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Supervised pathway analysis of blood gene expression profiles in Alzheimer's disease

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ABSTRACT

Early identification and treatment of Alzheimer's disease (AD) is hampered by the lack of easily accessible biomarkers. Currently available fluid biomarkers of AD provide indications of the disease stage; however, these are measured in the cerebrospinal fluid, requiring invasive procedures, which are not applicable at the population level. Thus, gene expression profiling of blood provides a viable alternative as a way to screen individuals at risk of AD. Previous studies have shown that despite the limited permeability of the blood–brain barriers, expression profiles of blood genes can be used for the diagnosis and prognosis of several brain disorders. Here, we propose a new approach to pathway analysis of blood gene expression profiles to classify healthy (control [CTL]), mildly cognitively impaired (mild cognitive impairment [MCI]; preclinical stage of AD), and AD subjects. In the pathway analysis, gene expression data are mapped to pathway scores according to a predefined gene set instead of considering each gene separately. The robustness of the analysis enables detection of weak differences between groups owing to the inherent dimension reduction. Our proposed method for pathway analysis takes advantage of linear discriminant analysis for identifying a linear combination of features best separating groups of subjects within each gene set. The gene expression data were retrieved from Gene Expression Omnibus (batch 1: GSE63060; batch 2: GSE63061). Predefined gene sets for pathway analysis were obtained from the Broad Institute Collection of Curated Pathways. The method achieved a 10-fold cross-validated area under receiver operating characteristic curve of 0.84 for classification of AD versus CTL and 0.80 for classification of mild cognitive impairment versus CTL. These results reveal the good potential of blood-based biomarkers for assisting early diagnosis and disease monitoring of AD.

1. Introduction

Alzheimer's disease (AD) is a common form of dementia that occurs most frequently in the aged population (>65 years). More than 30 million people worldwide have AD, and due to the increasing life expectancy, that number is likely to triple by 2050 (Barnes and Yaffe, 2011; Prince et al., 2013). Consequently, with no curative treatment available, the economic burden of AD-related health care will dramatically increase, not to mention more human suffering. Despite great efforts to develop therapeutic agents for AD, a large number of AD drug trials have failed. The failure of current clinical trials is proposed to be in part due to the fact that these trials are started too late in disease development. The current diagnosis of AD is made via clinical and neuropsychological examination that yields a diagnosis of probable or possible AD dementia (McKhann et al., 2011). Due to uncertainty in the diagnosis of AD and long-term progression of the disease, research on AD is difficult, especially in the initial stages of the disease. Thus, the demand for the development of early-stage diagnostic biomarkers that possess high sensitivity and specificity is at the center of attention in the medical and patient communities (Blennow et al., 2010; Hampel et al., 2010).

In the last decades, extensive research on AD has resulted in various candidate biomarkers for the early diagnosis of AD and for identifying high-risk individuals as well as for assessing the disease status and understanding the pathophysiological processes during disease progression. Such biomarkers include neuroimaging biomarkers (Rathore et al., 2017; Woo et al., 2017) obtained by magnetic resonance imaging (Bron et al., 2015; Coupé et al., 2015;

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Frisoni et al., 2010; Huang et al., 2017; Moradi et al., 2015) and positron emission tomography (Cho et al., 2016; Firbank et al., 2016; Jack et al., 2018; Rowe et al., 2008) as well as cerebrospinal fluid biomarkers (Cedazo-Minguez and Winblad, 2010; Galasko and Shaw, 2017; Olsson et al., 2016; Reiber and Peter, 2001). In spite of considerable progress in demonstrating the associations between those biomarkers and AD-related pathology, practical use of these biomarkers is limited because of their invasive nature and/or high cost and limited accessibility. Therefore, there is an urgent need for identifying noninvasively obtained and more cost-effective blood-based biomarkers that can aid in the early diagnosis of AD and monitoring the disease status. Despite blood–brain barriers (Sweeney et al., 2018), some studies have shown the utility of blood-based biomarkers for studies on various brain disorders (Goldsmith et al., 2016; Kawata et al., 2016; Lewczuk et al., 2017) because these markers are readily accessible and suitable for repeated measurements to monitor the course of a disease or to evaluate the effects of treatments within the timeframe of an interventional study.

Significant effort has been devoted to identification of peripheral-blood biomarkers in AD (Ashton et al., 2017; Burnham et al., 2014; Cedazo-Minguez and Winblad, 2010; Nakamura et al., 2018), in particular, for differentiation of patients with AD or mild cognitive impairment (MCI) from healthy elderly controls (CTLs) (Doecke et al., 2012; Lunnon et al., 2012, 2013; Ray et al., 2007; Voyle et al., 2016). Such biomarkers are crucial for the management of AD because most pathobiological events occur several years before symptom onset, underscoring the need for a simple diagnostic screening tool to identify and characterize healthy individuals at risk of AD (MCI is a preclinical stage of AD) (Jack et al., 2010). Nonetheless, cross-validation attempts and a meta-analysis revealed inconsistencies among studies, providing only a handful of markers capable of accurately differentiating CTL subjects from patients with MCI or AD (Olsson et al., 2016a; O'Bryant et al., 2017).

In the current work, our purpose was to test whether the changes in expression of certain genes in blood cells can serve as a diagnostic marker for early detection of AD. In particular, we aimed to develop a novel blood gene expression–based approach for discrimination of patients with AD or MCI from healthy elderly people by means of advanced machine learning algorithms. For classification of gene expression data, existing procedures have mainly focused on the gene-level models by classification of all available genes in a data set. A potential problem with such approaches is the reliance on individual genes and ignoring the available biological information about the genes and their relations. Moreover, methods involving single–gene-level classification suffer from the curse of dimensionality because of a large number of available genes for analysis. This large number of genes may hinder detection of weak but significant differences between groups.

To address these challenges, a promising alternative is to analyze gene expression data at the level of groups of genes, that is, pathways that are known to be related in advance. In pathway analysis, gene expression data are mapped to pathway scores according to a predefined gene set instead of considering each gene separately. Such an approach represents a promising direction of the analysis of gene expression data via targeting overall changes in the expression level of predefined gene sets that perform a particular cellular or physiological function. Moreover, analyzing gene expression data at the pathway level allows us to use the available biological information in conjunction with the statistical analysis of gene expression data.

In this article, we present a new supervised approach to pathway analysis based on linear discriminant analysis (LDA) for studies on gene expression data. We apply the proposed pathway analysis method to the data from Gene Expression Omnibus (GEO) from 2 batches of a data set (GSE63060 and GSE63061) and use blood gene expression data for discrimination of AD from CTL and MCI from CTL. For this purpose, we developed a two-stage approach to learning, consisting of a pathway analysis step that is based on LDA—for finding a linear combination of features best separating groups of subjects within each pathway—and a second learning stage which uses ridge logistic regression (RLR) for integrating pathway scores derived from the first stage. This way, first, a single real-valued score is derived for each pathway, and thereafter, these scores are concatenated as a new feature set for the RLR classifier, which acts as a combiner. Moreover, in the experimental section, we demonstrate the superiority of the proposed pathway level analysis to single–gene-level analysis for gene expression data in terms of classification of both cases under study, that is, discrimination of AD from CTL and MCI from CTL. In addition, our results indicate a potential role of blood gene expression data as a disease biomarker for assisting the early diagnosis of AD and disease monitoring in patients with AD.

2. Materials and methods

2.1. Gene expression data

Gene expression data used in this study were retrieved from GEO from 2 batches of a data set provided from the AddNeuroMed consortium, which involves 6 study sites across Europe (London, Kuopio, Lodz, Perugia, Thessaloniki, and Toulouse) or the London Dementia Case Register (London). Patient selection, design, and clinical data have been reported in the studies by Lovestone et al., 2009 and Lunnon et al., 2012. The diagnosis of possible or probable AD was performed using the National Institute of Neurological and Communicative Disease and Stroke and Alzheimer's disease (NINCDS-ADRDA) and Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) criteria. Subjects with neurological or psychiatric illness other than AD, unstable systematic illness or organ failure, or a geriatric depression rating scale score $\geq$ 4/5 were excluded from the study. All MCI subjects reported problems with memory, corroborated by an informant, but had normal activities of daily living as specified in the Petersen's criteria for amnestic MCI (Lovestone et al., 2009; Lunnon et al., 2013), and scored 0.5 on the Total Clinical Dementia Rating Scale or had a memory score of 0.5 or 1 (Morris, 1993). All subjects underwent a structured interview and a battery of neuropsychological assessments, including the mini–mental state examination and Clinical Dementia Rating Scale. CTL, and MCI subjects were assessed based on the CERAD battery (Sood et al., 2015). Detailed information on subject recruitment and assessments has been reported in the studies by Lovestone et al., 2009 and Snyder et al., 2014.

As described in the study by Sood et al., 2015, RNA was taken from whole venous blood that was collected from the subjects fasting 2 hours before collection into a PAXgene Blood RNA tube (Becton & Dickinson, QIAGEN Inc, Valencia, CA, USA). The tubes were frozen at −20 °C overnight before long-term storage at −80 °C. RNA was extracted using PAXgene Blood RNA Kit (QIAGEN) conforming to the manufacturer's instructions. Gene expression data were prepared using Illumina Human HT-12 v.3 Expression BeadChips for the first batch and Illumina Human HT-12 v4 Expression BeadChips for the second batch. cDNA was synthesized from 200 ng total RNA using the TotalPrep RNA Amplification Kit (Ambion) followed by amplification and biotinylation of cRNA and hybridization. The expression data were transformed using variance stabilization and quantile-normalized using the LUMI package in R. Details of the characteristics of the samples used in this work can be found in Table 1.

Table 1
Characteristics of the data sets used in this work

	AD	MCI	CTL
Batch 1 (GSE63060)			
No. of subjects	145	80	104
Age, mean (standard deviation)	75.4 (6.58)	74.45 (5.99)	72.37 (6.34)
Males/females	46/99	41/39	42/62
Batch 2 (GSE63061)			
No. of subjects	139	110	135
Age, mean (standard deviation)	77.89 (6.67)	78.24 (7.38)	75.41 (6.17)
Males/females	54/85	45/65	81/54

Key: AD, Alzheimer's disease; CTL, control; MCI, mild cognitive impairment.

2.2. An overview of the methodology

The overview of the proposed procedure for LDA-based pathway level analysis of gene expression data for classification of individuals into different phases of AD is illustrated in Fig. 1. The model was designed by combining ComBat (Johnson et al., 2007)—for adjusting batch effects on gene expression data—followed by the proposed pathway analysis. At the pathway analysis stage, first, the gene expression data were subdivided into separate pathways according to the Broad Institute Collection of Curated Pathways. We included only pathways containing between 10 and 500 genes, thus obtaining 3921 gene sets. In each pathway, the genes with low variance in the training data were filtered, and then LDA was applied to the remaining genes within the pathway. At the final stage, we carried out RLR for combining the pathway scores generated by the LDA-based pathway analysis step into the final decision value.

For comparing performance of our LDA-based approach to pathway analysis, we additionally performed Pathway Level Analysis of Gene Expression (PLAGE) pathway analysis (Tomfohr et al., 2005) for discrimination of patients with AD or MCI from CTL subjects. In this method, the pathway scores are derived from the first eigenvector during singular value decomposition of the gene expression data matrix. Given that PLAGE is an unsupervised procedure and does not use label information, the pathway analysis step with PLAGE was performed on all subjects before we subdivided the data into training and test sets. We selected the PLAGE method for comparison purposes because it was tested in another study (Voyle et al., 2016) for discrimination of patients with AD from CTL subjects on the basis of blood gene expression data. Moreover, one study by Tarca et al. (2013) revealed the superiority of PLAGE—in terms of sensitivity, specificity, and prioritization—to other single-sample pathway analysis methods.

2.3. LDA-based pathway analysis

LDA is a dimensionality reduction technique commonly implemented as a preprocessing step for classification tasks in high-dimensional data settings. We propose the LDA approach for mapping/converting gene sets into pathway scores so that the separation between the 2 groups is maximal. Just as principle component analysis (PCA), LDA is a linear transformation method that projects data onto lower-dimensional space; however, the difference is that LDA is a supervised technique and uses label information to compute linear discriminants, whereas PCA is an unsupervised technique and ignores label information. In particular, LDA computes linear discriminants by maximizing the ratio of between-class variance to within-class variance and thus attempts to maximize the separation between different classes.

We denote gene expression data corresponding to a gene set by $\mathbf{X} \in \mathbb{R}^{N \times D}$ belonging to C classes, and in our problem (MCI vs. CTL or AD vs. CTL) $C = 2$. Here, N is the number of subjects, and D is the number of genes in the corresponding gene set. According to the

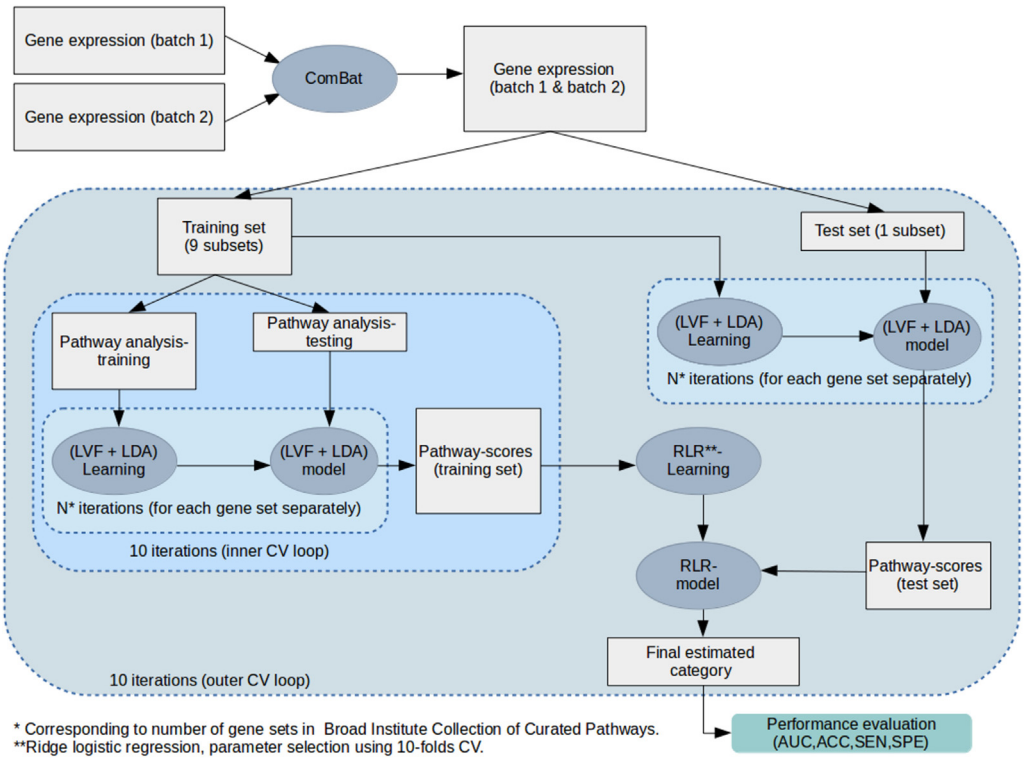

Fig. 1. The overall framework for pathway level analysis–based classification of preclinical and clinical stages of Alzheimer's disease. Abbreviations: LDA, linear discriminant analysis; RLR, ridge logistic regression, AUC, area under the receiver operating characteristic curve; ACC, accuracy; SPE, specificity; SEN, sensitivity; CV, cross-validation; LVF, low variance filtering.

criteria defined for gene set selection, D varied from 10 to 500 (see Section 2.2). The same process is applied to all the 3921 gene sets being considered; we omitted the gene set index for clarity. The goal of the LDA method is to find linear projection W onto lower dimensional space by maximizing the ratio of between-class variance to within-class variance (Welling, 2005), as formulated below:

$$\widehat{\mathbf{W}} = \underset{\mathbf{w}}{\operatorname{argmax}} \frac{\mathbf{W}^T \mathbf{S}_b \mathbf{W}}{\mathbf{W}^T \mathbf{S}_w \mathbf{W}}, \quad (1)$$

where $\mathbf{S}_b$ is the between-class variance defined as $\mathbf{S}_b = \sum_c (\boldsymbol{\mu}_c - \boldsymbol{\mu})(\boldsymbol{\mu}_c - \boldsymbol{\mu})^T$, and $\mathbf{S}_w$ is within-class variance, which is defined as $\mathbf{S}_w = \sum_c \sum_{i \in c} (\mathbf{x}_i - \boldsymbol{\mu}_c)(\mathbf{x}_i - \boldsymbol{\mu}_c)^T$. The linear projection matrix (W) of the LDA can be calculated by means of the generalized eigenvalue problem as

$$\mathbf{S}_w \mathbf{W} = \lambda \mathbf{S}_b \mathbf{W}. \quad (2)$$

Therefore, the projection matrix (W) is the set of eigenvectors associated with this problem. Basically, at most $C-1$ eigenvectors are useful to discriminate C classes. Thus, $C-1$ eigenvectors associated with the highest eigenvalues are used to project data onto lower dimensional space that yields the best possible separation between different groups (Tharwat et al., 2017). Because our task is a two-class classification problem, the resulting useful number of eigenvectors is 1. This way, the dimensionality of each gene set is reduced to one by projecting to a new dimension, which serves for determining pathway scores for further analysis.

2.4. Regularized logistic regression

After calculating the pathway scores at the step of LDA-based pathway analysis for 3921 gene sets, we used a logistic regression classifier for combining the 3921 pathway scores into the final decision value for classification purposes. From the LDA-based pathway analysis step, we have pathway score $p_{i,j}$ for subject i and pathway j. The pathway scores are concatenated as a long vector, p_i, for creating the new feature set for subject i. Because the number of pathway scores is greater than the number of subjects, simple logistic regression may lead to overfitting (Huttunen et al., 2013; Tohka et al., 2016). Therefore, we added a regularization term to prevent overfitting. Given the pathway score matrix $\mathbf{P} \in \mathbb{R}^{N \times M}$ from $C = 2$ classes, where N is the number of subjects and M is the number of pathways, for logistic regression models (Friedman et al., 2010a), the conditional probability for each class is expressed as follows:

$$Pr_c(\mathbf{p}) = \frac{exp(\beta_{c0} + \boldsymbol{\beta}_c^T \mathbf{p})}{\sum_{k=1}^{C} exp\left(\beta_{k0} + \boldsymbol{\beta}_k^T \mathbf{p}\right)}, \quad (3)$$

where β_{c0} and $\boldsymbol{\beta}_c = \{\beta_{c1}, \beta_{c2}, \ldots, \beta_{cM}\}$ are the coefficients of the model. After addition of the regularization parameter, the training of LR consists of estimating the model coefficients by maximizing the following regularized log likelihood function:

$$\sum_{i=1}^{N} log(Pr_{c_i}(\mathbf{p_i})) - \lambda \left|\left|\boldsymbol{\beta}\right|\right|_2^2, \quad (4)$$

where $\lambda > 0$ is the regularization parameter for controlling the strength of the regularization effect, which is determined by cross-validation. The regularization term is the ℓ_2 norm of coefficient vector $\boldsymbol{\beta_c}$, which shrinks the coefficients of the variables correlating with each other and assigns similar coefficient values to them.

2.5. Implementation and performance evaluation

For subdividing data into 2 training sets (pathway analysis training and pathway analysis test) and test sets, we chose 2 nested and stratified cross-validation loops (10-fold for each loop). In the inner cross-validation loop, the pathway analysis—training set was used to construct the pathway analysis model, and the pathway analysis test set served for constructing pathway scores for every training subject; we did not subject the same data set both to learning the pathway analysis model and to computation of pathway scores to avoid overfitting. The training set (a combination of pathway analysis training and pathway analysis test) was used to train the RLR model. We redivided the training set into 10-fold for finding the optimal λ for the model. The test data were used only for evaluating the model. The performance of the final model was evaluated for the area under the receiver operating characteristic curve (*AUC*), accuracy (*ACC*, the number of correctly classified samples divided by the total number of samples), sensitivity (*SEN*, the number of correctly classified case subjects divided by the total number of case subjects), and specificity (*SPE*, the number of correctly classified CTL subjects divided by the total number of CTL subjects) using the test subset of the outer loop. The pooling strategy was chosen for computing AUCs (Bradley, 1997). The reported results are averages over 100 nested 10-fold cross-validation runs to minimize the effect of the random variation. To compare the mean AUCs of 2 learning algorithms, we computed a p value for the 100 AUC scores with a permutation test (Anderson and Robinson, 2001). The nonparametric permutation test is selected as it provides a flexible and intuitive methodology for the statistical analysis with only requiring minimal assumptions for validity. Permutation methods are becoming more popular because of their flexibility and ease of concern compared with parametric tests, which rely on assumptions that may be difficult to meet in the data (Good, 2013; Ludbrook and Dudley, 1998; Nichols and Holmes, 2002).

The pathway analysis method (LDA) was implemented by means of the MASS package in the R software. The features were standardized before applying LDA, so that they have the properties of a standard normal distribution. Moreover, for identifying outliers in data, PCA was used; however, there was not observed any serious outlier for leaving it out from the analysis. The implementation of PLAGE was carried out by the GSVA R package (Hänzelmann et al., 2013), and the implementation of RLR was conducted using the GLMNET library (Friedman et al., 2010b), and the regularization parameter λ was selected via 10-fold cross-validation in the training data. For stratified cross-validation, we used the CARET package (Kuhn, 2017), and for plotting the figures, we used ggplot2 package (Wickham, 2016).

3. Results

3.1. Classification performance

We first examined the performance of the proposed pathway analysis procedure on discrimination of patients with AD or MCI from healthy subjects on the basis of peripheral blood gene expression data. Next, for demonstrating the advantage of the pathway level analysis of gene expression data over single–gene-level analysis, we performed computational experiments by excluding the pathway level analysis step and applying RLR to all the gene expression data after adjustment for batch effects by means of ComBat. Furthermore, to evaluate effectiveness of the LDA-based pathway level analysis of gene expression data, we repeated the experiments via the PLAGE approach (Tomfohr et al., 2005) for pathway analysis. The results of all these computational experiments are listed in Table 2. These results are the average over

Table 2
A comparison of the performance of our proposed pathway level analysis with PLAGE pathway analysis and gene level classification in the problems AD versus CTL and MCI versus CTL

	Pathway level (LDA + RLR)	Pathway level (PLAGE + RLR)	Gene level (RLR)
AD versus CTL			
AUC	0.842 (0.005)	0.82 (0.004)	0.817 (0.004)
ACC	0.764 (008)	0.72 (0.0075)	0.74 (0.008)
SEN	0.811 (0.01)	0.797 (0.008)	0.826 (0.01)
SPE	0.708 (0.013)	0.634 (0.013)	0.638 (0.14)
MCI versus CTL			
AUC	0.794 (0.007)	0.766 (0.005)	0.765 (0.007)
ACC	0.73 (0.01)	0.697 (0.009)	0.70 (0.01)
SEN	0.663 (0.017)	0.643 (0.014)	0.645 (0.016)
SPE	0.78 (0.012)	0.74 (0.011)	0.742 (0.011)

The results are averages over 100 computation repetitions. For the classification accuracy (ACC), the chance level is 54.3% for classification of AD versus CTL and 55.7% for classification of MCI versus CTL.
Key: AD, Alzheimer's disease; AUC, area under the receiver operating characteristic curve; CTL, control; LDA, linear discriminant analysis; MCI, mild cognitive impairment; RLR, ridge logistic regression; SPE, specificity; SEN, sensitivity.

100 independent experiments for each method, along with standard deviation in brackets. Moreover, the box plots for the performance measures of different methods are illustrated in Fig. 2. According to the results (shown in Table 2 and Fig. 2), the classification performance is better in both cases under study (AD vs. CTL and MCI vs. CTL) with our method of LDA-based pathway analysis compared with both PLAGE pathway analysis ($p < 0.001$) and gene level model ($p < 0.001$). In addition, the AUC was highly significant with all 3 approaches (LDA and PLAGE pathway level assessment and gene level model) for both classification problems (AD vs. CTL and MCI vs. CTL). These results indicated the power of the blood gene expression data for diagnosis of AD at different stages. We also repeated our experiments for classification of patients with AD versus MCI. The experiments yielded the average AUC of 0.63 with LDA pathway level assessment and AUC of 0.65 for single–gene-level model. It appeared that classification of AD versus MCI is more difficult than both AD versus CTL and MCI versus CTL classification problems, based on blood gene expression data. Moreover, our LDA pathway analysis does not provide improvement for this problem; one possible reason could be that the data are quite unbalanced for classification of AD versus MCI (284 AD subjects and 190 MCI subjects), which might have a negative effect on the performance of LDA pathway analysis step (Xie and Qiu, 2007).

3.2. Top pathways

We provided an estimate of pathway importance for classification of AD versus CTL and MCI versus CTL. The importance of pathways is calculated from the average magnitude of regression coefficients in RLR, that is, the final step for combining pathway scores within 100 independent computation repetitions of 10-fold cross-validation. Because we standardized the data before applying RLR, the magnitude of regression coefficients denotes the importance of each predictor (pathways) in the classification model. We selected only pathways with the average magnitude higher than 0.01. Supplementary Figures 1 & 2 show the importance of top pathways for classification of AD versus CTL and MCI versus CTL, respectively. Because the classification of AD versus CTL is a much easier problem in comparison with MCI versus CTL, a smaller number of pathways effectively contributed to the classification model.

To further explore the discriminative power of the top selected pathways, we constructed a parallel coordinate plot for visualization of the 9 most useful (important) pathways. Fig. 3 illustrates the

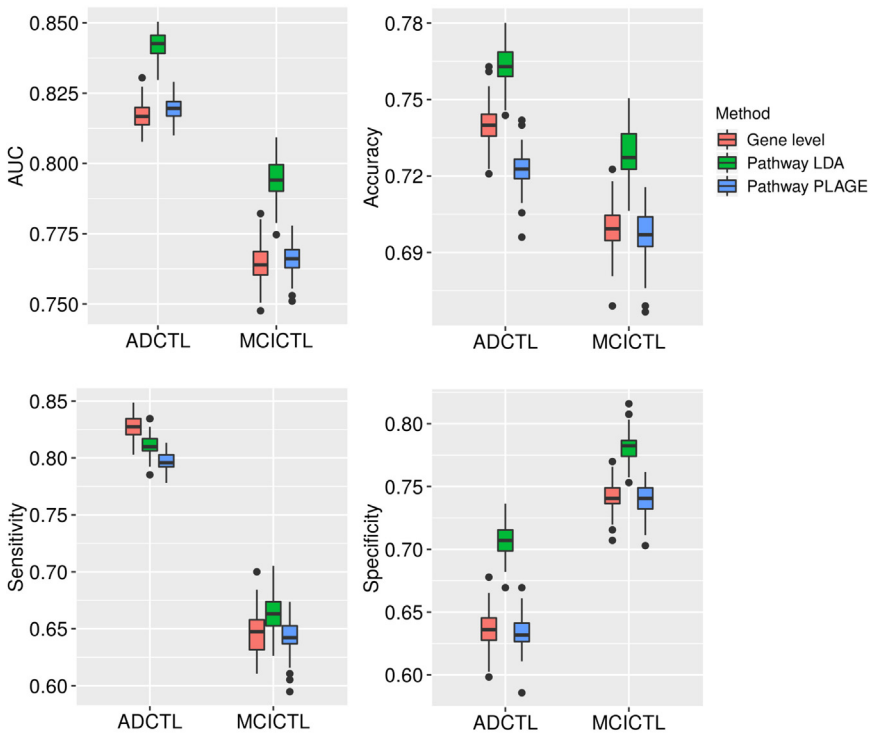

Fig. 2. Box plots for AUC, ACC, SEN, and SPE within 100 computation repetitions of our proposed pathway analysis procedure (pathway + LDA), PLAGE (pathway + PLAGE), and the gene level model in terms of classification of AD versus CTL and MCI versus CTL subjects. In each box, the central mark shown in black is the median, the edges of the box are the 25th and 75th percentiles, whereas the whiskers extend to the most extreme data points not considered outliers, and outliers are plotted as dots. Abbreviations: AD, Alzheimer's disease; AUC, area under the receiver operating characteristic curve; ACC, accuracy; CTL, control; LDA, linear discriminant analysis; SPE, specificity; SEN, sensitivity; MCI, mild cognitive impairment; PLAGE, Pathway Level Analysis of Gene Expression.

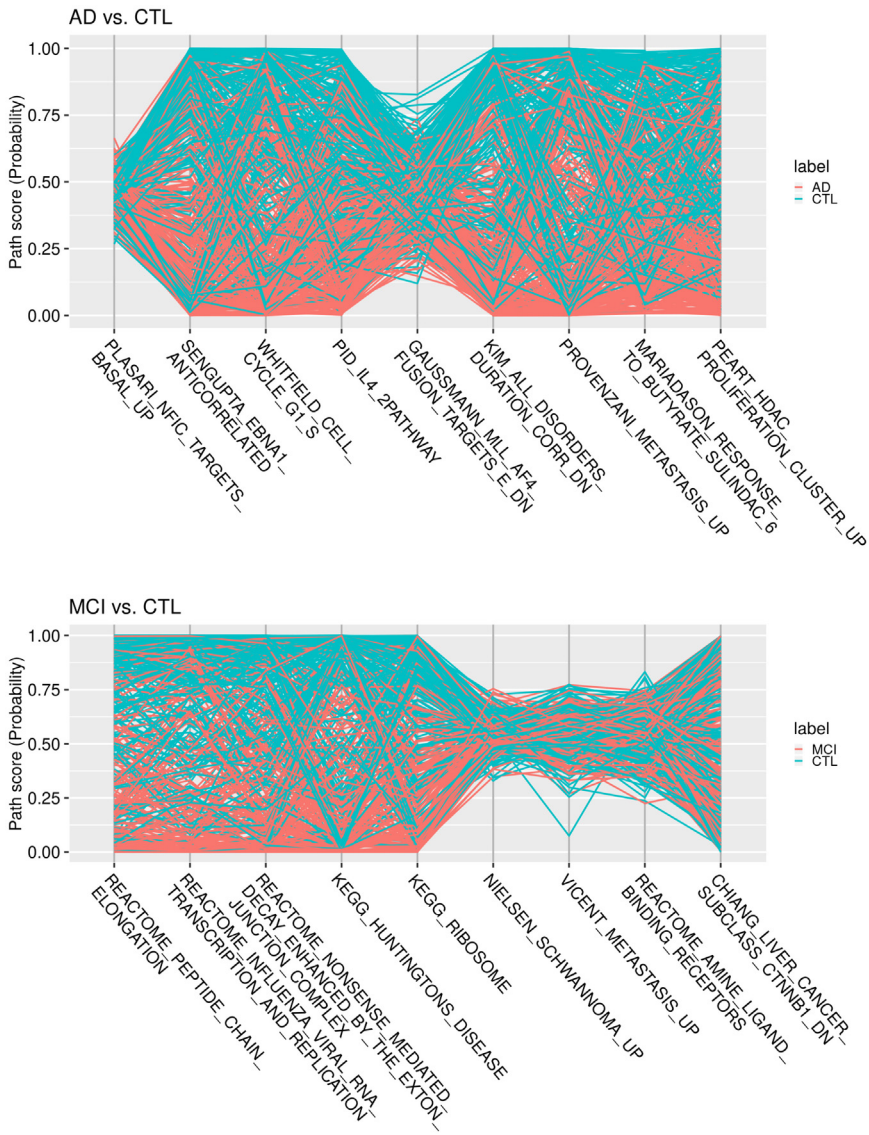

Fig. 3. A parallel coordinate plot for top 9 pathway scores on discrimination of AD from CTL (top) and MCI from CTL (bottom) for visualizing discriminative power of the top pathways. Abbreviations: AD, Alzheimer's disease; CTL, control; MCI, mild cognitive impairment.

parallel coordinate plot for path scores (the probability score for each category derived at the step of LDA-based pathway analysis) in terms of discrimination of AD from CTL (top) and MCI from CTL (bottom). According to Fig. 3 (top), the first (PLASARI_NFIC_TARGETS_BASAL_UP) and fifth (GAUSSMANN_MLL_AF4_FUSION_TARGETS_E_DN) pathways have lower discriminative power compared with the other presented pathways in terms of discrimination of patients with AD from healthy subjects. Nevertheless, as for discrimination of MCI from CTL, the first 5 pathways have higher discriminative power as compared with the last 4 pathways. It should be noted that LR is a multivariate classification algorithm that takes into account the effect of all variables on the response variable and does not separately consider the discriminative power of each variable alone. Therefore, although some pathways are not highly discriminative alone, they may provide significant information together with other pathways. Moreover, the LR classifier is used with a ridge penalty that tends to shrink the coefficients of predictors correlating with each other, thereby resulting in smaller coefficients for correlating predictors, despite having high discriminative power (Jang et al., 2013). Therefore, the coefficients of the highly correlating pathways are very well associated with each other.

In addition, we calculated the percentage of genes (from the total number of genes) in the overlap between the top selected pathways to show how independently the top pathways contribute to the discrimination of patients with AD or MCI from CTL subjects. According to Fig. 4, there is a minimal overlap in genes among the 9 selected pathways for discrimination of AD from CTL subjects. This finding in turn indicates that those pathways contributed to the classification task independently. On the other hand, Fig. 5 shows high percentages of genes in the overlaps between some pathways in terms of discrimination of MCI from CTL subjects. The percentage of genes in the overlap is maximal between the 2 most significant pathways, having 88% of common genes.

To look through pathways and find how differently pathways are expressed between 2 groups of subjects, we calculated the difference in average gene expression levels for each single gene that is included in the top pathways. Fig. 6 shows the box plots of the difference in the average gene expression level between groups AD and CTL (top) and between groups MCI and CTL (bottom) for the

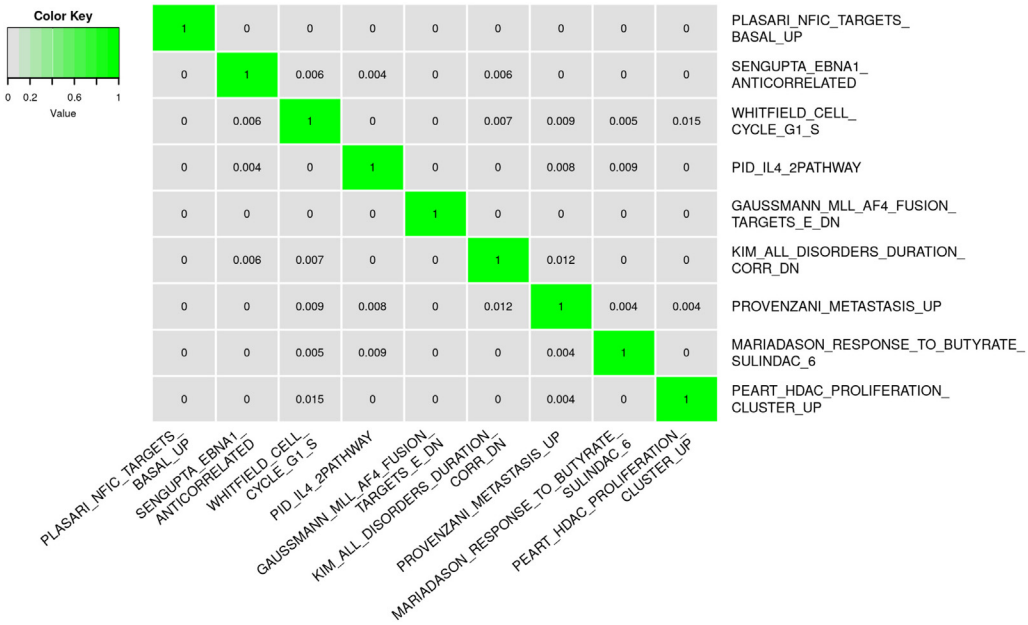

Fig. 4. The percentages of genes (relative to the total number of genes) in the overlaps between the 9 pathways most helpful for classification of AD versus CTL subjects. Abbreviations: AD, Alzheimer's disease; CTL, control.

top pathways. We omitted the genes that are excluded by low variance filtering at the pathway analysis step.

Moreover, we provided a subset of the most discriminative genes within the top 9 selected pathways for classification of AD versus CTL and MCI versus CTL. The discriminative power of each gene within the top pathways is calculated from the average magnitude of the discriminant coefficients at the step of LDA-based pathway analysis, within 100 independent computation repetitions of 10-fold cross-validation. Supplementary Figures 3 & 4 illustrate the top 10 discriminative genes for top 9 selected pathways for classification of AD versus CTL and MCI versus CTL, respectively.

Although the pathway-level model performs better than gene-level model in terms of classification performance, we studied the selected features by the gene-level model with enrichment analysis of genes corresponding top selected features. The Go analysis (biological process [BP]) was carried out through ToppGene (Chen et al., 2009) and the GO categories with FDR corrected ($p < 0.05$) were selected. Supplementary Fig. 5 illustrates the results corresponding to GO analysis of selected genes by the gene-level model in classification of AD versus CTL. As illustrated in this figure, an enrichment for the terms "nucleotide-excision repair, precision complex assembly," "cellular response to glucocorticoid stimulus," and "cellular response to corticosteroid stimulus" is seen. Given the

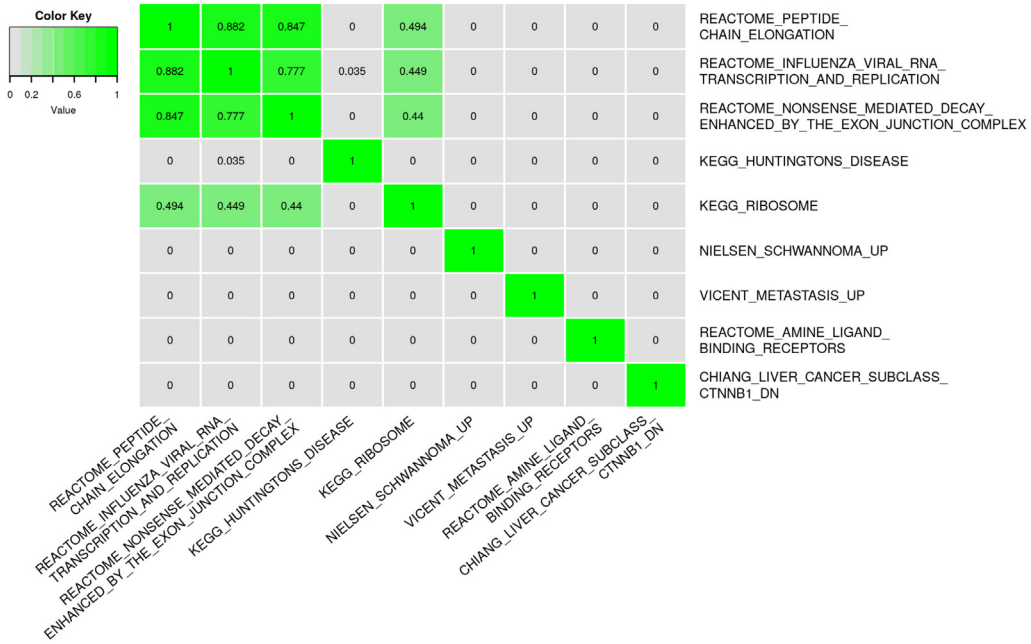

Fig. 5. The percentages of genes (relative to the total number of genes) in the overlaps between the 9 pathways most helpful for classification of MCI versus CTL subjects. Abbreviations: CTL, control; MCI, mild cognitive impairment.

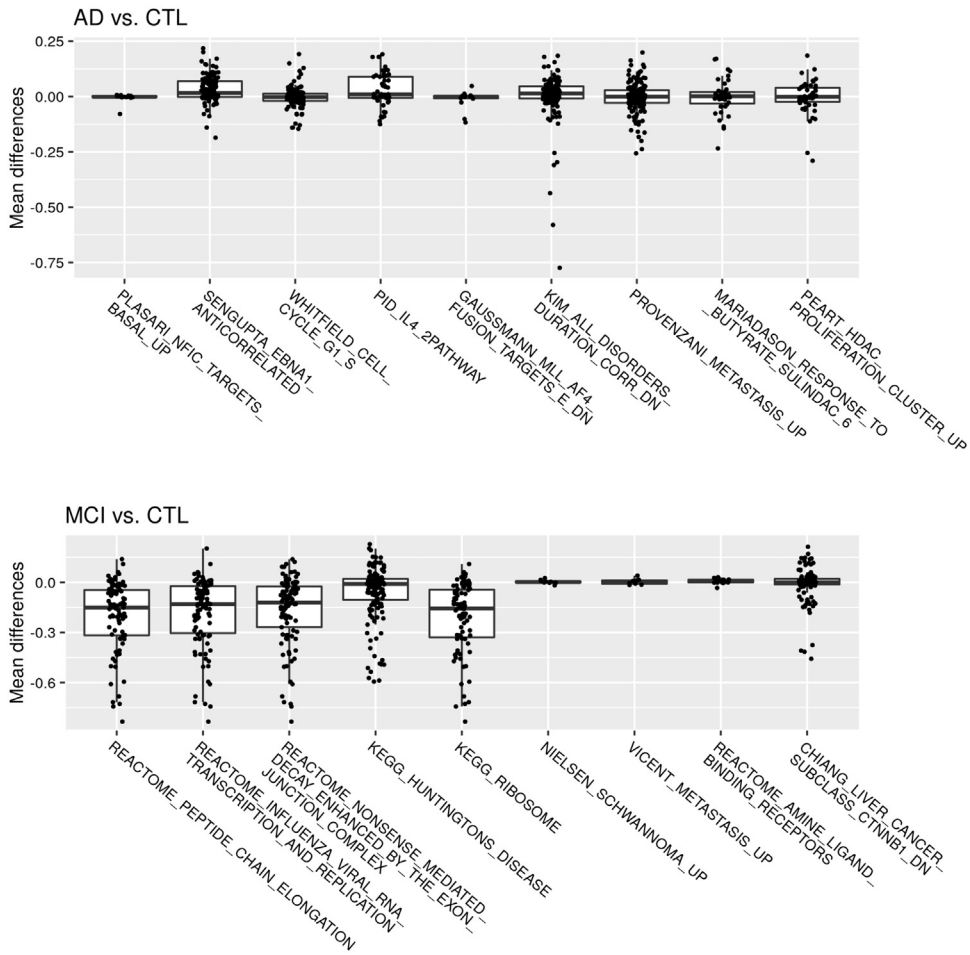

Fig. 6. Box plots for the difference in the average gene expression level between groups AD and CTL (top) and between groups MCI and CTL (bottom) in the 9 selected top pathways. Each dot indicates an individual gene. In each box, the central mark is the median, the edges of the box are the 25th and 75th percentiles, and the whiskers extend to the most extreme data points not considered outliers. Abbreviations: AD, Alzheimer's disease; CTL, control; MCI, mild cognitive impairment.

immense oxidative stress on neurons in Alzheimer's disease, an abundance in DNA damage is inevitable, potentially explaining the enrichment for nucleotide-excision repair complex assembly. In turn, increased corticosteroid response may be a result of increased corticosteroid production as an immunosuppressive response to neurodegeneration-associated inflammation observed in our pathway-level analysis. For MCI versus CTL model, the Go analysis (BP) could not provide any category that be significantly enriched after correction.

4. Discussion

The purpose of this study was twofold: to devise a novel approach to pathway level analysis for studies on gene expression data and to investigate the potential utility of blood gene expression data for diagnosis of AD. In this study, we introduced a new supervised pathway level analysis for gene expression data based on LDA and validated this method in terms of discrimination of AD or MCI patients from healthy elderly subjects. The idea was to test whether discrimination of patients with AD or MCI from CTL subjects is more accurate using pathway level analysis of gene expression data as compared with single–gene-level analysis. In particular, pathway level analysis of gene expression data has several advantages over single–gene-level analysis (Curtis et al., 2005), including robustness of the analysis owing to the inherent dimension reduction that also enables detection of weak differences between groups. Moreover, summarizing genes into biological pathways provides greater power of identification of a specific biological mechanism and may lead to implication of previously unknown biological processes.

We evaluated the proposed pathway analysis on data from GEO from 2 batches of a data set and used blood gene expression data to discriminate patients with AD or MCI from healthy subjects. To this end, we developed a novel two-stage approach to learning, consisting of a pathway analysis step that involves LDA for finding a linear combination of features (genes within a pathway) best separating groups of subjects within each pathway, and the second learning stage, which involves RLR for integrating pathway scores derived from the first stage. This way, first, a single real-valued score is derived for each pathway, and thereafter, these scores are concatenated as a new feature set for the RLR classifier, which acts as a combiner. As the results show (see Table 2), the proposed two-stage learning method performed markedly better than did a single–gene-level model ($p < 0.0001$) and a PLAGE-based pathway level model ($p < 0.0001$).

Several studies have investigated the usefulness of peripheral-blood gene expression data for diagnosis of AD (Doecke et al., 2012; Lunnon et al., 2013; Ray et al., 2007; Voyle et al., 2016). For instance, a recent study by Lunnon et al., 2013 defined an AD diagnostic classifier based on peripheral blood gene expression on AddNeuroMed data. They used 78 AD and 78 CTL samples as training data and 26 AD, 26 CTL, and 118 MCI as validation group.

Their 48 gene classifier based on Random Forest model achieved accuracy of 70% for classification of AD versus CTL subjects. They further compared the results of blood-based diagnostic classifier with the magnetic resonance imaging–based classifier, which was 85%. Another study by Rye et al., 2011 investigated the discriminative performance of blood gene expression data in 103 AD and 105 CTL samples and validated the results on 2 different validation sets. The performance reached to accuracy of 71.6% and 71.5% in the 2 separate validation sets. A more recent and similar study to us by Voyle et al., 2016 evaluated the use of blood gene expression data for classification of preclinical and clinical stages of AD. They implemented the PLAGE method of pathway analysis for classification of AD versus CTL subjects and compared the results with a gene level model. They showed that the PLAGE-based pathway level model performed similarly to the gene-level model regarding classification of AD versus CTL subjects. They achieved an AUC of 0.729 for classification of AD versus CTL via the PLAGE-based pathway model and 0.724 with the gene-level model. Our computational analyses yielded similar results; the findings of the present study show similar performance on classification in both problems (AD vs. CTL and MCI vs. CTL) for the gene-level model and PLAGE-based pathway-level model. Nevertheless, we achieved better discriminative performance for classification of AD versus CTL subjects using both the PLAGE-based pathway-level model (AUC = 0.82) and the gene-level model (AUC = 0.817). The improved classification performance of our versions of the procedures may be due to a more suitable method for learning a model from the gene or pathway scores (RLR in our experiments in contrast to random forests used by Voyle et al., 2016). Apart from those results showing similar performance for the gene-level model and PLAGE-based pathway-level model, our newly developed LDA-based pathway analysis notably improved the classification performance in both problems under study. We computationally demonstrated that the LDA-based pathway-level model shows significantly higher classification performance as compared with both the gene-level model and PLAGE-based pathway-level model.

It is essential to know which pathways are more discriminative and contain more information for discrimination of 2 groups because these data may provide insight into new biological findings. Here, we found a subset of pathways helpful for classification of AD versus CTL and MCI versus CTL subjects on the basis of the absolute value of the regression coefficient of RLR in the second learning stage. In addition, we identified important genes within the pathways that are most discriminative in the classification of 2 groups according to the discriminant coefficient at the pathway analysis stage. Because each pathway consisted of many genes, it is not straightforward to determine upregulated or downregulated pathways. For this purpose, we provided a box plot for the top selected pathways that are helpful for discrimination of AD or MCI from CTL subjects, illustrating a difference in the average gene expression level between group AD or MCI and group CTL. This way, we can determine the upregulated or downregulated pathways according to the median value of the difference in the average gene expression level between the 2 groups, among all the genes in each pathway. The top 5 pathways differentiating MCI from CTL subjects consist largely of ribosomal proteins with a large percentage (77%–84%) of genes overlapping among the top 3 and the fifth pathways. Elsewhere, it was shown that mRNA levels of ribosomal proteins significantly decrease at the early stages of AD, before the onset of cell death (Hernándes-Ortega et al., 2015; Marttinen et al., 2019). Furthermore, it has been reported that pathological tau protein associates with ribosomes in the AD brain, resulting in an overall decrease in translation of vital synaptic proteins (Meier et al., 2016). Memory formation and synaptic integrity are in particular reliant on the dynamic control of mRNA translation initiation and elongation with defects in these processes being linked to several cognitive disorders (Kapur et al., 2017). Thus, this may underscore a significantly undermined process affected at the early phases of AD. However, given the broad spectrum of neurodegenerative diseases showing defects in proteins associated with mRNA translational regulation raises the question of the specificity of these findings for AD, requiring further follow-up–based work. In line with these previous findings of the importance of mRNA translational regulation on synaptic integrity and function, we observed a modest downregulation of synaptic proteins (e.g., NRXN1, NLGN4X, L1CAM, and PLP1) in the NIELSEN_SCHWANNOMA_UP pathway, differentiating MCI versus CTL subjects. In addition, a decrease in the expression of genes associated with energy production (e.g., NADH dehydrogenase [ubiquinone] 1 subunits, cytochrome C oxidase subunits, ATP synthase subunits) was seen in the KEGG_HUNTINGTON_DISEASE pathway, in agreement with the notion that defects in synaptic and mitochondrial functions are among the earliest pathological events in AD (Du et al., 2010; Gillardon et al., 2007; Marttinen et al., 2019). Consequently, an increase in expression of inflammation-associated PID_IL4_2PATHWAY genes (e.g., IL10, PIK3R1 [PI3K subunit p85], SPI1, AKT1) differentiated AD from CTL subjects, possibly reflecting the onset of inflammation and neurodegeneration. PI3K-AKT signaling has previously been described to take part in regulating mTOR signaling and autophagy, inflammatory response, and regulation of tau phosphorylation via GSK3β activation regulation (Kim and Feldma, 2012). The observed increase in PIK3R1 and AKT1 may thus reflect the activation of a recently identified subset of microglia-denoted disease-associated microglia (DAM), as PI3K-AKT signaling is directly downstream of TREM2, a key receptor in the activation and initiation of transition from homeostatic microglia to DAM microglia (Keren-Shaul et al., 2017). Another key gene associated with DAM microglia is SPI1, which was observed to be increased in patients with AD in comparison with CTL subjects (Krasemann et al., 2017). Concomitantly, an increased proportion of cell cycle–related genes (SENGUPTA_EBNA1_ANTICORRELATED (e.g., NIN, SCYL1), WHITFIELD_CELL_CYCLE_G1_S) and mitochondrial energy production–associated genes (KIM_ALL_DISORDERS_DURATION_CORR_DN (NFUDA1, COX7C)) was observed in patients with AD in comparison with CTL subjects. This may further reflect the activation and proliferation of microglia in the brain. In addition, part of the genes showing high predictive value in KIM_ALL_DISORDERS_DURATION_CORR_DN is associated with microtubules and intracellular trafficking, and lysosomal acidity, potentially portraying an autophagosomal response to increased aggregation of amyloid-β and neurofibrillary tangles. The increase in cell cycle–associated genes in blood may also be the result of neurons being exposed to oxidative stress, DNA-damaging agents, or specific neurotoxins (including amyloid-β), causing neurons to undergo mitotic pressure resulting in an increased number of cell cycle events (Herrup, 2010). Nonetheless, the direct causal relation between an increase in cell cycle events and AD-associated neuron death has not been shown. In conclusion, our method identified key pathways described to be altered in AD, highlighting alterations in specific genes to have high predictive capability in differentiating CTL subjects from MCI and AD subjects. Moreover, our results indicate a decrease in expression for a substantial number of ribosomal genes in MCI subjects in comparison with CTL subjects, warranting further research on the role of these alterations in the early stages of AD pathology.

The results presented in this study are particularly exciting because they reveal the potential role of a blood gene expression profile as a diagnostic biomarker for AD. Although AD is a brain disorder, pathological changes due to AD are not limited to the brain. In recent years, an increasing number of studies addressed

disease-related changes that are detectable in blood (Goldsmith et al., 2016; Kawata et al., 2016; Lewczuk et al., 2017; Liew et al., 2006). They have found that peripheral blood as a tissue contains significant information for detecting different physiological and pathological changes. Thus, gene expression analysis of blood samples may offer an opportunity to study disease-related changes in noninvasively obtained samples in large quantities. Here, we used only blood gene expression profiles for detecting AD; however, the integrative analysis of various blood-associated metrics such as gene expression, protein expression, and metabolites may significantly improve the accuracy of blood-based biomarkers for AD detection. Although here we evaluated gene expression profiles only from blood, the classification accuracy was highly significant for discrimination of both AD and MCI patients from CTL subjects. In particular, the highly significant classification accuracy for distinguishing patients with MCI from healthy elderly people is quite interesting. Given that MCI is regarded as the earliest (preclinical) detectable stage subject to progression toward AD and because the disease-related changes are weak, detection of such changes and classification of patients into this stage are quite difficult. Nonetheless, our results indicate that alterations in a blood gene expression profile are detectable even at the MCI stage.

It bears repeating that the procedure of LDA-based pathway analysis and the presented learning framework are suited not only for blood-based gene expression data but also for any similar information such as RNA-seq data. The presented results helped demonstrate the validity of our methods and of their use for pathway analysis and for classification at the pathway level instead of a single-gene level. On the other hand, the gene expression measurements do not have to be based on blood, and the classification problem does not have to be limited to AD.

Disclosure

The authors have no actual or potential conflicts of interest.

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Appendix A. Supplementary data

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