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Prediction of Mild Cognitive Impairment that Evolves into Alzheimer’s Disease Dementia within Two Years using a Gene Expression Signature in Blood: A Pilot Study

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Abstract

Background: The focus on Alzheimer’s disease (AD) is shifting from dementia to the prodromal stage of the disorder; to a large extent due to increasing efforts in trying to develop disease modifying treatment for the disorder. For development of disease-modifying drugs, a reliable and accurate test for identification of mild cognitive impairment (MCI) due to AD is essential.

Objective: In the present study, MCI progressing to AD will be predicted using blood-based gene expression.

Material and Methods: Gene expression analysis using qPCR was performed on blood RNA from a cohort of patients with amnestic MCI (aMCI; n = 66). Within the aMCI cohort, patients progressing to AD within 1 to 2 years were grouped as MCI converters (n = 34) and the patients remaining at the MCI stage after 2 years were grouped as stable MCI (n = 32). AD and control populations were also included in the study.

Results: Multivariate statistical method partial least square regression was used to develop predictive models which later were tested using leave-one-out cross validation. Gene expression signatures that identified aMCI subjects that progressed to AD within 2 years with a prediction accuracy of 74%–77% were identified for the complete dataset and subsets thereof.

Conclusion: The present pilot study demonstrates for the first time that MCI that evolves into AD dementia within 2 years may be predicted by analyzing gene expression in blood. Further studies will be needed to validate this gene signature as a potential test for AD in the predementia stage.

Keywords: Alzheimer’s disease, biomarkers, diagnostic tests, gene expression signatures, mild cognitive impairment

INTRODUCTION

The World Alzheimer Report in 2009 estimated that 36 million people worldwide are living with dementia, with numbers nearly doubling every 20 years to 66
In 2012 by Koyama et al. [31] concluded that plasma 
no significant differences between AD and controls 
H9252
in A
in neurological diseases [16–24]. L¨onneborg [21] sum-
blood-based test could be used for diagnostic profiling 
invasive and reliable, yet still highly accurate [12–15].

develop biomarkers that are practical to use, minimally 
screening times. Methodological and technical varia-
als provided both considerable cost savings and shorter 
FDG-PET from the enrichment strategy for clinical tri-
demonstrated that removal of both CSF biomarkers and 
ple blood test. A cost benefit analysis by Yu et al. [10] 
are either more invasive, time-consuming, procedure 
such as amyloid-
(A
) and total-tau have been pro-
posed in the early diagnosis of AD [9]. However, these 
are either more invasive, time-consuming, procedure 
complex, or very expensive compared to that of a sim-
blood test. A cost benefit analysis by Yu et al. [10] 
demonstrated that removal of both CSF biomarkers and 
FDG-PET from the enrichment strategy for clinical tri-
als provided both considerable cost savings and shorter 
screening times. Methodological and technical varia-
tion has also been described [11]. Thus, there is need to 
develop biomarkers that are practical to use, minimally 
vasive and reliable, yet still highly accurate [12–15].

Several independent studies have indicated that a 
blood-based test could be used for diagnostic profiling 
in neurological diseases [16–24]. Lomneborg [21] sum-
marized in 2008 that of several studies that investigated 
plasma Aβ levels in AD, one study showed an increase 
in Aβ levels [25] and the majority of studies found no significant differences between AD and controls 
[26–30]. Later, a systematic review and meta-analysis in 
2012 by Koyama et al. [31] concluded that plasma 
Aβ(AβB) ratio may predict development of AD, 
however, significant heterogeneity in the meta-analysis 
underlines the need for substantial further investiga-
tion of plasma Aβ levels as a preclinical biomarker. 
Thambisetty et al. have identified plasma clusterin as a 
potential biomarker for AD [32] and present data that 
suggests that plasma clusterin is associated with rate 
of atrophy in MCI [33].

As the first company in the world, DiaGenic devel-
oped a CE marked blood-based diagnostic test for AD 
(ADect®) which identifies patients with mild to mod-
erate AD with an accuracy of 72%. The development 
and validation of this 96-assay gene expression signa-
ture has been described in Booij et al. and Rye et al. [34, 
35]. This test detects the blood-based gene expression 
signature in blood samples and adopts a combination 
of multiple gene expression assays to obtain a predic-
tion value for disease classification. Alterations in a 
gene expression signature in peripheral blood are pos-
tulated as a result of a systemic disease response in AD 
as verified by many previous studies [34–38].

Based on the same approach, DiaGenic has inves-
tigated the gene expression signature in blood of 
patients with amnestic MCI (aMCI) who within 2 
years progress to AD dementia and compared this with 
the gene expression pattern in stable MCI. Diagnost-
ic performance (prediction accuracy, sensitivity, and 
specificity) of the identified multivariate biomarker is 
presented.

MATERIAL AND METHODS

Participants

Patients with MCI (n = 66) were recruited from 8 
centers in Norway and Sweden from January 2007 until 
January 2011. All patients were clinically examined 
longitudinally on a yearly basis.

MCI patients progressing to AD within 2 years were 
grouped as MCI converters (n = 34) and the patients 
maintaining an MCI diagnosis after 2 years (i.e., at the 
3rd visit) were grouped as stable MCI (n = 32).

The clinical investigations were performed by clini-
cians with expertise in diagnosing MCI and dementia. 
Clinical interview and medical examination were per-
formed, and physical diseases and drug use were 
recorded. Imaging (either MRI or CT) and routine 
blood tests were performed for all subjects to exclude 
other causes of MCI or dementia than MCI or AD, 
respectively.

The cognitive and functional test battery included 
an overall cognitive screening test (Mini-Mental State
Examination, MMSE) [39] and a set of standardized cognitive tests assessing verbal and non-verbal memory, processing speed and executive function (shifting attention): The Clock Test [40, 41], Kendrick OLT test [42], 10-word test [43, 44], and Trail Making test A and B [45]. In addition, the clinical dementia rating (CDR) scale [46] was scored based on a detailed clinical interview with the patient and a caregiver.

MCI (amnestic MCI) was diagnosed in accordance with the Petersen criteria [47] with symptoms present for at least 6 months, and a diagnosis of AD was in accordance with International Classification of Diseases (ICD) adopted by the WHO member states in 1994 (ICD-10) and the National Institute of Neurological and Communication Disorders and Stroke/Alzheimer’s Disease and Related Disorders Association (NINCDS-ADRDA) criteria [48].

All diagnoses and the staging of cognitive impairment/dementia were evaluated blindly by an expert group of clinicians having access to all relevant clinical, laboratory, and imaging longitudinal patient data in order to independently establish a consensus clinical diagnosis.

The procedures included blood sample collection and were approved by local ethics committees. The blood samples were obtained at the clinical centers at the time of clinical evaluation. Written informed consent was obtained from each included subject.

Sample collection

Venous blood samples (2.5 mL) were drawn into PAXgene™ tubes (Becton & Dickinson, Qiagen Inc., Valencia, CA) according to the manufacturer’s instructions. From each subject sample, 2,210 ng RNA was used to prepare adequate amounts of cDNA for gene expression analysis on the complete set of MFCs (3 × 384-assay cards). The final concentration of RNA in the cDNA reaction mixture was 8.5 ng/μl. This concentration was tested and shown to be below the limit of inhibition of the cDNA synthesis. The cDNA was stored at −70°C or below until analysis.

Assay selection and micro-fluidic card (MFC) layout

A total of 1,239 gene probes have previously been selected [35] and served as a basis for the identification of informative genes. The 1,239 gene probes were selected based on an AD classifier developed following a whole genome screen comprising in total 126 clinically diagnosed AD patients, 98 age-matched cognitively healthy controls, 28 young controls, 28 Parkinson’s disease patients, and 10 subjects with MCI. TaqMan assays with adequate efficiency, expression level in blood RNA, and absence of signal from genomic DNA, were available for 970 of the 1,239 initial genes (Applied Biosystems, Foster City, CA). In addition, reference assay candidates and assays for transcripts identified by an extended literature search were also included. A total of 1,152 assays distributed on three different micro-fluidic cards (MFCs) were custom ordered from Applied Biosystem (Foster City, CA).

In addition to the study MFCs, a DiaGenic technical control MFC (4 × 96 assay format) was used to monitor the temporal stability during the gene expression analyses.

RNA extraction and cDNA synthesis

Total RNA was extracted from blood samples using PAXgene™ Blood RNA kit for manual extraction (PreAnalytix, Hombrechtikon, Switzerland) according to the manufacturer’s instructions. Total RNA was stored at −70°C or below until analysis. The RNA was assessed for quality using the NanoDrop ND-1000 spectrophotometer (NanoDrop, Wilmington, DE) and the Agilent 2100 BioAnalyzer (Agilent, Santa Clara, CA)., with sample acceptance limits RIN ≥7.3; 28S/18S ≥1.0; A260/A230 ≥1.0; A260/A280 ≥1.8; RNA concentration ≥30 ng/μL. The cDNA was prepared according to a predefined randomization scheme using the High-Capacity cDNA Reverse Transcriptase kit (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. From each subject sample, 2,210 ng RNA was used to prepare adequate amounts of cDNA for gene expression analysis on the complete set of MFCs (3 × 384-assay cards). The final concentration of RNA in the cDNA reaction mixture was 8.5 ng/μl. This concentration was tested and shown to be below the limit of inhibition of the cDNA synthesis. The cDNA was stored at −70°C or below until analysis.

Real time qPCR

Real time qPCR (RT-qPCR) was performed using the TaqMan® Universal PCR Master Mix II (2X) with uracil-N-glycosylase according to the manufacturer’s instructions in the standard run mode on 2 Viia7 Dx systems (Life Technologies, Carlsbad, CA) equipped with MFC blocks and robotic feeders (TwisterII, Caliper, Hopkinton, MA). The Viia7 systems were operated by the Viia7 Software version 1.0 (Life Technologies). The two instruments used during the study were qualified according to internal procedures prior to use and normalized against each other.
Samples were run on MFCs according to a predefined randomization scheme different from that used for cDNA synthesis. The donors were randomized independently for each of the MFCs. All samples were run on a complete set of MFCs, i.e., $3 \times 384$-assay cards. Prepared cDNA was diluted 1/10 in the PCR reaction mixture, which was tested and shown to be below the level of inhibition of the qPCR. Each of the 8 lanes of each card was loaded with 97/$H_{9262}$ PCR reaction mixture. Five MFCs were filled simultaneously and loaded into the RT-qPCR using the robotic feeder. To avoid potential fading of the fluorescent dye and to ensure stability of the reaction mixture on the MFCs, the instruments were in a room with dimmed light and temperature control.

During the study RNA reference samples were run at regular time intervals randomized among and processed as the study samples. The reference samples were used to monitor technical aspects such as instrument variation, inter-card and inter-day variability. Analysis of the gene expression of the reference material demonstrated no significant difference between the two instruments for the 20 assay set with a $p = 0.3$ using multivariate fmanova (an R package implementing fifty-fifty ANOVA) [49].

Reference samples were both run on study MFCs and on technical control MFCs. Temporal drift was investigated by monitoring the average $C_T$ of the technical replicates of the reference material. No systematic drift of average $C_T$ value was observed following analysis of the technical replicates (Fig. 1), and the average $C_T$ was in the range of 1 $C_T$.

**Data analysis**

The present study was performed as a retrospective analysis. Subject data collection and blood draw were both performed prior to inclusion in the present study. Optimal thresholds were set by the ViiA7 software for determination of $C_T$ for each assay. Missing values were imputed using k-Nearest Neighbors (k-NN) [50]. A panel of reference assays was investigated. Beta-actin demonstrated to be stably expressed and was used for reference gene normalization of the data. Partial Least Square Regression (PLSR) and Leave One Out Cross-Validation (LOO-CV) were used to build the gene expression algorithm (model) and to estimate the diagnostic performance. Jack-knife feature [51] was used for variable selection. A number of models were developed and evaluated, investigating complete datasets which excluding reference assays constituted 1,123 assays or subsets thereof. For each model the LOO-CV estimates of sensitivity, specificity, prediction accuracy, and area under the receiver operating characteristic curve (AUC) were used as a measure of the models performance as compared to the clinical diagnosis. The statistical computing language R version 2.10.0 was used [52].

**Clinical interferences**

Clinical interferences were studied in the study population. Subjects with co-morbidities such as cancer, hypertension, diabetes, coronary disease, depression, and allergies were compared with subjects without a history of chronic illness using PCA score analysis. This was done to evaluate if co-morbidities were likely to affect the test result.

**Biological significance**

To investigate the biological significance of proteins encoded by the selected gene assays pathway analyses were performed. The pathway analyses were performed using Ingenuity Pathway Analysis software (IPA, Ingenuity Systems, Redwood City, CA). Benjamini-Hochberg multiple testing correction [53] at $p<0.05$ was used to correct for multiple testing when identifying biological functions and canonical pathways significantly associated with assay sets.
The functional categories used in IPA were developed by Ingenuity staff of Ph.D. scientists who are experts across various domains of biology. These categories strive to encompass all types of biological functions that are most important to researchers using IPA.

The dynamic Canonical Pathways are well-characterized metabolic and cell signaling pathways that have been curated and hand-drawn by Ph.D. level scientists based on previous literature findings. The information contained in Canonical Pathways comes from specific journal articles, review articles, text books, and HumanCyc.

RESULTS

Subject characteristics

The included individuals in this study were recruited through DiaGenic blood collection studies at 8 clinical centers in Norway and Sweden using a standardized protocol. Clinical and demographic features of the study population are shown in Table 1. A total of 66 subjects were included in the present study and blood collected at baseline was used to investigate the gene expression signature.

Prediction of MCI that evolves into AD dementia within two years in subjects with aMCI

For development of the gene expression algorithm, PLSR and LOO-CV were used both to build the model and to estimate the diagnostic performance of the generated models. A number of models were developed and evaluated, and, similarly, a number of different datasets were investigated. The selection of these datasets is described below.

Our first model was developed using the complete dataset compiled of gene expression data obtained from 1,123 assays. Using the gene expression data from the stable MCI and the MCI converters, a PLSR model was developed discriminating the two classes. The model was evaluated by LOO-CV from which a prediction accuracy of 74% was demonstrated. The model performance characteristics are summarized in Table 2.

A large assay set of 1,123 assays is unpractical and costly for clinical use. A reduction of the number of assays used by investigating a subset of the data was
explored aiming at maintaining the most informative assays. Using a jack-knife procedure, a total of 225 of the most informative or significant assays ($p < 0.2$) were identified. The gene expression data obtained from this data subset was used for statistical modeling using PLSR for the discrimination of stable MCI from MCI converters. A model demonstrating a prediction accuracy of 77% following LOO-CV was obtained. The performance characteristics of this model based on the 225 assay set are summarized in Table 2.

No further jack-knife reduction was performed for the present dataset as subsequent product development did not aim at final assay selection at that time. For product development, further adaptation of the qPCR protocol for regulatory approval purposes is planned prior to locking of the algorithm; moreover a larger number of assays into the optimization phase is preferred. However, in this study we wanted to investigate a subset of 20 informative assays that we have previously identified in independent in-house studies (results not shown) during the development of ADtect® [34, 35]. The identification of these assays was based on the discrimination of AD and cognitively healthy controls. Based on this, these 20 assays were included as part of the complete assay set in the present study. Hence, the 20 assays were not a jack-knife product of the complete assay set as the 225 assays. The applicability of these 20 informative assays for the prediction of MCI that evolves into AD dementia within 2 years in an aMCI population was investigated. Using this exploratory dataset, a PLSR model was developed using the gene expression data for the discrimination of stable MCI from MCI converters. The developed algorithm using the 20 informative assays selected based on AD pathology demonstrated a prediction accuracy of 77%. The model performance when discriminating MCI converters from stable MCI is summarized in Table 2. This demonstrates that it is possible to retain the same level of prediction accuracy even when reducing the assay set to only a fraction of the initial dataset, in this case 20 assays. A summary of the origin of the investigated assay sets is shown in Fig. 2.

### Clinical interferences

In the present study, the possibility of co-morbidity interferences was investigated, including a history of, for example, cancer, hypertension, diabetes, coronary disease, depression, and allergies. In the cohort of stable MCI patients, 11 of the 34 subjects reported a history of chronic disease. Similarly, 13 of 32 of the MCI converters had a history of chronic disease.

### Table 2

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Complete dataset</th>
<th>Reduced dataset</th>
<th>Exploratory dataset</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prediction accuracy</td>
<td>74</td>
<td>77</td>
<td>77</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>70</td>
<td>73</td>
<td>70</td>
</tr>
<tr>
<td>Specificity</td>
<td>77</td>
<td>81</td>
<td>84</td>
</tr>
</tbody>
</table>

PCA scores revealed no clear effect for individuals suffering from at least one co-morbidity. It was therefore concluded that co-morbidities did not affect the results in the present study.

The use of medication could not be investigated in the present study as only 3 of 32 stable MCI and 3 of 32 MCI converters were on acetylcholinesterase inhibitor.

### Biological significance

For all assays selected, i.e., both the complete dataset and data subsets, the assays were chosen based on the predictive ability in an algorithm and not selected based on the individual informative nature of the genetic origin of the targeted transcripts. Still, we find that a majority of the genes included in the whole assay set as well as in the 225 assay set and the 20 assay set encode proteins with biological functions associated with AD and AD-related biological processes.

Of the 225 assays, 207 encode proteins with known identity. Adjusting for multiple testing using Benjamini-Hochberg correction [53], Aβ/H9252 processing is the canonical pathway that separates from other canonical pathways with the lowest $p$-value ($-\log 3.599$). Seven of the 54 proteins (ratio = 0.13) included in this canonical pathway are among the 207 proteins with a
known identity (Fig. 3) suggesting that this pathway has a pronounced influence on the model. Cell death and survival is the most significant biological function represented by these proteins (Fig. 4). Of the proteins, 73 are associated with cell death and survival and 64 of these proteins are associated with apoptosis.

Due to the limited number of proteins encoded by the 20 assays, no reliable analysis of significant canonical pathways or biological functions represented by these proteins could be done. Therefore the pathway analysis was done on the larger 225 assay set. Of the proteins encoded by the 20 assay set, 10 are directly associated with and cover all biological processes that have been listed above for the 225 assay set. Among the 207 proteins with known identity, 157 are directly associated with AD-related proteins.
A multiple genes and alterations in gene expression involved AD as a multifactorial sporadic disorder [54] with multistudies [34–38]. The results also support the concept of well in agreement with similar findings in previous AD detected using gene expression in blood. This is also reflected in a systemic response that can be these processes not only are affected in brain tissue but also in other parts of the body.

DISCUSSION

We and others have previously shown that disease biomarkers to classify AD with dementia from cognitively healthy individuals are present in blood [16, 23, 35] and an algorithm based on the gene expression of a selected set of genes can be used to identify those with an AD dementia [34–36]. We have now further extended these findings to also include AD in the pre-dementia stage of the disease. The results of the present study demonstrate that information to classify MCI due to AD in an aMCI population is present in blood. The results show the potential of using a prediction algorithm based on the expression of a limited set of expressed genes in blood for identification of MCI that evolves into AD dementia within 2 years among individuals with aMCI. When balancing the sensitivity and specificity, a prediction accuracy ranging from 74% to 77% was obtained for PLSR models built using gene expression data from stable MCI and MCI that evolves into AD dementia within 2 years.

A majority of the gene assays included in both the 225 assay set and in the 20 assay set encode proteins that are directly associated with biological processes that has been associated with AD. This suggests that these processes not only are affected in brain tissue but also are reflected in a systemic response that can be detected using gene expression in blood. This is also well in agreement with similar findings in previous AD studies [34–38]. The results also support the concept of AD as a multifactorial sporadic disorder [54] with multiple genes and alterations in gene expression involved [55, 56]. AP processing appears to be a significant canonical pathway and apoptosis a significant biological function represented among the proteins encoded by the 225 assay set. However, it is worth noting that there are several proteins encoded by the assay set that do not appear to be involved in any of the processes that has been associated with AD and it could be of interest to explore these proteins further to see if novel biological functions may be found that are essential to extend our understanding of the development of this devastating disease.

The clinical applicability of a multivariate index assay test based on gene expression in blood as described here is as a selection tool in clinical trials by enriching the MCI population with higher risk to progress to AD within the next few years as well as a clinician tool as an aid in the diagnosis for early treatment, future planning, and disease management. A blood-based biomarker is a patient-friendly, less invasive, and more affordable alternative to other investigated biomarkers such as CSF biomarkers or amyloid imaging using PET that are either invasive or very expensive [12, 14].

In the present study the possibility of co-morbidity interferences was investigated, but not found to affect the results. However, it cannot be ruled out that some of the co-morbidities may result in an alteration in gene expression. Future investigation may reveal clearer which co-morbidity has the highest impact of gene expression and if the magnitude has an effect on results from downstream analysis. Parnetti et al. recently developed CSF biomarker models based on logistic regression analysis for the prediction of MCI that evolves into AD dementia within 2 years among individuals with MCI [57]. Using the CSF biomarkers Aβ1-42 and p-tau for the model development, they obtained a sensitivity of 75% and specificity of 96%. In a multimodal classification approach combining PET-FDG, MR features following volumetric MR imaging, and the level of selected CSF biomarkers, Zhang et al. achieved a sensitivity of 92% and specificity of 73% which was superior to the performance obtained when investigating the individual modality of biomarkers [58]. The sensitivity and specificity using either CSF or CSF in combination with PET-FDG and volumetric MRI are higher than what is achieved with either of our blood-based gene expression models. However, our prodromal AD biomarker is the first blood-based gene expression biomarker described, and further refinement of the biomarker both by assay selection and increased sample size may demonstrate the full potential of such a biomarker. Still, the use of a blood-based gene biomarker is both a non-invasive low-risk procedure as well as cost effective compared to that of lumbar puncture, making blood-based gene expression biomarker tests an attractive alternative to CSF biomarkers.

In the present study a few donors had been followed beyond the 3rd visit. Of these there were 5 donors with stable MCI for 2 years that were diagnosed with AD at the 4th visit (3 years after baseline) during the longitudinal trial. The present PLSR model predicted...
all of these donors as stable MCI. However, all these subjects may be considered slow converters that possibly is somewhere in between the stable MCI and MCI converters. The subgroup of stable MCI is likely a heterogeneous group and as time passes a fraction of these are expected to progress to AD. The prediction accuracy is therefore dependent on where the follow-up cutoff is set. For the present study we have chosen 2 year follow-up as a cut-off to identify MCI in an aMCI population that will progress to AD within 2 years. Although a substantial portion of MCI progress to AD within 1–2 years, the progression from MCI to AD has been demonstrated to take as much as 10 years [59]. Compared to CSF biomarkers, our prodromal AD biomarker has a dynamic characteristics compared to the more static feature of CSF biomarkers. Our test predicts changes to occur within 2 years, which is a relatively short period of time. CSF biomarkers may have a longer time perspective. This static feature was recently reported by Buchhave et al. [59] who demonstrated that baseline CSF Aβ42 levels were equally reduced in patients with MCI who converted to AD within 0 to 5 years (early converters) compared with those who converted between 5 and 10 years (late converters).

When building a PLSR model including only the available clinical parameters (scores from the neuropsychological test battery), a prediction accuracy of 67% was obtained, which is inferior to that obtained for the gene expression model. Including the clinical parameters in the gene expression model only marginally improved the diagnostic performance by increasing the prediction accuracy from 74% to 75%. This is in contrast to that observed by Gomar et al. [60]. They obtained a prediction accuracy of 71% using cognitive markers only and concluded these to be generally stronger predictors than investigated biomarkers including brain volume, CSF biomarkers, and APOE genotype.

The present study was a pilot study with a limited number of samples. The aim of the study was to demonstrate the potential of using the prodromal AD gene expression signature for the development of a multivariate index assay test for the prediction of MCI that evolves into AD dementia within 2 years in an aMCI population. The regulatory requirement to further optimize, technically verify and clinically validate the prodromal AD gene expression signature is strongly emphasized.

To conclude, we have shown for the first time that it is possible to detect MCI that evolves into AD dementia within 2 years before the onset of dementia by analysis of the gene expression in blood and that gene expression in peripheral blood is sensitive to a pathological process manifesting in the brain.

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