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Gene expression analysis in plasma of patients with Alzheimer's disease

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Abstract: Alzheimer's disease (AD), which is a neurodegenerative disease, cannot be noticed until severe symptoms are observed. This poses a global challenge as the average human lifespan increases, making it a concern for the entire world population. Early diagnosis can play a crucial role in slowing the progression of the disease, thereby enhancing the quality of life for both the patient and their relatives. AD has been linked to alterations in mRNA expressions. The objective of the presented study was to determine whether there were significant differences in gene expression in blood plasma between Alzheimer's patients and healthy controls. *MAPT, APP, Tubb3, TrkB,* and *CDC42* genes were selected as target genes due to their potential associations with AD. To analyse mRNA expression levels in the control group and AD patients, the real-time PCR (qPCR) method was performed. The findings indicate that *MAPT*, *APP, Tubb3* and *CDC42* genes' expression levels were significantly downregulated by 1.09, 1.08, 1.09 and 1.14 times, respectively ($p<0.05$) in AD patients. Although the *TrkB* gene expression appeared to be downregulated by 1.03 times in the AD group, it is not statistically different. Given the molecular associations between the pathways of the target genes and AD, changes in the expression of these genes may contribute to the pathogenesis of AD. They may represent potential biomarkers for early diagnosis.

Keywords: Alzheimer's disease; Gene expression; mRNA profiling

1.0 INTRODUCTION

As the demographic characteristics of societies continue to shift towards an ageing population, the prevalence of dementia is on the rise. Globally, it is estimated that the total number of individuals with dementia will reach 75 million by 2030 and 135 million by 2050 (Robinson et al., 2015). It is known that Alzheimer's disease (AD) is the most common of all dementia types, and it accounts for 60 to 80% of dementia cases (Ashraf et al., 2016).

AD is a disease pathologically characterised by senile plaques and neurofibrillary tangles in certain parts of the brain, in addition to the degeneration of some types of neurons in the brain, resulting in severe neuronal loss. It is characterised by deterioration in other

cognitive functions as the disease progresses (Terry et al., 1991). AD usually begins at later ages and is considered to be the most common cause of cognitive impairment in individuals over the age of 65. The disease lasts about 3-20 years until death (Zvěřová, 2018).

Although it is known that oxidative stress and genetic and environmental factors are among the risk factors of the disease, the main leading cause of the late onset of the disease has not yet been elucidated. For this reason, the disease can only be diagnosed by observing severe symptoms, which often causes an early diagnosis not to be made (Albert et al., 2011). Given the lack of a current treatment or prevention method for AD, the most effective approach is to implement early intervention strategies aimed at delaying disease progression and ultimately decreasing the incidence of AD (Kim et al., 2014).

Therefore, biomarkers need to be identified for early diagnosis. These molecular markers offer valuable insights into the fundamental pathological processes of the condition, enabling earlier and more precise diagnosis. Timely detection plays a pivotal role in Alzheimer's, given that interventions tend to be more effective during the initial stages of the disease. Moreover, biomarkers play a vital role in tracking disease progression and evaluating the effectiveness of treatments.

Amyloid precursor protein (*APP*) and microtubule-associated protein tau (*MAPT*) genes are implicated in AD, with *APP* influencing beta-amyloid production and *MAPT* influencing tau protein regulation, contributing to the neuropathological processes associated with the condition. APP, which is synthesised by the *APP* gene, one of the genes determined to have a relationship with AD in studies in the literature, is a type 1 single-pass transmembrane protein family member. Proteolytic cleavage of APP is known to cause accumulation of β-amyloid peptide in the brains of AD patients (Aydin et al., 2012).

In parallel, the *MAPT* gene encodes the Tau protein, a crucial component in stabilising the axonal cytoskeleton (Coupland et al., 2014). This protein, discovered in 1975, is expressed in neurons and is responsible for cerebral microtubule polymerisation and stabilisation mediators. Previous investigations have revealed that the Tau protein involves various processes such as axonal transport, synaptic plasticity and function, and safeguarding nucleic acids. The accumulation of hyperphosphorylated tau proteins in degenerated neurons suggests that Tau proteins have functional importance in neurodegenerative diseases (Caillet-Boudin et al., 2015).

The gene product synthesised by the cell division cycle 42 (*CDC42*) gene is the small GTPase protein that mediates cytoskeletal rearrangements and cell cycle signalling pathways (Nicole et al., 1999). The neurotrophic receptor tyrosine kinase 2 (*TrkB)* gene encodes for the TrkB protein, which belongs to the protein tyrosine kinase family and functions as a receptor for the neurotrophins brain-derived neurotrophic factor and neurotrophin 4/5 (Stoilov et al., 2002). The tubulin beta 3 class III (*Tubb3)* gene synthesises the β-tubulin protein. This protein is a member of the tubulin protein family responsible for cell shape and movement, forming the cell structure called microtubules (Chew et al., 2013).

This study aimed to investigate the genes that can be used as potential biomarkers in diagnosing AD by performing expression analyses of *APP, MAPT, CDC42, TrkB*, and *Tubb3* genes in patients and control groups. The selection of these genes as target genes in this experiment is grounded in their explained associations with AD. Analysing the expression of these genes aims to identify potential biomarkers by elucidating the complex molecular mechanisms that contribute to AD.

2.0 MATERIALS AND METHODS

2.1 Sample Processing

For this study, blood samples were collected from 22 Alzheimer's patients and 17 healthy individuals (Table 1). The patients' diagnosis of Alzheimer's disease was confirmed through established clinical criteria by the neurologists at the Neurology Clinic, Umraniye Research and Training Hospital. For blood collection materials, the ethics committee approval was obtained from the Üsküdar University, Non-Invasive (Clinical and Human) Researches Evaluation Committee (dated 27.10.2021 and numbered 61351342/October 2021-51).

2.2 Total RNA Isolation

Hybrid-RTM Blood RNA Kit (GeneAll, cat. No: 315-150) was used to isolate RNA from blood collection material. Total RNA isolation was performed according to the manufacturer's protocol. The quantity and quality of the total RNAs acquired were quantified and assessed using the NanoPhotometer NP80 (Implen, Germany) instrument.

2.3 cDNA Synthesis

OneScript cDNA Synthesis Kit (ABM, cat. No: G236) was used to perform cDNA synthesis of isolated total RNA, following the manufacturer's protocol.

2.4 Gene Expression Analysis

The analysis of target gene expressions with real-time PCR (qPCR) was conducted using a LightCycler® 96 Instrument (Roche) using Blastaq 2x qPCR MasterMix (Cat. no: G891). A total of 39 samples were analysed in the study, comprising 22 samples from AD patients and 17 samples from healthy controls, utilising the qPCR method. The absolute quantification of *MAPT, APP, Tubb3, TrkB* and *CDC42* genes normalisation was accomplished using the *GAPDH* housekeeping gene. **Table 2** presents the primer sequences used in the study.

2.5 Statistical and Bioinformatical Analysis

GraphPad Prism 8.3.0 biostatistical database was used to prove the precision and accuracy of the results obtained after normalisation. The qPCR results were statistically evaluated with the non-parametric Mann-Whitney Test, and Receiver Operating Characteristics (ROC) curves were created. STRING database is a database that uses interactions between proteins to identify interactions between genes (https://string-db.org/). This study analysed the interactions between genes using the multiple protein search option. Then, the analysis option determined the pathways associated with the target genes. The GeneMANIA database searches many comprehensive, publicly available biological datasets to find gene networks. The method used in this study is the default analysis method (http://genemania.org/).

3.0 RESULTS

This study analysed gene expression in 39 samples, including 17 healthy controls and 22 AD patients. The mean ages of the groups were 68.41±7.93 and 72.41±6.801, respectively. Statistical analysis indicated no significant difference in age between the groups (p=0.099) (**Table 1**). The analysis unveiled significant differences between the patient and control groups in *MAPT, APP, Tubb3*, and *CDC42* gene expressions. The alterations in mRNA expression levels of the target genes are illustrated in **Figure 1**. The qPCR data was analysed using GraphPad Prism (version 8.3.0). The results of the statistical analyses are presented in **Table 3**, and the ROC curve analysis graphs are illustrated in **Figure 2**.

Table 1. The mean age of Alzheimer's disease patients and control individuals involved in the study.

	AD (n=22)	Control (n=17)	P-value
Mean of age ± SD	72.41 ± 6.801	68.41 ± 7.93	0.099

SD: standard deviation.

Table 2. The list of target genes' primers.

Target genes	Oligonucleotide sequence (5'-3')	Melting temp. (°C)
MAPT	F: GACTATCAGGTGAACTTT R: GCCAGCGTCCGTGT	43.7 55.5
APP	F: GAACTACATCCCGCTCTGC R: CGCGGACATACTTCTTTAGC	58.4 57.7
Tubb3	F: CATCCAGAGCAAGAACAGCA R: CTCGGTGAACTCCATCTCGT	60.1 60.3
TrkB	F: CCAAGAATGAGTATGGGAAGG R: GCATAGACCGAGAGATGTTCC	58.5 58.8
CDC42	F: CCATCGGAATATGTACCGACTG R: CTCAGCGGTCGTAATCTGTCA	61.5 61.4
GAPDH	F: AGGGCTGCTTTTAACTCTGGT R: CCCCACTTGATTTTGGAGGGA	59.4 65.4

Table 3. Statistical analysis of genes' mRNA expression.

Genes	Mann-Whitney	ROC analysis			
	p-value	AUC	SD	p-value	%95 CI
MAPT	0.01	0.72	0.09	0.01	0.54-0.90
APP	0.01	0.74	0.08	0.01	0.57-0.90
Tubb3	0.0005	0.81	0.06	0.0008	0.68-0.94
TrkB	0.33	0.59	0.09	0.32	0.40-0.78
CDC42	0.0003	0.82	0.06	0.0005	0.69-0.95

ROC: receiver operating characteristic; AUC: area under curve; SD: standard deviation; CI: confidence interval.

Protein interactions of the target genes were analysed using the STRING database. In contrast, gene interactions between them were analysed using the GeneMANIA database. The results are presented in **Figures 3 and 4**, respectively.

The protein interactions analysis showed that APP and MAPT are involved in the AD pathway in the KEGG and DISEASES databases. While Tubb3, TrkB and CDC42 directly affect related genes through protein interactions (**Figure 3**). MAPT and Tubb3 activate AMP-activated protein kinase (AMPK) downstream of N-methyl-D-aspartate receptors (NMDARs) (STRING, 2023). Overactivation of AMPK in neurons can lead to dendritic spine loss and is involved in the synaptotoxicity of beta-amyloids in AD

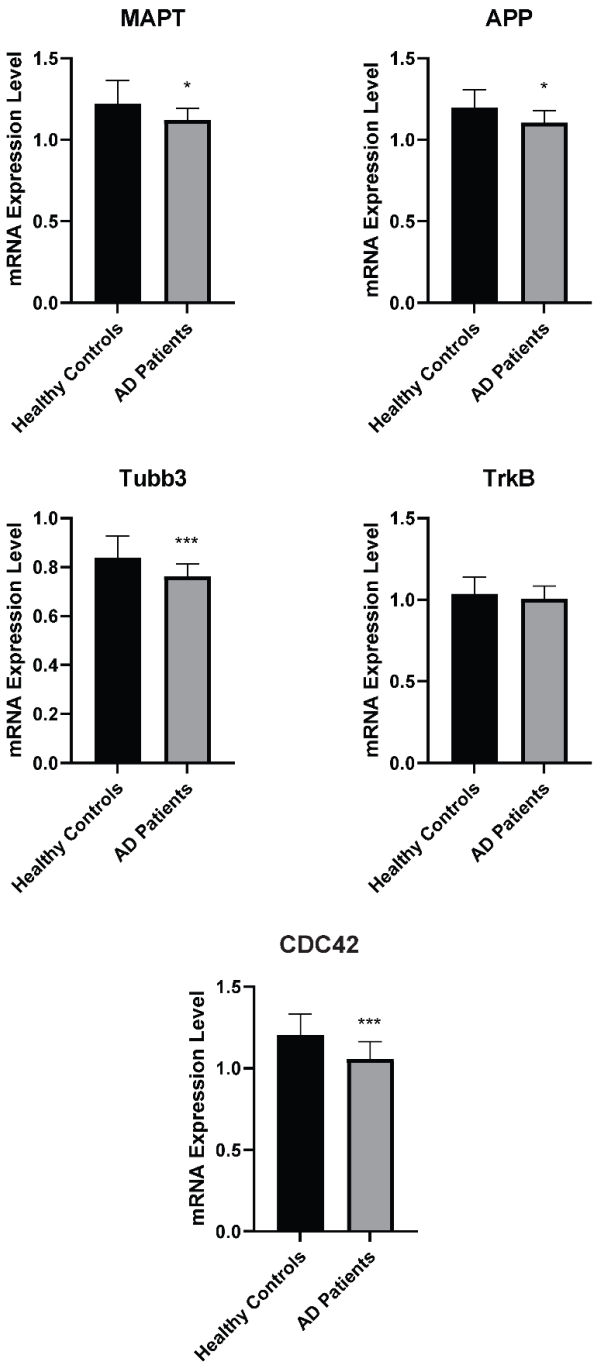

Figure 1. Target genes' mRNA expression levels. The *MAPT, APP, Tubb3, TrkB* and *CDC42* genes' expression levels were downregulated in AD patients compared to healthy controls by 1.09, 1.08, 1.09, 1.03 and 1.14-fold, respectively. Healthy controls (*n=17*); AD patients (*n=22*); *: p < 0.05; ***: p < 0.001.

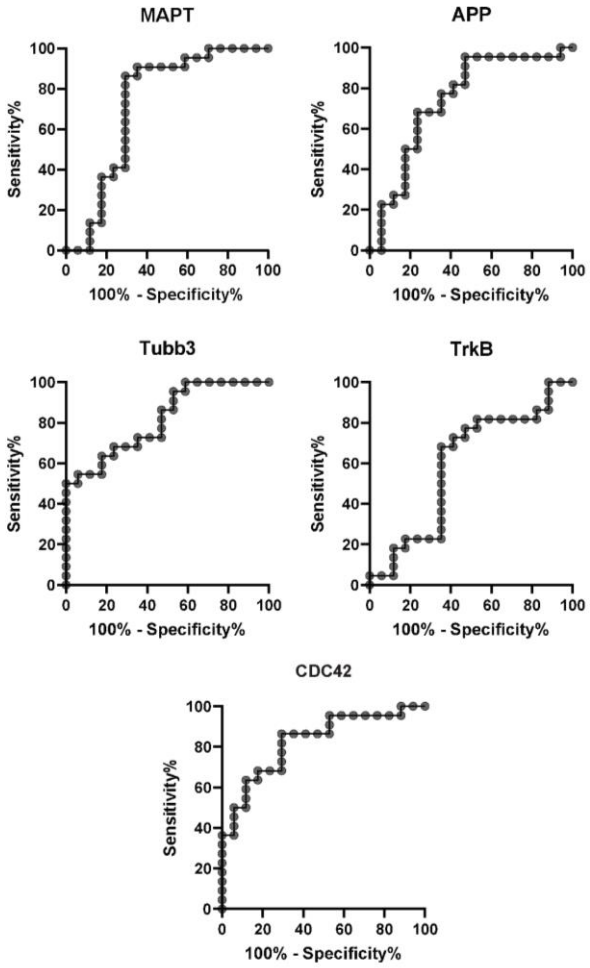

Figure 2. ROC curve analysis shows the statistical performance of target genes in distinguishing between Alzheimer's patients and healthy controls.

(Mairet-Coello et al., 2013). The analysis with the GeneMANIA database revealed 20 genes and 293 associations to which the target genes are linked (**Figure 4**).

The findings of this study reveal a significant difference in the expression levels of the four selected target genes between AD patients and the control group. Specifically, the expression levels of *MAPT*, *APP, Tubb3* and *CDC42* genes were down-regulated by 1.09 times ($p<0.05$), 1.08 times ($p<0.05$), 1.09 times ($p<0.001$) and 1.14 times ($p<0.001$) respectively, in comparison to the control group. However, it is noteworthy that the expression of the *TrkB* gene did not exhibit any statistically meaningful difference between the patient group and the control group.

4.0 DISCUSSION

The exploration of gene expression alterations in AD assumes significance in the context of identifying potential biomarkers for early detection. Biomarkers are pivotal in advancing our understanding and management of various diseases. These molecular indicators provide valuable insights into the underlying pathological processes of the condition, allowing for earlier and more accurate diagnosis. Early detection is

crucial in Alzheimer's, as interventions are often more effective in the initial stages of the disease. Biomarkers also contribute to monitoring disease progression and assessing treatment efficacy. Additionally, they facilitate the identification of individuals at a higher risk of developing AD, enabling proactive interventions and personalised care plans.

This study investigated the mRNA expressions of *MAPT, APP, Tubb3, TrkB*, and *CDC42* genes and revealed a remarkable downregulation in AD patients compared to healthy controls. As these gene expression dynamics are unravelled, the identified changes may serve as essential clues in the broader AD landscape, positioning them as potential biomarkers for early diagnosis.

The *MAPT* gene is responsible for encoding the microtubule-associated protein tau (MAPT), whose transcript undergoes intricate and regulated alternative splicing, resulting in various types of mRNA. *MAPT* transcripts exhibit distinct expressions within the nervous system, contingent on the neuronal maturation stage and neuron type (GeneCards, 2023a). The *MAPT* gene, which is involved in MAPK signalling, Alzheimer's disease, and neurodegeneration pathways (KEGG Pathway, 2023a), is one of the essential genes related to AD. Fukasawa and colleagues analysed the expression of the *MAPT* gene in brain samples from 26 Alzheimer's patients and 24 healthy elderly individuals (Fukasawa et al., 2018). Like our study, a reduction in the expression of the *MAPT* gene was observed in patients. Because AD pathology is limited to the brain, cerebrospinal fluid (CSF) may be the best body fluid for biomarker research of the disease. However, it is more difficult to apply because it requires invasive procedures. Blood biomarkers are less invasive and cost-effective than neuroimaging and CSF scans (Tzen et al., 2014; Waragai et al., 2012).

Tau is a microtubule-binding protein that undergoes an increase in expression and phosphorylation in AD, constituting the principal component in AD tangles and neurite pathology. Certain studies have demonstrated that total tau and specific phosphorylated-tau isoforms are significantly elevated in the CSF of individuals with AD. However, it has been found that plasma tau and CSF tau levels exhibit a weak correlation with each other (Guo et al., 2021; Ossenkoppele et al., 2021). According to Mattsson and colleagues, plasma tau is proposed to reflect AD pathology partially, yet there exists considerable overlap between normal ageing and AD, particularly in patients without dementia (Mattsson et al., 2016).

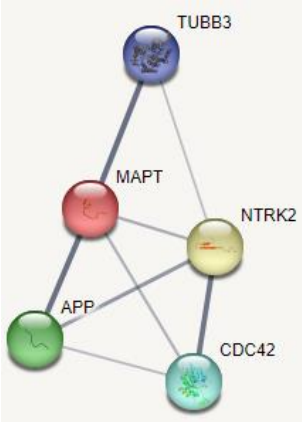

Figure 3. Protein interactions of the target genes. The edges indicate functional and physical protein associations, and the line thickness indicates the strength of data support. *NTRK2*: alias for *TrkB* gene. Created by STRING (STRING, 2023).

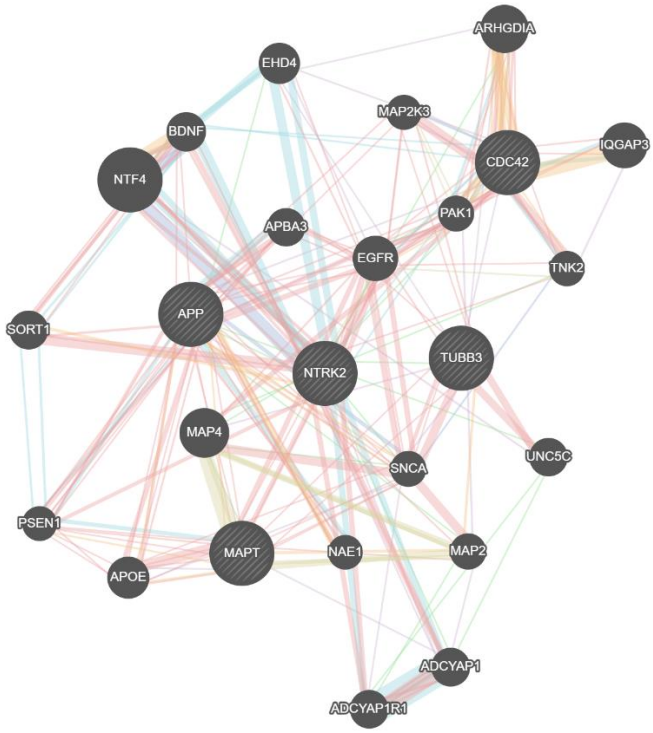

Figure 4. Gene interactions between the target (*MAPT, APP, Tubb3, TrkB* and *CDC42*) and their associated gene networks. Created by GeneMANIA (GeneMANIA, 2023).

On the other hand, a separate study demonstrated that plasma levels of total tau and pTau181 were higher in individuals with AD when compared to cognitively unimpaired individuals. Their study found that plasma pTau181 correlated more strongly with Aβ and Tau PET (Mielke et al., 2018). Moreover, Thijssen and colleagues discovered that plasma pTau181 could identify amyloid β-PET positive individuals irrespective of their clinical

diagnosis, and it also showed a correlation with cortical tau protein deposition assessed through 18F-Flortaucipir PET imaging (Thijssen et al., 2020). There are controversial results on the correlation between plasma tau levels and disease progression in AD patients. More correlated results were found in the pTau181 level than in the total tau level (Mielke et al., 2018; Thijssen et al., 2020).

Therefore, different transcripts of *MAPT* gene expression levels may provide clues for understanding Tau pathology in AD patients. Contrary to these findings, another study in the literature suggests that *MAPT* mRNA expression levels and the methylation status in the blood may not serve as effective biomarkers for Alzheimer's disease (AD). The findings indicate that the examined CpG sites do not exhibit genetic significance concerning *MAPT* gene expression or the pathology associated with AD. This highlights the complexity and variability in identifying reliable biomarkers for AD, emphasising the need for further research and exploration of alternative markers (Mori et al., 2022).

Amyloid precursor protein (APP) is a type I membrane protein known to play a crucial role in AD pathology. The processing of APP occurs via two distinct pathways, namely, the amyloidogenic and nonamyloidogenic pathways. APP is cleaved by β site APP cleaving enzyme 1 (BACE1) in the amyloidogenic pathway. As a result, C99 and soluble APPβ fragments are produced. After C99, the γ-secretase complex (presenilin 1, PEN-2, APH-1 and nicastrin) leads to the release of fragment Aβ from the membrane. Most of these fragments are fragments of Aβ1-40. However, a smaller proportion of highly cohesive and toxic Aβ-42 fragments are formed. These fragments constitute the primary components of senile plaques found in the brain. A-secretase and γ-secretase break down APP in the nonamyloidogenic pathway. APP undergoes cleavage by α-secretase, releasing soluble APPα and C83 fragments in the nonamyloidogenic process. Subsequently, C83 undergoes cleavage by the γ-secretase complex. That sequential processing does not result in the production of Aβ (Yun et al., 2020).

The APP operates as a cell surface receptor, playing essential roles in physiological functions associated with neurite outgrowth, neuronal adhesion, and axonogenesis on neuron surfaces. Furthermore, it triggers an AGER-dependent pathway, activating p38 MAPK, which leads to the internalisation of amyloid-beta peptide and induces mitochondrial dysfunction in cultured cortical neurons (GeneCards, 2023b). The *APP* gene has been found to have a possible link to AD in previous studies, and it encodes for the production of a type 1 transmembrane protein called APP. The build-up of β-amyloid peptide in the brains of AD patients is known to occur due to the cleavage of APP (Aydin et al., 2012). As in the results of our study, some studies indicate that the *APP* gene's expression decreases with age (Kern et al., 2006; Sun et al., 2018).

The *Tubb3* gene encodes for class III beta-tubulin protein. Beta tubulins are prominently expressed in neurons and likely play essential roles in neurogenesis, axon guidance, and maintenance (GeneCards, 2023c). In the gene expression analysis study conducted from the blood of AD patients, similar to our results, it was determined that the expression of the *Tubb3* gene responsible for cell shape was significantly decreased by 1.09 times in AD compared to healthy controls (Chew et al., 2013; Wang et al., 2021).

The *TrkB* gene encodes a neurotrophic tyrosine receptor kinase (NTRK) family member. Functioning as a membrane-bound receptor, this kinase undergoes phosphorylation upon binding with neurotrophins and activates members of the MAPK pathway. The receptor tyrosine kinase assumes a fundamental role in the developmental and maturation processes of the central and peripheral nervous systems, governing vital aspects such as neuron survival, proliferation, migration, differentiation, and actively participating in synapse formation and plasticity (GeneCards, 2023d). Several studies have revealed decreased *TrkB* gene expression in neurodegenerative diseases such as AD and Huntington's (Hock et al., 2000; Zuccato et al., 2008). Our study found that the expression of the *TrkB* gene decreased 1.03 times in the AD group compared to healthy controls.

The MAPK pathway, which involves the *CDC42* gene, regulates apoptosis, and an upregulation in the expression of this gene can lead to cellular death (KEGG Pathway, 2023b). In a study comparing the gene expression levels of AD patients and healthy individuals, it was determined that the expression of the *CDC42* gene, which is involved in the cell cycle signalling pathway, decreases gradually over time in AD patients (Nicole et al., 1999; Zhang & Niu, 2022). Few studies have explored the expression of the *CDC42* gene in individuals with AD. However, the available research aligns with our findings, as it suggests that the expression of the *CDC42* gene is reduced by 1.14 times ($p < 0.05$) in the patient group.

The complexity of Alzheimer's disease pathology presents many challenges in diagnosis and therapy. Therefore, functional screens are critical for understanding disease-specific signalling pathways and target proteins. To determine efficient treatment procedures for AD, it is essential to define key factors that act in the early stages of neurodegeneration and identify indicators to detect changes in these key factors. These findings are also necessary for early diagnosis and identification of new drug targets.

5.0 CONCLUSION

This study sheds light on the critical role of gene expression changes in Alzheimer's disease. The research focused on *MAPT, APP, Tubb3, TrkB* and *CDC42* genes. It revealed significant downregulation of the expression levels of four of these target genes, except *TrkB*, in AD patients compared to healthy controls. The findings underscore the importance of early diagnosis in mitigating disease progression and improving the quality of life for individuals affected by AD and their families.

Furthermore, these identified alterations in gene expression patterns may serve as promising biomarkers for early AD detection. Exploring the intricate molecular pathways and interactions involving these genes could unveil novel therapeutic targets, paving the way for innovative interventions and advancing our understanding of AD pathophysiology.

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Author Contributions: YD and ADY were involved in patient selection and sample collection. SKG, IGA and BAA contributed to the selection of target genes. BAA designed the experiments, while SKG and IGA performed the isolation of RNA and gene expression analysis. SKG, IGA and BAA conducted data analysis. All authors participated in manuscript writing and approved the final version of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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