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Evaluation of the Ankylosing Spondylitis Transcriptome for Oxidative Phosphorylation Pathway: The Shared Pathway with Neurodegenerative Diseases

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ABSTRACT

Ankylosing spondylitis (AS) is a systemic inflammatory disorder of joints and entheses. Recent studies have reported an increased prevalence of dementia in AS patients. However, data for exploring the association between dementia and AS remain uncertain.

In this study, enriched pathways and differentially expressed genes (DEGs) were identified in whole blood transcription data of AS patients obtained from the gene expression omnibus (GEO) database; using gene set enrichment analysis (GSEA) and differential expression analysis.

Four pathways, including oxidative phosphorylation, Alzheimer's, Parkinson's, and Huntington's diseases were significantly enriched in AS patients compared to the controls. We identified 22 common genes among the pathways that showed an increasing trend in AS compared to the controls. Five of them including *COX7B*, *NDUFB3*, *ATP5PF*, *UQCRB*, and *NDUFS4* were the most significant genes which were selected for gene expression analysis; using real-time PCR on RNA contents of peripheral blood mononuclear cells (PBMCs) of AS patients and controls (20 samples from each group). The gene expression analysis indicated considerable overexpression of *COX7B* ($p<0.0001$) and *ATP5J* ($p=0.0001$) genes in AS patients group in comparison to the control samples.

The role of oxidative phosphorylation has previously been established in dementia pathogenesis. Given that AS patients have also a remarkably higher prevalence of dementia than the their healthy counterparts, hence our results may propose that the common pathway of oxidative phosphorylation can be regarded as a possible shared contributing factor in the etiopathogenesis of AS and dementia.

Keywords: Ankylosing spondylitis; Dementia; Gene expression; Oxidative phosphorylation

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INTRODUCTION

Ankylosing spondylitis (AS) is an autoimmune disease that mainly affects the spine, sacroiliac joints, and, occasionally, peripheral joints. It is an inflammatory rheumatologic disease that can progress to fundamental functional disabilities.[1-3] Based on the genetic stratification of different populations, the outbreak of AS varies, and men are more affected than women by the disease.[1,4-6]

AS patients have several comorbidities, including psychiatric disorders and cardiovascular diseases.[7,8] According to a recent report, AS patients have a higher prevalence rate of Alzheimer's and overall dementia than the total population.[8] Although numerous hypotheses have attempted to clarify the relation between AS and dementia, like an excessive amount of serum amyloid or chronic inflammation, the exact reason is still unclear.[9-11]

Despite several genetic markers like human leukocyte antigen (HLA) B27 and endoplasmic reticulum aminopeptidase (ERAP), genes encoding transcription factors, cytokine receptors, transport proteins, and signaling molecules have already been implicated in the pathogenesis of AS, the precise cause of the disease is unknown.[12,13] Therefore, much remained to be done to comprehend the potential biomarkers and significant pathways in AS.

In the current study, we initially downloaded the whole blood transcription data of AS patients from the Gene Expression Omnibus (GEO) database. Then, using gene set enrichment analysis (GSEA) enriched pathways were identified. Uniform trend in GSEA enrichment plot for oxidative phosphorylation and neurodegenerative diseases pathways results in exploring common significant genes in these pathways. Finally, the expressions of interest genes were experimentally measured in AS and healthy groups using Real-time polymerase chain reaction (PCR).

MATERIALS AND METHODS

Microarray Dataset Analysis

The publicly accessible microarray dataset GSE25101 and its GPL6947 Illumina Human HT-12 V3.0 expression platform were downloaded from the National Centre for Biotechnology Information GEO. The dataset included 16 AS patients and 16 healthy controls and the platform contained 49576 probes.[14]

Gene Set Enrichment Analysis (GSEA)

GSEA was applied on the normalized expression data using the GSEA software available from the Broad Institute Website (http://www.broadinstitute.org/gsea). GSEA estimates how genes in predefined sets of genes are dispensed based on the fold change (AS versus Control) ordered list generated by our data (all probe sets included).

GSEA calculates four principal statistics including enrichment score (ES), normalized enrichment score (NES), nominal *p*-value, and false discovery rate (FDR) for the gene set enrichment analysis report. A positive or negative ES indicates the gene set enrichment at the top or bottom of the ranked list, respectively. The NES is estimated based on the ES for all dataset permutations and could be utilized to compare the results throughout the gene sets. Furthermore, the nominal *p*-value computes the statistical significance of the ES for a single gene set. But in the case of multiple gene sets evaluation, multiple hypothesis testing and the correction of gene set size are required. Therefore, the FDR is employed for estimated probability. Based on the GSEA report, the FDR less than 25% is appropriate, although the smaller value of FDR could be selected for a more stringent cutoff.[15]

To identify pathways that correlate with the AS phenotype, a Gene sets C2-KEGG database with 1,000 gene set permutations was implemented. Gene sets in this database are derived from the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database.[16]

Differential Expression Analysis

Differential expression analysis was performed using R (version 3.5.2; http://CRAN.R-project.org/bin/windows/base) statistical computing environment and BioConductor (version 3.9).[17] The limma package,[18] with empirical Bayes method was used to assess the differentially expressed genes between AS and control groups in normalized and log2 transformed of series matrix file. Then, the probe IDs basing on the annotation files which were downloaded from GEO were converted into gene symbols. The genes meeting our criterion (adjust p-value<0.05) were regarded to be differentially expressed genes.

Sampling from Ankylosing Spondylitis Patients and Healthy Controls

For this study, 20 AS patients and 20 age and gender-matched controls (each group contains 5 females and 15 males) were enrolled from the Rheumatology Research Center (RRC) outpatient clinic, Shariati Hospital. In conformity with modified New York Criteria (MNYC) for AS, patients were diagnosed by a rheumatologist. The average disease duration of AS patients was 8.7±9.8 years and the frequency of HLA-B27 positive in patients was 80 percent. The mean ±SD age of healthy controls and AS group was 36±7.4 and 35±11.3, respectively. The healthy group did not have a history of autoimmune disease in themselves and their families (more demographic features are shown in Table 1).

This study was performed based on the Declaration of Helsinki guidelines and was approved by the Ethics Committee of Tehran University of Medical Sciences (Approval No: IR.TUMS.VCR.REC.1398.1000) and all subjects signed a consent form for participation. From all participants, 5 mL of peripheral blood was obtained into ethylenediaminetetraacetic acid (EDTA)-anticoagulated tubes; using venipuncture.

Peripheral Blood Mononuclear Cell (PBMC) Isolation, RNA Extraction, and cDNA Synthesis

Ficoll-Hypaque gradient (Innotrain, Germany) was employed to isolate PBMCs from whole blood samples. Consequently, total cellular RNA was extracted; utilizing a high pure RNA Isolation Kit (Progen lab, Germany). The quantification of RNA was determined by spectrophotometry (NanoDrop ND-2000 C, Thermo Fisher Scientific, USA). According to the manufacturer's protocols, isolated RNA was used to synthesize complementary DNA (cDNA) by Transcriptor First Strand cDNA Synthesis Kit (Roche, Germany).

Quantitative Real-time PCR

SYBR Green (Ampliqon, Odense, Denmark) and a StepOnePlus Real-time PCR system (Applied Biosystems, Foster City, USA) were employed for quantitative PCR. The reaction mixture contained 7.5 µL SYBR Green PCR master mix, 1.5 µL cDNA template, 1.5 µL of primers (0.75 µL each), and 4.5 RNase free water to a total volume of 15 µL. Details of primers are listed in Table 2. The basic local alignment search tool on the NCBI website was used for analyzing the specificity of primers. The real-time PCR conditions were as follows: 95°C for 15 minutes (holding stage), 40 cycles of 95°C for 15 seconds, and 63°C for 1 minute, and then 95°C for 15 seconds (extension stage), 60°C for 1 minute, and finally 95°C for 15 seconds.

The comparative CT method was utilized for the relative gene expression analysis[19,20] *Hypoxanthine phosphoribosyltransferase 1* (*HPRT1*) mRNA transcript level as an internal control gene was used for normalizing the relative amount of target gene transcript levels.

Statistical Analysis

Normal distribution of the variables was evaluated by the Kolmogorov–Smirnov test and subsequently, the Mann–Whitney or independent sample *t*-test was used for abnormal or normal data, respectively. GraphPad Prism 8.0 (GraphPad Software Inc, CA, USA) was used to plot the graphs. The result with a *p*-value <0.05 was considered statistically significant.

Table 1. Baseline characteristics of ankylosing spondylitis (AS) patients and healthy controls

Property	AS patients (n=20)	Healthy controls (n=20)
Male	15 (75%)	15 (75%)
Female	5 (25%)	5 (25%)
Age	35 ± 11.3	36 ± 7.4
BASDAI	6.4 ±2	-
BASFI	4.5 ±2.6	-
BASMI	3.2 ±1.2	-
Disease duration	8.7	-

BASDAI, Bath Ankylosing Spondylitis Disease Activity Index; BASFI, Bath Ankylosing Spondylitis Functional Index; BASMI, Bath Ankylosing Spondylitis Metrology Index.

Table 2. The sequence of primers used in a real-time polymerase chain reaction (PCR).

Gene	Forward primer	Reverse primer	Product size (bp)
COX7B	5′-AGCGCACTAAATCGTCTCCA-3′	5′-TGTACGTTTCTGGTGGCTCT-3′	72
ATP5J	5′-TCAGCCGTCTCAGTCCATTT-3′	5′-ACTAGCATCAACAGGTCCTCC-3′	150
UQCRB	5′-ATCAGCCCATTGAGTGTCCC-3′	5′-TGCAGTTCAAGGGGTGAGAG-3′	73
NDUFS4	5′-TGCTCGCAATAACATGCAGTC-3′	5′-GATCAGCCGTTGATGCCCAA-3′	113
NDUFB3	5′-GCTGGCTGCAAAAGGGCTA-3′	5′-CTCCTACAGCTACCACAAATGC-3′	146
HPRT1	5′-GGTGAAAAGGACCCCACGAA-3′	5′-AGTCAAGGGCATATCCTACAACA-3′	92

RESULT

The normalized expression dataset of AS was significantly enriched in the eight biological pathways by utilizing the GSEA method. We used FDR<0.1, and *p*-value<0.01 as the cutoff point to determine whether KEGG pathways were significantly enriched. Figure 1 shows 22 shared genes involved in oxidative phosphorylation (ES=0.6, NES=2.3), Alzheimer's disease (AD) (ES=0.5, NES=2), Parkinson's disease (PD) (ES=0.6, NES=2.2), and Huntington's disease (HD) (ES=0.4, NES=1.6) pathways, which were significantly enriched.

Differential expression analysis determined nine of the common genes were up-regulated significantly with adjusted *p*-value <0.05 in AS patients compared to the control group. According to the highest absolute logarithm fold change (LogFC) and the lowest adjusted *p*-value, five genes were selected for further analysis. As shown in the heatmap plot (Figure 2a), five selected genes, including *Cytochrome C Oxidase Subunit 7B* (*COX7B*), *NADH dehydrogenase 1 beta subcomplex, 3* (*NDUFB3*), *ATP5J* or *ATP synthase peripheral stalk subunit F6* (*ATP5PF*), *Ubiquinol-cytochrome c reductase binding protein* (*UQCRB*), and *NADH: ubiquinone oxidoreductase subunit S4* (*NDUFS4*) were significantly over-expressed in AS group compared with the control group (Log FC=1.05, 0.93, 0.78, 0.70 and 0.6, respectively). Principal component analysis (PCA) was applied to reduce dimensionality by using function "prcomp" from the stats package (version 3.6.1). The top three principal components (PC) of the selected genes were investigated to display the separation rate between two groups of patients and controls. The PCA figure (Figure 2b) was generated with R package Scatterplot3d (version 0.3-41).[21] In this graph x, y, z coordinates specifying PC2, PC1, and PC3, respectively, and it revealed the ability of interest genes to relatively distinguish the AS samples (indicated by orange circles) and the control samples (indicated by blue circles). Since these five genes had significantly different expressions between the two groups (adjusted *p*-value <0.05), they were selected for further investigation; using the real-time PCR method.

Based on our experimental analyses, the mRNA expression level of *COX7B* in AS patients was significantly higher than healthy group (FC=1.73, *p*<0.0001; Figure 3a). Furthermore, PBMCs from AS patients expressed the *ATP5J* mRNA higher than control group (FC= 1.36, *p*=0.0001; Figure 3b). The mRNA expression levels of *NDUFB3* (FC=1.04, *p*=0.73; Figure 3c), *NDUFS4* (FC= 1.10, *p*=0.21; Figure 3d), and *UQCRB* (FC= 0.7, *p*=0.19; Figure 3e) were not different between two groups.

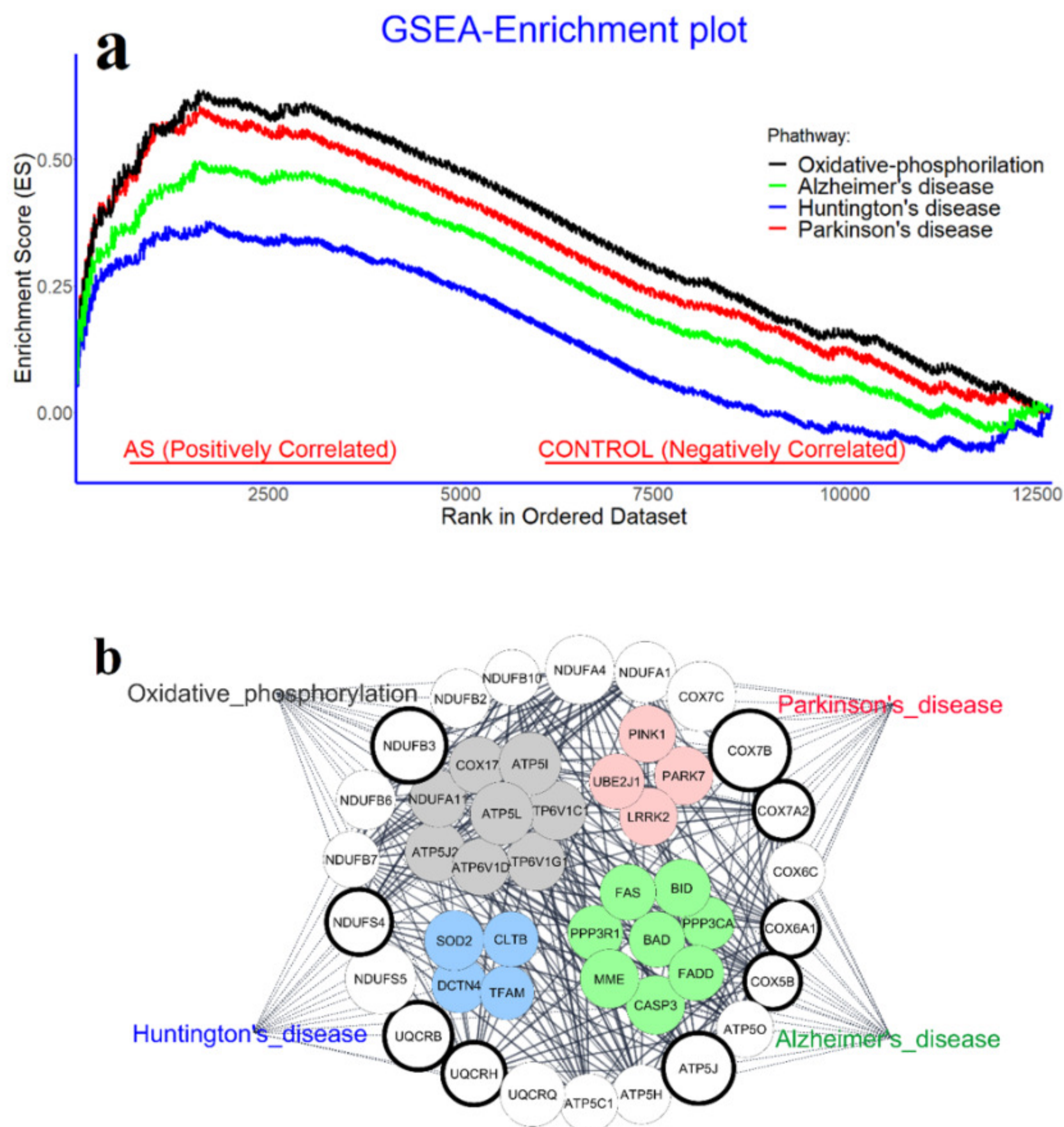

Figure 1. a) Enrichment plot. Genes expression in four pathways including oxidative phosphorylation, Parkinson's disease (PD), Huntington's disease (HD), and Alzheimer's disease (AD) are positively correlated in the ankylosing spondylitis (AS) patients compared to the controls. b) Network of enriching genes of the four mentioned pathways. The colorless circles contained shared genes in Gene set enrichment analysis (GSEA) methods and the circles in gray, red, blue, and green included the exclusive genes of oxidative phosphorylation, PD, HD, and AD pathways, respectively. The size of colorless circles indicates absolute LogFc and their borders' thicknesses related to adjusted *p*<0.05 in differential expression analysis. The gene connections were made by stringApp in Cytoscape.[22]

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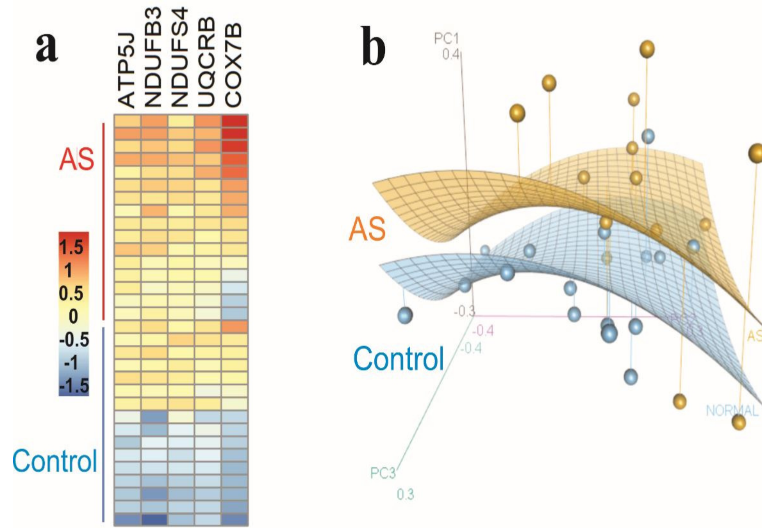

Figure 2. Heatmap and principal component analysis (PCA) plots. a) Revealed that the expression of *COX7B*, *NDUFB3*, *ATP5J*, *UQCRB*, and *NDUFS4* were expressed at a higher level in ankylosing spondylitis (AS) than the control group. b) PCA plot shows that the expression of 5 interested genes could relatively be clustered between two groups. AS and control groups were indicated by orange and blue colors, respectively (The graphs were generated with the R packages of "pheatmap" and "scatterplot3d").

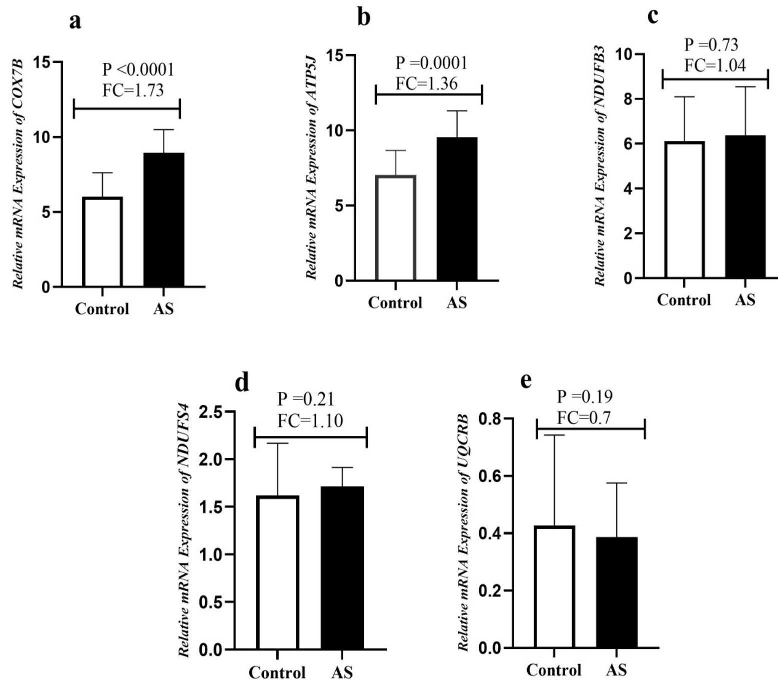

Figure 3. Bar graphs demonstrate the relative mRNA expression of (a) *COX7B*; (b) *ATP5J*; (c) *NDUFB3*; (d) *NDUFS4*; and (e) *UQCRB*, in 20 ankylosing spondylitis (AS) patients *vs*. 20 healthy controls (FC: Fold Change, P: *p*-value based on Mann Whitney or independent T-test).

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DISCUSSION

AS patients have several comorbidities like increased risk of cardiovascular (CV) diseases.[22] Bath AS Disease Activity Index (BASDAI), Bath AS Functional Index (BASFI), and Bath AS Metrology Index (BASMI) scores of AS patients have been established to correlate with depression and anxiety.[23] They could help the physicians to find the AS psychological complications like sleep disorders.[24] Based on a recent cohort study, AD (0.99%) and overall dementia (1.37%) have a higher prevalence rate in AS compared to the control group.[8] The inflammation hypothesis of common mental disorders suggests that chronic inflammation in AS patients contributes to the pathophysiology of the disease. Due to the paucity of studies about the effects of chronic inflammation on the co-occurrence of mental disorders in AS, it has not yet been determined whether the dependence of inflammation and common mental disorders is the result of acute or chronic inflammation.[10] In this study, we propose a probable association between AS and neurodegenerative diseases.

Mitochondrial oxidative phosphorylation, which is an assembly of respiratory enzyme complexes I, II, III, IV, and V, is one of the critical cellular processes that provide ATP for biological activities.[25,26] The prevalence of mitochondrial respiratory chain diseases is approximately 1 per 5000 subjects in children and adults.[27] Previous studies have reported that mitochondrial dysfunctions, particularly the electron transport chain, are responsible for many neurodegenerative diseases, such as AD, PD, and HD.[28,29] Another study suggested that only the neurons with overexpressed cytochrome oxidase go through oxidative damage in AD brains.[30] In addition, Zhang et al showed that the most significant pathway in AD was oxidative phosphorylation.[29]

In the current study, the GSEA method resulted in the identification of 22 enriched genes that were common in the four pathways of oxidative phosphorylation, PD, HD, and AD [(data is accessible in the pathway of homo sapiens-electron transport chain in wikiPathways database (Figure 4)]. This figure was constructed using a plug-in of the Cytoscape software (version 3.6.1), wikiPathways.[32] The expression levels of five interest genes of this pathway, including *COX7B*, *ATP5J*, *NDUFB3*, *NDUFS4*, and *UQCRB* were assessed quantitatively; using the StepOnePlus real-time PCR system. The result of the Real-time PCR has shown that the expression of *COX7B* and *ATP5J* were significantly increased in AS patients in comparison with healthy subjects. Interestingly, *COX7B*, *NDUFB3*, and *ATP5J2* genes were reported to be differentially expressed in an Alzheimer's dataset.[29]

Cytochrome c oxidase (COX) is a critical cellular enzyme with an essential function in oxidative metabolism. The mammalian COX complex consists of 13 different polypeptide subunits.[31,32] Defective COX biogenesis affects tissues with high energy demand and has been associated with severe mitochondrial diseases.[33-35] *COX7B* is a nuclear-encoded protein that develops a complex with three mitochondrial-encoded proteins (*COX1*, *COX2,* and *COX3*). *COX7B* gene includes three coding exons and codes for an 80 amino acid mitochondrial protein. The role of this gene in Microphthalmia with linear skin lesions (MLS) and an X-linked dominant male-lethal disorder has been demonstrated.[36] Moreover, the expression of *COX7B* is increased in AD brains and its overexpression in cells enhanced toxicity of Amyloid-beta peptide (1-40).[37]

ATP5J is a protein that connects F0 and F1 components of ATP synthase.[38] It is required for the interactions of the catalytic and proton-translocating segments.[39] The *ATP5J* association has been reported with several disorders, such as hypertension and insulin resistance and it is among the top genes identified by integrated analysis that affect the hippocampus in Alzheimer's disease.[29,40] On the other hand, decreased prostacyclin level in coronary heart disease may be a consequence of an increased *ATP5J* level. Therefore, as a potential risk factor for the disease, *ATP5J* might have a vital clinical significance.[41] In end-stage renal disease, *ATP5J* is a novel risk factor for ischemic heart disease.[42] Furthermore, over-expression of this gene was associated with cell migration in colorectal cancer.[43] On the other hand, increased occurrence of cancer, insulin-dependent diabetes mellitus, and cardiac complications have been found in AS patients.[44-46] This suggests that the oxidative phosphorylation pathway is probably involved in comorbidities of AS with other diseases.

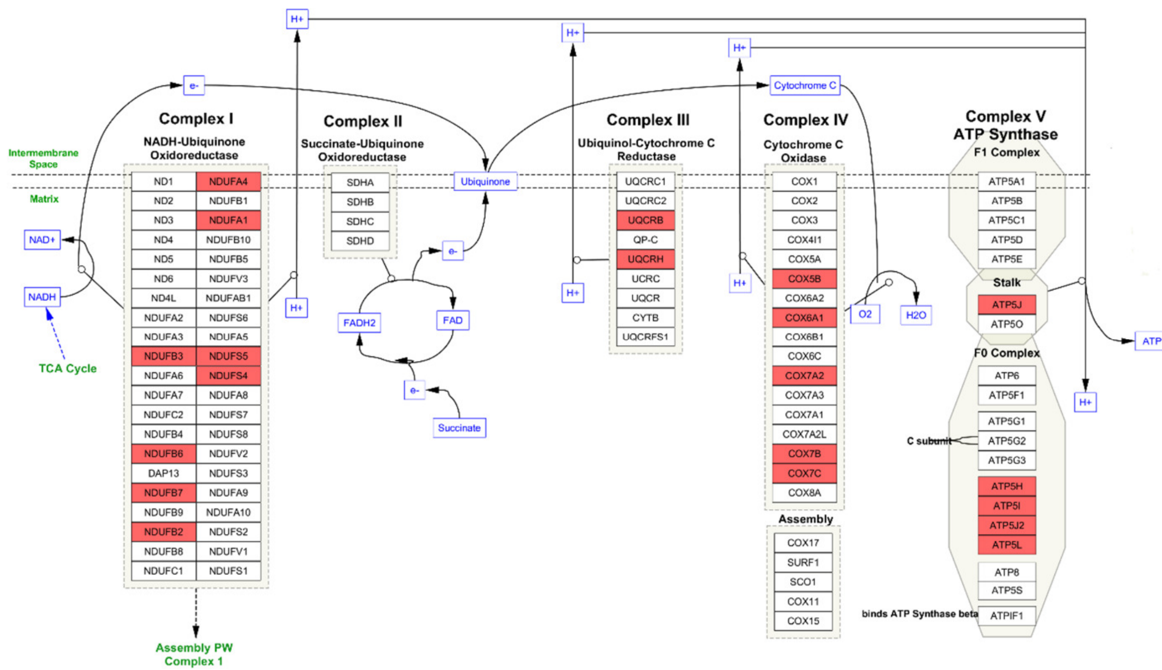

Figure 4. Electron transport chain pathway. The red rectangles contain the common enriching genes in oxidative phosphorylation, Parkinson's disease (PD), Huntington's disease (HD), and Alzheimer's disease (AD) pathways in the GSEA method.

In addition, oxidative phosphorylation is related to the generation of integral signaling molecules, like reactive oxygen species (ROS).[47] Previous studies revealed the inappropriate release of ROS in the bloodstream as well as in synovial fluid of AS patients.[48] Cathepsin activity may be inhibited by ROS by affecting the major histocompatibility complex (MHC) II function and, consequently, the possibility of cross-presentation will be increased.[49] Cross-presentation of the intracellular pathogen *Salmonella Typhimurium* is boosted through the ROS overrepresentation by MHC molecules.[50] Studies have determined that ROS modifications in an inflammatory environment increase the antigenicity of collagen.[51] Moreover, it was shown that autoantibodies against type I and II collagens were highly produced at the cartilaginous sites of AS patients.[52] Mitochondria-derived ROS is involved in autoimmunity and inflammation. It seems mitochondrial ROS have a major role in activating the NACHT, LRR, and PYD domains-containing protein 3 (*NLRP3*) inflammasome, which is essential for activation of pro-inflammatory IL-1 family cytokine.[49,53-55] Therefore, impairments in the oxidative phosphorylation pathway may culminate in the dysregulation of inflammatory pathways in AS patients.

In consideration of all, we explored critical pathways and genes that likely were involved in the pathogenesis of AS in an expression dataset obtained from the GEO database. Four pathways, including oxidative phosphorylation, AD, PD, and HD were significantly enriched and increased in AS patients compared with the control group. Real-time PCR gene expression analysis indicated significant overexpression of *COX7B* and *ATP5J* genes in AS group in comparison to the control samples. We propose the common pathway of oxidative phosphorylation as a possible cause of comorbidity between AS and dementia. Findings in this study might improve our understanding of the importance of the oxidative phosphorylation pathway in AS disease. Consequently, anti-oxidants could help improve the disease manifestations of AS patients.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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