m1), IL8 (hs99999034_m1), ICAM-1 (hs99999152_m1), CCL5 (hs00174575_m1), CSF1 (hs00174164_m1), TNFα (hs99999043_m1), IL3 (hs00174117_m1), IL1a (hs99999028_m1), PDGFB (hs00234042_m1). The RT-PCR’s were set up according to the manufacturers instructions (Geneamp® EZ RTth RNA PCR reagent set, Applied Biosystems, Inc.). RT-PCR reactions were performed in 20 µl with 1x Taqman EZ Buffer, 3 mM manganese acetate, 300 µM dATP, 300 µM dCTP, 300 µM dGTP, 600 µM dUTP, 1 µl Taqman gene assay mix (20x), 0.1 U/µl RTth DNA Polymerase, 0.01 U/µl AmpErase UNG, 100 ng total RNA. After 2 min 50°C, 30 min 60°C and 5 min 95°C, 40 PCR cycles were performed as follows: 94°C 20 sec (denaturation), 60°C 1 min (annealing and elongation). Each TaqMan RT-PCR assay was performed in triplicate in 96-well plates and ROX as passive reference. Each plate had a reference RNA (100ng) and a negative control, milliQ water. mRNA expression was given as cycle threshold (CT), where a higher CT indicates lower mRNA expression. All flag settings were used and wells with Ct <36, bad ROX passive reference, standard deviation >0.5, no amplification, relative noise >4, were omitted. The delta CT (ΔCT) was calculated with the StatMiner software (Integromics, Inc.), using GAPDH as endogenous control.

**Data analyses**

For statistical analysis, SPSS version 15.0 (for Windows) was used. Results are expressed as means (SD) unless indicated otherwise. Frequency distributions for categorical variables were compared with chi-squared tests and Student t-tests were used to compare continuous variables. MANOVA for repeated measures was used.
DEVELOPMENT OF NEW BIOMARKERS FOR ALZHEIMER’S DISEASE

with mRNA expression levels (ΔCT) of the individual genes as within subjects variable and diagnosis as between subjects variable. Subsequently, mRNA expression (ΔCT) was compared between groups for the individual mRNA genes using Student t-tests. For the purpose of graphical representation, relative expression (RQ) was calculated with the formula $2^{-\Delta\Delta CT}$, with $\Delta\Delta CT$ as the mean difference of ΔCT between AD patients and controls. Pearson correlations were used to determine correlations among the different mRNA genes (using ΔCT), and between each mRNA gene and MMSE, age and levels of CSF biomarkers Aβ42, tau and ptau-181. Correlations were determined first for the whole group, and then after stratification for diagnosis. In general, statistical significance was set at $p<0.05$. Interactions were considered significant if $p$-values were lower than 0.10.

RESULTS

Table 1 presents the patient characteristics according to diagnosis. AD patients had a lower score on the MMSE, had lower levels of CSF Aβ42 and higher levels of tau and ptau-181 than controls (all $p<0.001$).

The expression of mRNA of IGFB6, PDGFB, IL1A and IL3 was detectable in less than half of the patients, which made analyses unreliable. ANOVA for repeated measures revealed that mRNA expression levels of ICAM1, IL8, CCL5, CSF1 and TNF were in general, albeit non-significantly, lower in AD patients than in controls ($p=0.07$). In addition, there was an interaction for diagnosis*mRNA genes ($p<0.10$), indicating that pattern of expression of mRNA genes differed by group. Post hoc analyses of individual mRNA genes showed that expression of mRNA CCL5 was lower in AD patients than in controls ($p<0.005$), while there were no other significant differences between groups (Figure 1).

Across groups, there were correlations between CCL5 and TNF ($r = 0.42, p<0.005$) and between TNF and IL8 ($r = 0.35, p<0.05$). In addition, there were correlations for CCL5 with CSF tau levels ($r = 0.39, p<0.05$), CSF ptau-181 levels ($r = 0.38, p<0.005$) and with MMSE ($r = -0.31, p<0.05$), indicating, that lower CCL5 mRNA expression

Table 1. Baseline characteristics

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<thead>
<tr>
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<th>Control N=23</th>
<th>Alzheimer’s disease N=23</th>
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<tbody>
<tr>
<td>Female, n (%)</td>
<td>10 (44%)</td>
<td>11 (48%)</td>
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<tr>
<td>Age, years</td>
<td>64±7</td>
<td>64±7</td>
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<tr>
<td>MMSE</td>
<td>28±1</td>
<td>20±4*</td>
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<tr>
<td>CSF Aβ42, pg/mL</td>
<td>829±278</td>
<td>463±99*</td>
</tr>
<tr>
<td>CSF Tau, pg/mL</td>
<td>292±155</td>
<td>646±329*</td>
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<tr>
<td>CSF Ptau-181, pg/mL</td>
<td>56±21</td>
<td>98±49*</td>
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Data are provided as mean ± SD, unless indicated otherwise. Controls were patients with subjective memory complaints. * Data available for 20 controls and 21 AD patients.

* $p<0.001$
(higher ΔCT) was associated with higher tau and ptau-181 levels and lower MMSE. Also for TNF there were correlations with CSF tau levels ($r = 0.32$, $p<0.05$), CSF ptau-181 levels ($r = 0.33$, $p<0.05$) and with MMSE ($r = -0.33$, $p<0.05$).

After stratification for diagnosis, correlations between CCL5 and TNF remained significant for controls ($r = 0.46$, $p<0.05$). In addition there were also correlations between IL8 and CCL5 ($r = -0.42$, $p<0.05$), and IL8 an Aβ42 levels ($r = -0.51$, $p<0.05$). In the group of AD patients there was a correlation for IL8 with TNF ($r = -0.45$, $p<0.05$), while correlations with age, MMSE or CSF biomarker levels disappeared.

**DISCUSSION**

We have shown that a panel of five mRNA genes differed between AD patients and controls of similar age and sex. Further investigation showed that this effect was mostly attributable to mRNA expression of CCL5, which was lower in AD patients compared to cognitively healthy subjects. The results of this study, using Q-RT-PCR to measure mRNA expression in blood samples, did not confirm the report of an 18-analyte multiplexed plasma protein panel that reportedly differentiated AD from controls with high accuracy.

We used a novel approach to validate a previously published panel of signaling proteins for the differentiation of AD patients from controls. With this method we were able to set up a quantitatively molecular multimarker analysis by measuring signaling activity at mRNA level. In our study, nine of the 18 earlier reported markers were measured, since these nine seemed most related to AD. Our results did not confirm the results of this previously reported panel which showed 90% diagnostic accuracy for the differentiation of AD from controls. In our study, unfortunately expression

![Figure 1](image-url)

**Figure 1.** Relative expression (RQ) was defined as expression in AD patients compared to controls. The RQ was calculated with the formula $2^{-\Delta CT}$, with ΔCT as the mean difference of ΔCT between AD patients and controls (expression of controls was defined as 1). The error bars represent the 95% confidence interval around the estimated RQ. The black bars represent the controls (reference value; by definition scaled to 1) and the grey bars the AD patients. * $p<0.005$
levels of four mRNA genes (IGFB6, PDGFB, IL1A and IL3) were not available for analyses. The concentration of mRNA of these genes was probably too low for the protocol we used. Two other groups also attempted to replicate the results of the 18-analyte multiplexed plasma panel, and obtained negative results as well, as is shown in Table 2.\textsuperscript{10,11} One study showed no differences of protein levels between groups, and poor diagnostic accuracy of the panel.\textsuperscript{11} The second replication study showed lower levels of TNF and higher levels of CCL5 in AD as compared to controls, also with low diagnostic accuracy.\textsuperscript{10} It is disappointing that the excellent discriminative value of the reported 18-analyte multiplexed plasma panel has not been validated, by either of the two replication studies, or by our study. It appears, therefore, that the results of the initial study reporting the 18-analyte multiplexed plasma panel should at least be considered with reservations.

We measured signaling activity at mRNA level instead of protein levels, to be closer to the source. But also with this direct approach, we did not succeed in replicating the promising results of the earlier study with one exception. We found lower CCL5 expression, which was congruent with the initial study,\textsuperscript{6} but not with both other studies.\textsuperscript{10,11} It has been found that CCL5 levels are up-regulated in microglia and cerebrovascular tissue in the first phase after exposure to amyloid,\textsuperscript{12,13} while after a longer period of exposure a decrease of CCL5 was shown.\textsuperscript{14} Furthermore, CCL5 has been associated with the prevention of amyloid associated cell death.\textsuperscript{15} It could be hypothesized that in an early phase of exposition to amyloid, microglia cells respond by producing CCL5 to inhibit apoptosis, while in later stages this system fails and patients suffer from an insufficient production of CCL5, which leads to the pathologic process of AD. Next to lower CCL5, we also found lower TNF mRNA expression in patients with a higher tau and ptau-181 levels and lower MMSE, e.g. in patients with a typical AD profile. Lower levels of TNF were also seen in the initial study.\textsuperscript{6} Lower expression of TNF in AD has also been shown before by several other studies.

Table 2. Comparison of results of studies evaluating signaling proteins in blood samples for the differentiation of Alzheimer patients and control subjects

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<tbody>
<tr>
<td>CCL5</td>
<td>⊘</td>
<td>⊗</td>
<td>NS</td>
<td>⊘</td>
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<tr>
<td>CSF1</td>
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<td>NS</td>
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<td>NS</td>
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<td>ICAM1</td>
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<td>IL8</td>
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<td>TNF</td>
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<td>IGFBP6</td>
<td>⊘</td>
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<td>IL1A</td>
<td>⊘</td>
<td>NS</td>
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<td>IL3</td>
<td>⊘</td>
<td>NS</td>
<td>NS</td>
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<td>PDGFB</td>
<td>⊘</td>
<td>NS</td>
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 ⊘ and ⊗ = Levels of protein or mRNA expression were significantly increased, resp. decreased in AD compared to controls, NS = levels of protein or mRNA expression were not significantly different between AD and controls, - = no data available
studies, and TNF was shown to be decreased in brain areas of AD patients. It seems possible that mRNA expression of the signaling proteins CCL5 and TNF are indeed related to AD pathology. Further support for the notion that measuring mRNA is a promising direction to seek for new AD biomarkers is provided by the observed associations between CCL5 and TNF levels and CSF biomarkers for AD pathology. It is tempting to speculate that mRNA expression of signaling proteins in blood was indeed related to AD-associated brain changes. In addition, RNA expression profiling permits rapid screening of markers by assays that have an easy set up, compared to ELISA or proteomics studies, while the only requirement is information on the target sequence. We have now examined a small seemingly promising group of signaling mRNA markers, but probably many more signaling processes are involved in the pathogenesis of AD, and further exploration of these processes would be essential. To this end future studies should be performed in an unbiased approach using a genome wide manner, and for this RNA expression profiling seems promising since it has adequate sensitivity due to the use of an PCR amplification step. Therefore, by adapting the molecular approach to the genome wide level, novel molecular blood markers forming a ‘signature for AD’ can hopefully be identified.

REFERENCES


