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ORIGINAL RESEARCH

Whole Blood Expression Pattern of Inflammation and Redox Genes in Mild Alzheimer's Disease

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Background: Although Alzheimer's disease (AD) is associated with alterations of the central nervous system, this disease has an echo in blood that might represent a valuable source of biomarkers for improved diagnosis, prognosis and for monitoring drug response.
Methods: We performed a targeted transcriptomics study on 38 mild Alzheimer's disease (AD) patients and 38 matched controls for evaluating the expression levels of 136 inflammation and 84 redox genes in whole blood. Patients were diagnosed as mild AD based on altered levels of total TAU, phospho-TAU and Abeta$_{(1–42)}$ in cerebrospinal fluid, and Abeta$_{(1–40)}$, Abeta$_{(1–42)}$ and total TAU levels in plasma. Whenever possible, blood and brain comparisons were made using public datasets.
Results: We found 48 inflammation and 34 redox genes differentially expressed in the blood of AD patients vs controls (FC >1.5, p < 0.01), out of which 22 pro-inflammatory and 12 redox genes exhibited FC >2 and p < 0.001. Receiver operating characteristic (ROC) analysis identified nine inflammation and seven redox genes that discriminated between AD patients and controls (area under the curve >0.9). Correlations of the dysregulated inflammation and redox transcripts indicated that *RELA* may regulate several redox genes including *DUOX1* and *GSR*. Based on the gene expression profile, we have found that the master regulators of inflammation and redox homeostasis, NFκB and NRF2, were significantly disturbed in the blood of AD patients, as well as several zinc finger and helix-loop-helix transcription factors.
Conclusion: The selected inflammation and redox genes might be useful biomarkers for monitoring anti-inflammatory therapy in mild AD.
Keywords: oxidative stress, neuroinflammation, gene expression, dementia, NRF2, NFkappaB

Introduction

The two most important unmet needs in the management of Alzheimer's disease (AD) are to find a disease modifying therapy and to develop novel biomarkers for early disease, which could be used to improve diagnosis, prognosis and monitoring, as well as to predict patient's response to therapy. Moreover, if these biomarkers would be mechanistically connected with the onset or progression of AD, then early pharmacological interventions aimed at regulating their function may provide a tremendous therapeutic benefit.

Measurement of amyloid (A)β$_{(1–42)}$, total TAU (TAU) and phosphorylated TAU (p-TAU^{T181}) in cerebrospinal fluid (CSF), complemented by radioactive tracing of fibrillary Aβ protein loads in the brain[1] and volumetric analysis of the hippocampus and entorhinal cortex, has proven to be useful in the early diagnosis of AD.[2,3] However, these biomarkers are not convenient for disease prognosis and

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monitoring, or for predicting the response to therapy because the clinical correlations are lost while disease is progressing.[4,5] Other molecules are now being explored as AD-monitoring biomarkers in the CSF, including neurofilament light polypeptide (NFL), neurogranin and visinin-like protein 1,[6] that appear to be promising candidate markers for monitoring AD, albeit not being highly specific for this disease.[7]

Compelling evidence exists that AD, along with other neurodegenerative diseases and non-communicable diseases, is underlined by low-grade chronic inflammation, both at local and peripheral level.[8,9] The critical role of neuroinflammation in the pathogenesis of AD is well documented by clinical and preclinical studies.[10] In response to local brain damages, a persistent immune response is elicited locally by various brain cells (resident macrophages – microglia, astrocytes, newly recruited myeloid cells, etc), and further sustains neurodegeneration.[11] For instance, transcriptomic data from 1633 post-mortem brain samples have identified a cluster of AD patients with a strong neuroinflammatory signature linked to microglia function and the TREM2/TYROBP signaling pathway, an important mediator of neuroinflammation.[12] Moreover, neuroinflammation correlates with cognitive deficits in AD. Thus, a multimodal and multivariate neuroimaging approach demonstrated a spatially distributed neuroinflammation in connection with changes in large-scale functional connectivity and cognitive deficits.[13] Albeit not being specific to AD, neuroinflammation represents not only a promising therapeutic target,[14] but also a source of potential markers in CSF. For instance, a meta-analysis performed on 2629 patients and 2049 controls showed that the levels of TGF-β (transforming growth factor-β), MCP-1 (monocyte chemoattractant protein-1) and YKL-40 were significantly elevated in the CSF of AD patients as compared to controls.[15] Additionally, a study on AD cohorts from the DIAN and ADNI multicentric studies (1246 participants)[16] found that higher levels the immunomodulatory progranulin in CSF in late-onset AD were associated with more advanced disease stages and cognitive impairment. Meanwhile, progranulin correlated with high levels of soluble TREM2 (triggering receptor expressed on myeloid cells 2) only when there was underlying pathology. These new inflammatory CSF candidate markers might complement conventional AD biomarkers for better stratification of patients. However, they must be further validated in large and well-characterized cohorts, in connection with the disease stage for future translation into the clinical practice.

In addition to neuroinflammation, redox alterations comprising both increased oxidative activity and deficient antioxidant protection have been also conclusively reported in AD brains,[17,18] correlating with the brain regions where AD-specific injuries occur.[19] Various oxidative markers related to DNA oxidation/damage and lipid peroxidation, as well as antioxidant molecules such as glutathione, thioredoxin and superoxide dismutase, have been identified in AD patients, part of them being detected both in brain and CSF.[20]

Altogether, the interplay between inflammation and redox changes[21] sustains the hypothesis that correlated low-grade inflammation and oxidative disturbances underlie pathological mechanisms in AD.[22]

Blood, collected by a minimally invasive procedure, is an alternative to CSF. Investigations in blood would allow the screening of large populations at risk, with reduced time intervals between evaluations, hence supporting personalized therapeutic strategies and their timely update.[23] Due to the recent development of ultrasensitive immunoassays,[24] some of the most relevant molecules in AD, such as Aβ, TAU and NFL, can now be measured in the plasma of AD patients, albeit the much lower concentrations than in CSF. However, little has been done in the search for blood inflammatory biomarkers. This is probably due to the fact that such biomarkers are not likely specific for AD, and the results might be confounding due to other inflammatory processes deriving from co-morbidities, infections or ageing.[25]

Blood is a window for investigating the inter-related systemic and local immune responses, inflammation and redox signaling alterations in chronic pathologies.[18,26] The data reported by the few transcriptomic studies performed up to now in the blood of AD patients have revealed that peripheral blood mononuclear cells exhibit molecular changes that occur also in the AD brain,[27] indicating new immunologic facets of the blood–brain crosstalk.[28] Moreover, the meta-analysis of three public datasets (ADNI, AddNeuroMed 1 and 2) emphasized that AD-related genes in blood were indeed enriched with inflammation, mitochondria and Wnt signaling pathways,[29] hence emphasizing the link between peripheral immune disturbances and AD pathogenesis.

Despite this evidence, blood-based cellular inflammation and oxidative biomarkers have not been validated so far in AD. For the moment, most results obtained in the blood of AD patients vs matched controls have not yet been broadly reproduced across research laboratories,

possibly due to diagnostic misclassification, low number of investigated patients at different disease stages, variable health status, the type of the investigated biological samples (whole blood or peripheral blood mononuclear cells) or different methods used for assessing and normalizing gene expression levels.[30]

In this work, we carried out a case–control study for investigating through qRT-PCR the expression profile of critical inflammation and redox genes in the whole blood of mild AD patients and controls. Our results indicated highly distinctive expression levels of several inflammation and redox genes. Novel therapies aimed at mitigating chronic low-grade inflammation and oxidative stress in

mild AD patients could take advantage of these new markers for drug target engagement and disease monitoring.

Materials and Methods
Patients and Controls

The case–control study was performed on 38 mild AD patients and 38 controls that were recruited and diagnosed at the Hospital Universitari Santa Maria-IRB Lleida, Lleida, Spain, based on the values of the CSF markers described in Table 1. The patient, the responsible caregiver and the legal representative (when different from the responsible caregiver) signed an informed consent form. The inclusion criteria comprised acetylcholinesterase

Table 1 Demographic, Clinical and Biochemical Characteristics of the Investigated Patients and Controls

	Mild AD (N=38)	Controls (N=38)	p value
Demographic data			
Age (mean ± SD)	76.58±5.86	74.18±7.29	p=0.119
Sex (% F)	26.31%	47.36%	X^2=3.619, p=0.057
Education in years (mean ± SD)	7.81±2.73	9.76±4.33	**p=0.025**
Comorbidities			
Diabetes (% affected)	21.05%	21.05%	X^2=0.000, p=1.000
Hypertension (% affected)	55.26%	47.36%	X^2=0.474, p=0.491
Hypercholesterolemia (% affected)	34.21%	18.42%	X^2=2.443, p=0.118
Anxiety (% affected)	18.42%	26.31%	X^2=0.682, p=0.409
Depression (% affected)	26.31%	13.15%	X^2=2.077, p=0.150
Cognition			
MMSE SCORE (mean ± SD)	23.4 ± 2 0.6	29.3 ± 1.1	**p<0.001**
CSF markers (N=34)			
• AB$_{(1-42)}$ (PG/ML)	489.7 ± 148.6	979± 419*	**p<0.0001**
• P-Tau ^{T181} (PG/ML)	79.5 ± 25.8	< 60**	-
• Tau (PG/ML)	548.0 ± 277.3	212± 122***	**p<0.0001**
Plasma markers (N=37)			
• TAU (PG/ML)	2.81 ± 0.95	2.74± 0.76^	ns
• AB$_{(1-40)}$ (PG/ML)	166.44 ± 44.55	272±52^^	**p<0.0001**
• AB$_{(1-42)}$ (PG/ML)	10.60 ± 2.92	39.7±10.5^^	**p<0.0001**
APOE alleles			
E2/E3	N = 2	nd	
E3/E3	N = 16	nd	
E4/E2	N = 2	nd	
E4/E3	N = 15	nd	
E4/E4	N = 3	nd	

Notes: Whenever possible, results are presented as mean ± SD (nd=not determined, ns=not significant; p values in bold and italics are statistically significant according to the Student's *t*-test). *Normal values from the meta-analysis made by Sunderland et al (N=427).[48] **Threshold established by Skillback et al.[47] ***Normal values from meta-analysis by Sunderland et al (N=1054).[48] ^Normal values established by Fossati et al (N=68).[49] ^^Values established by Hanonet al (N=122).[50]

inhibitor-naïve individuals aged over 60 years who were diagnosed with AD according to the NIA-AA criteria.[2] Additionally, only patients with mild cognitive impairment (mini-mental state examination [MMSE]≥20) were included. The exclusion criteria comprised 1) the presence of visual and/or communication problems that could render compliance with the study procedures difficult; 2) comorbidities such as cancer, severe renal or hepatic insufficiency, severe cardiac or respiratory failure; 3) no acute episodes of inflammation within 1 month prior to investigation; 4) excessive alcohol intake (>280 g/week); 5) MRI evidence of hydrocephalus, stroke, a space-occupying lesion, or any clinically relevant central nervous system disease other than AD; 6) the presence of mental disorders according to DSM-V-TR™ criteria; 7) the presence of untreated (or treated for less than 3 months prior to the screening visit) vitamin B12 or folate deficiency; and 7) the presence of untreated thyroid disease. Cognition was assessed using a broad neuropsychological battery. The MMSE score was used to include only patients with mild cognitive impairment. All tests were run by the same neurologist. The MMSE questionnaire includes questions to evaluate different domains, such as attention, time and place orientation, and word recall. The scores of this test ranged from 0 to 30, and a higher score indicates better cognitive function.[31] The demographic, clinical and biochemical characteristics of the investigated mild AD patients and controls are presented in Table 1.

AD-Specific Biomarkers in CSF and Plasma

CSF samples were collected between 8:00 and 10:00 a.m. to avoid variations related to the circadian rhythm. The samples were collected in polypropylene tubes, centrifuged at 2000 × g for 10 min at 4°C, immediately frozen and stored within 4 hours in a −80°C freezer. The disease-specific biomarkers amyloid beta ($A\beta_{(1-42)}$), total TAU and Thr181-phosphorylated TAU protein ($p\text{-}TAU^{T181}$) in cerebrospinal fluid (CSF) were quantified using dedicated ELISA kits (Innogenetics), according to the manufacturer's instructions. The cutoff values for these biomarkers were determined in an independent cohort of AD patients and controls in our laboratory. The cutoff values for Aβ42, total TAU, and phospho-TAU were <600 pg/mL, >425 pg/ mL, and >65 pg/mL, respectively. Total TAU, $A\beta_{(1-40)}$, and $A\beta_{(1-42)}$ levels were quantified in the plasma of AD patients using the Human Neurology 3-Plex A assay

(N3PA) on the ultrasensitive Simoa HD-1 analyzer (Quanterix, Billerica, MA, USA).

APOE genotyping: DNA (20 μL) was extracted from buffy coat cells using a Maxwell® RCS blood DNA kit (Promega, USA), and APOE genotyping was performed by qRT-PCR.

Gene Expression

From each subject, 2.5 mL of venous blood were collected in PAXgene Blood RNA Tubes (Qiagen) for total RNA isolation with the PAXgene Blood miRNA Kit (Qiagen). Reverse transcription using 400 ng of RNA was performed with the RT^2 First Strand Kit (Qiagen) according to the manufacturer's protocol. The expression of 168 inflammatory genes was evaluated with the RT^2 Profiler™ PCR Array Human NF-κB Signaling Pathway (PAHS-025Z, Qiagen) that addresses mainly genes involved in NFκB signaling (Supplementary Table 1), and with the RT^2 Profiler™ PCR Array Human NFκB Signaling Targets (PAHS-225Z, Qiagen) that contains NFκB target genes (Supplementary Table 2). The arrays shared 22 overlapping genes that yielded essentially similar results. Eighty-four key genes involved in oxidative stress/antioxidant response (Supplementary Table 3) were analyzed with the RT^2 Profiler™ PCR Array Human Oxidative Stress Plus (PAHS-065Y, Qiagen). The SYBR Green chemistry on the ABI-7500 fast instrument (Applied Biosystems) was used. The expression level of each gene was normalized with the geometric mean of *HPRT1* and *RPLP0*, and $2^{-\Delta CT}$ mean values as well as and the fold change (FC) were calculated as previously described.[32]

Statistical Analysis

Results are presented as mean ± standard deviation (SD) or standard error of the mean (SEM). Statistical analysis was performed using SPSS 20 and GraphPad Prism 8. Since data were not normally distributed (Kolmogorov–Smirnov, p<0.05), the differences in gene expression between patients and controls were evaluated using the nonparametric Mann–Whitney *U*-test. The difference in gene expression was considered significant with an FC >1.5 and p < 0.05. Correlations between gene expression levels were calculated using the Pearson coefficient (r), and were considered significant with $r > 0.6$ and $p \leq 0.01$. Receiver operating characteristic (ROC) curve was created and area under the curve (AUC) was calculated to assess the potential value of the selected transcripts in discriminating patients from controls. Principal component

analysis (PCA) was performed using the web tool ClustVis[33] for visualizing clustering of multivariate data. The power of our study was calculated using the G*Power 3.1.9.7 tool.[34]

GEO Data Mining

Gene expression data were retrieved from the Gene Expression Omnibus database (GEO) from two datasets provided from the AddNeuroMed consortium. Patient selection, design, and clinical data have been reported in previous studies.[35,36] Employing the GEO2R online tool, we identified the dysregulated genes (DEGs) comparing 239 blood samples from healthy subjects (104 and 135 from GSE63060 and GSE63061, respectively) with 245 from early diagnosed patients (110 and 135 from GSE63060 and GSE63061, respectively). The intersected 5.289 DEGs with FC > 2 and Adj (p-value) <0.05 were grouped with the functional clustering tool DAVID (Database for Annotation, Visualization and Integrated Discovery) v6.7.[37] Functional enrichment was considered for pathways with FC> 2 and Adj (p-value (FDR/BH) <0.05). Pathway analysis was performed with the Gene Ontology Biological Process (GOTERM_BP_DIRECT). The GSE122063 gene expression dataset reporting data on frontal and temporal cortex in human post-mortem AD and control brains was analyzed using GEO2R (http://www.ncbi. nlm.nih.gov/geo/geo2r/). Genes were considered differentially expressed with FC >1.5 and adjusted p < 0.05. The adjusted p value was calculated using the Benjamini and Hochberg procedure (the false discovery rate method) to identify the important few from the trivial many effects tested.[38]

Transcription Factors Analysis

Dysregulated genes (FC>1.5-fold and Adj (p-value<0.05) from inflammatory and redox panels were analyzed with the oPOSSUM (v.3.0) human single-site analysis online tool[39] to determine enrichment scores and genomic positions of binding motifs for known transcription factors. The selected JASPAR core and PBM profile specificity was 8 bits. The selected parameters to perform the search were as follows: 0.4 conservation cut-off, 0.85 matrix score threshold, 5000 bp upstream and downstream of the consensus site.

Identification of NFκB Binding Sites in Redox Genes

A script for the search for transcription factor binding sites was developed in Python 3.6 and is reported in Supplementary file 1. The script uses as input a BED file containing the ChIP-seq peaks for the transcription factors of analysis, a text file containing a list of RefSeq transcripts accession numbers, and a position frequency matrix (PFM) file from the JASPAR database containing the consensus transcription factor bindingsites to be computed. Additionally, it makes use of the BED file at the UCSC Genome Browser, Table Browser resource (https:// genome.ucsc.edu/cgi-bin/hgTables) containing the locations of every transcript and its RefSeq accession number in the genome. For the overlapping with active regions, a combined segmentation BED file was generated by concatenating Combined Segmentations at the UCSC Genome Browser, using BEDTools.[40] This track combines segmentations of ChromHMM and Segway methods, using CTCF binding sites, histone marks (H3K27ac, H3K27me3, H3K36me3, H3K4me1, H3K4me3, H4K20me1 and H3K9ac) DNAse-seq and FAIRE-seq data from six core human cell lines in ENCODE (GM12878, H1-hESC, HeLA-S3, HepG2, HUVEC, K562). The script retrieves the genomic coordinates for the desired transcripts, extends them 5000 bp upstream of the transcription start site and intersects them with the genomic regions downloaded from ChIP-Atlas[41] using the wrapper of BEDTools for Python, pybedtools.[42] In this analysis, all the available sites for binding to RELA, RELB and NFKB1 were downloaded and intersected with the extended transcripts of *NFKBIA, GSTP1, DUOX1, SQSTM1, GSR*, and *NCF1*. Then, the sequences of the peaks were extracted using pybedtools from the FASTA file of the hg19 Human Genome. The profiles for human RELA, RELB and NFKB1 were downloaded from the JASPAR database[43] in PFM format from the entries MA0107.1, MA1117.1, MA0105.2. Absolute frequencies are turned into a PSSM (position specific-scoring matrix), containing scores through the log2 (odds-ratio) (odds ratio: observed frequency/expected frequency). One unit was added as a pseudo-count to each absolute frequency to avoid log(0). Scoring of each site followed a similar procedure as we have previously described.[44] Briefly, a sliding window of a width dependent of the profile to be used was passed over the extracted sequences. Each nucleotide in the sliding window received a score according to the PSSM and then, the score from each nucleotide was added up in order to provide an absolute score for the site. The relative score, the maximal and minimal scores were obtained with a given PSSM and computed as (absolute score + | minScore|)/(|maxScore|+|minScore|). Sites with a relative
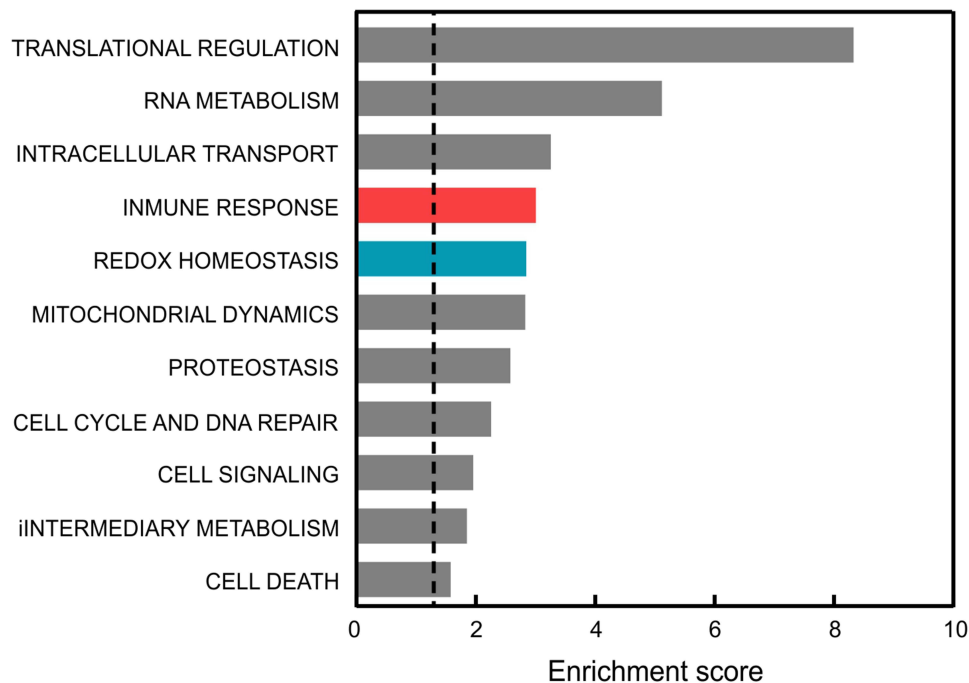
Figure 1 Comparison of the functional pathways altered in the blood of AD patients vs controls. Dysregulated genes were averaged from those reported in GSE63060 and GSE63061 microarray data after GEOR2 analysis. The functional annotation corresponding to biological processes (GOTERM_BP_DIRECT) was analyzed using the DAVID database.[37] Significant associations (adjusted p-value (FDR/BH) <0.05) are plotted against the enrichment score.

score below 0.9 were discarded, and the remaining ones were provided as a BED file. In order to detect active regions, the script makes use of pybedtools to intersect the segmentation file with the regions described as transcription start sites (TSS), weak enhancers (WE), enhancers (E) and promoter flanking (PF) regions, and returns only the sites that are active according to these criteria.

Results

We firstly analyzed whether brain damage in mild AD patients could correlate with consistent gene expression changes in peripheral blood. By pooling data from GSE63060 and GSE63061 microarray datasets,[45,46] we obtained a list of dysregulated genes in the blood of 245 patients vs 239 controls. These dysregulated genes, with FC >2 and adjusted p-value (FDR/BH) <0.05, were further classified by GOTERM_BP_FAT category in functional clusters of biological processes. The analysis highlighted that genes involved in immune responses and in redox metabolism might be amongst the most dysregulated genes in the blood of AD patients (Figure 1). Therefore, we further investigated by qRT-PCR if alterations in the expression of inflammation and redox genes might occur in mild AD.

Altered Expression Pattern of Inflammation and Redox Genes in the Blood of Mild AD Patients

We conducted a case–control study on 38 mild AD patients and 38 controls that were highly characterized and matched in terms of age and comorbidities (Table 1). Patients were recruited during the first visit to the neurologist, before starting specific AD medication. The MMSE score, used to evaluate cognitive capabilities, exhibited lower values in AD patients as compared to controls, tended to decline progressively in these patients along consecutive years (Figure 2A). Patients exhibited in CSF abnormally high levels of TAU and phospho-TAU (p-TAU^{T181}), along with low levels of A$\beta_{(1-42)}$ (Figure 2B and Table 1) as compared to normal values reported in the literature.[47,48] Additionaly, plasmatic levels of the amyloid peptides A$\beta_{(1-42)}$ and A$\beta_{(1-40)}$, determined with an ultra-sensitive Quanterix-Simoa immunoassay, further demonstrated abnormally low levels of these markers in the plasma of patients (Figure 2C and Table 1).[49,50]

A targeted gene expression screening was performed by qRT-PCR using pathway-focused arrays of 136 inflammation and 84 redox genes (Supplementary Tables 1–3). As shown in the gene expression heat maps (Figure 3), we
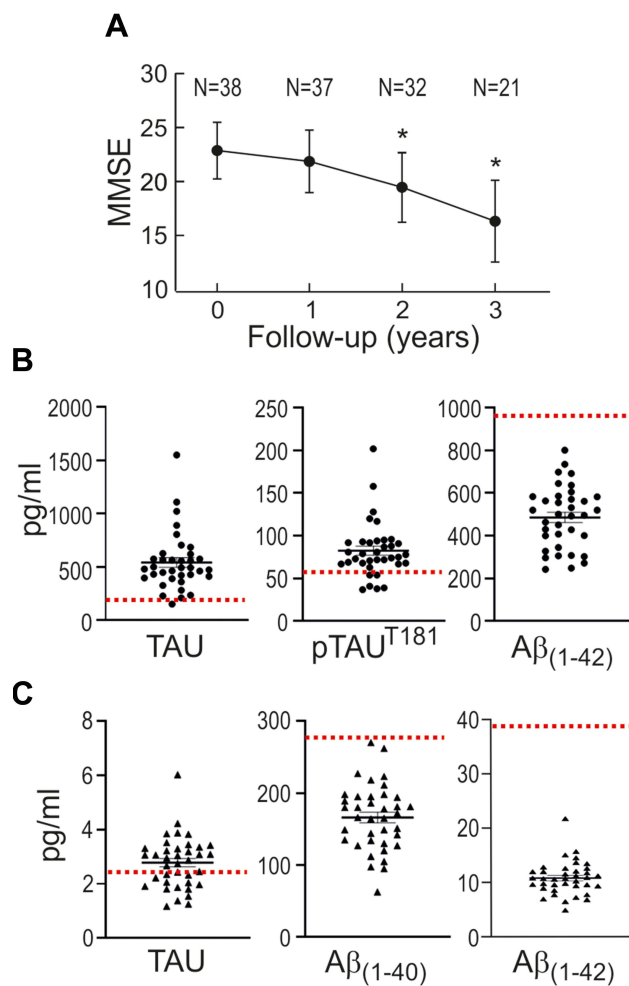
A

B

C

Figure 2 Disease-specific markers in the group of mild AD patients. (**A**) Evolution of the MMSE score. Patients were recruited during the first visit to the neurologist (0 time point), before starting specific AD medication. Results are expressed as mean ± SD. N is the number of patients tested for MMSE at consecutive yearly visits. *p<0.001 denotes statistically significant difference vs visit 0 (Pairwise Wilcoxon signed-rank test). (**B**) TAU, phosphoTAU (p-TAU^{T181}) and Aβ$_{(1-42)}$ levels in CSF. Expected normal values are as follows: TAU, 212±122 pg/mL [47]; p-TAU^{T181} <60 pg/mL;[46] Aβ$_{(1-42)}$, 979±419 pg/mL.[47]. (**C**) Total TAU, Aβ$_{(1-40)}$ and Aβ$_{(1-42)}$ levels in plasma. Expected normal values are as follows: TAU, 74±076 pg/mL,[48] Aβ(1–40), 272±52 pg/mL,[49] Aβ$_{(1-42)}$, 39.7±10.5 pg/mL.[49] For B and C, values are expressed as mean ± SEM.

identified 48 inflammation genes (Figure 3A) and 34 redox genes (Figure 3B) that were significantly overexpressed (FC>1.5, p<0.01) in the blood of patients vs controls. Heat maps of inflammation and redox genes with FC value <1.5 are also presented in Supplementary Figures 1 and 2, respectively.

We compared significant alterations in gene blood expression with available GSE122063 microarray datasets of frontal and temporal cortex of AD patients and controls.[51] Thirteen inflammatory and six redox genes appeared to be upregulated both in the blood and in the brain of AD patients as compared to controls (Table 2).

In silico Identification of Transcription Factors Associated with the Altered Gene Expression Pattern Evidenced in the Blood of Mild AD Patients

The dysregulated gene expression profile indicates that the activity of some transcription factors must be altered. To identify such transcription factors, we used the oPOSSUM 3.0 software[39] which provides over-represented transcription factor binding sites in the genes with altered expression (Figure 4). Among the inflammation-related transcription factors, we found sites for binding to the REL family (*RELA*, generic NFKB, *NFKB1, REL*), the ETS family (*ELK1, GABPA, FEB* and *SPI1*), the zinc finger superfamily (*KLF4, SP1* and *ERG1*) and *STAT1*. Regarding redox-related transcription factors, we found binding sites for the helix-loop-helix family (*ARNT: HIF1A, ARNR:AHR, MAX, MYF* and *EBF1*), the zinc finger superfamily (*ZNF354C, ERG1, ZFP423* and KLF4), two REL sites (generic NFKB and *NFKB1*), as well as binding sites for transcription factors encoded by *NFYA, FOXF2, SRY, E2F1, MYB* and *NFE2L2* (Supplementary Tables 4 and 5). These results confirm the dysregulation of the NFκB pathway found in the blood of these patients, and further extend these findings to several Zinc finger and helix-loop-helix transcription factors involved in homeostatic functions. Of note, a dysregulated transcription factor found in the redox group was *NFE2L2*, encoding NRF2 which is a master regulator of redox homeostasis (see Discussion for a detailed analysis of altered transcription factors).

Selection of Dysregulated Inflammation and Redox Genes

In a more restrictive gene expression analysis, we highlighted 22 inflammation genes (Figure 5) that had FC>2 and p<0.001. The overexpressed inflammation genes contribute to the canonical (*NFKBIA*) and non-canonical (*RELB. LTA, NFKB2, TRAF2, CD40*) NFκB activation pathways (Figure 5A), modulate apoptosis: *TNFRSF1B, CCND1, BCL2L1, TP53, FASLG*, and *AKT1* (Figure 5B), are part of the interferon signalling pathway: *STAT1, IRF1* and *TBK1* (Figure 5C) or are involved in other signalling pathways (*STAT5B*) (Figure 5C), are receptors or ligands (*CSF2RB, IL2RA, CFB, LTB*) that are signalling via NFκB (Figure 5C) or participate in coagulation (*FB* and *PLAU*) (Figure 5D). We also detected 12 redox genes with FC > 2
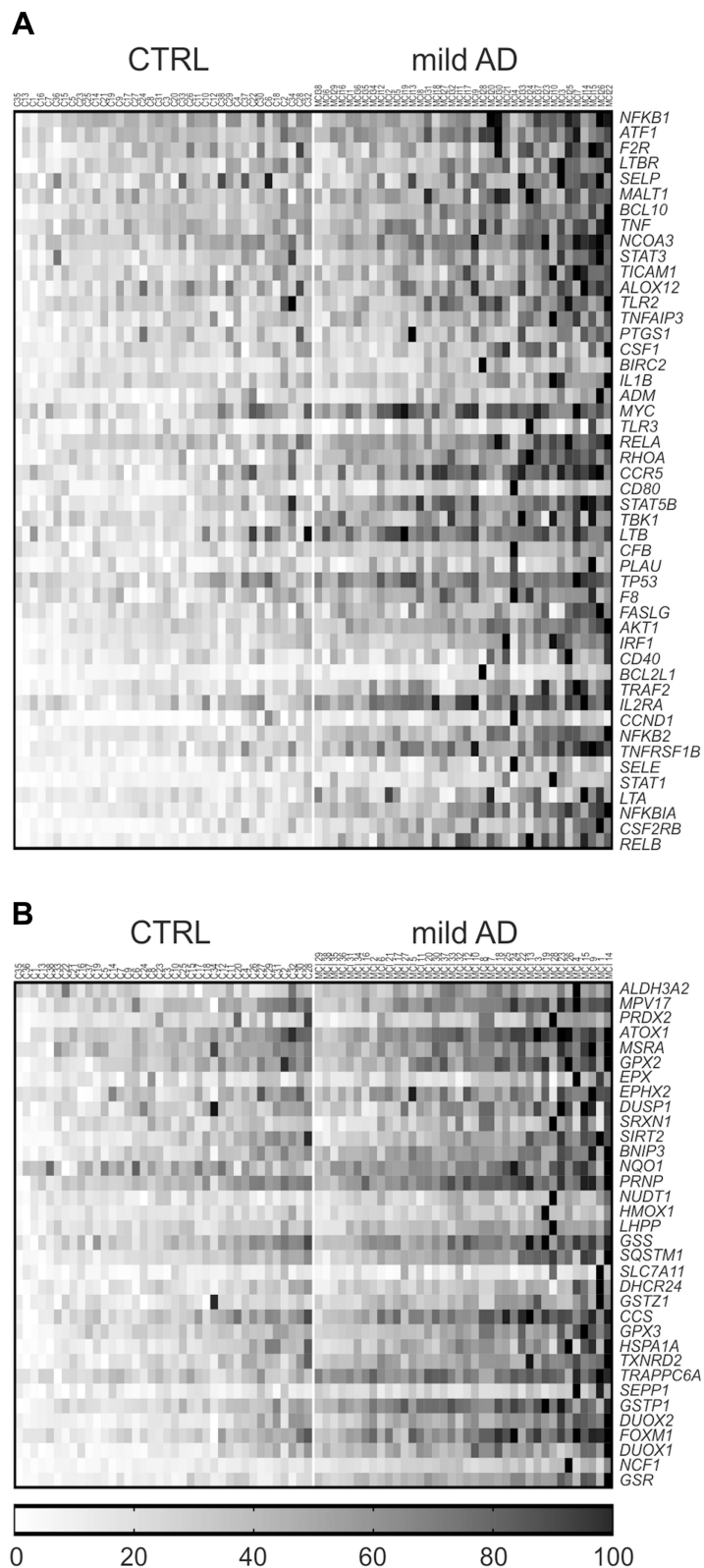
A

B

Figure 3 Heat maps of individual gene expression data in the blood of mild AD patients and controls (CTRL). (**A**) Inflammatory genes. (**B**) Redox genes. Only genes with mean FC value >1.5 and p<0.01 in the AD group vs the control group are represented. Individual FC values for each gene were obtained by dividing the $2^{-\Delta Ct}$ value of each subject to the mean $2^{-\Delta Ct}$ value of the control group. Data were normalized and ordered in the heat map according to the average expression levels of all the considered genes, and were scaled considering the highest value as 100%. Heat maps of inflammation and redox genes with FC value<1.5 are presented in Supplementary Figures 1 and 2, respectively.

Table 2 Comparison of Genes Upregulated in the Blood of AD Patients (FC>1.5, p<0.01, Figure 3) and in Post-Mortem Samples of AD Brain from the GSE122063 Microarray Data Set

Genes		GSE122063 (12 AD vs 11 CTRL)				Our Results in Blood (38 AD vs 38 CTRL)	
		Frontal Cortex		Temporal Cortex		Blood	
		FC	Adjusted p value	FC	Adjusted p value	FC	p value
Inflammation genes	*ADM*			>1.5	0.0375	1.798	<0.001
	BCL2L1	>2.0	0.0051	>2.5	0.0021	2.666	<0.001
	CCR5	>1.5	0.00117	>1.5	0.00209	1.929	<0.001
	CFB			>1.5	0.0486	2.080	<0.001
	CSF2RB	>1.5	0.0256			3.656	<0.001
	FASLG			>1.5	0.0212	2.343	<0.001
	IL2RA			>2.0	0.0190	2.730	<0.001
	IRF1	>1.5	0.0043	>1.5	0.0242	2.411	<0.001
	NFKBIA	>1.5	0.0005	>1.5	<0.0050	3.651	<0.001
	TLR2			>2.0	<0.0050	1.701	<0.001
	TNFAIP3	>2.0	<0.001	>1.5	0.0002	1.738	<0.001
	TNFRSF1B	>1.5	0.00314	>1.5	0.0266	2.850	<0.001
	TP53			<0.33	0.0465	2.168	<0.001
Redox genes	**DUSP1**	>1.5	0.0179	>1.5	<0.0500	1.603	<0.001
	HMOX1	>1.5	<0.005	>2.0	<0.0010	1.743	<0.001
	HSPA1A	>2.5	<0.0001	>2.5	<0.0010	2.159	<0.001
	NQO1	>1.5	0.00521			1.649	<0.001
	NCF1			>1.5	<0.0500	3.208	<0.001
	SEPP1	>1.5	<0.05	>1.5	<0.0010	2.477	<0.001

Notes: Genes in brain samples were considered differentially upregulated with FC>1.5 and adjusted p values < 0.05. Genes in bold font exhibit AUC>0.9 in the ROC analysis (Figure 7).

and $p<0.001$ in the patients group as compared to controls (Figure 6). These genes encode members of the NADPH oxidases family (*NCF1, DUOX1,* and *DUOX2*) that are involved in ROS production (Figure 6A), regulate the metabolism of glutathione (*GSR, GSTP1* and *GPX3*) and thioredoxin (*TXNRD2*) (Figure 6B), or perform other redox functions (*FOXM1, SEPP1, HSPA1A, TRAPPC6A1A,* and *CCS*) (Figure 6C). The fact that we found upregulation of genes involved in ROS production and detoxification, indicated a global dysregulation of redox homeostasis in the blood of mild AD patients.

Receiver operating characteristic (ROC) analysis of inflammatory and redox genes with FC>2 was used for selecting those genes whose expression levels could discriminate well between patients and controls. The computation of the Area Under the Curve (AUC) evidenced nine inflammatory genes (*AKT1, CSF2RB, IL2RA, NFKB2, RELB, STAT5B, TBK1, TNFRSF1B* and *TP53*) and seven redox genes (*DUOX1, FOXM1, GSR, GSTP1, NCF1, TRAPPC6A* and *TXNRD2*) that have AUC values > 0.9 (Figure 7, Supplementary Table 6). In addition, we identified three genes with 1.5< FC <2 that also yielded high AUC values (AUC > 0.9) and might be considered potential candidates for discriminating between patients and controls (Supplementary Table 6). These genes were *CCR5* (FC = 1.93, p<0.001; AUC = 0.905), *RHOA* (FC
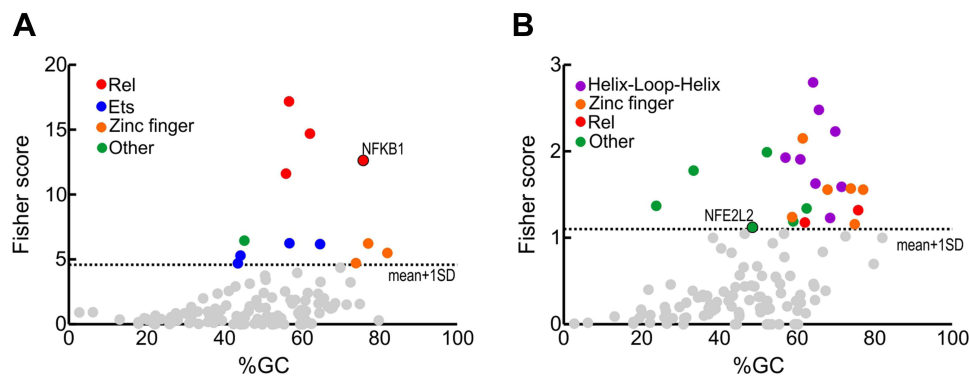
Figure 4 Transcription factors whose binding sites are enriched in the regulatory sequences of the genes with altered expression (FC>1.5 and p<0.05) comparing AD vs control subjects. (**A**) inflamatory genes from Figure 3A. (**B**) redox genes from Figure 3B. Identification of enriched transcription factors was done with the oPOSSUM software.[39] Fisher scores are plotted vs the GC composition of the TF profile. Dashed line represents one standard deviation above the mean. Transcription factor binding with scores higher than 1±SD are highlighted in colors as indicated.
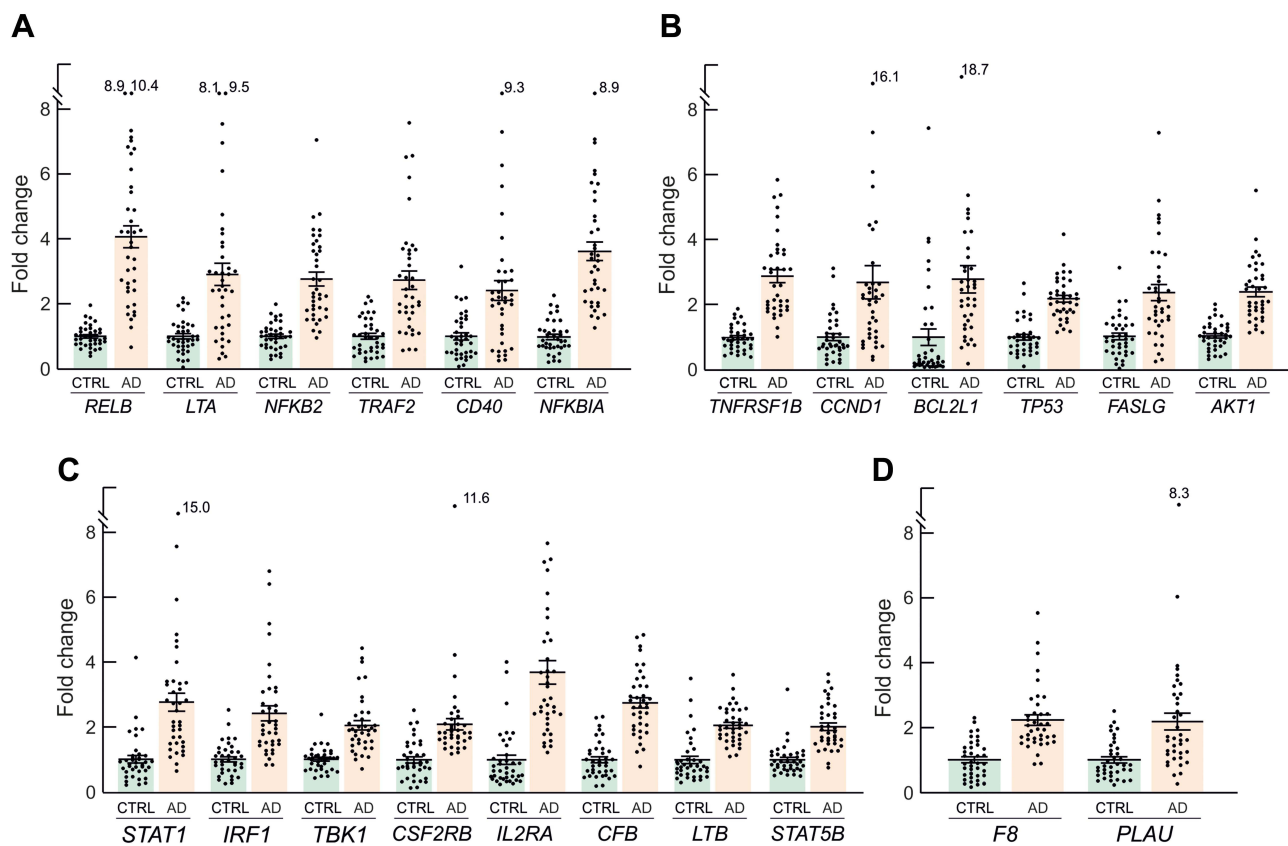
Figure 5 Inflammation genes with FC>2 and p<0.001, grouped according to their functional role. (**A**) Genes of the canonical and non-canonical NFκB pathway. (**B**) Genes involved in modulation of apoptosis. (**C**) Genes of the interferon and other inflammatory signalling pathways. (**D**) Genes that participate in coagulation. Mean FC values and SEM for each individual gene in AD patients and controls are also represented, as well as outlier values.

= 1.92, p<0.001; AUC = 0.950), and *GSTZ1* (FC = 1.99, p<0.001; AUC = 0.904). Moreover, PCA for the selected inflammatory and redox genes with FC >2 and p<0.001 showed a partial separation of patients and controls, with some overlap in Principal component 1 (PCA1) that explains 66.7% of the total variance, evidencing some connections between the selected genes and mild AD. (Figure 8).
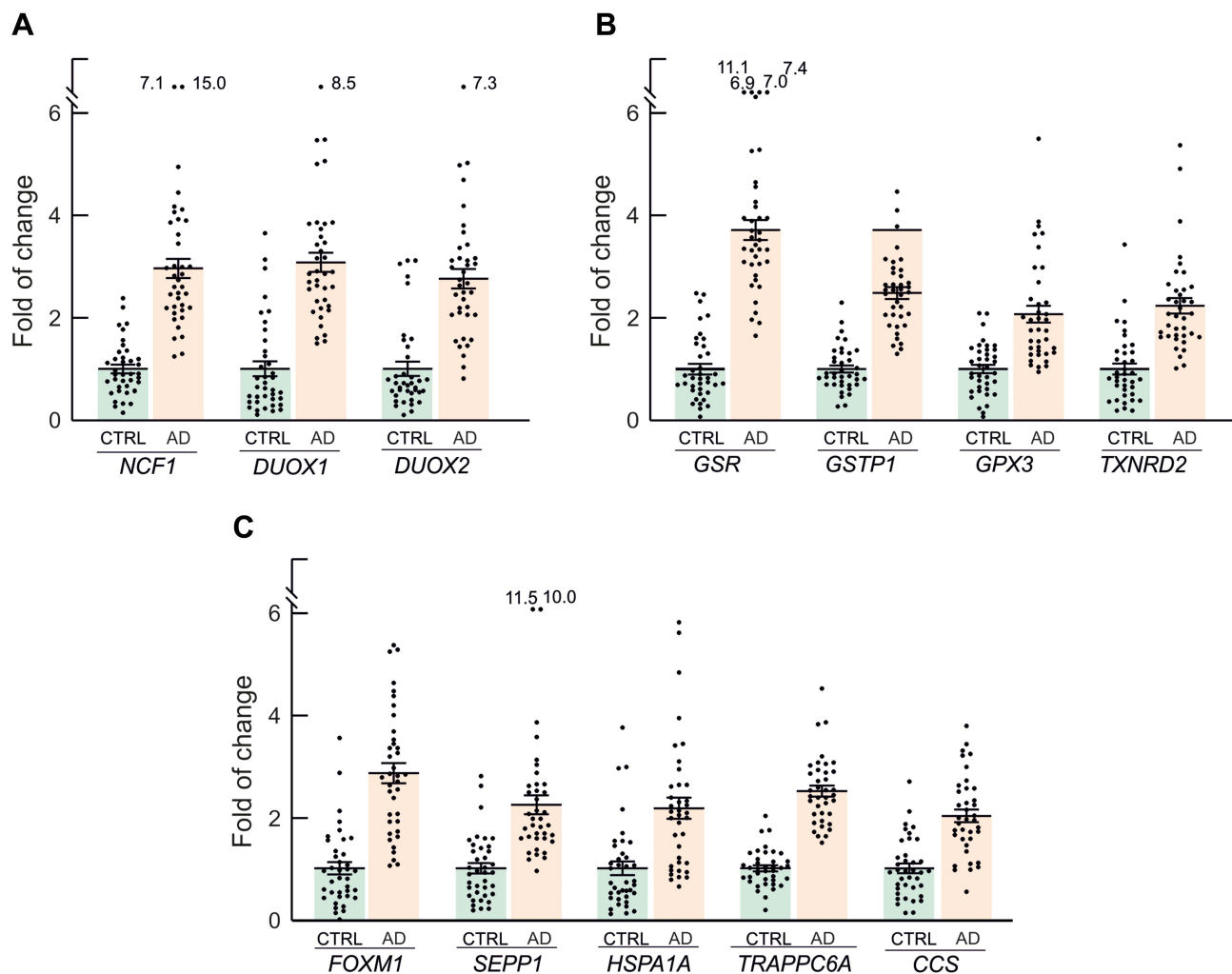
Figure 6 Redox genes with FC>2 and p<0.001, grouped according to their functional role. (**A**) Genes of the NADPH oxidase family, involved in ROS production. (**B**) Genes of glutathione and thioredoxin pathways. (**C**) Other genes involved in redox metabolism. Mean FC values and SEM for each individual gene in AD patients and controls are also represented, as well as outlier values.

Associations Between Candidate Inflammation and Redox Genes

Considering that we have found alterations in both inflammation and redox genes, and that the oPOSSUM analysis suggests that some of these genes might share common transcription factors, we analyzed inter-pathway correlations between some of the most significantly overexpressed inflammation and redox genes. Among inflammation genes, *NFKBIA* was chosen because it is also upregulated in the AD frontal and temporal cortex (Table 2), and RELB because of its high FC value (FC>3). Albeit not being clearly upregulated in blood, *RELA* was analyzed because it is a master regulator of the canonical NFκB activation pathway and a critical regulator of *NFKBIA* expression.[52,53] Regarding redox metabolism, we chose some of the most well-established genes

regulated by the transcription factor NRF2, which is the master regulator of antioxidant defense and is critically involved in resolution of inflammation.[54] These genes included *GSR* (FC=3.93, p<0.001), *GSTP1* (FC=2.48, p<0.001) and *SQSTM1* (FC=1.88, p<0.001). Moreover, we analyzed two genes involved in ROS production: *NCF1* (FC = 3.21, p<0.001) and *DUOX1* (FC = 3.13, p<0.001). As shown in Figure 9, the selected inflammatory and redox genes exhibited good correlations (Pearson r>0.6 and p<0.001) that indicate an association between these dysregulated inflammation and redox genes.

To elucidate if there are also mechanistic relationships in addition to the associations presented above, we conducted an analysis of the transcription factor binding sites for RELA/p65, RELB and NFKB1/p105 (Supplemental Table 7). We used the ChIP-seq experimental data from
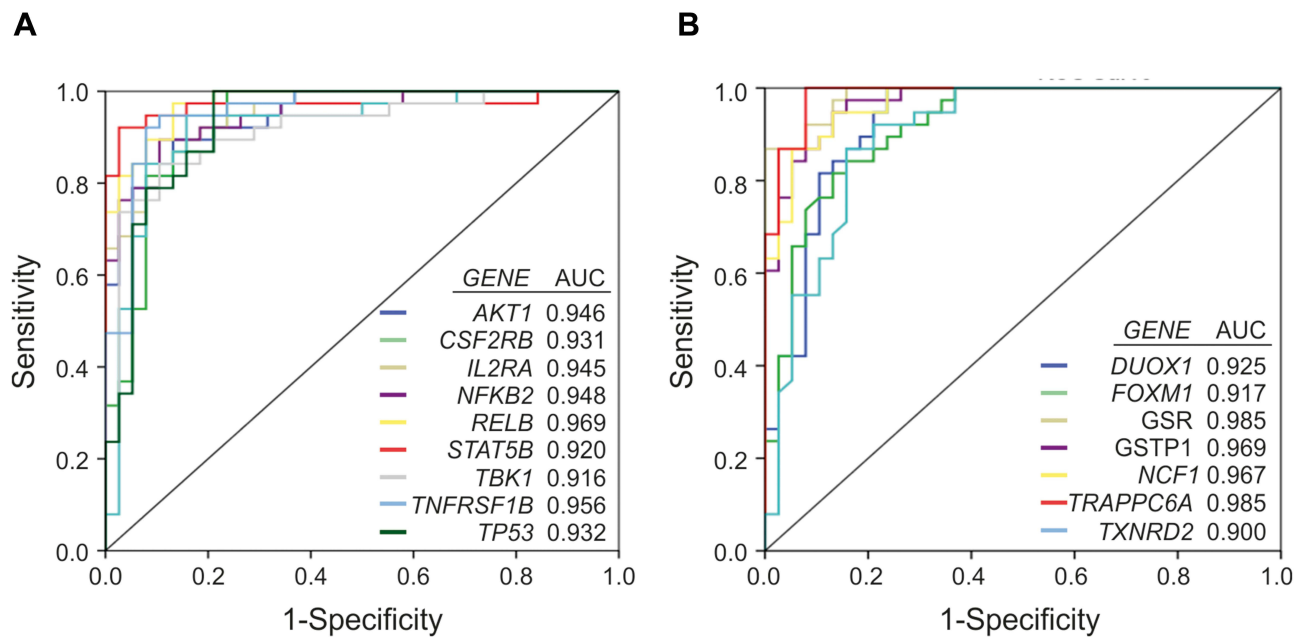
A

B

Figure 7 Receiver operating curve (ROC) analysis of the inflammation and redox genes that were significantly overexpressed (FC>2) in the group of AD patients vs controls, and exhibit area under the curve (AUC) values ≥ 0.9. A, inflammatory genes. B, redox genes.
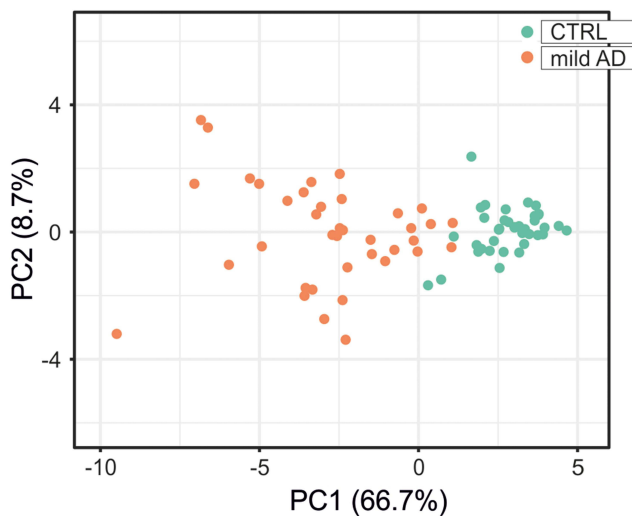

Figure 8 Principal component analysis for the expression level of the 19 inflammatory and redox genes selected from the ROC analysis (Figure 7). Green dots, control subjects; orange dots, AD patients. X and Y axis show principal component 1 (PCA1) and principal component 2 (PCA2) that explain 66.7% and 8.7% of the total variance, respectively.

ChIP-Atlas,[41] and we developed a Python script able to scan a set of genomic regions using a position-specific scoring matrix (PSSM) computed from profiles in the JASPAR database[43] for transcription factor binding sites that are active according to the Combined ChromHMM and Segway segmentation.[55] We then scanned the ChIP-seq sites within RELA, RELB and NFKB1 with their respective PSSMs. Our script retrieved several already known transcription binding sites for RELA, RELB and NFKB1 (relative score >90%) in the *NFKBIA* gene,[56–59] that we used as a positive control. We also found an already described binding site for RELA and NFKB1 in the promoter of *GSTP1*[60] and two additional binding sites: one overlapping with the first, but in the antisense direction, and another one ~2 kb upstream. One binding site for RELA was detected for the *DUOX1* gene in an enhancer region, and four novel binding sites for NFKB1 were found in the promoter region of the autophagy-related gene *SQSTM1*. This evidence suggests that *GSTP1, DUOX1* and *SQSTM1* are NFκB-regulated genes and their expression was found upregulated in the blood of mild AD patients (Figure 3B and Figure 6A and B).

Discussion

This study aimed to determine if transcriptional alterations of inflammation and redox genes in whole blood could provide a molecular signature for mild AD patients as compared to age-matched controls. Because these gene expression changes might be common to various other chronic diseases, the observed alterations cannot be used for diagnostic purposes but rather as 1) evidence that blood leukocytes develop systemic inflammatory and oxidative responses in these patients, 2) identification of the most dysregulated inflammatory and redox pathways in
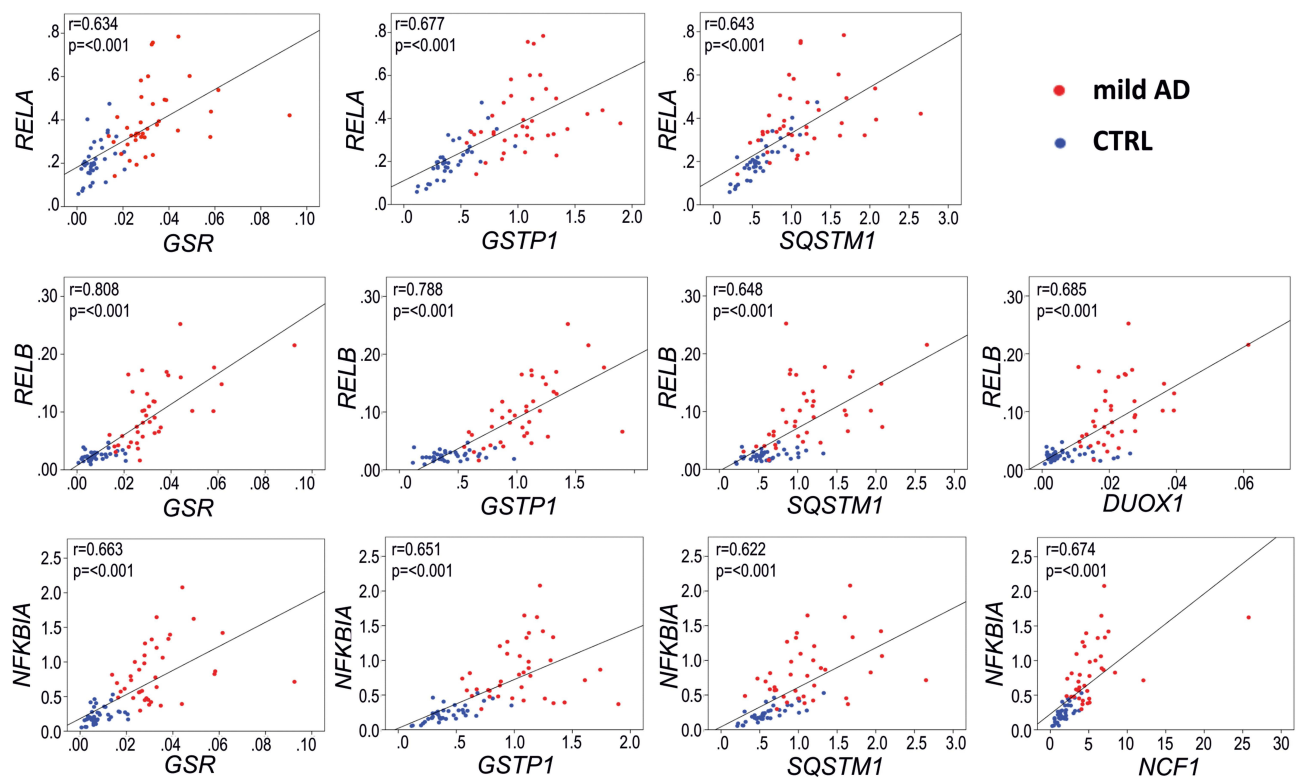
Figure 9 Representative Pearson correlations between inflammation and redox genes in the blood of AD patients.with the most significantly overexpressed (FC>3, p<0.001, and AUC>0.9) inflammation and redox genes. Note the high correlation in expression between the inflammation-related genes *RELB* and *NFKBIA*, regulated by transcription factor RELA, and three redox genes, *GSR, GSTP1* and *SQSTM1*, regulated by the transcription factor NRF2. Blue dots, control group; red dots, AD group. Gene expression data are presented as $2^{-\Delta Ct}$ values.

the blood of mild AD patients, 3) correlation with reported brain alterations in AD, 4) identification of the transcription factors responsible for these alterations.

This clinical case–control study pointed out a distinctive gene expression profile in the blood of mild AD patients. We identified 48 inflammation genes and 34 redox genes that were upregulated in the blood of these patients.

In an attempt to identify the transcription factors that might account for these alterations, we used the oPOSSUM software that identifies common transcription factor binding sites in the dysregulated genes. As expected, we found the participation of NFκB among the inflammation genes but, interestingly, also among the redox genes. The involvement of the transcription factor NRF2, master regulator of redox homeostasis, was expected but, somewhat surprisingly, its transcriptional activity was at the limit of statistical significance. This probably reflects that most of the investigated redox genes already have a robust basal expression, and NRF2 induces most likely a modest but still very relevant response to inflammation. We found that several inflammation and redox genes dysregulated in the investigated cohort

presented binding sites for zinc fingers proteins. It has been long known that trace metals, such as zinc, are impacting on protein aggregation, synaptic signaling, mitochondrial function, oxidative stress and inflammation, ultimately resulting in synapse dysfunction and neuronal loss in the AD brain.[61] It was shown that zinc status alters AD progression through NLRP3-dependent inflammation,[62] and zinc dyshomeostasis has been reported in the plasma of AD patients.[63] Another intriguing finding was the very significant contribution of helix-loop-helix transcription factors to the dysregulated redox gene expression pattern. It is particularly interesting the case of ARNT/HIF1α, related to the hypoxic response,[64] and of ARNT/AHR,[65] involved in ageing and inflammation. Increased HIF-1-mediated expression of target genes, such as those related to glycolysis or blood flow, may be an adaptive response to oxidative stress in AD pathology.[66] Regarding AHR, its levels were shown to be increased in astrocytes of hippocampal post-mortem tissues and in the serum of AD patients.[67]

Substantial overexpression (FC>2, p<0.001) was registered in the blood of the investigated AD patients for 22 inflammation and 12 redox genes that are involved in

several distinctive functions. Very relevant is the dysregulation of the non-canonical NFκB signaling pathway, including the CD40 receptor and the members of the tumor necrosis factor family, lymphotoxin alpha and beta,[68] as well as core modulators of inflammatory genes such as *RELB, NFKB2* and *TRAF2*. Considering that the non-canonical pathway requires *de novo* protein synthesis[69] overexpression of these genes points out an ongoing inflammatory process. On the other hand, the *NFKBIA* gene, that inhibits the canonical NFκB pathway, was found significantly upregulated in our blood analysis as well as in the frontal and temporal cortex of AD patients (Table 2). *NFKBIA* contains several κB-responsive elements that participate in a negative feedback control of the canonical pathway, and therefore *NFKBIA* upregulation might reflect an activation of the canonical pathway. This pathway is widely regulated through post-transcriptional protein processing by phosphorylation, sumoylation and ubiquitination of RELA, and not at transcriptional level.[70] Accordingly, our results do not exclude a combined activation of both canonical and non-canonical NFκB signaling pathways. In fact, because non-canonical NFκB activation requires an early contribution of the canonical signaling cascade,[71] results indicate that the canonical NFκB pathway might be indeed activated in the blood of the investigated patients.

We found dysregulation of the interferon signaling pathway at the level of *IRF1, TBK1* and *STAT1* in the blood of the investigated AD patients. High expression levels of *IRF1* were also found in the AD brain, in the frontal and temporal cortex (Table 2). The TANK binding kinase 1 (TBK1) is an IκB kinase (IKK) involved in the regulation of type I interferons and NFκB signal transduction triggered by pathogens and endogenous stimuli.[72] In the brain, TBK1 may promote TAU hyperphosphorylation and neuronal loss in AD and related tauopathies. Our findings also evidence the enhanced transcription of two genes under the NFκB transcriptional control, that encode members of the STAT protein family of transcription factors (*STAT1* and *STAT5*) and are involved in many aspects of cellular immunity, proliferation, apoptosis, and differentiation.[73] Moreover, STAT1 can regulate the expression of β secretase 1 for the generation of Aβ peptides,[74] while STAT5 seems to regulate microglial activation.[75]

Apoptosis-modulating NFκB targets were also found overexpressed in the blood of the investigated AD patients, including the genes encoding the pro/anti-apoptotic Bcl-2-like protein 1 (BCL2L1) and AKT1, as well as the apoptosis inducer Fas ligand (FASLG). GEO data mining evidenced that *BCL2L1* and *FASLG* have been found overexpressed also in the cortex of AD patients (Table 2), contributing to cell death and neurodegeneration. In addition, it has been shown that higher counts of apoptotic peripheral leukocytes were registered in AD patients, probably accounting, at least partially, for their increased susceptibility to infections.[76]

Besides inflammation, a distinctive redox status was evidenced at transcriptional level in the blood of the investigated AD patients. Significant overexpression of genes involved in superoxide metabolism included the NADPH oxidases *NOX2* (the p47phox component encoded by *NCF1*), *DUOX1* and *DUOX2*, as well as the copper chaperone for superoxide dismutase (*CCS*) involved in the activation of cytoplasmic copper/zinc superoxide dismutase-1. These results point towards an increased production of reactive oxygen species (ROS) in the blood of mild AD patients. Consistent with this, we found overexpression (FC>1.5) of several antioxidant genes that are directly or indirectly regulated by the transcription factor NRF2. Among bona fide NRF2 target genes,[77] we identified several genes involved in glutathione (GSH) metabolism, such as the cysteine-glutamate exchanger Xc(-) that provides cysteine for GSH synthesis (*SLC7A11*),[78] GSH regeneration (*GSR*)[79] and GSH use in antioxidant responses (*GPX2, GPX3, GSTP1, GSTZ1*).[80] Noteworthy, it has been recently demonstrated in vivo that glutathione levels in specific brain regions, measured by magnetic resonance spectroscopy, is a clinically relevant biomarker in AD.[81] We also found increased expression of genes involved in thioredoxin metabolism (*TXNRD1*),[77] or encoding antioxidant peroxiredoxins (*PRDX2*),[82] NAD(P)H quinone oxidoreductase (*NQO1*),[83] heme oxygenase 1 (*HMOX1*),[83] and autophagy cargo receptor p62 (*SQSTM1*).[84] Among these NRF2 responsive genes, at least *NQO1* and *HMOX1* were found to be overexpressed also in post-mortem frontal or temporal cortex of AD patients.[85]

The stepwise selection procedure applied to the investigated inflammation and redox genes brought to the forefront a panel of nine inflammation and seven redox genes that were significantly overexpressed (FC>2), and discriminate between patients and controls according to the ROC and PCA analyses. Moreover, the inter-pathway correlation studies indicated potential functional association between at least some of these inflammation and redox genes. Mechanistically, we have highlighted for the first time the regulation by *RELA* of two genes involved in ROS production (*DUOX1*) and in GSH regeneration (*GSR*). Moreover, two NRF2 target genes, the antioxidant *GSTP1* gene and the autophagy-related *SQSTM1*

gene, were correlated with *NFKB1* expression level, indicating a sophisticated crosstalk between oxidative stress, inflammation and autophagy.[86] Autophagy impairment has been shown to play a critical role in AD by accretion of noxious proteins in the brain,[87] and also in shaping both the systemic innate and adaptive immune responses, including antigen presentation.[88]

A coordinated modulation of ROS metabolism and inflammation in the blood of mild AD patients is suggested. Nevertheless, the present study, which is based on gene expression, can only evidence gene associations but cannot substantiate functional interferences between the identified inflammation and redox genes, beyond the known redox-control of the transcriptional activity of NFκB.[89,90]

Summarizing, the reported alterations provide a gene expression signature in the blood of mild AD patients that, albeit not being specific for this pathology, certainly emphasizes the relevance of blood inflammatory and redox disturbances in AD. The gene expression changes registered in the blood of mild AD patients are indicative of possible systemic immune disturbances, especially at the level of the innate immunity. These disturbances are seemingly not due to ageing, considering that age-matched individuals constituted the control group.

Despite the well-characterized and homogeneous cohorts used in this study, the main limitation is represented by the small sample size. Power analysis results (Power 1-β error=0.56, α=0.05), obtained using the G*Power tool, impose future investigations in larger independent cohorts for validating the most relevant findings of this study and for avoiding the risk of over-fitting. Additionally, investigations at protein and cellular functional level are needed to clearly demonstrate the impact of the identified gene expression changes on the peripheral immune response and the connection with the pathological processes in AD brain,[91] in the context of leukocyte trafficking to the brain through the compromised blood–brain barrier.[92]

Conclusion

This gene expression study brings new evidence on the activation of inflammatory pathways accompanied by increased redox activity and regulation in the blood of mild AD patients. The reported alterations of signaling pathways in blood leukocytes might have consequences on immune competence and neurodegeneration in mild AD, but this has to be demonstrated in future studies. Besides NFκB, several other transcription factors, including zinc finger and helix-loop-helix proteins, as well as the cytoprotective NRF2 transcription factor may underlie the altered transcriptional profile of inflammation

and redox genes in mild AD. We highlight for the first time the potential contribution of *RELA* to the enhanced expression of the redox genes *DUOX1* and *GSR*. Altogether, the selected nine inflammation and seven redox genes might be promising therapeutic targets, and, in conjunction with disease-specific markers, could also represent valuable tools for monitoring new adjunctive therapeutic approaches targeted towards inflammation and redox disturbances in mild AD.

Availability of Data and Materials

Supporting data are available from the corresponding authors upon reasonable request

Ethics Approval and Consent to Participate

The study was conducted in accordance with the Declaration of Helsinki and was approved by the ethics committee Hospital Arnau de Vilanova de Lleida, Lleida, Spain – CE 1218. Written informed consent was obtained from all study participants at the moment of recruitment and blood collection.

Author Contributions

All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, agreed to the submitted journal, and agree to be accountable for all aspects of the work. Elena Milanesi and Maria Dobre contributed equally and thus share first authorship.

Disclosure

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