



## Global expression profiling of cognitive level and decline in middle-aged monozygotic twins



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### ABSTRACT

Only few studies have investigated the genomewide transcriptome of normative cognitive aging. We therefore aimed at investigating blood gene expression patterns associated with cognitive aging using a population-based sample of 235 middle-aged monozygotic twin pairs with longitudinal data on cognitive function. This unique setup enabled examination of gene expression differences associated with individual and intrapair differences in cognitive level and change while controlling for underlying genetic variation and shared early environment. Overall, increased expression of several gene sets was found to strongly correlate with a lower cognitive level and cognitive decline. The most significantly correlated gene sets were related to protein metabolism, translation, RNA metabolism, infectious disease, and the immune system, which are all processes previously linked to transcription signatures of pathological and normal brain aging, and aging in blood. The results of our study thus suggest that gene expression patterns of cognitive level and decline in our sample mirror those seen in cognitively impaired individuals, which could point toward a more generic response to cognitive aging and aging in general.

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### 1. Introduction

A better understanding of the mechanisms underlying pathological as well as nonpathological cognitive aging is of great importance for the preservation of mental health in late life. To this end, genomewide gene expression studies offer an excellent approach for the identification of the molecular features that are in part responsible for individual differences in the maintenance of cognitive function.

Most previous expression profiling studies of cognitive aging have had a clinical focus and have characterized abnormalities in transcriptional patterns in patients with Alzheimer's disease (AD). As AD is a disease of the brain, these studies have often involved the investigation of various postmortem brain tissues (e.g.,

Avramopoulos et al., 2011; Blalock et al., 2004; Clement et al., 2016; Colangelo et al., 2002; Diedrich et al., 1991; Loring et al., 2001; Lukiw, 2004; Silva et al., 2012; Tan et al., 2010; Weeraratna et al., 2007; Wilmot et al., 2008; Zhang et al., 2013), whereas gene expression studies in blood have been implemented mainly with the aim of identifying biomarkers for early detection of AD (Booij et al., 2011; Fehlbaum-Beurdeley et al., 2010; Han et al., 2013; Kalman et al., 2005). Although there is no requirement that a transcriptional signature in blood accurately mirrors signatures of the brain in order for it to be a good biomarker, there does seem to be a reasonably high agreement between genes expressed in blood and genes expressed in the brain (Rollins et al., 2010; Sullivan et al., 2006; Tylee et al., 2013). In accordance, several studies performing transcriptional profiling of blood have documented a more systemic nature of expression changes in AD that correlates well with brain changes, and thus can be informative about underlying biological mechanisms (Han et al., 2013; Lunnon et al., 2012; Maes et al., 2007; Naughton et al., 2015).

Across studies of blood and brain tissue the differential expression of genes involved in one or more of numerous processes

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have been highlighted, including (but not limited to) genes related to energy metabolism, protein metabolism, immunity and inflammation, neurological functions, signaling pathways, various cellular functions, stress response, DNA damage/repair, and apoptosis (Avramopoulos et al., 2011; Blalock et al., 2004; Booij et al., 2011; Colangelo et al., 2002; Fehlbauer-Beurdeley et al., 2010; Han et al., 2013; Kalman et al., 2005; Loring et al., 2001; Lukiw, 2004; Lunnon et al., 2012; Maes et al., 2007; Naughton et al., 2015; Silva et al., 2012; Tan et al., 2010; Weeraratna et al., 2007; Wilmot et al., 2008; Zhang et al., 2013).

Besides AD, some reports of transcriptomic profiling have included also individuals with mild cognitive impairment (MCI) and incipient dementia (Berchtold et al., 2014; Lunnon et al., 2012), and others have characterized the course of change in brain gene expression following age, that is, mirroring normal cognitive aging (Berchtold et al., 2008, 2013; Cribbs et al., 2012; Lu et al., 2004; Nikas, 2013). Interestingly, such studies have found a widespread deregulation of genes enriched for several of the aforementioned processes.

With the exception of the study by Harries et al. (Harries et al., 2012), where blood gene expression patterns were related to cognitive level and rate of change assessed by the Mini-Mental State Examination in 688 individuals with a mean age of 72.6 years at intake and 9 years of follow-up, most studies relating transcriptional changes to brain aging generally have not attempted to associate the profiles to measures of cognitive functioning during the normative cognitive aging of individuals in the middle-aged and young elderly age groups. Consequently, in this study, we therefore aimed at identifying transcriptional changes correlated with differences in cognitive ability as assessed by a battery of 6 cognitive tests representing tasks that are sensitive to normative aging. Our sample was 470 middle-aged monozygotic (MZ) Danish twins (235 twin pairs), with 2 measures of general cognitive ability in a 10-year interval. Genomewide expression profiling was performed at follow-up enabling studies of both level and (preceding) rate of change of cognitive function. The use of an MZ twin sample allowed us to ascertain possible associations irrespective of genetic liabilities to changes in gene expression.

## 2. Materials and methods

### 2.1. Study population

The study sample consisted of 235 monozygotic (MZ) twin pairs drawn among participants in the study of Middle-Aged Danish Twins, a longitudinal population-based and nationwide investigation of twins who were randomly selected among eligible pairs from birth cohorts 1931–1952. Middle-Aged Danish Twins was initiated in 1998, with the baseline study sample comprising 4314 twins aged 46–68 years (mean age 56.8 years, SD = 6.4 years) of which 51.3% were males. The study included an in-person interview comprising an extensive questionnaire including several questions on mental and physical health as well as cognitive and physical tests. Approximately 10 years later, from 2008 to 2011, 2402 of the surviving baseline twins participated in a follow-up assessment. Mean age at follow-up was 66.5 years (SD = 6.2 years) and 54.0% were males (Skytthe et al., 2013). Zygosity was established by means of 4 questions on physical similarity, which has a sensitivity of more than 95% (Christiansen et al., 2003).

Across the 470 individuals included in the present study, the average interval between the baseline and follow-up assessments was 10.7 years (SD = 0.88 years). Within the twin pairs, the average difference in interval between the baseline and follow-up assessments between twins was 0.36 years (SD = 0.56 years).

Written informed consents were obtained from all participants. Collection and use of biological material and survey information were approved by the Regional Scientific Ethical Committees for Southern Denmark, and the study was approved by the Danish Data Protection Agency.

### 2.2. Assessment of cognitive function and blood cell counts

The cognitive assessment consisted of 6 brief cognitive tests, of which 3 (task 1–3) were adapted from the neuropsychological assessment proposed by the Consortium to Establish a Registry for Alzheimer's Disease (Morris et al., 1989). The testing protocol was the same at both assessments: 1) a verbal fluency test (the number of animals named in a minute), 2) an immediate word recall test (from a 12-word list), 3) a delayed word recall test (recall of items from the 12-word list after approximately 10 minutes), 4–5) an attention and working memory test (digits forward and digits backward), and 6) a processing speed test (digit-symbol replacement) (McGue and Christensen, 2002). Scores from each of the 6 tasks were standardized using the means and standard deviations of the participants who were less than 50 years of age at baseline, and finally, a cognitive composite score was computed by aggregating the 6 standardized scores. If one item was missing, the score was multiplied by 6/5 and if more than one item was missing, the composite score was coded as missing.

Blood leukocyte subtypes (basophils, eosinophils, lymphocytes, monocytes, and neutrophils) were counted using a Coulter LH 750 Hematology Analyzer (Beckman Coulter, Woerden, the Netherlands).

### 2.3. RNA extraction and gene expression profiling

Whole blood was collected in PAXgene Blood RNA Tubes (Pre-AnalytiX GmbH, Hombrechtikon, Switzerland) and total RNA was extracted using the PAXgene Blood miRNA kit (QIAGEN) according to the manufacturer's protocol. The concentration of the extracted RNA was determined using a NanoDrop spectrophotometer ND-8000 (NanoDrop Technologies), and the quality was assessed by the Agilent 2100 Bioanalyzer (Agilent Technologies).

Gene expression profiling was performed using the Agilent SurePrint G3 Human GE v2 8 × 60K Microarray (Agilent Technologies). This array contains 62,976 60-mer probes, including 96 × 10 and 10 × 32 positive controls, 900 × 10 replicates of biological probes, and 50,599 biological probes representing 21,316 unique genes. Sample labeling and array hybridization were carried out according to the "Two-Color Microarray-Based Gene Expression Analysis—Low Input Quick Amp Labeling" protocol (Agilent Technologies). Samples were labeled with Cy5 and the reference consisting of a pool of 16 samples was labeled with Cy3. Hybridization, washing, scanning, and quantification were performed according to the array manufacturer's recommendations.

### 2.4. Data preprocessing

Agilent Feature Extraction software v. 10.7.3.1 (Agilent technologies) was used to analyze acquired array images. The raw intensity data were background-corrected using the normexp method and was then within-array normalized by Loess normalization method and between-array normalized by Quantile normalization (Yang et al., 2002; Yang and Thorne, 2003). The normalized values were used to calculate log<sub>2</sub>-transformed Cy5/Cy3 ratios. Missing expression values were imputed by k-nearest neighbors averaging, and replicate probes were collapsed calculating the median. Data preprocessing was performed using the R package *limma* (Ritchie et al., 2007). All the probes on the Agilent SurePrint G3 array

were reannotated using GENCODE v.25 gene annotation database ([www.genecodegenes.org](http://www.genecodegenes.org)) (Harrow et al., 2012).

### 2.5. Statistical analysis

The association between gene expression, as the dependent variable, and cognitive level assessed at follow-up or cognitive change during the preceding 10-year period as the independent variable was investigated using 2 different approaches: individual-level (see [Supplementary Material, Script 1](#)) and intrapair (see [Supplementary Material, Script 2](#)) analyses.

In the individual-level analyses, all individuals were considered as singletons while performing regression analyses using linear mixed models to adjust for twin-pair relatedness. The twin-pair identifier was coded as a random effect. The over time cognitive change was calculated by subtracting the cognitive level at intake from the cognitive level at follow-up.

In the intrapair analyses, within twin-pair differences were investigated using linear regression models. The twins within a pair were randomly assigned the number 1 or 0 and the differences in gene expression values, cognitive level or cognitive change over time, and relevant covariates were calculated by subtracting the values for twin 0 from those of twin 1.

All analyses were adjusted for sex, age, and leukocyte cell composition (the percentwise distribution of blood leukocyte subtypes). Nested analyses without adjusting for leukocyte cell composition were also performed.

In all analyses, adjustment for multiple testing was performed by the Benjamini & Hochberg false discovery rate (FDR) correction method (Benjamini and Hochberg, 1995).

All analyses were carried out in R.

### 2.6. Gene set enrichment analysis

To gain insight into the biological processes involving the differentially expressed genes, we performed gene set enrichment analysis (GSEA) using the Gene Set Enrichment Analysis software (Mootha et al., 2003; Subramanian et al., 2005). Genes were pre-ranked using the  $\beta$ -values obtained in the association analyses, and reference gene sets were derived from the Reactome Pathway Database (Fabregat et al., 2018) through the Molecular Signature Database (Liberzon et al., 2011; Subramanian et al., 2005).

The results of the GSEA were organized in networks using the Cytoscape (Shannon et al., 2003) plug-in “Enrichment Map” (Merico et al., 2010). In the networks, gene sets are represented by nodes, with the size of the node indicating the number of genes included in the gene set (the larger the node, the more genes are included in the gene set). Edges represent the overlap between the gene sets, with the thickness of the edge marking the overlap in genes between the connected gene sets (the thicker the edge, the larger the overlap between connected gene sets). Only highly significant gene sets ( $p < 0.001$  and  $FDR < 0.05$ ) were included in the networks. Gene sets with a negative normalized enrichment signal (NES) were visualized in blue with the color gradient indicating the absolute size of the NES (darker colors represent lower NES and hence, generally, a more significant enrichment), and gene sets with a positive NES were visualized in red. As only a few gene sets with positive NES made the significance threshold, no color gradient was applied there.

The overall function of the different gene sets were determined by looking up the individual gene sets and their overall function on the Reactome webpage (<https://reactome.org>).

## 3. Results

Descriptive characteristics of the study participants are provided in [Table 1](#). As expected, the mean cognitive function of the study participants declines during the 10-year follow-up period. The absolute intrapair difference declines as well, although only modestly. This suggests that the twins within a twin pair become slightly more similar with age, even though the range suggests that the variation among the entire study population increases.

### 3.1. Single-transcript analysis

Altogether, 4 different analyses were performed as the association between gene expression differences and individual and intrapair differences in cognitive level and cognitive change over time was explored. Depending on the analysis, up to 28,505 protein-coding transcripts were studied. Among these, no single transcripts were found to significantly associate ( $FDR < 0.05$ ) with individual or intrapair cognitive level or change (results not shown). However, when applying a less-stringent cutoff ( $FDR < 0.20$ ), 2 suggestive hits were found: a negative association between individual cognitive level and expression of the *POU6F1* gene ( $FDR = 0.09$ ) and a negative association between individual cognitive change and expression of the *MAD2L1* gene ( $FDR = 0.16$ ).

If focusing on the effect estimates instead of the significance level, a clear tendency of lower effect sizes among the negatively associated genes was found across the different analyses. However, a nearly 50:50 distribution between positively and negatively associated genes was seen among the nominally significant transcripts. The 20 most positively and negatively associated genes ( $p < 0.05$ ) sorted by the size of the effect estimates are shown in [Supplementary Material, Table S1–S4](#).

### 3.2. Gene set enrichment analysis

The results of the GSEA for the 4 different models (association between gene expression differences and individual and intrapair differences in cognitive level and cognitive change over time, respectively) are shown in [Fig. 1A–D](#). Only gene sets significant after applying rather conservative thresholds ( $p < 0.001$  and  $FDR < 0.05$ ) are shown in the figures. Lists of the gene sets depicted in the figures are shown in [Supplementary Material, Tables S5–S8](#), with gene sets sorted by the numerical size of the NES. Overall, only negatively associated gene sets were found to meet the stringent significance thresholds.

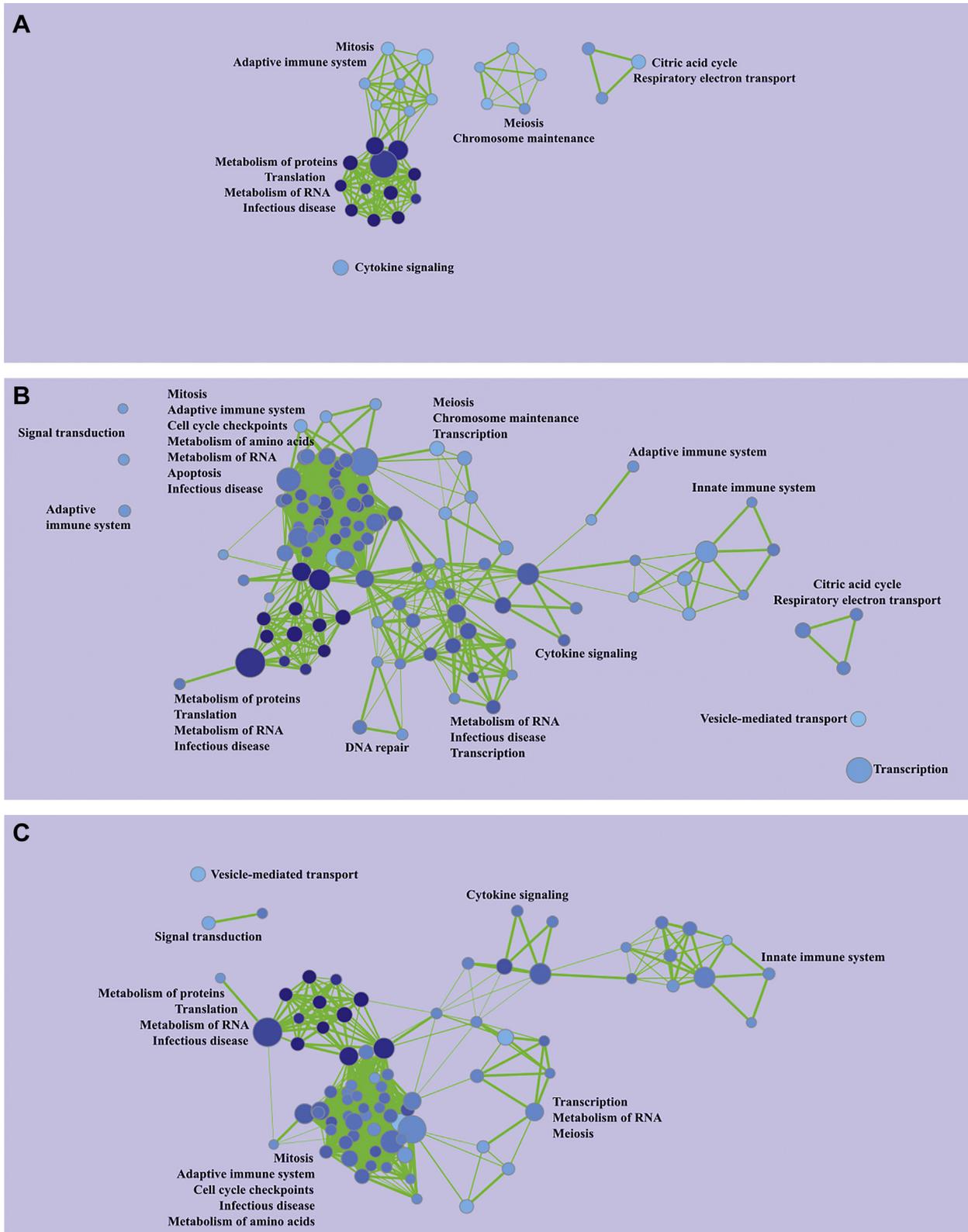
Generally, there is a large degree of overlap between the significantly associated gene sets across the 4 analyses. In accordance, from the figures and the tables, it is evident that the most significant gene sets are practically identical. The top hit in all 4 instances is “SRP-dependent co-translational protein targeting to

**Table 1**  
Study population characteristics

	Assessment	
	Intake	Follow-up
N	470	470
Male pairs/female pairs	129/106	129/106
Age, mean (SD)	55.0 (6.0)	65.7 (6.1)
Age range	45.0–67.0	55.0–79.0
Cognitive composite score, mean (SD)	−0.50 (3.73)	−2.10 (3.78)
Cognitive composite score, range	−9.41; 11.94	−16.75; 12.53
Intra-pair cognitive difference, mean (SD) <sup>a</sup>	2.60 (2.10)	2.47 (2.02)
Intra-pair cognitive difference, range <sup>a</sup>	0.02; 10.74	0.00; 12.55

The cognitive function of the twins included in this study was assessed at 2 occasions; in 1998 (intake) and again from 2008–2011 (follow-up).

<sup>a</sup> Absolute difference.



**Fig. 1.** Gene sets and pathways significantly associated ( $p < 0.001$  and  $FDR < 0.05$ ) with individual cognitive level (A), with individual cognitive change (B), with intrapair cognitive level (C), and with intrapair cognitive change (D). Nodes represent gene sets, with the size of the node indicating the number of genes included in the gene set (the larger the node, the more genes are included in the gene set). Edges represent the overlap between the gene sets, with the thickness of the edge marking the overlap in genes between the connected gene sets (the thicker the edge, the larger the overlap between connected gene sets). Gene sets with a negative normalized enrichment signal (NES) are shown in blue with the color gradient indicating the absolute size of the NES (a darker color corresponds to a lower NES). The figures were prepared using the Cytoscape (Shannon et al., 2003) plug-in “Enrichment Map” (Merico et al., 2010), and the overall function of the different gene sets were determined by looking up the individual gene sets and their overall function on the Reactome webpage (<https://reactome.org>). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

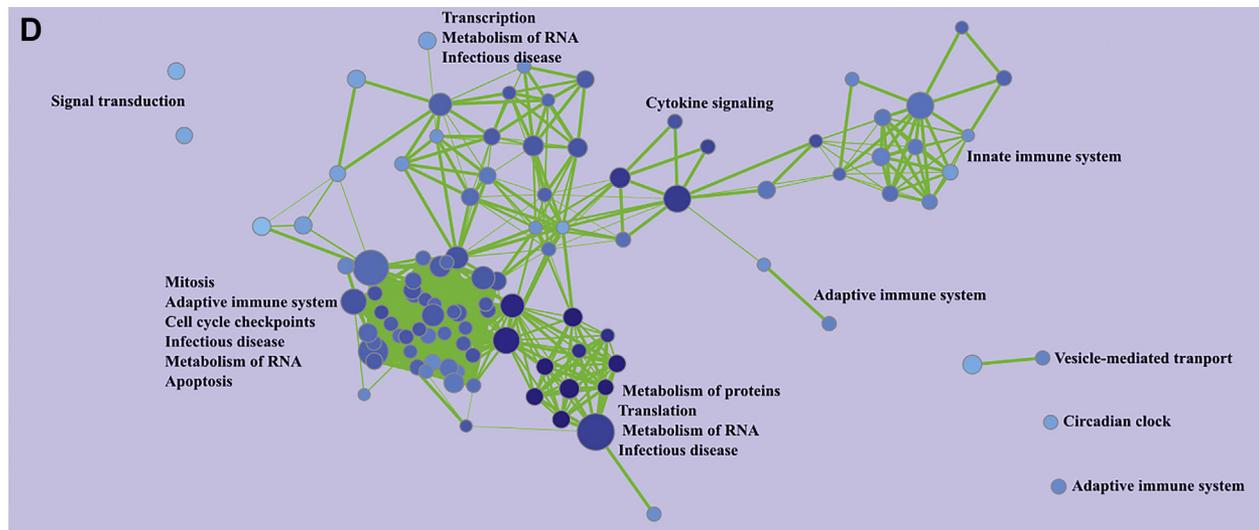


Fig. 1. (continued).

membrane”, which primarily includes genes encoding ribosomal proteins. Other overlapping top gene sets and pathways are related to protein metabolism, translation, RNA metabolism, and infectious disease. In addition, as expected, gene sets related to the immune system (adaptive and innate immune system and cytokine signaling) appear to play a prominent role.

### 3.3. Sensitivity of adjusting for leukocyte cell composition

When studying expression profiles in whole blood, it has in recent years become customary to adjust for leukocyte cell counts. However, to enable comparison of our results with the results of studies not adjusting for cell counts, analyses including only sex and age as covariates were also performed (see [Supplementary Material, Figure S1A–S1D](#)). Reassuringly, these results mirror to a large degree those obtained when cell composition is considered, although the significance of gene sets and pathways related to cytokine signaling increases. In addition, as evident from the figures, the complexity of the results decreases in the sense that less gene sets remain associated with cognitive function, at least when applying our rather stringent criteria for significance. Interestingly, positively associated pathways were identified when omitting leukocyte cell counts as covariates.

## 4. Discussion

In this study, we aimed at investigating blood gene expression patterns associated with normative cognitive aging in a population-based sample of middle-aged monozygotic twins.

Although the differential expression of no single transcripts was significantly associated with cognitive function, a suggestive negative association was found between individual cognitive level and expression of the *POU6F1* gene (FDR = 0.09). The *POU6F1* gene encodes the POU Class 6 Homeobox 1 transcription factor, which was recently found to influence neuropeptide-dependent plasticity of the adult brain in mice, supporting our finding of a possible role of this gene in human cognitive function (McClard et al., 2018). In addition, a suggestive negative association between individual cognitive change over 10 years and expression of the *MAD2L1* gene (FDR = 0.16) was identified. According to GeneCards (<https://www.genecards.org>), the *MAD2L1* gene encodes a protein known to be involved in the mitotic spindle assembly checkpoint, and the potential role of this gene in cognitive function is thus less obvious.

However, cell cycle deregulation is known to play a role in AD (Raina et al., 1999).

Contrary to the single-transcript analysis, the exploration of the biological function of the differentially expressed genes through GSEA revealed a substantial number of gene sets significantly correlated with general cognitive level and cognitive change over time, even when using quite stringent significance cutoffs. In addition, a large degree of congruence in results was found across the 4 analyses. In general, gene sets with biological functions related to protein metabolism, translation, RNA metabolism, and infectious disease were among the most enriched. In addition, gene sets related to the immune system (adaptive and innate immune system and cytokine signaling) were found to be predominant. This is consistent with the findings of previous studies of blood and postmortem brain tissue focusing on gene expression changes related to dementia and normal brain aging (Avramopoulos et al., 2011; Blalock et al., 2004; Booij et al., 2011; Colangelo et al., 2002; Fehlbauer-Beurdeley et al., 2010; Han et al., 2013; Kalman et al., 2005; Loring et al., 2001; Lunnon et al., 2012; Naughton et al., 2015; Tan et al., 2010; Weeraratna et al., 2007; Wilmot et al., 2008; Zhang et al., 2013), as well as with a recent study that found that peripheral blood leukocyte transcripts related to cell stress, inflammatory systems and cell cycle/apoptosis were able to distinguish AD from control samples (Delvaux et al., 2017). Perhaps not surprisingly, there also is a large degree of overlap between the findings of this study and the findings of 2 recent studies looking into blood gene expression patterns of normal aging (Frenk and Houseley, 2018; Peters et al., 2015). Based on these studies, it seems that inflammation as well as translation and mRNA metabolism are common denominators of normal aging and cognitive aging.

Across the 4 different analyses, the most enriched gene set was “SRP-dependent co-translational protein targeting to membrane”. This gene set primarily consists of genes encoding ribosomal proteins. A deregulation of ribosomal genes has previously been associated with aging and pathological and nonpathological decreased cognitive function (Berchtold et al., 2008; Frenk and Houseley, 2018; Peters et al., 2015). However, in contradiction to our findings, all of these studies observe a downregulation of the ribosomal genes with age or deteriorating cognition. The fact that we instead see a downregulation with increasing cognitive function (which in general should be equivalent to lower age), could be a consequence of the study sample being comprised of middle-aged

individuals that are not under the same selection pressure as demented or highly aged individuals. In support of this, lower levels of ribosomal proteins have been suggested to improve health in animal studies (Frenk and Houseley, 2018).

Interestingly, all of the highly significant gene sets identified in this study were found to be negatively correlated with cognitive function, that is, an increased expression associates with a lower cognitive function or a higher cognitive decline. This finding is in keeping with previous studies comparing cognitively impaired cases (e.g., individuals with AD or MCI) with healthy controls, where the general picture is a surplus of upregulated transcripts in the cases (Berchtold et al., 2014; Blalock et al., 2004). Other studies, however, see a general transcriptional downregulation in patients with AD (Colangelo et al., 2002; Maes et al., 2007). It has been speculated that the upregulation could be a response rather than a mechanism, that is, a temporary compensatory state attempting to circumvent the cognitive aging or deterioration (Berchtold et al., 2008; Maes et al., 2007). In addition, previous studies have suggested that transition states or states of more gene expression variability exist during midlife (Lu et al., 2004), and in cognitively normal individuals aged 20–99 years, Berchtold et al. (2008) found that the largest change, by number, of differential expression in different parts of the brain was observed in the shift from age 40–59 years to age 60–79 years, suggesting that these are critical transition phases. This indicates that the upregulation seen in our study and in the studies focusing on individuals with MCI could also be related to age. Finally, it has been suggested that the trajectory of change in the brain is nonlinear in the transition from a cognitively normal state to MCI to AD (Lunnon et al., 2012), as seen in the study by Berchtold et al. (2014) where the expression pattern of MCI was not correlated well with the expression changes seen with aging, and apparently did not reflect an evolution from normal aging to AD.

Historically, studies on blood transcriptomics have been performed without adjusting for differential cell counts. However, in recent years, it has become customary to do so. In this study, we did both, and while the main focus was on the results of the analyses performed with adjustment for cell composition, the overall conclusions would reassuringly have been the same had the focus been on the results of the analyses performed without cell composition adjustment (see [Supplementary Material, Figure S1A–S1D](#)). However, when including this adjustment, the number of significant gene sets increases and gene sets related to inflammation becomes less significant. As the leukocyte cell composition may change in response to inflammation, this is not surprising. As such, it is difficult to differentiate if the cell composition changes because of inflammation or because of the change in cognitive function or vice versa. Thus, this is yet another reason to perform such an adjustment. The fact that the number of highly significant gene sets increases when correcting for cell composition can be speculated to be a consequence of the removal of the confounding effect of cell composition. On the other hand, it may also be that adjusting for cell composition results in a less well fitting model causing an inflation of the results.

A considerable strength of this study is the use of a quite unique study sample of monozygotic twins with longitudinal data on cognitive function. This enabled us to scrutinize gene expression differences associated with individual and intrapair differences in cognitive level as well as cognitive change while controlling for the possible influence of underlying genetic variation and shared early environment (Tan et al., 2015). This significantly increases the power of the study (Tan et al., 2017). However, despite this and the fact that this study includes a rather high number of individuals compared with most previous studies, the lack of significantly associated single transcripts could be a consequence of a lack of power to detect the relatively small differences between individuals

in the applied cognitive composite measure, which reflects variation in cognitive function within the normal range rather than clinically manifest cognitive impairment. On the other hand, despite applying very conservative thresholds, a remarkable number of significant gene sets were identified in the GSEA, illustrating the gain in power achieved when focusing on biological processes instead of single genes.

In conclusion, the findings of this study indicate that cognitive level and cognitive change in middle-aged individuals are negatively associated with the expression of numerous gene sets and seem to associate with the same gene sets and overall biological functions as identified in previous studies of nonpathological and pathological cognitive change, brain aging, and normal aging. This could suggest a more generic gene expression response to cognitive aging and aging in general, irrespective of any pathologies, but further studies are needed to disclose the potential impact of our findings.

### Data statement

According to the Danish legislation, transfer and sharing of individual-level data requires prior approval from the Danish Data Protection Agency and that data sharing requests are dealt with on a case-by-case basis. For these reasons, the data cannot be deposited in a public database. However, we welcome any enquiries regarding collaboration and individual requests for data sharing.

### Disclosure

The authors declare no conflicts of interest.

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.neurobiolaging.2019.08.019>.

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