ISTANBUL MEDENIYET UNIVERSITY
INSTITUTE OF GRADUATE STUDIES
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

Uncovering Dysregulated miRNA-mRNA Networks in Alzheimer's Disease Through Integrated Bioinformatics and Their Potential as Blood-Based Biomarkers

Master's Thesis

Birgül Çolak Al

June 2023

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THESIS JURY APPROVAL

This MSc thesis titled "Uncovering dysregulated miRNA-mRNA networks in Alzheimer's Disease through integrated bioinformatics and their potential as blood-based biomarkers" written by Birgül Çolak Al at the Department of Molecular Biology and Genetics was accepted by our jury.

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STATEMENTS

Style and Reference Manual Statement

Having reviewed this thesis written under my supervision, I confirm that it has been written in accordance with Vancouver Manual of Style and used its reference format consistently throughout the entire text.

Signature

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Declaration of Originality

I hereby declare that all information in this dissertation has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conducts, I have fully cited and referenced all material and results that are not original to this work.

Signature

Birgül Çolak Al

ÖZET

Alzheimer Hastalığı'nda Entegre Biyoinformatik Yöntemler Kullanılarak Disregüle miRNA-mRNA Ağlarının Ortaya Çıkarılması ve Kan Temelli Biyobelirteç Olarak Potansiyellerinin Araştırılması

Çolak Al, Birgül

Yüksek Lisans Tezi, Moleküler Biyoloji ve Genetik Programı

Danışman: Doç. Dr. Nagehan Ersoy Tunalı

Haziran, 2023

Alzheimer Hastalığı karmaşık bir nörodejeneratif hastalıktır ve ileri düzeyde teşhis ve tedavi yöntemlerine ulaşmak için detaylı moleküler araştırmalara ihtiyaç vardır. Bu çalışmada Alzheimer Hastalığı'nın moleküler detaylarını keşfetmek amacıyla kapsamlı bir biyoinformatik akış kullandık. Farklı beyin bölgelerinde ileri düzeyde Alzheimer Hastalığı olan bireyler ile sağlıklı kontroller arasındaki gen ifade farklarını analiz ederek, ortak farklı ifade edilen genleri belirledik. Ayrıca, protein-protein etkileşim ağı oluşturarak GFAP'nin yukarı regüle olan merkezi gen, SNAP-25'in ise aşağı regüle olan merkezi gen olduğunu ortaya koyduk. Ardından, bu merkezi genlere yönelik bir ilaç yeniden konumlandırma stratejisi izledik. Daha sonra, moleküler kenetleme analizi uygulayarak, dronabinol ve permethrin'in GFAP'ye, lestaurdinib, scriptaid ve alvocidib'in ise SNAP-25'e yüksek bağlanma affinitesine sahip olduğunu bulduk. Ayrıca, beyin ve serum örneklerindeki farklı ifade edilen miRNA'ları örtüştürerek Alzheimer Hastalığı için potansiyel miRNA biyobelirteçleri tespit ettik ve oluşturduğumuz mRNA-miRNA ağı, özellikle GFAP ve miR-7977, SNAP-25 ve miR-129-5p arasındaki güçlü bağı ortaya çıkardı. Son olarak, ön çalışmamızda ileri düzeyde Alzheimer Hastalığı olan bireylerin plazma örneklerinde kantitatif gerçek-zamanlı polimeraz zincir reaksiyonu doğrulaması gerçekleştirdik, böylece mikro RNA'lar ve hedef genlerinin ifade düzeylerini inceledik. Çalışmamızda, miR-7977 ile hedef geni GFAP arasında tersine bir ifade düzeni tespit ettik, burada miR-7977 düşük düzeyde ifade edilirken GFAP yüksek düzeyde ifade edildiğini gözlemledik. Benzer şekilde, miR-129-5p ile hedef geni SNAP-25 arasında da tersine bir ilişki gözlemledik; miR-

129-5p yüksek düzeyde ifade edilirken SNAP-25 düşük düzeyde ifade edilmektedir. Özetle, ön çalışmamız, plazma örneklerinde düşük ifade edilen miR-7977 ve yüksek ifade edilen miR-129-5p'nin Alzheimer Hastalığı için umut verici biyobelirteç potansiyelini ortaya koymaktadır. Alzheimer Hastalığı tanı ve tedavisinde kişiselleştirilmiş yaklaşımların ilerlemesine katkı sağlamak adına, bu bulguları doğrulamak için ileri moleküler analizler önemlidir.

Anahtar Kelimeler: Alzheimer Hastalığı, biyoinformatik, farklılaşmış gen anlatımı, mikroRNA, biyobelirteçler, ilaç yeniden konumlandırma, moleküler kenetleme.

ABSTRACT

Uncovering Dysregulated miRNA-mRNA Networks in Alzheimer's Disease Through Integrated Bioinformatics and Their Potential as Blood-Based Biomarkers

Çolak Al, Birgül

Master's Thesis, Department of Molecular Biology and Genetics

Supervisor: Assoc. Prof. Dr. Nagehan Ersoy Tunalı

June, 2023

Alzheimer's Disease is a complex neurodegenerative disease, requiring detailed molecular investigations in order to reach advanced diagnostic and therapeutic modalities. In this study, we employed a comprehensive bioinformatics framework to explore the molecular intricacies of Alzheimer's Disease. By analyzing differential gene expression in various brain regions implicated advanced Alzheimer's Disease compared with healthy controls, we identified a set of shared differentially expressed genes. Furthermore, construction of a protein-protein interaction network revealed GFAP as an up-regulated hub gene and SNAP-25 as a down-regulated hub gene. Subsequently, we pursued a drug repositioning strategy targeting these hub proteins, and molecular docking studies indicated high binding affinity of repositioned drugs dronabinol and permethrin to GFAP, and lestaurdinib, scriptaid, and alvocidib to SNAP-25. Moreover, we identified potential miRNA biomarkers for Alzheimer's Disease by overlapping differentially expressed miRNAs from brain and serum samples, revealing miRNAs present in both compartments. The constructed mRNA-miRNA network highlighted promising interactions, particularly between GFAP and miR-7977, as well as SNAP-25 and miR-129-5p. Finally, in our pre-liminary study, we conducted quantitative real-time polymerase chain reaction validation on plasma samples from advanced Alzheimer's Disease patients to examine the expression levels of mRNAs and their target miRNAs. We found inverse expression pattern between miR-7977 and its target gene GFAP, where miR-7977 is down-regulated, while GFAP is up-regulated. Similarly, miR-129-5p and its target gene SNAP-25 also displayed an

inverse correlation, with miR-129-5p being up-regulated, while SNAP-25 is down-regulated. To summarize, our study highlights the diagnostic potential of down-regulated miR-7977 and up-regulated miR-129-5p expression levels in plasma samples as promising biomarkers for Alzheimer's Disease. Further molecular assays are crucial to corroborate these findings, paving the way for advancements in personalized Alzheimer's Disease diagnosis and treatment.

Keywords: Alzheimer's Disease, bioinformatics, differential gene expression, microRNA, biomarkers, drug repositioning, molecular docking.

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A heartfelt thanks to my dear husband, Mehmet, whose unwavering faith in my abilities and endless patience has been the bedrock of this journey. His emotional support has been a vital element during this pursuit.

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With deepest gratitude,

Birgül Çolak Al

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LIST OF SYMBOLS

≥: greater than or equal

>: greater

≤: Less than or equal

<: less

° C: centrigrate

µg: micro gram

µl: micro liter

µM: micro molar

kcal/mol: kilocalorie per mole

ng/µl: nanogram per microliter

LIST OF ABBREVIATIONS

AML: Acute Myeloid Leukemia

Alzforum: Alzheimer Research Forum website

A-IADL-Q: Amsterdam IADL Questionnaire

Aβ: Amyloid beta

APs: Amyloid plaques

APP: Amyloid precursor protein

APOE: Apolipoprotein E

BP: Biological process

CC: Cellular component

CNS: Central nervous system

CSF: Cerebrospinal fluid

cDEMs: Common differentially expressed miRNAs

cDNA: Complementary DNA

CLIP: Cross-linking immunoprecipitation

CDK: Cyclin-dependent kinase

DAVID: Database for Annotation, Visualization, and Integrated Discovery

DEG: Differentially expressed gene

DEGs: Differentially expressed genes

DEMs: Differentially expressed miRNAs

DGIdb: Drug Gene Interaction database

EC: Entorhinal Cortex

NMDA: Extrasynaptic N-methyl-D-aspartate

FDR: False discovery rate

FAD: Familial Alzheimer's Disease

FDG: Fluorodeoxyglucose

FLT3: FMS-like tyrosine kinase 3

FDA: Food and Drug Administration

FC: Frontal Cortex

GO: Gene ontology

GSK-3β: Glycogen synthase kinase 3β

HIP: Hippocampus

AlzData: Integrated Alzheimer's Disease database

JAK2: Janus kinase 2

KPI: Kunitz Protease Inhibitor

KEGG: Kyoto Encyclopedia of Genes and Genomes

MRI: Magnetic resonance imaging

mRNA: Messenger RNA

miRNAs: MicroRNAs

MAPs: Microtubule-associated proteins

MF: Molecular function

MPNs: Myeloproliferative neoplasms

NCBIGEO: National Center for Biotechnology Information, Gene Expression Omnibus

NIAGADS: National Institute on Aging Genetics of Alzheimer's Disease Data Storage Site

NFTs: Neurofibrillary tangles

NMDA: N-methyl-D-aspartate

PET: Positron emission tomography

PTM: Posttranslational modification

PSEN1: Presenilin 1

PSEN2: Presenilin 2

pri-miRNA: Primary miRNA

PDB: Protein Data Bank

PPI: Protein-Protein Interaction

PMID: PubMed Identifier

qRT-PCR: Quantitative real-time PCR

RISC: RNA-induced silencing

SAD: Sporadic Alzheimer's Disease

TTBK1: Tau-tubulin kinase 1

TC: Temporal Cortex

UTR: Untranslated region

WHO: World Health Organization

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1. INTRODUCTION

1.1 History, Prevalence and Epidemiology of Alzheimer's Disease

Alzheimer's Disease is defined as a complicated, deteriorating neurodegenerative condition that affects millions of human beings globally, and assaults the brain gradually and leads cognitive decline, severe disability and ultimately death (1). In 1906, Alois Alzheimer, a German physician discovered the clinicopathological characteristics of Alzheimer's Disease. The disease is named by Dr. Kraepelin, after Dr. Alois Alzheimer. Originally, Alzheimer's Disease was referred as 'presenile dementia'. However, the term was later expanded to also include 'senile dementia' (2). Alzheimer's Disease is the widespread contributor to dementia, making up a significant majority of approximately 70% of dementia cases (3). According to reports, the number of Alzheimer's cases will triple by 2050, suggesting that the disease is becoming more common and severe (4). According to the World Health Organization (WHO), an estimated 50 million people worldwide have dementia, with Alzheimer's Disease being the most common cause. The prevalence of Alzheimer's Disease increases with age, with the majority of cases occurring in people over 65 years old. In high-income countries, the prevalence of dementia is around 5-7% in people aged 60 years and over, while in low- and middle-income countries, the prevalence is around 2-3%. However, these estimates may be conservative due to underdiagnosis and underreporting in some countries (5). It was estimated that the annual societal costs associated with the 55.2 million people globally suffering dementia amounted to around US $1313.4 billion in 2019. This works out to approximately US $23,796 per person. US $213.2 billion (or 16%) of this total was spent on direct medical expenses, US $448.7 billion (or 34%) on direct social sector spending (including long-term care), and US $651.4 billion (or 50%) was spent on informal care. This leads to a situation where families and healthcare systems around the world are under a lot of strain due to the high costs connected with dementia. Despite the fact that low-income and middle-income countries have the majority of dementia patients, high-income countries bear the brunt of the greatest total and per-patient costs (6). Overall, these factors clearly indicate the importance of studying Alzheimer's Disease holds significant importance not just for patients, but also for their families and the global

world economy. Despite intense research on understanding the molecular mechanisms of Alzheimer's Disease, the specific cause of the condition is still unknown. However, it is thought to be a combination of genetic, environmental, and lifestyle factors. While cure depends on early diagnosis and identification, there is currently no known effective therapy. Further research is required to explore the mechanisms behind the disease.

1.2 Neuropathology of Alzheimer's Disease

Alzheimer's Disease is one of the widely studied neurodegenerative diseases that gradually causes damage to the nervous system. Alzheimer's Disease is characterized by neuropathological changes that are categorized as positive lesions and negative lesions in the literature (7). The first category is referred to as "positive lesions" and is used to describe the physical alterations as a result of substance buildup in the brain. Numerous elements, including neurofibrillary tangles (NFTs), amyloid plaques (APs), dystrophic neurites, neuropil threads, and other deposits, are present in these accumulations. In this sense, the word "positive" denotes an abundance or presence of abnormal entities rather than something good. These accumulations indicate toxic buildups that have a detrimental effect on brain function, especially in the context of Alzheimer's Disease. For instance, amyloid plaques are protein aggregates that accumulate between neurons, and NFTs are tangled bundles of fibers found inside neurons. Both instances interfere with regular brain functions and are signs of Alzheimer's Disease. The second type is known as "negative lesions," which are characterized by severe brain shrinkage caused by the results of loss of neurons, synaptic connections, and the neuropil, which is a dense network of intertwined nerve fibers. Here, the word "negative" refers to a lack or disappearance of the brain's typical structural components. The steady deterioration of cognitive abilities that is characteristic of Alzheimer's Disease is caused by the decrease in brain matter and connections (7, 8).

1.2.1 Amyloid Precursor Protein

In the pathophysiology of Alzheimer's Disease, the amyloid precursor protein (APP) is the key player. Three primary isoforms of the APP gene, which is found on human

chromosome 21, arise through alternative splicing (9). These are identified as APP695 (consists of 659 amino acids), APP751 (consists of 751 amino acids), and APP770 (consists of 770 amino acids). APP751 and APP770 have been demonstrated to be expressed in a wide variety of TISSUES. Their extracellular parts possess a Kunitz Protease Inhibitor (KPI) domain that is composed of 56 amino acids (10). APP695 lacks the KPI domain and is mainly present in neuronal tissue (11). The protein and mRNA levels of APP isoforms that contain the KPI are increased in Alzheimer's Disease brains and correlated to elevated amyloid beta (Aβ) deposition (12). Extrasynaptic N-methyl-D-aspartate (NMDA) receptor activation (one of the brain's main excitatory neurotransmitters) that is prolonged in neurons can change the expression of APP from APP695 to APP isoforms that contain KPI, and this leads to an increase in the synthesis of Aβ (13). Moreover, the pathogenesis of the disease may be aided by dysregulated splicing of the *APP* RNA (14). APP, coupled with the amyloid precursor-like proteins (APLP1 and APLP2) in mammals are classified as part of a family of related proteins. Both APLP1 and APLP2 are type 1 transmembrane proteins and share the same process (15).

The pathogenesis of Alzheimer's Disease is mediated by the small peptide Aβ, that is produced when APP is processed through proteolysis as indicated in Figure 1 (16). APP is processed via two ways: non-amyloidogenic pathway and amyloidogenic pathway. The non-amyloidogenic pathway is mostly used to process APP once it is delivered to the plasma membrane via the endoplasmic reticulum-Golgi secretory pathway. N-terminal fragment and a C-terminal fragment that is bound to membrane are produced by the process. N-terminal fragment, abbreviated as APPs α, is a soluble fragment, whereas membrane-bound C-terminal, abbreviated as C83 fragment, is not soluble. Therefore, C83 is further cleaved by γ-secretases to produce a soluble extracellular peptide known as p3. This prevents the synthesis of intact Aβ (17). In the amyloidogenic pathway, β-secretase cleaves APP, produces a membrane-bound C-terminal fragment that is not soluble and a soluble N-terminal fragment (18). The C-terminal segment is then cut by γ-secretase, resulting in the release of AICD and Aβ peptides. AICD is a cytoplasmic polypeptide, and functions as transcription factor when it is delivered to nucleus. Meanwhile Aβ peptides are released into the extracellular environment when the endosome recycles to the cell surface (19).

When γ-secretase cleaves an Aβ peptide, it produces molecules with 38 to 43 residues. Aβ40 and shorter peptides are generally harmless, but Aβ42 and Aβ43 are extremely self-aggregating among the various Aβ species. Early-onset familial Alzheimer's Disease (FAD) caused by APP and presenilin mutations lead to elevated Aβ42/Aβ40 ratios. Aβ42 readily forms neurotoxic oligomers, fibrils, and plaques. Aβ oligomers are more toxic than fibrils and may contribute more to Alzheimer's Disease progression than amyloid plaques. Toxic oligomers, fibrils, and senile plaques can develop because of abnormal APP processing by β- and γ-secretases. Pathogenic presenilin mutations disrupt -secretase-APP connections and increase Aβ peptide synthesis (20). These findings suggest that Alzheimer's Disease treatment strategies should better include stabilizing the γ-secretase-Aβn complex (16).

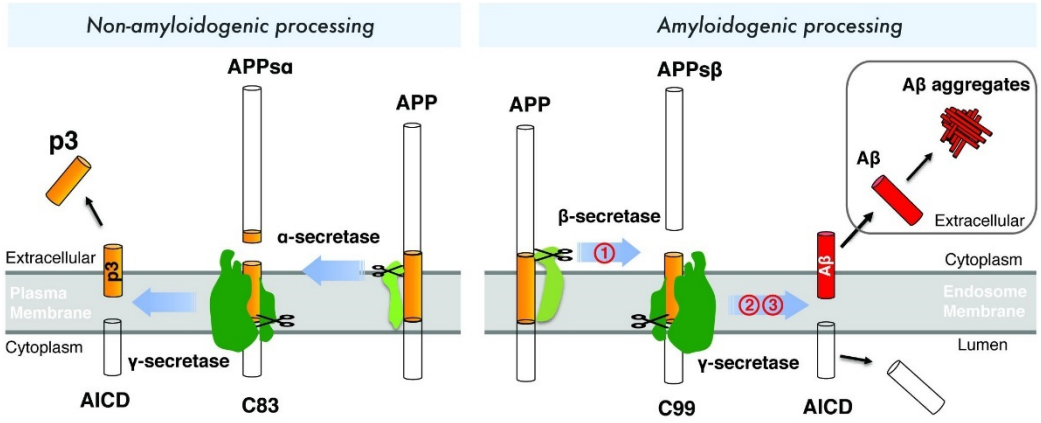


Figure 1. APP mechanisms: non-amyloidogenesis on the left and amyloidogenesis on the right. (16).

Figure 2 indicates the strategy of modulating Aβ generation, which involves blocking APP amyloidogenic processing. β- and γ-secretases, which catalyse APP intramembrane proteolysis, are the most important Alzheimer's Disease therapeutic targets. Various β- and γ--secretase inhibitors/modulators have been shown to inhibit or regulate APP amyloidogenic processing, either reducing total Aβn or changing Aβ synthesis to shorter and milder Aβ species (16).

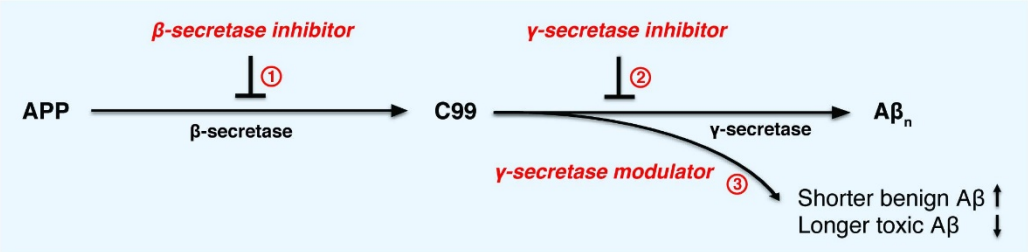


Figure 2. β- and γ-secretase-targeted drug development (16).

1.2.2 Neurofibrillary Tangles

Tau functions is a microtubule-binding protein to control microtubule assembly, and mainly found in the axonal tracts of neurons (21). Tau also affects axonal transport by competing with kinesin and dynein for microtubule binding (22), and function in elongation of axons and neurogenesis in adults (23). Tau is known as microtubule-associated proteins (MAPs) which are evolutionary conserved. MAP gene encodes tau, located in chromosome 17, with 16 exons (24). Posttranslational modification (PTM) motifs with certain characteristics strictly control the cellular activities and biochemical characteristics of tau spatiotemporally. The tau sequence has more than 60 residues that are phosphorylated, acetylated, methylated, ubiquitinated, nitrated, and glycosylated among other PTMs. Dysregulation of tau PTM is linked to a variety of neurological conditions that are all incurable and referred to as tauopathies (25). Insoluble aggregates formed by tau types that have been misfolded accumulate in the central nervous system (CNS) because of tauopathies. Numerous levels of neuronal physiology process are thought to be affected by these inclusions, which ultimately results in neuronal loss. NFTs, also known as tau deposits, were first identified in the brains of patients with Alzheimer's Disease as dense bundles close to the cell surface of afflicted neurons (26). Since insoluble deposits first form preceding hyperphosphorylated tau, which is isolated from NFTs and other inclusions, this atypical PTM is thought to be the main driver of aggregation (27).

Tau can be transferred from neuron to neuron (28). The mechanisms that are involved in tau propagation can be broken down into three primary steps: firstly, the pathological form of tau is getting released into the extracellular space from the donor cell, secondly, the pathological form of tau is being taken up by recipient cells, and

thirdly, the pathological form of tau being taken up by recipient cells building new intracellular aggregates (Figure 3) (29).

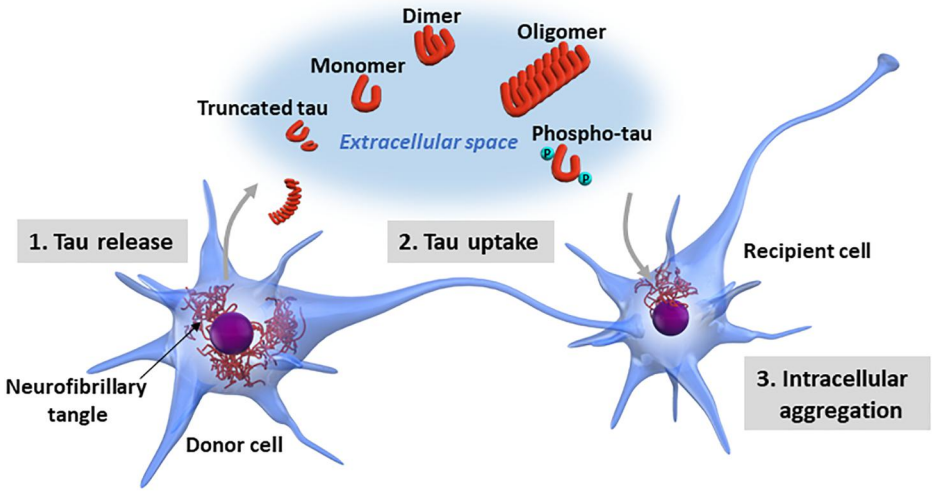


Figure 3. The three steps in the process of tau transfer across neurons (29).

Tau may appear in many different forms, each with their own unique biochemical properties. These forms consist of monomeric, oligomeric, truncated, and phosphorylated tau. Beside this, tau has the potential to experience a wide variety of posttranslational modifications, such as acetylation, glycation, isomerization, nitration, SUMOylation, and ubiquitination, or a combination of these posttranslational modifications. However, it remains unknown that which types are involved in tau propagation and which are the ones that are released into the extracellular space (29). Therefore, to comprehend the pathophysiology of Alzheimer's Disease, it is crucial to study the mechanism underlying NFTs.

1.2.3 Synapse Loss in Alzheimer's Disease

One of the characteristics of Alzheimer's dementia is synaptic dysfunction and loss, which highlights the need of studying more in deep the synapses processes in relation with Alzheimer's Disease. Research have shown that synaptic loss is a characteristic of Alzheimer's Disease in both the neocortex and the hippocampus (30). Additionally, in Alzheimer's Disease, as is the case in animal models of dementia, synapses are shown to gradually disappear over time (31). The association of Aβ oligomers with

synapses leads to a loss in synaptic function and is also responsible for changes in their size, shape, and composition (32). Recent studies have shown that tau may play a part in the control of synaptic function (33). Studying synaptic alterations and dysfunction is essential to understand the pathogenesis processes of Alzheimer's Disease. This knowledge could bring light on therapeutic approaches to potentially prevent or reverse synapse loss.

1.2.4 Neuron Loss in Alzheimer's Disease

The decrease in the number of neurons in the brain occurs as a natural process of neuron death, primarily through apoptosis, which is a programmed cell death mechanism. This neuronal loss is known to increase with age and is further exacerbated in Alzheimer's Disease (34). Alzheimer's Disease kills neurons through different pathways that are apoptosis, necrosis and necroptosis and pyroptosis. Excitotoxicity, DNA damage, loss of growth factor and death receptors trigger apoptosis of neurons during Alzheimer's Disease. Death receptors, and reactive oxygen species stimulate neuron death through necrosis. Lysosomal stress and ion flux affects pyroptosis resulting in neuron loss in Alzheimer's Disease (35). Therefore, the cellular-molecular mechanism of neuron death in Alzheimer's Disease needs to be investigated in the future.

1.3 Familial vs Sporadic Alzheimer's Disease

Alzheimer's Disease is caused by genetic and environmental factors. Alzheimer's Disease can be classified into two subtypes as sporadic Alzheimer's Disease (SAD) and familial Alzheimer's Disease (FAD) based on pathogenic mechanisms (36). SAD makes up the great majority of all cases of the disease and often affects people who are older than 65 years. APOE (apolipoprotein E) gene is involved in the progression of SAD. Additionally, Alzheimer's Disease risk is elevated in those with the APOE 4ε allele. On the other hand, FAD is an uncommon form of the disease, that is genetically inherited. Genetic susceptibility factors, lifestyle, and environmental factors are the main factors of progression of SAD. FAD is caused by genetic mutations that are passed down through families and can lead to early onset Alzheimer's Disease, where symptoms appear before the age of 65 years. The genes APP, PSEN1 (Presenilin 1)

and PSEN2 (Presenilin 2) play an important roles in the progression of FAD. Both familial and SAD, despite having different genetic origins, show comparable clinical characteristics (37). However, understanding the differences between SAD and FAD is critical to the development of effective diagnostic strategies and therapies for the treatment of these subtypes.

1.4 Genetic Factors of Alzheimer's Disease

Mutations in three genes have been identified as being highly related with the development of FAD. These genes are APP, PSEN1 and PSEN2. These gene mutations cause the buildup of beta-amyloid plaques and tau protein tangles, both of which are hallmark aspects of the pathophysiology of Alzheimer's Disease (9, 38, 39). Additionally, APOE gene is involved in the process of transport of cholesterol and lipids through the bloodstream. Some variants of the APOE gene are linked to an increased likelihood of getting Alzheimer's Disease in a later stage of the disease. There are three prevalent varieties of APOE in the 2, 3, and 4 alleles. People inherit two copies of the APOE gene, and the APOE 4 variant is the major risk factor for Alzheimer's Disease. Those who have one copy of the 4 variants have a higher risk, while those who have two copies have an even greater risk (40). On the other hand, the APOE 2 gene variant has been denoted as a preventive factor for the disease (41). It is possible that APOE 4 has a role in the progression of Alzheimer's Disease in several different ways, such as the development of amyloid plaques, the synthesis and clearance of beta-amyloid protein, inflammation, and oxidative stress in the brain (40). However, although some people with the APOE 4 allele are predisposed to developing Alzheimer's Disease, this is not always the case. Due to genetic and environmental factors may also play a role in the development of the disease (42).

1.5 Environmental Factors of Alzheimer's Disease

Alzheimer's Disease is a complicated condition that could be driven by a combination of both genetic and environmental factors. According to the findings of several studies, certain aspects of one's lifestyle, such as diet, physical activity, and social interaction, may either increase or decrease the likelihood of acquiring Alzheimer's Disease. For instance, a diet rich in fruits and vegetables may protect against Alzheimer's Disease,

whereas diets heavy in saturated fats and refined sugars may raise the chance of having the condition. Diets that are rich in whole grains may also reduce the risk of developing Alzheimer's Disease (42). Additionally, environmental factors, such as exposure to metals (43), solvents, pesticides (44), and electromagnetic fields (45), as well as environmental toxins and pollutants (44), may play a role in the development of disease. Besides, environmental variables such as nutrition, lifestyle, alcohol consumption, smoking, and pollution produce epigenetic alterations in important genes and pathways relevant to Alzheimer's Disease (42). In addition, epigenetic modifications are present throughout the various stages of development, which increases the likelihood of acquiring chronic disorders later in life, including Alzheimer's Disease (46). All these elements, including epigenetic changes, must be considered in order to accurately assessing the gene-environment interactions leading to Alzheimer's Disease.

1.6 Stages of Alzheimer's Disease

Millions of people throughout the world are afflicted with Alzheimer's Disease. The illness normally takes time to develop, and there are three phases of progression: early-stage (mild), middle-stage (moderate), and late-stage (severe) Alzheimer's Disease (47, 48).

Early-stage Alzheimer's Disease is typically diagnosed during the symptomatic stage, although it is extremely difficult to detect. The person may appear to be working properly with the exception of occasional memory lapses, such as forgetting the names of people or items, but other than that, they may seem to be remembering things regularly. In the early stage, it is common for people to experience symptoms such as forgetfulness of recent events or conversations, difficulty remembering newly learned information, organizing and planning, handling money, making decisions and solving problems, unusual mood swings, and becoming withdrawn. All these symptoms are indicative of early-stage dementia.

Individuals often enter the intermediate stage of Alzheimer's Disease when the disease reaches a more advanced degree. Damage to nerve cells makes it harder to communicate one's thoughts and to carry out daily activities without support. During

this stage, the symptoms grow more visible and start to cause problems in day-to-day living. The middle stage, which can persist for many years, is the longest stage. The middle stage of Alzheimer's Disease is characterized by a number of common symptoms, including an increase in forgetfulness of essential details such as the address or phone number of home, difficulty with even simple mental arithmetic, confusion and disorientation, difficulty in performing routine tasks, issues with speech, including difficulty in finding words, completing sentences, or articulating thoughts, and changes in mood and behaviour that are quite noticeable.

The late stage of Alzheimer's Disease is characterized by a gradual but steady worsening of the disease symptoms. The individual may require substantial assistance with activities of daily life such as bathing, using the toilet, and clothing. Extreme memory loss, difficulty communicating (including being non-verbal at times), difficulty recognizing family members and close friends, difficulty with mobility, including walking, increased risk of falls and accidents, difficulty swallowing, weight loss, changes in sleeping patterns, and prominent personality changes are some of the common symptoms of late-stage Alzheimer's Disease. The phases of Alzheimer's Disease give a basic framework for understanding what to predict, despite the fact that the development of the disease course might differ. As with any diseases, early detection and intervention can help manage the symptoms and improve the quality of life for people with Alzheimer's Disease and their loved ones (49).

1.7 Diagnosis of Alzheimer's Disease

Primary care clinicians must confirm the patient's medical history and family history, review the patient's medication for possible causes of cognitive impairment, perform blood tests to rule out reversible causes of cognitive impairment, and perform a quick clinical assessment to confirm the presence of cognitive impairment in order to diagnose early Alzheimer's Disease. (50). Further cognitive, behavioural, functional, and imaging assessments can be carried out in a specialist setting (51). Specialists are able to discover the reasons of impairment and confirm a diagnosis by conducting a thorough neuropsychological test battery that includes major cognitive domains, a comprehensive testing battery, and using additional clinical exams and biomarkers taking into account which cognitive domains are impaired in the early stages.

Generally, episodic memory, executive function, visuospatial function, and language are the most commonly impacted cognitive areas in early-stage Alzheimer's Disease. The amount of activity and cognitive reserve of a patient must be taken into consideration by clinicians when they select suitable, sensitive tests that can identify minor cognitive abnormalities found in the early stages of Alzheimer's Disease. Clinicians can employ the Quick Dementia Rating System and the Amsterdam IADL Questionnaire (A-IADL-Q) and Fast Tool to detect cognitive impairment and evaluate a patient's functional capacity (52). Cognitive evaluations, brain imaging studies including magnetic resonance imaging (MRI), and cerebrospinal fluid analyses are some of the typical procedures used to diagnose Alzheimer's Disease (53).

Patients can benefit from a wide range of chances if they got the diagnosis of Alzheimer's Disease at an earlier stage. They are able to work together with their families, carers, physicians, and other members of a larger support team to design advanced care plans for themselves. They have the option of seeking early intervention in the form of symptomatic treatment, changes to their lifestyle, and risk-reduction efforts, all of which have the potential to deliver clinically substantial reductions in cognitive, functional, and behavioural deterioration (54). Additionally, it can assist in lowering expenses and limitations within the healthcare system (55). The progression of Alzheimer's Disease patient diagnosis will require a coordinated effort that must begin in the primary healthcare system. This is because the prevalence of Alzheimer's Disease is expected to continue to rise in the coming years (56). As a result, clinical history, neuropsychological and laboratory tests, and neuroimaging are among the tools that should be utilized to establish an accurate diagnosis of Alzheimer's Disease.

1.8 Treatment of Alzheimer's Disease

The currently available treatment for Alzheimer's Disease offers only symptomatic alleviation and, to a certain extent, disease-modifying effects. The newly developed disease-modifying medicines for Alzheimer's Disease are more likely to prevent the progression of the disease than to reverse its effects (57). The current treatment for Alzheimer's Disease comprises the development of cholinergic inhibitors (58), Aβ disaggregation causing drugs (59), tau inhibitors (60), and a variety of antioxidants

(61). As a consequence of this, it is very necessary to carry out extensive research on Alzheimer's Disease treatment that takes into consideration the disease's pathophysiology and investigates a variety of chemical scaffolds in order to develop a therapeutic candidate that is capable of effectively treating this illness.

1.9 Biomarkers

In 1998, the National Institutes of Health Biomarkers Definitions Working Group created a definition stating that "biomarker" means an objectively measurable characteristic used as an indicator for normal biological processes, pathogenic developments, or pharmacological reactions associated with therapeutic interventions (62, 63). Medical symptoms refer only to perceptions by patients regarding their health condition or ailment. Therefore, biomarkers are extremely important for improving diagnostic accuracy, determining how quickly a disease is progressing, and contributing to the creation of treatments that are currently available as well as those that will be developed in the near future for a wide range of conditions, including Alzheimer's Disease.

1.9.1 Biomarkers in Alzheimer's Disease

Blood-based biomarkers for Alzheimer's Disease are demanded because they provide a reliable approach that is minimally intrusive, reasonably priced, capable of monitoring the progression of the disease, and determining the efficacy of proposed treatments (64). Biomarkers can be categorized in different types such as genetic, molecular, biochemical, or imaging-based, and blood-based proteins, cerebrospinal fluid (CSF) biomarkers, and neuroimaging techniques (65). In Alzheimer's Disease research, biomarkers like tau protein (66, 67), Aβ (68), and neurofilament light chain have received significant attention (69). Currently, diagnosing Alzheimer's Disease requires cognitive and behavioural assessments, neuroimaging studies (such as MRI(70) or positron emission tomography (PET) (71) scans), and sometimes CSF sampling. These methods are costly, invasive, and not always accessible to everyone, making it difficult to screen large numbers of people or to monitor patients regularly over time (72). According to Figure 4, the amyloid-tau-neurodegeneration (AT(N)) classification, "A" biomarkers are defined as amyloid PET, CSF A42 and CSF

A42/A40, "T" refers to tau PET and CSF p-Tau, and "N" is shown by structural magnetic resonance imaging, fluorodeoxyglucose (FDG), PET (NFL). This figure outlines the most important findings obtained from analysing both traditional and cutting-edge biomarkers using either neuroimaging or biological fluids (73).

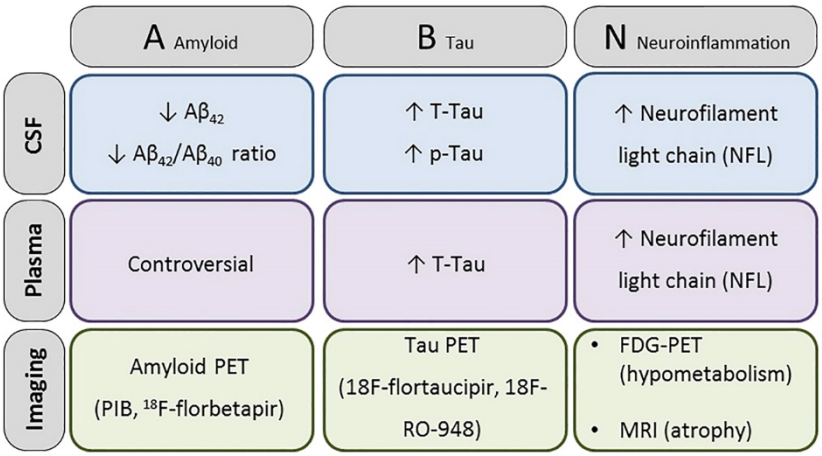


Figure 4. Overview of biomarkers in Alzheimer's Disease (73).

To recap, in addition to enabling the early detection of Alzheimer's Disease, biomarkers provide the potential for monitoring and tracking the disease as well as determining how well it reacts to treatment. Among the biomarkers, microRNAs (miRNAs) are involved in the control of multiple biological pathways, which are disturbed in Alzheimer's Disease, hence miRNAs should be investigated as potential biomarkers for the disease. They are able to be identified in readily available biofluids like blood or CSF, and they have the potential to be regarded as non-invasive biomarkers that are dependable for early diagnosis, disease progression, and therapy response (74).

1.10 microRNAs

MicroRNAs (miRNAs) are short, non-coding RNA molecules that play a crucial role in post-transcriptional gene regulation. MiRNAs were first discovered in the early 1990s, but it wasn't until 1990s that their biosynthesis and function were thoroughly studied. In the late 1990s, researchers started to delve into and understand these small but mighty RNA molecules at a deeper level. Initially, they were assumed to be molecular garbage without any actual value. Scientists first began to uncover the

complex mechanisms by which miRNAs form and how they utilize within cells for essential biological processes such as the regulation of gene expression or the control of protein synthesis. Our knowledge of miRNAs has expanded greatly, and it has been discovered that these molecules play an important role in a wide variety of biological activities (75, 76).

The biogenesis of miRNAs involves a complex multi-step process that includes transcription, cleavage, transport, and processing (Figure 5) (77). The mechanism begins with a multi-step process that begins with transcription by RNA polymerase II. This is followed by cleavage of the primary miRNA (pri-miRNA) by Drosha and DGCR8. The produced precursor miRNA, known as pre-miRNA, is then processed by Dicer after being moved from the nucleus to the cytoplasm by Exportin-5. This processing allows the production of mature miRNAs, which are then incorporated into the RNA-induced silencing (RISC) complex. After this, they attach to target mRNAs, which either prevents the mRNA from being translated or causes it to be degraded(78).

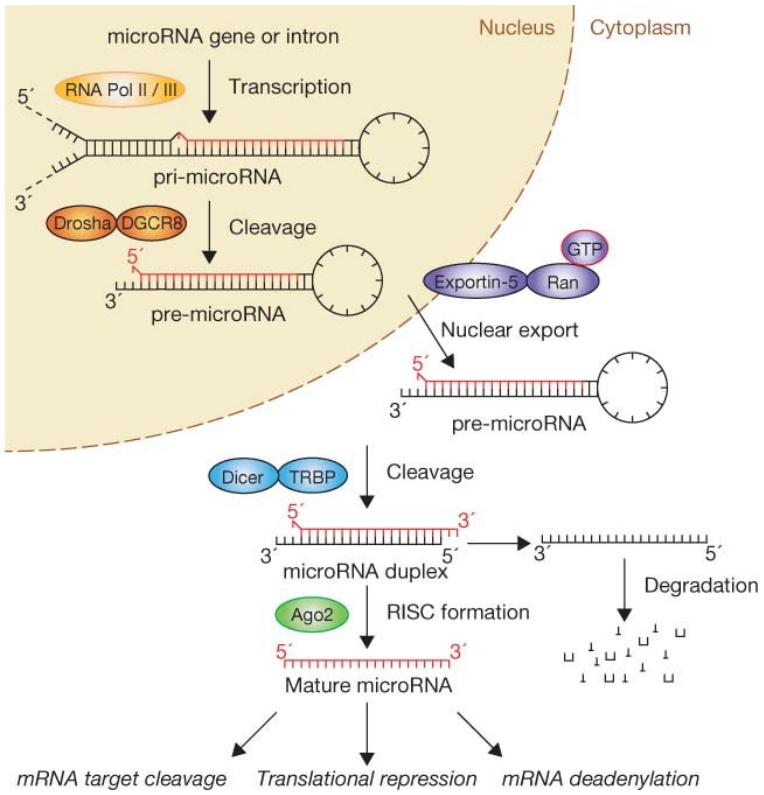


Figure 5. Biogenesis of miRNAs (77).

miRNAs can interact with the 3' UTR (79) and 5' UTR (80) of target genes, including the coding sequence and gene promoters (81). This post-transcriptional mechanism plays a vital role in the regulation of a variety of cellular processes, including cell death, differentiation, and development. In recent years, one field of research that has attracted attention is the potential involvement of miRNAs in diseases such as cancer, cardiovascular disease, cardiac failure, inflammatory disease, liver disease, neurodegenerative disorders such as Alzheimer's Disease (82). Various research has indicated that miRNAs may play an essential role in the development of Alzheimer's Disease pathology. The role that miRNAs play in regulating neuronal survival, differentiation, and synaptic plasticity is related to the relationship between miRNA and Alzheimer's Disease. In addition, research has shown that abnormal regulation of miRNAs may be responsible for the disruption of normal physiological processes that are associated with Alzheimer's Disease. (83, 84, 85, 86).

1.10.1 The Role of miRNAs in Alzheimer's Disease

MiRNAs are particularly significant small molecules for the progression of Alzheimer's Disease since they are dysregulated in many brain regions due to the fact that they have a dysregulated presence in several areas of the brain. They have an effect on many of the important proteins that have been connected to Alzheimer's Disease and can both promote and inhibit the progression of the disease (87). Because of this, miRNAs play an important part in the process of identifying Alzheimer's Disease as biomarkers.

Research showed that miRNAs function in the activation of enzymes that have a role in APP process. For example, a lot of miRNAs such us miR-29, miR-107, and miR-124, miR-195 regulate the activity of BACE1, one of the β-secretases (88). Aβ metabolism is also affected by miRNA as down-regulation of miR-103 and miR-107 in hippocampus results in the increase of Aβ and APP phosphorylation, leading to deterioration (89, 90). In the same sense, abnormally high quantities of Aβ are able to influence the expression of miRNAs as up-regulation of APP reduces the production of miR-107 (90).

MiRNAs play an essential role in affecting tau processing as well (91). Tau is directly phosphorylated by tau-tubulin kinase 1 (TTBK1) at three sites and results in the tau accumulation (92), whereas tau is regulated by glycogen synthase kinase 3β (GSK-3β) (93). MiR-219-5p suppresses tau phosphorylation by targeting TTBK1 and GSK-3β (91).

MiRNAs maintain synaptic plasticity in many ways. MiR-431 rescues synapses and neurites against Aβ-induced negative impacts via the Wnt/β-catenin signalling cascade (94). Synaptic and cognitive impairments are reversed by accumulation of miR-188-5p (95).

There is a wide range of degrees of modulation that may be seen in the differentiation, development, growth, and maturation of neurons by miRNAs. Up-regulated in the brains of Alzheimer's Disease patients are four miRNAs called miRs 142a-5p, 146a-5p, 155-5p, and 455-5p. These miRNAs affect neuronal activity and may potentially play a role in brain development and neurodegeneration. of neurons to varying degrees (96).

As discussed before, miRNAs are involved in both the early stages of Alzheimer's Disease and its later, pathogenic stages. Patients with Alzheimer's Disease exhibit spatio-temporal variations in the levels of miRNA expression. Certain miRNAs, such as miR-9, miR-124, miR-125b, and miR-132, are only expressed in CNS, and abnormalities in their regulation have been linked to Alzheimer's Disease (87).

1.10.2 Role of miRNAs in Diagnosis of Alzheimer's Disease

Due to the increasing prevalence of Alzheimer's Disease, as well as the lack of accurate diagnostic techniques, there is a growing demand for non-invasive biomarkers that are capable of properly diagnosing Alzheimer's Disease. Recently, the use of miRNAs, which are small molecules of non-coding RNA that have the ability to alter gene expression, were proposed as potential diagnostic biomarkers for Alzheimer's Disease has been proposed. A number of studies have been carried out to investigate the potential diagnostic utility of miRNAs and their role in Alzheimer's Disease (97). MiRNAs can detect and bind to complementary ribonucleotide sequences in the 3' untranslated region of target messenger RNAs (98), thereby inhibiting the production

of the target mRNA. These miRNAs are used for prognosis, diagnosis and therapy in diseases including Alzheimer's Disease. In Alzheimer's Disease, miRNAs in serum, cerebrospinal fluid, and brain tissue have been studied. The expression levels differ between different stages of Alzheimer's Disease. MiRNAs target Aβ metabolism, abnormal function of tau, synapses, neural development and immune-inflammatory responses as seen in Figure 6 (99).

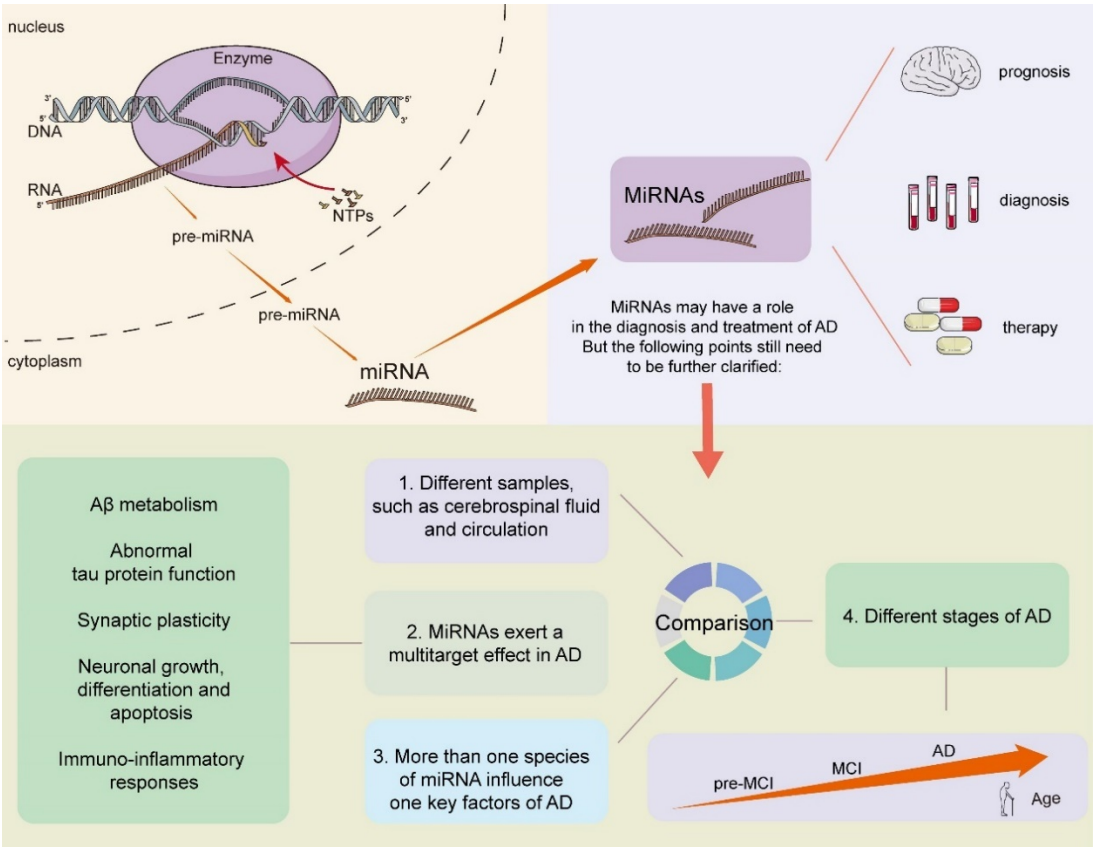


Figure 6. The role of miRNA in clinical applications (99).

In Alzheimer's Disease patients, the levels of miR-9–5p, miR-106a-5, and miR-106b-5p, among others, were found to be down-regulated in whole blood. In contrast, the levels of miR-28–3p, miR-128, and miR-455–3p, among others, were found to be up-regulated in serum, while miR-29a was found to be up-regulated in plasma (reviewed in (100)). These biomarkers are used as diagnostic markers for Alzheimer's Disease. In summary, miRNAs have a great deal of potential as non-invasive biomarkers for the Alzheimer's Disease diagnostic process. Their dysregulation has been connected to the

etiology of Alzheimer's Disease, and it has been observed that they are prevalent in the brain samples and bodily fluids of people who have the disease. Additional study is required to verify certain miRNAs as useful biomarkers and to investigate the possibility of these biomarkers as therapeutic targets for Alzheimer's Disease.

1.11 Bioinformatics in Alzheimer's Disease

Identifying the genetic and molecular origins of Alzheimer's Disease is crucial for creating reliable diagnostic tools and effective drugs (101). Bioinformatics enables a multifaceted structure for analysing and combining omics data, including genomes, transcriptomics, proteomics, and metabolomics, to research the complex molecular pathways underlying Alzheimer's Disease. (102).

Gene expression analysis among different groups of biological samples is used as an efficient method to investigate the molecular mechanisms underlying the related diseases. For instance, comparing the gene expression levels between diseased and healthy samples may reveal important pathways involved in the onset or progression of a disease (103). The methodologies for producing high-throughput gene expression data have continuously improved over the past three decades, which has caused a significant growth in the number of gene expression research being conducted. A sizable number of these research publish their datasets on various platforms to offer open access to gene expression datasets, such as NCBI GEO (National Center for Biotechnology Information, Gene Expression Omnibus) (104), AlzData (integrated Alzheimer's Disease database) (105), Alzforum (Alzheimer Research Forum website) (106), NIAGADS (National Institute on Aging Genetics of Alzheimer's Disease Data Storage Site) (107).

Drug repositioning is an approach for seeking alternative therapeutic uses for existing medications, which can save time and resources, and also lower the risk of toxicity and failure in clinical trials (108). Utilizing network analysis, machine learning, and deep learning models, researchers are able to find new drug targets and drug-gene-disease connections for Alzheimer's Disease utilizing the accumulated large-scale omics data that is already available (109).

Molecular docking is a computational method that predicts the binding affinity and orientation of small molecules such as drugs to target proteins (110). In Alzheimer's Disease research, molecular docking is used to identify potential drug candidates that target various proteins such as β-amyloid (111), phosphodiesterase (112), acetylcholinesterase (113), and monoamine oxidase (114).

Numerous research have investigated genes that are expressed differently in Alzheimer's Disease through bioinformatic methods (115, 116, 117, 118, 119). Recent research on Alzheimer's Disease serves as an illustration of a bioinformatic investigation. Distinct glial, immunological, neuronal, and vascular cell types were found by conducting single-cell RNA sequencing analysis in human Alzheimer's Disease brain tissues through differential expression patterns between Alzheimer's Disease patients and controls (120).

The correlation between lung cancer and Alzheimer's Disease was investigated with traditional bioinformatics techniques, such as differentially expressed gene (DEG) analysis. They observed a significant number of DEGs that function in the progression of Alzheimer's Disease (121).

In another study, data from genome-wide association studies were combined with information on the expression of genes in specific tissues in order to uncover co-expression networks, biological pathways, and possible candidates for drug repositioning for Alzheimer's Disease. This study demonstrates how powerful network-based analysis can be in identifying critical genes and pathways involved in the etiology of Alzheimer's Disease (122).

In addition to the well-known APOE locus, bioinformatic methods were utilized to find 11 additional susceptibility loci for Alzheimer's Disease showing how useful bioinformatics may be in identifying genetic variants that are connected with Alzheimer's Disease (123).

In general, Alzheimer's Disease-related biomarkers are possible to be identified using omics-based approaches. In many dimensions of the Alzheimer's Disease omics study, bioinformatics methods, including statistical and machine learning analysis, are applied to offer answers to comprehend the underlying developmental alterations in

Alzheimer's Disease (116). Simple workflow is seen in Figure 7 (116). Data can be genomics, transcriptomics, proteomics, or metabolomics. The obtained data need processing as selection of prob-per data, and normalization. Then data analysis is utilized by statistical analysis. As an output, the obtained mRNAs, miRNAs or others are identified as biomarkers related to disease or factors are identified as environmental factors related to disease (116).

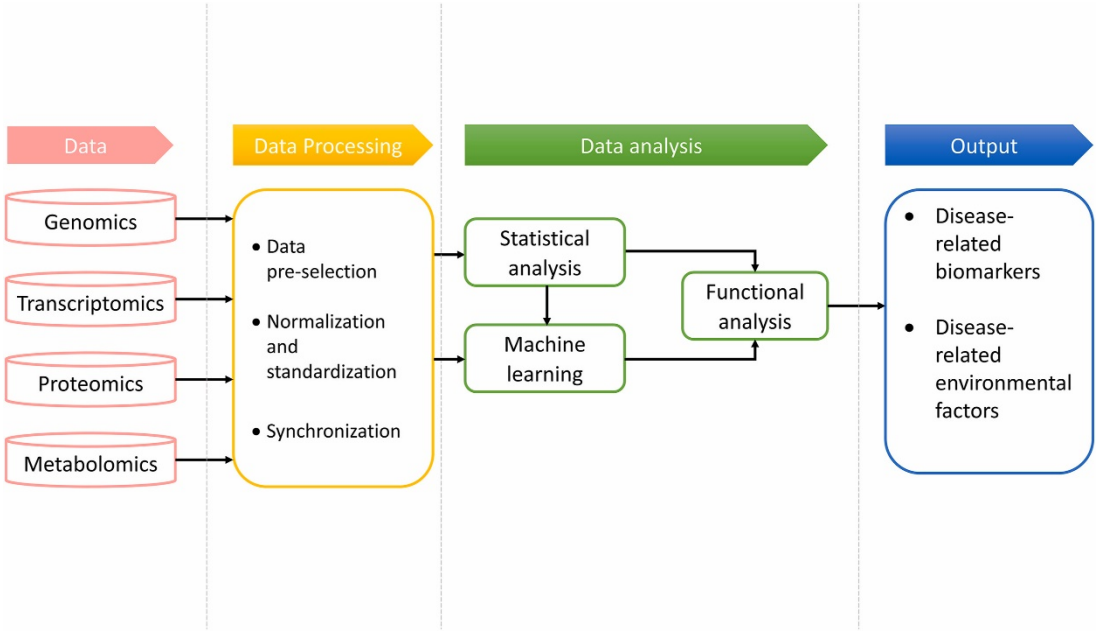


Figure 7. Workflow of the multi-omics approach (116).

In general, molecular docking, drug repositioning, and bioinformatics are three essential approaches for identifying new biomarkers, therapeutic targets, and drug candidates in Alzheimer's Disease. These techniques from different fields illustrate the need of integrating computational and experimental methodologies for researching complicated illnesses with several contributing factors, such as Alzheimer's Disease. In this thesis, bioinformatic methods such as differentially expressed gene analysis, drug repositioning and molecular docking methods are used to reveal miRNA-mRNA network and to test their potential as biomarkers in biological samples using quantitative real time PCR (qRT-PCR)

2. MATERIALS AND METHODS

Alzheimer's Disease is a complex neurodegenerative disorder that necessitates detailed molecular investigations to advance diagnostic and therapeutic approaches. In this study, we employed a comprehensive bioinformatics framework to unravel the molecular intricacies of Alzheimer's Disease. Our methodology encompassed several key steps aimed at understanding the underlying molecular mechanisms contributing to the disease progression, summarized in Figure 8. To begin, we conducted differential gene expression analyses in four brain regions implicated in Alzheimer's Disease, namely the hippocampus, entorhinal cortex, temporal cortex, and frontal lobe. Through this analysis, we aimed to identify genes that exhibit significant differential expression in Alzheimer's Disease-affected brain regions. Furthermore, we explored the overlap between the differentially expressed genes across these brain regions. Next, to gain insights into the functional implications of the identified differentially expressed genes, we performed functional enrichment analyses. This analysis provided valuable information about the biological processes and pathways associated with the genes that exhibit altered expression in Alzheimer's Disease. To further understand the molecular interactions underlying Alzheimer's Disease pathogenesis, we constructed a Protein-Protein Interaction (PPI) network. Within this network, we identified hub genes, which represent highly connected proteins potentially playing critical roles in the disease's molecular mechanisms. In line with our goal to identify potential therapeutic candidates, we implemented a drug repositioning strategy targeting the hub genes identified in our study. Utilizing molecular docking, a computational technique, we assessed the probability of drug-protein binding, which allowed us to identify potential drugs that could be repurposed for Alzheimer's Disease treatment. Simultaneously, we extended our investigation to explore the regulatory aspect of Alzheimer's Disease -associated genes. Leveraging online miRNA target databases, we identified potential miRNAs that may modulate the expression of the differentially expressed target genes found in our PPI analysis. These miRNAs hold the potential to serve as valuable biomarkers for detecting Alzheimer's Disease -associated gene expression changes, offering a non-invasive approach for early disease detection and monitoring. In parallel, we analysed serum samples dataset obtained from Alzheimer's

Disease patients to validate the findings observed in the brain regions. By comparing the miRNA expression profiles from the serum dataset with those from the brain regions, we identified a set of commonly differentially expressed miRNAs. These miRNAs were categorized as either up-regulated or down-regulated based on their expression levels in the serum dataset, providing insights into the systemic alterations in miRNA expression associated with Alzheimer's Disease. Subsequently, we utilized a specialized computational tool to predict target binding sites for the identified differentially expressed miRNAs on the corresponding differentially expressed genes. This allowed us to elucidate potential miRNA-mediated regulatory interactions that may contribute to the observed gene expression changes in Alzheimer's Disease. To further validate our findings, we selected specific miRNAs as targets for subsequent qRT-PCR experiments. This pre-liminary experimental approach enabled us to validate the differential expression of the selected miRNAs and provided their potential as blood-based biomarkers in Alzheimer’s Disease. By combining these methodologies, we aimed to unravel the complex molecular landscape of Alzheimer's Disease, from the identification of differentially expressed genes and miRNAs to the validation of their expression patterns and exploration of potential therapeutic interventions. The following sections provide a detailed description of each step in our methodology.

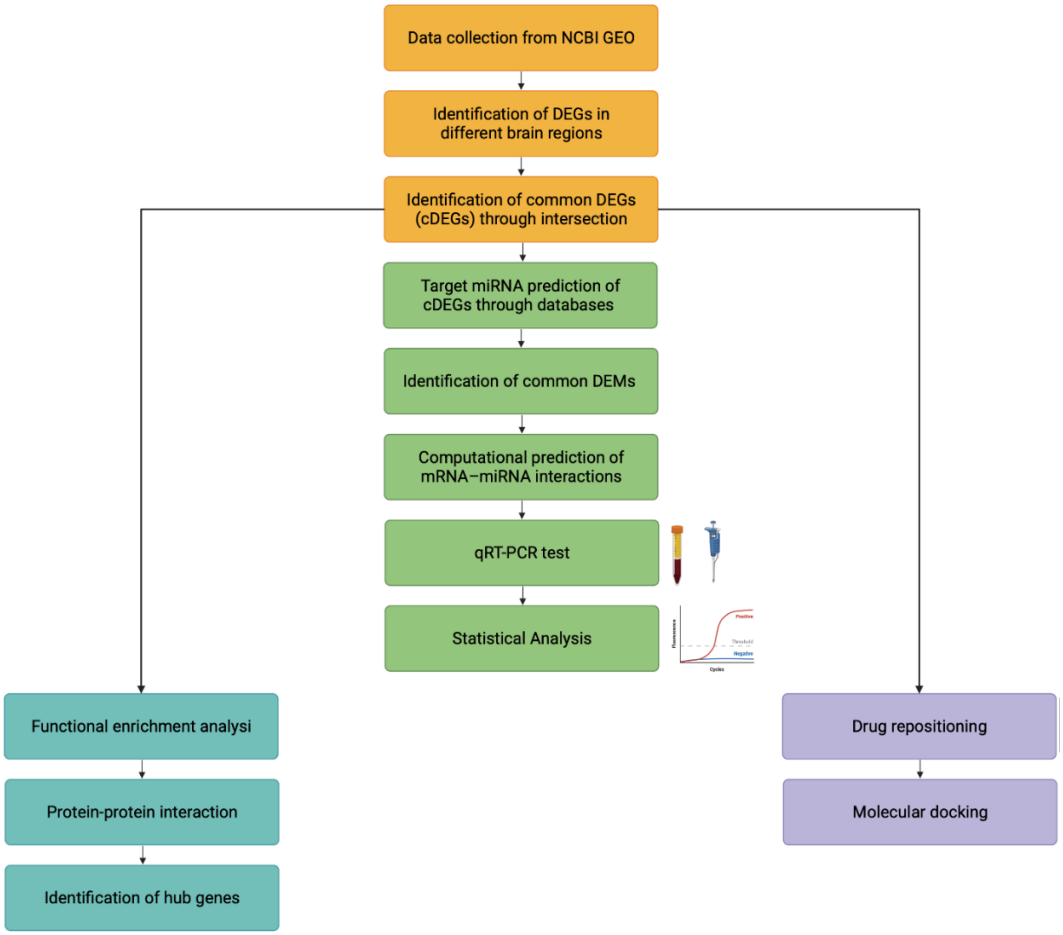


Figure 8. Workflow of the study.

2.1 Data Collection

To find relevant data from the beginning up to March 2021, a thorough investigation was conducted using the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) datasets (https://www.ncbi.nlm. nih.gov/geo/, accessed on 15 October 2022)(104). The following keywords were used: "Alzheimer", Alzheimer's Disease", "entorhinal", "hippocampi", "hippocampus", "brain", "post-mortem", "temporal", "frontal", "serum", and "plasma". Following this, three filters were applied, namely for organism "*Homo sapiens*"; for data type "Series"; for mRNA "expression profiling by array" and for miRNA "non-coding expression profiling by array". Data set types such as expression profiling by high throughput sequencing and non-coding RNA profiling by high-throughput sequencing are excluded due to GEO2R is not capable of doing the analysis of high throughput sequencing. Among

identified NCBI Gene Expression Omnibus (GEO) datasets, several datasets were selected for gene expression analysis. Afterwards, the number of datasets were reduced for more in-depth analysis, ensuring that only the most relevant ones were considered. This process allowed us to obtain high-quality data and exclude datasets that were not pertinent to our research goals. Table 1 provides an overview of comprehensive description of the microarray datasets including the details about data accession numbers, platform, experiment types, samples, sample size, age and their PMID numbers.

Table 1. Overview of datasets used in the study.

	Data Accession Number	Platform	Experiment type	Tissue	Samples	Sample Size		Age		PMID
						Controls	Patients	Controls	Patients	
mRNA										
1	GSE5281	GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array	Expression profiling by array	Brain	Entorhinal Cortex	3F, 10M	6F, 4M	79.6 ± 10.0	85.6 ± 6.0	17077275
					Hippocampus	3F, 10M	6F, 4M	79.6 ± 9.0	77.8 ± 5.4	17077275
					Medial Temporal Gyrus	4F, 8M	6F, 10M	80.1 ± 9.4	79.1 ± 6.2	29937276
					Superior Frontal Gyrus	4F, 7M	10F, 13M	79.3 ± 9.7	79.2 ± 7.4	18270320
2	GSE48350	GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array	Expression profiling by array	Brain	Hippocampus	12F, 13M	10F, 9M	80.7 ± 10.0	86.5 ± 5.3	18832152
					Entorhinal Cortex	9F, 9M	8F, 7M	82.0 ± 9.8	83.1 ± 8.2	23273601
					Superior Frontal Cortex	14F, 12M	14F, 7M	80.8 ± 10.1	87.3 ± 6.2	22824372
3	GSE122063	GPL16699 Agilent-039494 SurePrint G3 Human GE v2 8x60K Microarray 039381 (Feature Number version)	Expression profiling by array	Brain	Temporal Cortex	12F, 10M	22F, 6M	78.8 ± 8.4	81.0 ± 6.5	20838618
					Frontal Cortex	12F, 10M	22F, 6M	78.8 ± 8.4	81.0 ± 6.5	23999428
					Entorhinal Cortex	12F, 12M	23F, 14M	80.9 ± 7.9	84.0 ± 9.6	23206327
4	GSE118553	GPL10558 Illumina HumanHT-12 V4.0 expression beadchip	Expression profiling by array	Brain	Temporal Cortex	12F, 19M	29F, 23M	79.1 ± 8.8	82.4 ± 10.6	30990880
					Frontal Cortex	11F, 12M	26F, 14M	78.2 ± 7.6	83.0 ± 9.8	31063847
miRNA										
5	GSE16759	GPL8757 USC/XJZ Human 0.9 K miRNA-940-v1.0	Non-coding RNA profiling by array	Brain	Parietal Lobe	3F, 1M	3F, 1M	92.0 ± 1.9	87.5 ± 4.6	20126538
6	GSE157239	GPL21572 [miRNA-4] Affymetrix Multispecies miRNA-4 Array [ProbeSet ID version]	Non-coding RNA profiling by array	Brain	Temporal Cortex	5F, 3M	6F, 2M	79.9 ± 6.8	79.8 ± 9.0	missing
7	GSE120584	GPL21263 3D-Gene Human miRNA V21_1.0.0	Non-coding RNA profiling by array	Blood	Serum	137F,151M	714F, 307M	71.7 ± 6.3	79.2 ± 6.1	30820472 31666070 34686734

2.2 Analysis of Gene Expression

In this study, we performed differential expression analysis to investigate both gene and miRNA expression patterns between Alzheimer's Disease patients and healthy controls. The chosen datasets underwent preprocessing, curation, and individual analysis to extract differentially expressed genes (DEGs), including both up-regulated and down-regulated genes as well as differentially expressed miRNAs (DEMs), through the GEO2R online analysis tool (https://www.ncbi.nlm.nih.gov/geo/geo2r/), comparing Alzheimer's Disease patients with healthy control subjects. Benjamini-Hochberg was used to procedure to control the FDR (False-discovery rate) adjusted p-value at 0.05 (124, 125). Overlapped DEGs and DEMs were detected by Venn diagram tool (https://bioinformatics.psb.ugent.be/webtools/Venn/) for illustrating relationships between datasets. Overlapped DEGs were named as common DEGs (cDEGs) in between brain regions. To ensure rigorous statistical analysis and address the issue of multiple testing, we employed adjusted p-values for gene analysis, while utilizing regular p-values for miRNA analysis. The decision to adjust the p-values for gene analysis was driven by the vast number of genes being tested simultaneously, which increases the risk of false positives. By applying adjustment methods, such as the Bonferroni or False Discovery Rate (FDR) correction (126), we aimed to control the overall false positive rate and enhance the reliability of gene-level findings. On the other hand, for miRNA analysis, we opted for regular p-values due to the relatively lower number of miRNAs being investigated. Adjusting p-values for miRNAs may result in overly conservative thresholds and potentially lead to the omission of true positive findings. Thus, by employing different strategies for gene and miRNA analyses, we aimed to strike a balance between controlling false positives and ensuring sensitivity to identify biologically relevant miRNA associations. This approach enhances the interpretability and accuracy of our results. To sum up, DEGs in Alzheimer's Disease patients compared to healthy controls were selected based on the adjusted p-value < 0.05 as statistically significant. Furthermore, the utilization of logFC (log2 fold-change) differences as cut-off criteria for determining up-regulated and down-regulated DEGs and DEMs is justified by the need to assess effect size, establish biologically meaningful thresholds, maintain consistency with statistical significance, and adhere to standard practices in the field. These criteria ensure that

the identified genes and miRNAs exhibit both statistical significance and a substantial magnitude of change, supporting the robustness and biological relevance of our findings. A logFC difference of 1 or greater indicates a two-fold or higher change, which is often considered a significant alteration in expression. Overall, we enhanced the meaningfulness of their results by integrating the fold change and p-value approaches (127). In this study, logFC (log2 fold-change) ≥ 1 is considered as cut-off criteria to determine the up-regulated DEGs, whereas logFC ≤ -1 is considered as the cut-off criteria to determine the down-regulated DEGs. In cases where datasets exhibit low numbers of DEGs or DEMs, as in the case of this study, it is reasonable to consider using lower logFC thresholds to capture potentially relevant alterations. By employing a lower logFC threshold, such as logFC ≥ 0 or logFC ≤ 0, we aim to identify genes with smaller yet still meaningful changes in expression levels. DEMs in Alzheimer's Disease patients compared to healthy controls were selected based on the p-value < 0.05 as statistically significant. Among them, logFC ≥ 0 is considered as cut-off criteria for determining the up-regulated DEGs whereas logFC ≤ 0 is considered as cut-off criteria for determining the down-regulated DEGs.

2.3 Gene Ontology and Pathway Enrichment Analysis

Gene Ontology (GO) serves as a comprehensive computational model of biological systems, encompassing gene function information that spans from the molecular to the organism level. GO classifies gene functions into three main categories: biological process (BP), molecular function (MF), and cellular component (CC). The analysis of GO allows for large-scale investigations of functional enrichment, making it a commonly employed method (128, 129). On the other hand, the Kyoto Encyclopedia of Genes and Genomes (KEGG) is a database that incorporates diverse data related to four distinct categories: system information, genomic information, chemical information, and health information. Widely regarded as a reference database, KEGG plays a crucial role in the integration and interpretation of extensive datasets concerning biological pathways, diseases, chemical substances, and drugs (130). In this research study, the functions of cDEGs were analysed using Gene Ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. For the enrichment analysis of cDEGs, the Database for

Annotation, Visualization, and Integrated Discovery (DAVID) v6.8 (https://david.ncifcrf.gov/) was utilized. In both cases, a statistical significance threshold of p-value < 0.05 was applied to determine the significance of the results obtained from the enrichment analysis (131, 132). We also used EnrichR database for KEGG pathways, Reactome and Jensen Tissue analysis (133, 134). A p-value less than 0.05 was used to ascertain the statistical significance of the findings derived from these analyses.

2.4 Construction of Protein–Protein Interaction Network and Hub Genes

To predict Protein-Protein Interaction (PPI) network, the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING, v. 11.5) application was employed within the Cytoscape program (v. 3.10.0) (135). STRING is a resource that offers evidence of protein-protein interactions (PPIs), and these pieces of evidence help assess the reliability of the interactions. The confidence levels of protein interactions are determined based on various factors, such as conserved neighbourhood, cooccurrence, fusion information, co-expression, experiments, data from other databases, and text mining methods. Each protein interaction is supported by specific evidence, which is then used to calculate a confidence score. The tool provides four confidence levels: low confidence (0.15 and above), medium confidence (0.4 and above), high confidence (0.7 and above), and the highest confidence (0.9 and above). By focusing on interactions with medium confidence, one can prioritize more reliable interactions backed by accurate experimental evidence(136, 137). Cytoscape is an open source bioinformatic software platform specifically designed for visualizing molecular interaction networks. For the creation of the PPI network, the DEGs obtained in the previous section were used as seed proteins. Initially, a medium confidence score (score 0.400) was set to explore the interactions (135). Proteins without matches in the STRING database and proteins lacking interactions with other proteins within the network were excluded from the analysis. To identify the most densely connected regions within the PPI network based on topology, the cytoHubba (v 0.1), a Cytoscape plug-in, was utilized (138).

2.5 Drug Repositioning

Drug discovery is usually a slow and expensive process based on trial-and-error using *in vitro* experiments. An alternative approach is to use simulated molecular docking, an *in silico* method, which is faster and cheaper to identify potential drug candidates for further testing (139). Drug repositioning is used to search for small compounds that have the potential to reverse the gene expression profile for diseases as well as Alzheimer's Disease. In this study, to screen FDA (Food and Drug Administration) approved drugs that can bind and interact with the target proteins, we used Drug Gene Interaction database (DGIdb, v4.2.0) (140) and L1000CDS2 database (141), which is a tool for drug repositioning that can reverse the gene expression profile.

2.6 Molecular Docking

The 3D structures of the proteins allow researchers to utilize *in silico* approaches. *In silico* research can provide fast and cheap way of identifying the drug molecule candidates by compound screening from databases. The interaction patterns of ligands with target molecules can be predicted by molecular docking. Molecular docking is a powerful method to analyse interactions, which can decrease the number of in-vivo and *in vitro* studies for therapeutic assays. When using docking methods, an energy-based scoring system is used to determine the most favourable conformation of a ligand bound to a target. This is based on the assumption that lower energy scores indicate better protein-ligand interactions compared to higher scores. The process can be considered as an optimization problem, where the ultimate goal is to find the ligand-binding mode with the lowest energy (139). In this study, we utilized molecular docking techniques to observe binding characteristics of the drugs that we obtained from databases mentioned above with their target proteins. First of all, the 3D structures of proteins were obtained from RSCB Protein Data Bank (PDB) (142) and AlphaFold Protein Structure Data Base (143); the 3D structures of drugs are obtained from DrugBank (144). ChEMBL (145) is used to get amino acid sequences of drugs that had no experimentally approved 3D structures, and homology models of these drugs were obtained by SWISS-MODEL software (146). To predict binding sites of the target proteins and estimate druggability scores of these sites, DoGSiteScorer software, which is an underlying tool of Proteins*Plus*, is used (147, 148). The binding

sites with highest scores were selected for further analysis. Then, AutoDock Vina is used to predict the binding affinity of predicted small compounds and their targets such as proteins, or receptors (149, 150). For this purpose, all ‘pdb files’ are converted to ‘pdbqt files’ by using Open Babel, a free, open-source version of the Babel chemistry file translation program, since AutoDock Vina can read files in ‘pdbqt’. (151). Results of Autock Vina is visualized with molecular graphical system known as PyMol (152).

2.7 A Computational Framework for Predicting miRNA-Target Gene Interactions and Constructing mRNA-miRNA Regulatory Networks

Various online methods have been employed to determine mRNA-miRNA network. Among them, intersection of results obtained from two web-based tools called as miRNet (2.0) (153) and NetworkAnalyst (3.0) were selected to find the target miRNAs of up-regulated and down-regulated cDEGs (154). miRNet offers an extensive assessment of reliable miRNA-target interaction information sourced from 11 distinct miRNA databases. These databases encompass TarBase, miRTarBase, miRecords, miRanda, miR2Disease, HMDD, PhenomiR, SM2miR, PharmacomiR, EpimiR, and starBase (153). NetworkAnalyst is a potent web-based visual analytics platform for thorough profiling, meta-analysis, and systems-level interpretation of gene expression data (154). Further, STarMir tool was used to predict the mRNA-miRNA interaction. With the help of logistic prediction models created utilizing high-throughput miRNA binding data from cross-linking immunoprecipitation (CLIP) investigations (155), the STarMir tool's purpose is to predict miRNA binding sites on target mRNAs (156). In comparison to the established techniques, the models, which included thorough thermodynamic, structural, and sequence data, were found to produce better predictions of both seed and seedless sites (157). STarMir can be reached freely at (http://sfold.wadsworth.org/starmir.html).

2.8 RNA Isolation, cDNA Synthesis and qRT-PCR

In order to investigate the expression levels of selected miRNAs (miR-7977 and miR-129-5p) and mRNAs (GFAP and SNAP-25) quantitative real time PCR (qRT-PCR) method was used. Sample information are shown in Table 2. Ethical report number is

2018/0041, found on page 94. Firstly, plasma samples were collected from 6 advanced Alzheimer's Disease patients and 6 healthy controls for the study. Secondly, QIAGEN miRNeasy Serum/Plasma Kit (Cat. No: 217184) kit was used to isolate total RNA including miRNA from plasma samples of advanced Alzheimer's Disease patients and healthy controls according to manufacturer's protocol. Then, cDNAs were synthesized with two kits. miScript® II RT (Qiagen cat. no. 218193) Kit was used to synthesize cDNA from mature miRNAs. Reaction components are listed in Table 3. The reaction was incubated for 60 min at 37 ºC, then incubated for 5 min at 95ºC to inactivate miScript Reverse Transcriptase Mix. OneScript® Plus cDNA Synthesis Kit (Applied Biological Materials, Cat. No.: G236) was used to synthesize cDNA from mRNAs. The reaction components are shown in Table 4, reaction conditions are shown in Table 5.

Table 2. Sample distributions.

Samples	Average Age	Range	Female: Male
6 Advanced Alzheimer's Disease patients	83.86 ± 4.4 years	73-95 years	1.54
6 Healty Controls	59 ± 7.13 years	50-86 years	1.5

Table 3. cDNA synthesis components for miRNA detection using miScript® II RT Kit

Component	Volume/reaction

Table 3. Continued

5X miScript HiSpec Buffer	4 µl
10X miScript Nucleics Mix	2 µl
RNase-free water	Variable
miScript Reverse Transcriptase Mix	2 µl
Template RNA	Up to 20

Table 4. cDNA synthesis components for mRNA detection through OneScript® Plus cDNA Synthesis Kit

Components	**Volume (µl)**
5X RT Buffer	4
dNTP	1
Oligo(dT) (10 µM)	1
Random Primers (10 µM)	1
Total RNA (60 ng)	Variable
OneScript® Plus RTase	1
Nuclease-free H2O	Up to 20

Table 5. cDNA synthesis reaction conditions

	Temperature	Time
Synthesis of cDNA	52 °C	30 min
Stop Reaction	85 °C	5 min

Following cDNA synthesis, qRT-PCR was conducted in duplicates in a 36-well rotor in Rotor-Gene Q (QIAGEN) instrument. The 20 µL PCR reaction was prepared as described in Table 6. BlasTaq™ 2X qPCR MasterMix (Applied Biological Materials, Canada, Cat. No.: G891) was used as master mix. The primers for qRT-PCR were synthesized and are listed listen in Table 7. U6 was used for miRNAs and β-actin was used for mRNA as housekeeping internal controls. U6-2 Primer (Applied Biological Materials, Cat. No.: MPH00001) was used as forward primer for U6, and Universal 3' miRNA Reverse Primer (Applied Biological Materials, Cat. No. MPH00000) was used as a reverse primer in qRT-PCR. All qRT-PCR reactions were performed in triplicates. The qRT-PCR reaction conditions are seen in Table 8.

Table 6. Components qRT-PCR.

Components	Volume (µl)
BlasTaq™ 2X qPCR[1]	10
Forward Primer (10 µM)	0.5
Reverse Primer (10 µM)	0.5

Table 6. Continued

cDNA (1 µg)	Variable
MGW	Up to 20

[1] The reaction buffer contains 1.5 mM Mg2+.

Table 7. Primer sequences for qRT-PCR.

Target	Primers	5’ - 3’ Sequence
miR-7997	hsa-miR-7977_fwd	TTCCCAGCCAACGCACC
	hsa-miR-7977_rev	GAACATGTCTGCGTATCTC
miR-129-5p	hsa_miR-129-5p_fwd	CTTTTTGCGGTCTGGGCTTG
	hsa_miR-129-5p_rev	GAACATGTCTGCGTATCTC
β-Actin	hsa_β-actin_fwd	CACCATTGGCAATGAGCGGTTC
	hsa_β-actin_rev	AGGTCTTTGCGGATGTCCACGT
GFAP gene	hsa_GFAP_fwd	CTGGAGAGGAAGATTGAGTCGC
	hsa_GFAP_rev	ACGTCAAGCTCCACATGGACCT
SNAP-25 gene	hsa_SNAP-25_fwd	CGTCGTATGCTGCAACTGGTTG
	hsa_SNAP-25_rev	GGTTCATGCCTTCTTCGACACG

Table 8. Reaction conditions of qRT-PCR.

	Temperature and Time	Cycles
Hold	95 °C, 3 min	1
Cycling	Step 1: 95 °C, 15 s (not acquiring) Step 3: 60 °C/65 °C[2], 10 s acquiring to cycling A (Green)	45
Melt	Ramps from 50 °C to 99 °C Rising by 1 degree each step Wait for 90 sec of pre-melt on first step Wait for 5 sec on each step, Melt A on Green	

[2] 60 °C for miRNA, 65 °C for mRNA.

2.9 Statistical Tests

To calculate the expression levels of the miR-7977, miR-129-5p, GFAP and SNAP-25 relative to suitable references, $2^{-\Delta\Delta Ct}$ method was used (158). U6 was used as endogenous control for miRNAs, whereas β-actin was used as endogenous control for mRNAs. For the comparison of average expression levels vales in Alzheimer's Disease patients and healthy controls, student's t-test was used to compute p-values through the use of GraphPad PRISM software (Version 9.5.1, GraphPad Software, San Diego, CA). Levels of significance were designated as *$p<0.05$, **$p<0.01$, and ***$p<0.0001$.

3. RESULTS

3.1 Identification of Common Differentially Expressed Genes in Brain

The numbers of DEGs and DEMs obtained from each dataset with GEO2R are presented in Table 9 and Table 10 respectively. As seen in Table 9, GSE5281 (751 up-regulated DEGs vs 1921 down-regulated DEGs) and GSE118553 (115 up-regulated DEGs vs 23 down-regulated DEGs) datasets are used to identify DEGs in entorhinal cortex. GSE5281 (2342 up-regulated DEGs vs 1325 down-regulated DEGs) and GSE48350 (227 up-regulated DEGs vs 325 down-regulated DEGs) datasets are used to identify DEGs in hippocampus. GSE5281 (176 up-regulated DEGs vs 3286 down-regulated DEGs) and GSE122063 (404 up-regulated DEGs vs 710 down-regulated DEGs) datasets are used to identify DEGs in frontal cortex. GSE5281 (2139 up-regulated DEGs vs 5271 down-regulated DEGs) and GSE122063 (554 up-regulated DEGs vs 742 down-regulated DEGs) datasets are used to identify DEGs in temporal cortex.

Table 9. Differentially expressed genes datasets used in this study.

Sample Type	Accession Number	Adj p-value < 0.05	logFC ≥ 1	logFC ≤ -1
Entorhinal	GSE5281	2078	751	1921
	GSE118553	138	115	23
Hippocampus	GSE5281	3667	2342	1325
	GSE48350	552	227	325
Frontal	GSE5281	3462	176	3286
	GSE122063	1114	404	710

Table 9. Continued

Temporal	GSE5281	7410	2139	5271
	GSE122063	1296	554	742

As seen in Table 10, GSE16759 (40 up-regulated DEGs vs 65 down-regulated DEGs) is used to identify DEMs in parietal lobe. GSE157239 (94 up-regulated DEGs vs 96 down-regulated DEGs) is used to identify DEMs in temporal lobe. GSE120584 (741 up-regulated DEGs vs 139 down-regulated DEGs) is used to identify DEMs in the plasma samples of Alzheimer's Disease patients compared to healthy controls.

Table 10. Differentially expressed miRNA datasets used in this study.

Sample Type	Accession Number	P-value < 0.05	logFC > 0	logFC < 0
Parietal Lobe	GSE16759	105	40	65
Temporal Lobe	GSE157239	190	94	96
Serum	GSE120584	880	741	139

The selection of specific brain regions, namely the entorhinal cortex, hippocampus, frontal cortex, and temporal cortex, was based on the presence of a higher number of common genes among the different datasets. These overlapped DEGs of the same brain region, that are entorhinal cortex from GSE5281 and GSE118553 (18 up-regulated DEGs vs 4 down-regulated DEGs); hippocampus from GSE48350 and GSE5281 (76 up-regulated DEGs vs 61 down-regulated DEGs); frontal cortex from GSE5281 and GSE122063 (13 up-regulated DEGs vs 212 down-regulated DEGs); temporal cortex from GSE5281 and GSE122063 (74 up-regulated DEGs vs 285 down-regulated

DEGs) were selected. Then, we identified the shared molecular signatures across these brain regions, which could provide insights into the common pathological mechanisms underlying Alzheimer's Disease. Among the four brain regions (entorhinal cortex, hippocampus, frontal cortex, and temporal cortex), we selected specific combinations of regions based on the number of intersected common DEGs (cDEGs) to enhance the robustness and reliability of our findings (Figure 9). For up-regulated cDEGs, we focused on the intersection between the frontal cortex and temporal cortex, as they exhibited the highest overlap of up-regulated DEGs (7 cDEGs). Similarly, for down-regulated DEGs, we considered the intersection of the hippocampus, frontal cortex, and temporal cortex, which demonstrated the greatest number of shared down-regulated DEGs (14 cDEGs). Notably, the entorhinal cortex did not intersect with the other regions, likely due to a lower number of DEGs in this specific region. Therefore, we excluded it from the combined analysis of common DEGs. By strategically focusing on these brain regions with a substantial overlap of DEGs, we aimed his approach allowed us to identify a subset of genes that were consistently dysregulated across multiple brain regions in Alzheimer's Disease, strengthening the significance and relevance of our findings.

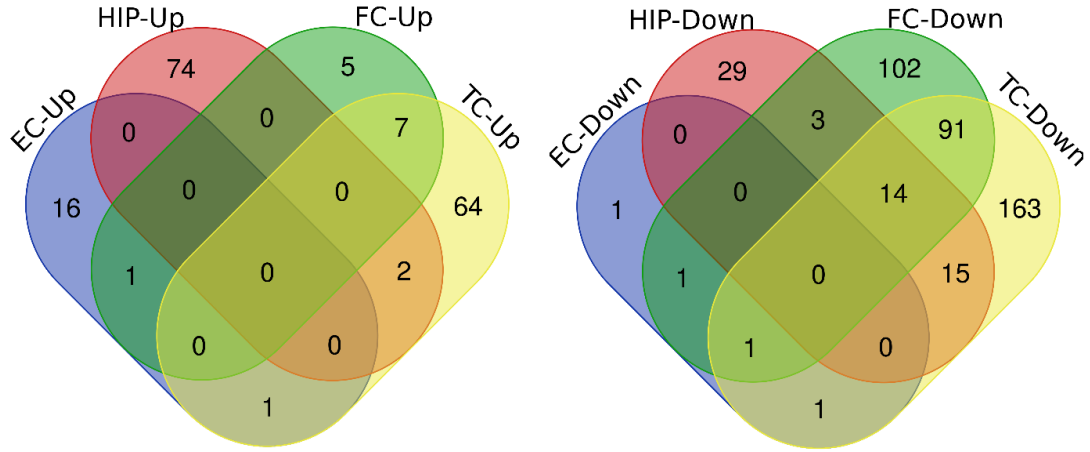


Figure 9. Venn diagram shows common DEGs that represent intersection of four brain regions (EC: Entorhinal cortex, HIP: Hippocampus, FC: Frontal Cortex, TC: Temporal Cortex). Venn diagram of common up-regulated DEGs are shown on the left, of common down-regulated DEGs are shown on the right.

The details of the cDEGs are shown in Table 11. Common up-regulated DEGs are listed as GFAP, LOC100131541, ITPKB, AEBP1, KCNE4, and NACC2; whereas common down-regulated DEGs are listed as NRXN3, SNAP-25, CDC42, ACOT7, CHGB, SULT4A1, CADPS, SVOP, NAP1L5, NECAP1, INA, DYNC1I1, GLS2, ATP8A2.

Table 11. Descriptions of cDEGs

Common DEGs (cDEGs)								
	Symbol	Ensembl Gene ID	Entrez	Type	Species	Chromosome	Position (Mbp)	Description
Common Up-regulated DEGs								
GFAP	GFAP	ENSG00000131095	2670	coding	Human	17	44.9032	glial fibrillary acidic protein
LOC100131541	NA	NA	NA	NA	NA	NA	NA	NA
ITPKB	ITPKB	ENSG00000143772	3707	coding	Human	1	226.6317	inositol-trisphosphate 3-kinase B
MYO10	MYO10	ENSG00000145555	4651	coding	Human	5	16.6619	myosin X
AEBP1	AEBP1	ENSG00000106624	165	coding	Human	7	44.1043	AE binding protein 1
KCNE4	KCNE4	ENSG00000152049	23704	coding	Human	2	223.0518	potassium voltage-gated channel subfamily E regulatory subunit 4
NACC2	NACC2	ENSG00000148411	138151	coding	Human	9	136.0065	NACC family member 2
Common Down-regulated DEGs								
NRXN3	NRXN3	ENSG00000021645	9369	coding	Human	14	78.1704	neurexin 3
SNAP25	SNAP25	ENSG00000132639	6616	coding	Human	20	10.2188	synaptosome associated protein 25
CDC42	CDC42	ENSG00000070831	998	coding	Human	1	22.0255	cell division cycle 42
ACOT7	ACOT7	ENSG00000097021	11332	coding	Human	1	6.2643	acyl-CoA thioesterase 7
CHGB	CHGB	ENSG00000089199	1114	coding	Human	20	5.9115	chromogranin B
SULT4A1	SULT4A1	ENSG00000130540	25830	coding	Human	22	43.8245	sulfotransferase family 4A member 1
CADPS	CADPS	ENSG00000163618	8618	coding	Human	3	62.3983	calcium dependent secretion activator
SVOP	SVOP	ENSG00000166111	55530	coding	Human	12	108.9077	SV2 related protein
NAP1L5	NAP1L5	ENSG00000177432	266812	coding	Human	4	88.6959	nucleosome assembly protein 1 like 5
NECAP1	NECAP1	ENSG00000089818	25977	coding	Human	12	8.0769	NECAP endocytosis associated 1
INA	INA	ENSG00000148798	9118	coding	Human	10	103.2771	internexin neuronal intermediate filament protein alpha
DYNC1I1	DYNC1I1	ENSG00000158560	1780	coding	Human	7	95.7725	dynein cytoplasmic 1 intermediate chain 1
GLS2	GLS2	ENSG00000135423	27165	coding	Human	12	56.4709	glutaminase 2
ATP8A2	ATP8A2	ENSG00000132932	51761	coding	Human	13	25.372	ATPase phospholipid transporting 8A2

3.2 Functional Enrichment Analysis

The GO terms functional enrichment analysis results from DAVID (132) are categorized according to p-value < 0.05, shown in Table 12. In the BP category of cDEGs, the GO terms were mainly enriched in neurotransmitter transport, regulation of neurotransmitter levels, establishment of organelle localization, organelle localization, intermediate filament cytoskeleton organization, intermediate filament-based process, establishment of vesicle localization, vesicle localization, regulation of cell projection organization, intracellular transport, single-organism intracellular transport, dense core granule exocytosis, regulation of transport, transport, signal release from synapse, neurotransmitter secretion, neurofilament cytoskeleton organization, calcium ion regulated exocytosis, presynaptic process involved in chemical synaptic transmission, establishment of localization, synaptic vesicle transport, establishment of synaptic vesicle localization, cell projection organization, vesicle-mediated transport in synapse, regulation of filopodium assembly, synaptic vesicle localization, cytoskeleton-dependent intracellular transport, establishment of localization in cell, single-organism cellular localization, synaptic vesicle exocytosis, single-organism transport, regulated exocytosis, single-organism localization, chemical synaptic transmission, anterograde trans-synaptic signaling, trans-synaptic signaling, localization, synaptic signaling, single organism cell adhesion, regulation of biological quality, neuron differentiation, substantia nigra development.

In the CC group of cDEGs, the GO terms were mainly enriched in synapse part, neuron part, synapse, presynapse, cytoplasmic ribonucleoprotein granule, cytoplasmic vesicle, intracellular vesicle, vesicle, cell projection, cytoplasmic part, bounding membrane of organelle, secretory vesicle, cytoskeleton, whole membrane, cytoplasm, ribonucleoprotein granule, cytoplasmic vesicle membrane, vesicle membrane, intracellular organelle, cytosol, neuron projection, secretory granule.

In the MF group of cDEGs, the GO terms mostly enriched in protein binding, calmodulin binding, spectrin binding, hydrolase activity, motor activity and voltage-gated potassium channel activity.

Table 12. GO terms of common differentially expressed genes (cDEGs) in brain

Category	Term	Genes	Number of Genes	%	P Value	Benjamini
	GO:0008536~neurotransmitter transport	SNAP25, CADPS, NRXN3, GFAP	4	20	0.0013	0.56
	GO:0001505~regulation of neurotransmitter levels	SNAP25, CADPS, NRXN3, GFAP	4	20	0.0015	0.56
	GO:0051656~establishment of organelle localization	CDC42, SNAP25, CADPS, NRXN3, DYNC1I1	5	25	0.002	0.56
	GO:0051640~organelle localization	CDC42, SNAP25, CADPS, NRXN3, DYNC1I1	5	25	0.0034	0.56
	GO:0045104~intermediate filament cytoskeleton organization	ATP8A2, INA, GFAP	3	15	0.0038	0.56
	GO:0045103~intermediate filament-based process	ATP8A2, INA, GFAP	3	15	0.0039	0.56
	GO:0051650~establishment of vesicle localization	SNAP25, CADPS, NRXN3, DYNC1I1	4	20	0.0043	0.56
	GO:0051648~vesicle localization	SNAP25, CADPS, NRXN3, DYNC1I1	4	20	0.0046	0.56
	GO:0031344~regulation of cell projection organization	CDC42, SNAP25, ATP8A2, MYO10, GFAP	5	25	0.0046	0.56
	GO:0044907~intracellular transport	CDC42, SNAP25, MYO10, CADPS, NRXN3, DYNC1I1, GFAP	7	35	0.0062	0.58
	GO:1902582~single-organism intracellular transport	CDC42, SNAP25, CADPS, NRXN3, DYNC1I1	5	25	0.0078	0.58
	GO:0190504~dense core granule exocytosis	SNAP25, CADPS	2	10	0.0078	0.58
	GO:0051049~regulation of transport	CDC42, SNAP25, ATP8A2, KCNE4, CADPS, NRXN3, GFAP	7	35	0.008	0.58
	GO:0006810~transport	CDC42, SNAP25, ATP8A2, NECAP1, SVOP, MYO10, KCNE4, CADPS, NRXN3, DYNC1I1, GFAP	11	55	0.0088	0.58
	GO:0099643~signal release from synapse	SNAP25, CADPS, NRXN3	3	15	0.0094	0.58
	GO:0007269~neurotransmitter secretion	SNAP25, CADPS, NRXN3	3	15	0.0094	0.58
	GO:0060052~neurofilament cytoskeleton organization	ATP8A2, INA	2	10	0.0098	0.58
	GO:0017156~calcium ion regulated exocytosis	SNAP25, CADPS, NRXN3	3	15	0.0098	0.58
	GO:0099531~presynaptic process involved in chemical synaptic transmission	SNAP25, CADPS, NRXN3	3	15	0.01	0.58
	GO:0051234~establishment of localization	CDC42, SNAP25, ATP8A2, NECAP1, SVOP, MYO10, KCNE4, CADPS, NRXN3, DYNC1I1, GFAP	11	55	0.012	0.64
Biological Process	GO:0048489~synaptic vesicle transport	SNAP25, CADPS, NRXN3	3	15	0.016	0.74
	GO:0097480~establishment of synaptic vesicle localization	SNAP25, CADPS, NRXN3	3	15	0.016	0.74
	GO:0030030~cell projection organization	CDC42, SNAP25, ATP8A2, MYO10, NRXN3, GFAP	6	30	0.016	0.74
	GO:0099003~vesicle-mediated transport in synapse	SNAP25, CADPS, NRXN3	3	15	0.016	0.74
	GO:0051489~regulation of filopodium assembly	CDC42, MYO10	2	10	0.017	1
	GO:0097479~synaptic vesicle localization	SNAP25, CADPS, NRXN3	3	15	0.017	0.74
	GO:0030705~cytoskeleton-dependent intracellular transport	CDC42, MYO10, DYNC1I1	3	15	0.018	0.77
	GO:0051649~establishment of localization in cell	CDC42, SNAP25, MYO10, CADPS, NRXN3, DYNC1I1, GFAP	7	35	0.019	0.79
	GO:1902580~single-organism cellular localization	CDC42, SNAP25, CADPS, NRXN3, DYNC1I1	5	25	0.02	0.79
	GO:0016079~synaptic vesicle exocytosis	SNAP25, CADPS	2	10	0.021	1
	GO:0044765~single-organism transport	CDC42, SNAP25, ATP8A2, KCNE4, CADPS, NRXN3, DYNC1I1, GFAP	8	40	0.021	0.8
	GO:0045055~regulated exocytosis	SNAP25, CADPS, NRXN3	3	15	0.025	0.92
	GO:1902578~single-organism localization	CDC42, SNAP25, ATP8A2, KCNE4, CADPS, NRXN3, DYNC1I1, GFAP	8	40	0.028	1
	GO:0007268~chemical synaptic transmission	SNAP25, CADPS, NRXN3, GFAP	4	20	0.031	1
	GO:0098916~anterograde trans-synaptic signaling	SNAP25, CADPS, NRXN3, GFAP	4	20	0.031	1
	GO:0099537~trans-synaptic signaling	SNAP25, CADPS, NRXN3, GFAP	4	20	0.032	1
	GO:0051179~localization	CDC42, SNAP25, ATP8A2, NECAP1, SVOP, MYO10, KCNE4, NACC2, CADPS, NRXN3, DYNC1I1, GFAP	12	60	0.033	1
	GO:0099536~synaptic signaling	SNAP25, CADPS, NRXN3, GFAP	4	20	0.034	1
	GO:0098602~single organism cell adhesion	CDC42, ITPKB, MYO10, NRXN3	4	20	0.038	1
	GO:0065008~regulation of biological quality	CDC42, ITPKB, SNAP25, ATP8A2, MYO10, KCNE4, CADPS, NRXN3, GFAP	9	45	0.04	1
	GO:0030182~neuron differentiation	CDC42, SNAP25, ATP8A2, NRXN3, GFAP	5	25	0.045	1
	GO:0021762~substantia nigra development	CDC42, INA	2	10	0.046	1
	GO:0044456~synapse part	CDC42, SNAP25, NECAP1, SVOP, CADPS, NRXN3, INA 20	7	35	0.00027	0.057
	GO:0097458~neuron part	CDC42, SNAP25, ATP8A2, NECAP1, SVOP, MYO10, CADPS, NRXN3	8	40	0.00078	0.082
	GO:0045202~synapse	CDC42, SNAP25, NECAP1, SVOP, CADPS, NRXN3, INA 20	7	35	0.0018	0.095
	GO:0098793~presynapse	SNAP25, NECAP1, SVOP, CADPS, NRXN3	5	25	0.0018	0.095
	GO:0036464~cytoplasmic ribonucleoprotein granule	CDC42, INA, DYNC1I1	3	15	0.0028	0.26
	GO:0031410~cytoplasmic vesicle	CDC42, SNAP25, ATP8A2, NECAP1, SVOP, CADPS, DYNC1I1, CHGB	8	40	0.006	0.21
	GO:0097708~intracellular vesicle	CDC42, SNAP25, ATP8A2, NECAP1, SVOP, CADPS, DYNC1I1, CHGB	8	40	0.0061	0.21
	GO:0031982~vesicle	CDC42, SNAP25, ATP8A2, ACOT7, NECAP1, SVOP, CADPS, AEBP1, DYNC1I1, CHGB 20	10	50	0.0081	0.24
	GO:0042995~cell projection	CDC42, ATP8A2, GFAP	3	15	0.012	0.37
	GO:0044444~cytoplasmic part	SNAP25, ACOT7, SVOP, ATP8A2, MYO10, GLS2, CADPS, GFAP, CDC42, ITPKB, NECAP1, NACC2, SULT4A1, INA, DYNC1I1, CHGB 20	16	80	0.012	0.31
Cellular Component	GO:0098588~bounding membrane of organelle	CDC42, SNAP25, ATP8A2, NECAP1, SVOP, CADPS, GFAP	7	35	0.015	0.33
	GO:0099503~secretory vesicle	CDC42, SNAP25, SVOP, CADPS, CHGB	5	25	0.016	0.33
	GO:0005856~cytoskeleton	CDC42, ITPKB, SNAP25, MYO10, INA, DYNC1I1, GFAP 20	7	35	0.02	0.38
	GO:0098805~whole membrane	SNAP25, ATP8A2, NECAP1, SVOP, CADPS, GFAP	6	30	0.025	0.39
	GO:0005737~cytoplasm	SNAP25, ACOT7, SVOP, ATP8A2, MYO10, GLS2, CADPS, AEBP1, GFAP, CDC42, ITPKB, NECAP1, NACC2, SULT4A1, INA, DYNC1I1, CHGB 20	17	85	0.026	0.39
	GO:0035770~ribonucleoprotein granule	CDC42, INA, DYNC1I1	3	15	0.028	0.39
	GO:0030659~cytoplasmic vesicle membrane	SNAP25, NECAP1, SVOP, CADPS	4	20	0.037	0.48
	GO:0012506~vesicle membrane	SNAP25, NECAP1, SVOP, CADPS	4	20	0.039	0.48
	GO:0043229~intracellular organelle	SNAP25, ACOT7, SVOP, ATP8A2, MYO10, GLS2, CADPS, AEBP1, NAP1L5, GFAP, CDC42, ITPKB, NECAP1, NACC2, SULT4A1, INA, DYNC1I1, CHGB 20	18	90	0.042	0.48
	GO:0005829~cytosol	CDC42, ITPKB, SNAP25, ACOT7, NECAP1, MYO10, CADPS, SULT4A1, DYNC1I1, GFAP	10	50	0.044	0.82
	GO:0043005~neuron projection	CDC42, SNAP25, MYO10	3	15	0.044	0.82
	GO:0030141~secretory granule	CDC42, SNAP25, CADPS, CHGB	4	20	0.048	0.5
	GO:0005515~protein binding	SNAP25, ACOT7, SVOP, ATP8A2, MYO10, KCNE4, GLS2, CADPS, NRXN3, AEBP1, NAP1L5, GFAP, CDC42, ITPKB, NECAP1, NACC2, SULT4A1, INA, DYNC1I1, CHGB	20	100	0.0048	0.93
	GO:0005516~calmodulin binding	ITPKB, MYO10, AEBP1	3	15	0.018	1
Molecular Function	GO:0030507~spectrin binding	MYO10, DYNC1I1 20	2	10	0.026	0.69
	GO:0016787~hydrolase activity	CDC42, ATP8A2, ACOT7, MYO10, GLS2, AEBP1, DYNC1I1	7	35	0.048	1
	GO:0003774~motor activity	MYO10, DYNC1I1 20	2	10	0.059	1
	GO:0005249~voltage gated potassium channel activity	SNAP25, KCNE4	2	10	0.062	1

The JENSEN tissues, KEGG pathways and Reactome analysis results from EnrichR (134) are categorized according to p-value < 0.05, shown in Figure 10. Tissues and

pathway enrichment of cDEGs with EnrichR. cDEGs are mostly enriched in tissues such as caudate nucleus, medulla oblongata, cingulate cortex, thalamus, cerebral peduncle, subthalamic nucleus, corpus callosum, amygdala, prefrontal cortex, and temporal lobe (Figure 10 a). KEGG pathway analysis from EnrichR database shows that the cDEGs are enriched in Fc gamma R-mediated phagocytosis, D-Glutamine and D-glutamate metabolism, pathogenic *Escherichia coli* infection, arginine biosynthesis, proximal tubule bicarbonate reclamation, *Salmonella* infection, biosynthesis of unsaturated fatty acids, fatty acid elongation, alanine, vasopressin-regulated water reabsorption (Figure 10 b). Reactome analysis from EnrichR database shows that the cDEGs are enriched in glutamate neurotransmitter release cycle, netrin-1 signaling, neurotransmitter release cycle, FCGR3A-mediated phagocytosis, regulation of actin dynamics for phagocytic cup formation, Fcgamma receptor (FCGR) dependent phagocytosis, RHO GTPases activate formins, inactivation of CDC42 and RAC1, neuronal system, autophagy, phase 3 - rapid repolarization, RHO GTPases activate KTN1, neurotoxicity of Clostridium toxins, RHO GTPases activate IQGAPs, CD28 dependent Vav1 pathway, glutamate and glutamine metabolism, DCC mediated attractive signaling, phase 2 - plateau phase, infectious disease, acetylcholine neurotransmitter release cycle, serotonin neurotransmitter release cycle, norepinephrine neurotransmitter release cycle, GABA synthesis, G beta: gamma signaling thru CDC42, chaperone mediated autophagy, RHO GTPases activate PAKs, cytosolic sulfonation of small molecules, dopamine neurotransmitter release cycle, other interleukin signaling, aggrephagy, synthesis of IP3 and IP4 in cytosol, transmission across chemical synapses, Leishmania infection, myogenesis, uptake and actions of bacterial toxins, RHO GTPase effectors, EGFR down-regulation, G-protein beta: gamma signaling, GPVI-mediated activation cascade, nuclear signaling by ERBB4, CD28 co-stimulation, EPHB-mediated forward signaling, COPI-independent Golgi-to-ER retrograde traffic, gene and protein expression by JAK-STAT signaling after interleukin-12 stimulation, mitochondrial fatty acid beta-oxidation, RHO GTPases activate WASPs and WAVEs, RHOV GTPase cycle, HSP90 chaperone cycle

for steroid hormone receptors (SHR) in presence of ligand, RHOU GTPase cycle, interleukin-12 signaling, inositol phosphate metabolism (Figure 10 c).

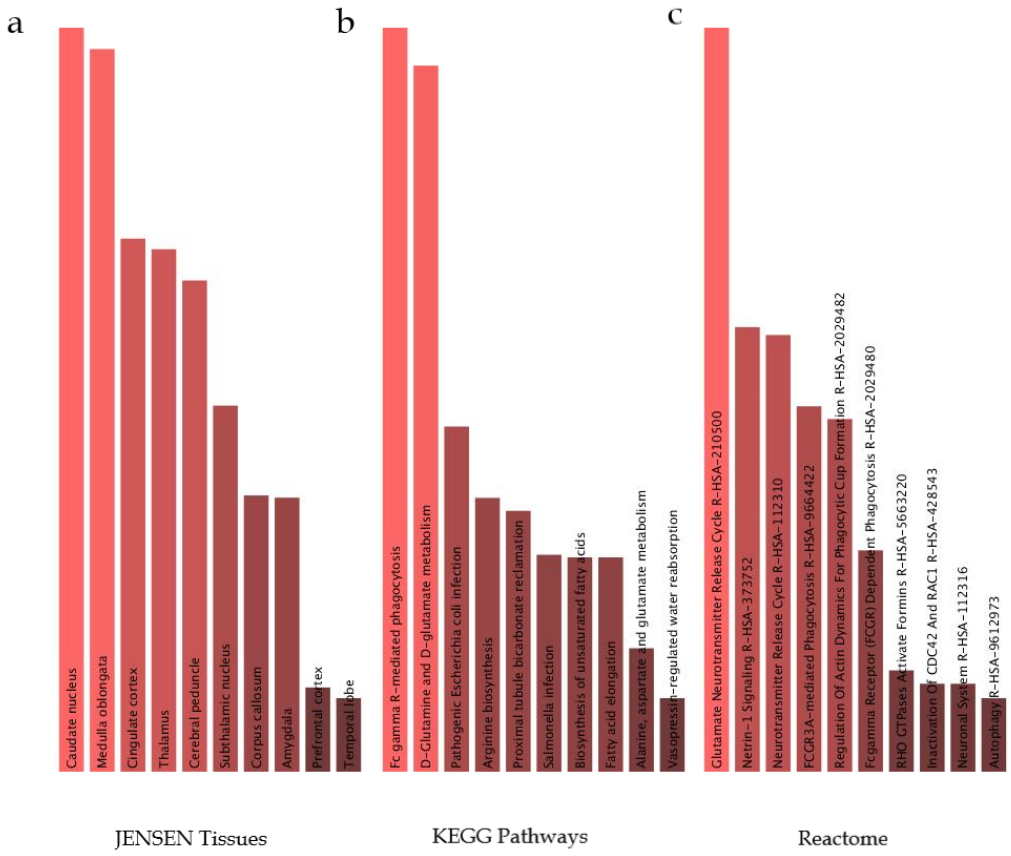


Figure 10. Tissues and pathway enrichment of cDEGs with EnrichR

3.3 Protein-Protein Interaction Network Construction and Hub Gene Analysis of Common Differentially Expressed Genes.

In our methodology, we constructed a protein-protein interaction (PPI) network using the STRING database (137) to analyse the cDEGs as seen in Figure 11. We set the minimum required interaction cutoff to a medium confidence level of 0.400, ensuring the inclusion of reliable interactions in the network. We focused the first shell of the PPI network only on the query proteins, excluding any additional proteins. Additionally, no proteins were included in the second shell. This approach resulted in a relatively small network, as we specifically prioritized the interactions involving the query proteins. It is important to note that including more proteins or expanding to the second shell can lead to larger networks that may require additional computational

time and downloading. Upon analysis, we observed that several proteins, namely ACOT7, ATP8A2, KCNE4, NACC2, NECAP1, GLS2, NRXN3, AEBP1, ITPKB, and NAP1LK5, were disconnected nodes in the network. Among the cDEGs, we found that GFAP from the up-regulated DEGs and MYO10, CDC42, SNAP-25, SULT4A1, NRXN3, DYNC1I1, INA, CADPS, CHGB, and SVOP from the down-regulated cDEGs formed a network within the PPI network.

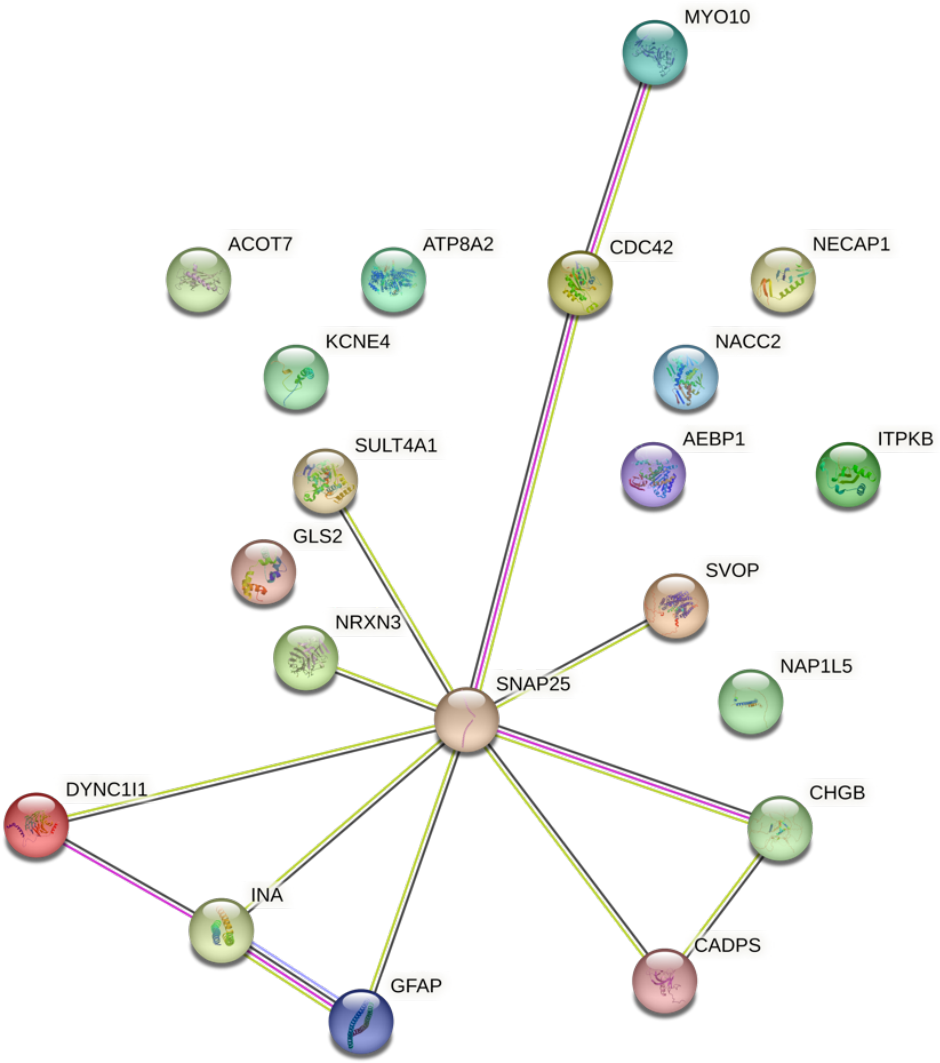


Figure 11. Protein-protein interaction network construction of cDEGs with STRING.

Then, the nodes and network is loaded into Cytoscape (135) to visualize and identify the highly connected PPI Network as seen in Figure 12. The selection criteria for

identifying the hub proteins are determined as degree cutoff. To limit the number of interactions displayed, we set the maximum number of interactions to the default setting, which is the 10 best-scoring hits. These top 10 hub genes in network STRING are ranked by Degree method in CytoHubba (138). Top 10 hub proteins are ranked by Degree method as SNAP-25, INA, CDC42, GFAP, CADPS, CHGB, DYNC1I1, NRXN3, MYO10, SULT4A1, respectively seen on the right side of Figure 12. By constructing the PPI network and analysing the connections among the cDEGs, we aimed to identify potential functional relationships and molecular interactions among the proteins associated with Alzheimer's Disease. The visualization of the PPI network provided insights into the protein interactions and highlighted specific proteins of interest within the network.

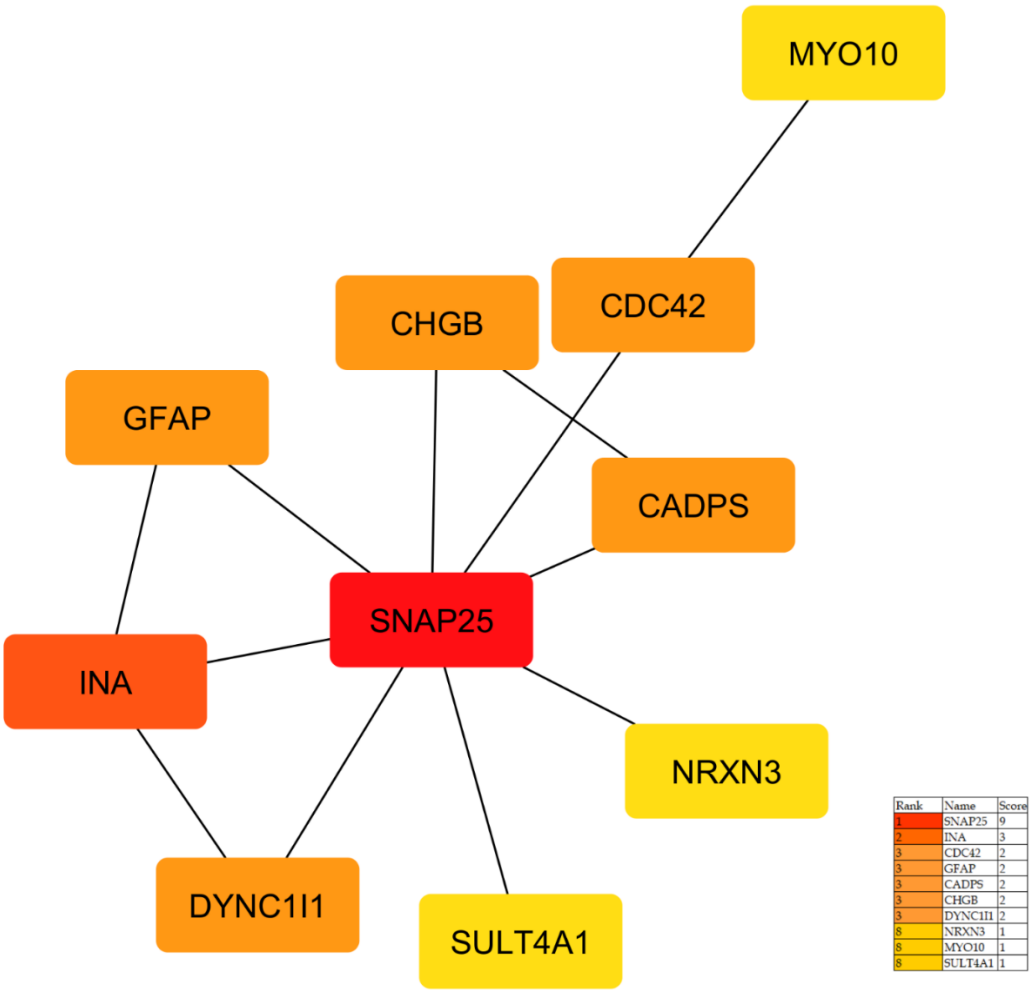


Figure 12. Hub gene visualization of cDEGs with Cytohubba plug-in of Cytoscape.

Upon analysing the network, we observed that the up-regulated cDEG, GFAP, and the down-regulated cDEG, SNAP-25, displayed significant connectivity within the PPI network. This suggests that these genes may play crucial roles in the development and progression of Alzheimer's Disease. The insights gained from visualizing the PPI network provided valuable information about protein interactions and highlighted specific proteins that warrant further investigation as potential therapeutic targets for Alzheimer's Disease. In particular, GFAP and SNAP-25 emerged as promising candidates for drug repositioning strategies to reverse their expression in Alzheimer's Disease. Additionally, the identification of hub proteins opens up new possibilities for the discovery of biomarkers associated with Alzheimer's Disease. Exploring the relative miRNAs that regulate GFAP and SNAP-25 expression could potentially lead to the development of non-invasive biomarkers for early Alzheimer's Disease detection and disease monitoring.

3.4 Drug repositioning and Molecular Docking

The implementation of *in silico* approaches in our research provided an efficient and cost-effective means of identifying potential drug candidates by conducting compound screenings from various databases. This method reduced the need for numerous *in vivo* and *in vitro* studies for therapeutic assays, thus saving time and resources. Firstly, to expand our drug candidate pool, we utilized two additional databases, the DGIdb (140) and the L1000CDS2 (141) database to screen FDA approved drugs that have potential interactions with target proteins that are GFAP and SNAP-25, thereby paving the way for drug repositioning that could potentially reverse disease-related gene expression profiles in Table 13 and Table 14. Table 13 presents a list of drug candidates identified from the DGIdb database, along with their respective target genes and relevant query and interaction scores. The query score reflects the relevance of the drug-gene interaction, while the interaction score represents the strength of the interaction (140). The table provides valuable insights into potential drugs that interact with GFAP and SNAP-25. For the target gene GFAP, which is associated with astrocyte activation and neuroinflammatory processes in Alzheimer's Disease (159), five drug candidates were identified. Sorbitol shows a query score of 2.87 and an interaction score of 1.65, indicating a moderate level of interaction with GFAP. Permethrin shows a query score

of 2.15 and an interaction score of 1.24, Permethrin also exhibits a moderate level of interaction with GFAP. Oprelvekin has a query score of 1.43 and an interaction score of 0.82, suggesting a lower but still noteworthy interaction with GFAP. Dronabinol has a query score of 1.08 and an interaction score of 0.62, Dronabinol also displays a lower level of interaction with GFAP. Amphetamine has a query score of 0.99 and an interaction score of 0.57, indicating a relatively weaker interaction with GFAP. For the target gene SNAP-25 that function in the exocytosis of neurotransmitters from synapses (160), four drug candidates were identified. Onabotulinumtoxina demonstrates a high query score of 4.3 and an impressive interaction score of 15.46, suggesting a strong and significant interaction with SNAP-25. Incobotulinumtoxina, similarly, with a query score of 4.3 and an interaction score of 15.46, Incobotulinumtoxina exhibits a robust interaction with SNAP-25. Abobotulinumtoxina shows a high query score of 4.3 and an interaction score of 15.46, signifying a strong interaction with SNAP-25. Diazoxide has a lower query score of 0.66 and an interaction score of 2.38, indicating a weaker but still notable interaction with SNAP-25.

Table 13. Drug candidates obtained from DGIdb database.

Drug	Gene	Query Score	Interaction Score
Sorbitol	GFAP	2.87	1.65
Permethrin	GFAP	2.15	1.24
Oprelvekin	GFAP	1.43	0.82
Dronabinol	GFAP	1.08	0.62
Amphetamine	GFAP	0.99	0.57

Table 13. Continued

Onabotulinumtoxina	SNAP-25	4.3	15.46
Incobotulinumtoxina	SNAP-25	4.3	15.46
Abobotulinumtoxina	SNAP-25	4.3	15.46
Diazoxide	SNAP-25	0.66	2.38

Table 14 presents a list of drug candidates obtained from the L1000CDS2 database (141), with a focus on their interactions with the target gene SNAP-25, which plays a critical role in synaptic vesicle fusion and neurotransmitter release (161). The table provides information on the drugs' names, their ranks within the database, and the corresponding scores indicating the strength of the drug-gene interactions. Among the identified drug candidates, Vorinostat and Trichostatin A hold the top ranks (1 and 3, respectively) with a score of 0.2222, suggesting significant interactions with SNAP-25. Furthermore, Scriptaid, Lestaurtinib, Alvocidib, Pracinostat, Nintedanib, and Mocetinostat also demonstrate potential interactions with SNAP-25, each sharing the same rank of 7, 14, 18, 20, 27, and 28, respectively, along with a score of 0.1667. Overall, Table 13 and Table 14 provides a comprehensive overview of potential drug candidates interacting with target genes relevant to Alzheimer's Disease. However, further in-depth research and experimentation are essential to validate and understand the therapeutic potential of these drug candidates in the context of Alzheimer's Disease, where synaptic dysfunction plays a pivotal role. These drug candidates needed further investigation to assess their potential therapeutic relevance and efficacy in the context of Alzheimer's Disease and synaptic dysfunction

Table 14. Drug candidates obtained from L1000CDS2 database.

Drug	Target Gene	Rank	Score
Vorinostat	SNAP-25	1	0.2222
Trichostatin A	SNAP-25	3	0.2222
Scriptaid	SNAP-25	7	0.1667
Lestaurtinib	SNAP-25	14	0.1667
Alvocidib	SNAP-25	18	0.1667
Pracinostat	SNAP-25	20	0.1667
Nintedanib	SNAP-25	27	0.1667
Mocetinostat	SNAP-25	28	0.1667

Secondly, we obtained the 3D structures of target proteins from the RSCB Protein Data Bank (PDB) (142) and the AlphaFold Protein Structure Data Base (143). Additionally, we retrieved the 3D structures of drugs from DrugBank (144), and for drugs without experimentally approved 3D structures, amino acid sequences were obtained from ChEMBL (145) for homology modeling. Although Onabotulinumtoxina, Incobotulinumtoxina, and Abobotulinumtoxina were generated using the SWISS-MODEL software (162) to obtain their 3D structures, we encountered a challenge during the molecular docking process. These drugs are considerably larger in size when compared to the target protein structure, making it impractical to apply

AutoDock Vina for docking analysis. Due to the size disparity between these drugs and the target protein, we made the decision to exclude them from the molecular docking experiments. Applying AutoDock Vina to such large drugs would not yield meaningful results and could potentially lead to inaccuracies in the analysis (163).

Molecular docking, a powerful method used to analyse ligand-target interactions, played a key role in predicting the interaction patterns of drugs with their respective target proteins (110). The 3D structures of the target proteins, GFAP and SNAP-25, were obtained from the PDB DataBank (142) and AlphaFold (143), respectively. For GFAP, we had access to only one published protein structure in the databases, identified as "6A9P." This structure represented the protein in its ligand-free state, without any associated ligands. On the other hand, for SNAP-25, we encountered multiple published structures with ligands and small molecules, given its nature as a SNARE complex molecule found in cells. However, to maintain consistency with GFAP, we sought a ligand-free structure for SNAP-25 as well. As no such structure was available in the PDB database (142), we opted to use a predicted model obtained from AlphaFold (143), which served as a suitable substitute for our analysis. As a result, we used the identifier code "6A9P" to retrieve the GFAP structure from the PDB DataBank (142), while the SNAP-25 structure with identifier code "AF-P60880-F1" was obtained from the AlphaFold Data Base (143).

To predict the binding sites of the target proteins and estimate druggability scores of these sites, we utilized the DoGSiteScorer software, which is an underlying tool of ProteinsPlus. The binding sites with the highest scores were selected for further analysis. Prediction of binding sites of each protein is calculated with DogSiteScorer (147). Among different dogsites of each protein, the sites that have highest drug scores were considered to analyse. Table 15, presents the drug scores of predicted binding sites of target proteins, namely GFAP (Glial Fibrillary Acidic Protein) and SNAP-25 (Synaptosomal-Associated Protein 25). The table provides information about different binding sites on these proteins and their corresponding drug scores, which indicate the likelihood of drug-protein interactions. For GFAP, three binding sites are listed as P_2, P_4, and P_1, with drug scores of 0.87, 0.85, and 0.83, respectively. These scores suggest that there is a high probability of drug binding to these specific sites on GFAP, making them potential drug targets. Similarly, for SNAP-25, two binding sites are

mentioned as P_0 and P_1, with drug scores of 0.84 and 0.63, respectively. These scores indicate the likelihood of drug-protein interactions at these sites on SNAP-25. Overall, Table 15 provides valuable information about the drug scores associated with predicted binding sites on GFAP and SNAP-25, offering insights into potential drug targets for these proteins. These findings may have implications for developing targeted therapies to modulate the function of GFAP and SNAP-25, and thereby impacting their roles in neurological conditions, such as Alzheimer's Disease, where these proteins are known to play crucial roles in synaptic function and neuroinflammation. However, further experimental validation and investigation are necessary to confirm the significance of these drug-protein interactions in the context of Alzheimer's Disease.

Table 15. Drug scores of predicted binding sites of target proteins.

Protein	Site Name	Drug Score
GFAP	P_2	0.87
	P_4	0.85
	P_1	0.83
SNAP-25	P_0	0.84
	P_1	0.63

In Figure 13, the predicted binding sites of each gene are visualized with DogSiteScorer (147). Binding site of GFAP which are P_2 is represented in cyan, P_4 is represented in orange and P_1 is represented in yellow. The binding site of SNAP-25 which are P_0 is represented in orange and P_1 is represented in yellow.

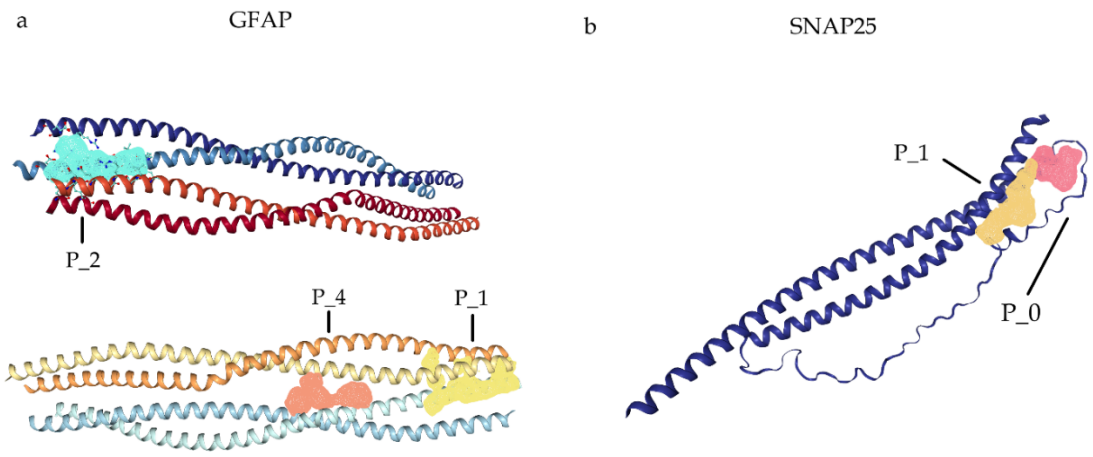


Figure 13. Visualization of predicted binding sites of target proteins and their scores with DogSiteSorer (147).

Subsequently, we employed AutoDock Vina to predict the binding affinity between the predicted small compounds and their target proteins or receptors. To facilitate this process, we converted all 'pdb files' to 'pdbqt files' using Open Babel, an open-source version of the Babel chemistry file translation program, as AutoDock Vina requires 'pdbqt' format files. The results obtained from AutoDock Vina were then visualized using the molecular graphical system known as PyMol (152), providing a clear representation of the binding characteristics of the drugs with their respective target proteins. We performed molecular docking of GFAP with various drug candidates, including Amphetamine, Sorbitol, Dronabinol, and Permethrin. The docking results were visualized in PyMol and are presented in Figure 14. Amphetamine, labelled in red, showed the lowest binding affinity to GFAP from the P_2 binding site with a score of -4.046 kcal/mol, from the P_4 binding site with a score of -4.177 kcal/mol, and from the P_1 binding site with a score of -3.924 kcal/mol. These scores indicate a high probability of binding for Amphetamine to GFAP at all three binding sites, with the P_4 site demonstrating the strongest binding. Sorbitol, represented in yellow, displayed the lowest binding affinity to GFAP from the P_2 binding site with a score of -3.882 kcal/mol, from the P_4 binding site with a score of -3.743 kcal/mol, and from the P_1 binding site with a score of -3.361 kcal/mol. The decreasing kcal/mol values indicate an increasing likelihood of binding for Sorbitol to GFAP, with the P_1 site having the strongest binding affinity. Dronabinol, depicted in green, exhibited the

lowest binding affinity to GFAP from the P_2 binding site with a score of -5.776 kcal/mol, from the P_4 binding site with a score of -6.266 kcal/mol, and from the P_1 binding site with a score of -6.457 kcal/mol. These negative scores imply a high probability of strong binding between Dronabinol and GFAP, with the P_1 site showing the highest affinity. Lastly, Permethrin, represented in blue, demonstrated the lowest binding affinity to GFAP from the P_2 binding site with a score of -5.371 kcal/mol, from the P_4 binding site with a score of -6.36 kcal/mol, and from the P_1 binding site with a score of -6.507 kcal/mol. The decreasing kcal/mol values indicate an increasing likelihood of binding for Permethrin to GFAP, with the P_1 site having the highest binding affinity.

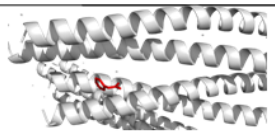
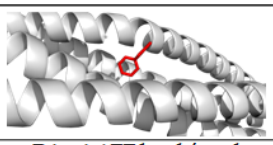
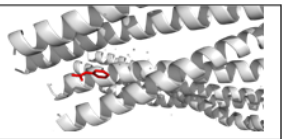
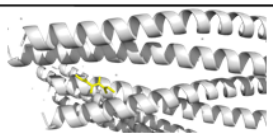
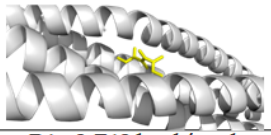
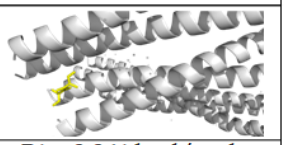
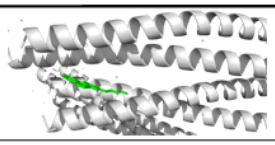
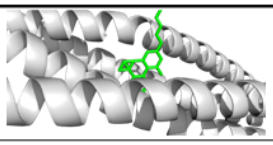
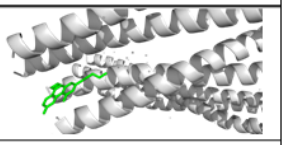
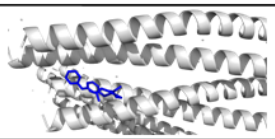
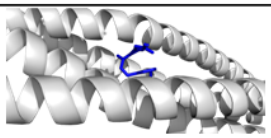
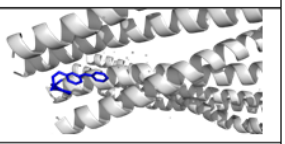
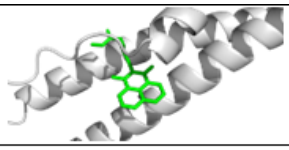
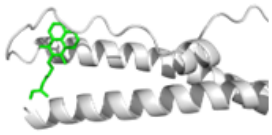
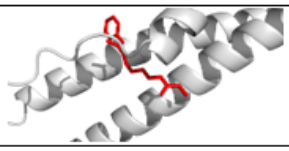
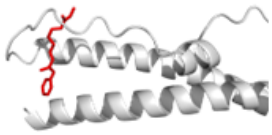
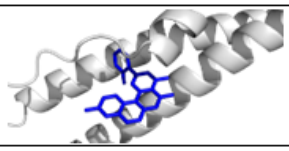
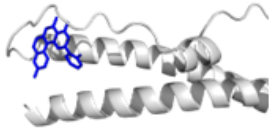
Amphetamine			
	P2: -4.046 kcal/mol	P4: -4.177 kcal/mol	P1: -3.924 kcal/mol
Sorbitol			
	P2: -3.882 kcal/mol	P4: -3.743 kcal/mol	P1: -3.361 kcal/mol
Dronabinol			
	P2: -5.776 kcal/mol	P4: -6.266 kcal/mol	P1: -6.457 kcal/mol
Permethrin			
	P2: -5.371 kcal/mol	P4: -6.36 kcal/mol	P1: -6.507 kcal/mol

Figure 14. Binding affinity results of GFAP and their interactions are visualized with PyMol (152).

The AutoDock Vina (163) results of SNAP-25 protein are visualized in PyMol (152), represented in Figure 15. Scriptaid is labelled red color. The lowest binding affinity of scriptaid to SNAP-25 from the P_0 binding site is -7.547 kcal/mol, and from the P_1 binding site, it is -6.113 kcal/mol. Vorinostat is highlighted in red. The lowest binding affinity of vorinostat to SNAP-25 from the P_0 binding site is -6.255 kcal/mol, and from the P_1 binding site, it is -4.705 kcal/mol. Alvocidib is highlighted in red. The

lowest binding affinity of alvocidib to SNAP-25 from the P_0 binding site is -7.132 kcal/mol, and from the P_1 binding site, it is -6.137 kcal/mol. Pracinostat is highlighted in red. The lowest binding affinity of pracinostat to SNAP-25 from the P_0 binding site is -6.268 kcal/mol, and from the P_1 binding site, it is -5.363 kcal/mol. Mocetinostat is highlighted in red. The lowest binding affinity of mocetinostat to SNAP-25 from the P_0 binding site is -7.113 kcal/mol, and from the P_1 binding site, it is 1095403.155 kcal/mol. Nintedanib is highlighted in red. The lowest binding affinity of nintedanib to SNAP-25 from the P_0 binding site is -6.276 kcal/mol, and from the P_1 binding site, it is 2577527.46 kcal/mol. estaurtinib is highlighted in red. The lowest binding affinity of lestaurtinib to SNAP-25 from the P_0 binding site is -6.415 kcal/mol, and from the P_1 binding site, it is -7.587 kcal/mol. Diazoxide is highlighted in red. The lowest binding affinity of diazoxide to SNAP-25 from the P_0 binding site is -5.421 kcal/mol, and from the P_1 binding site, it is -4.839 kcal/mol. Trichostatin A is highlighted in red. The lowest binding affinity of trichostatin A to SNAP-25 from the P_0 binding site is -5.588 kcal/mol, and from the P_1 binding site, it is -5.057 kcal/mol.

Scriptaid		
	P0: -7.547 kcal/mol	P1: -6.113 kcal/mol
Vorinostat		
	P0: -6.255 kcal/mol	P1: -4.705 kcal/mol
Alvocidib		
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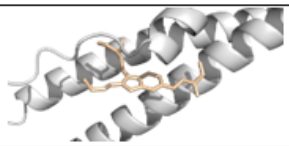
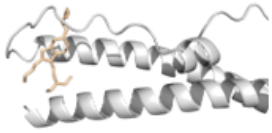
Pracinostat		
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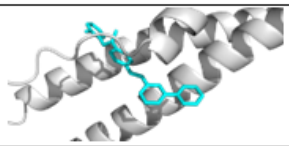
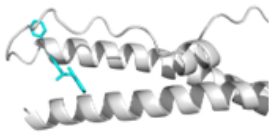
Mocetinostat		
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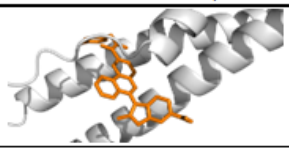
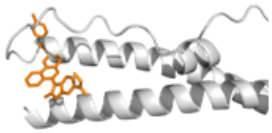
Nintedanib		
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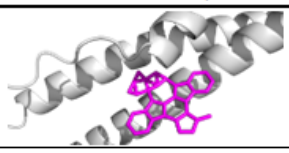
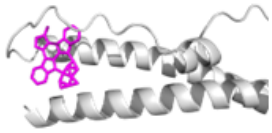
Lestaurtinib		
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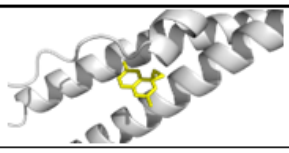
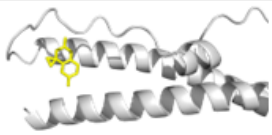
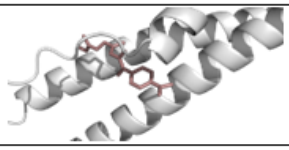
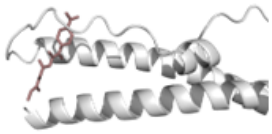
Diazoxide		
	P0: -5.421 kcal/mol	P1: -4.839 kcal/mol
Trichostatin A		
	P0: -5.588 kcal/mol	P1:-5.057 kcal/mol

Figure 15. Binding affinity results of SNAP-25 and their interactions are visualized with PyMol (152).

By utilizing these computational tools and methods, we gained valuable insights into the potential drug-protein interactions, which have important implications in drug discovery and the development of potential therapeutics for conditions like Alzheimer's Disease. Molecular docking and *in silico* approaches, combined with experimental validation, offer a promising path for identifying novel drug candidates and advancing precision medicine research.

3.5 An Integrated Computational Approach for Predicting miRNA-Target Gene Interactions and Constructing mRNA-miRNA Regulatory Networks

Understanding the relationship between up-regulated miRNAs and down-regulated genes in bioinformatics requires a combination of computational predictions, functional analyses, and experimental validations. This knowledge contributes to our understanding of the complex regulatory networks involved in gene expression and can provide crucial insights into disease mechanisms and potential therapeutic interventions. In this study, we aim to investigate potential non-invasive biomarkers for early Alzheimer's Disease detection and disease monitoring by exploring the relative miRNAs that regulate GFAP and SNAP-25 expression that exhibited remarkable interconnectedness within the PPI network. To achieve this objective, we constructed a mRNA-miRNA network, integrating miRNA expression profiles and mRNA expression data obtained from Alzheimer's Disease patients and healthy controls. In pursuit of this aim, we identified predicted target miRNAs for the cDEGs obtained from the intersection of different brain regions. Subsequently, we aim to determine the overlap between these predicted miRNAs and miRNA datasets derived from serum sample datasets, as well as samples from the parietal lobe and temporal lobe. Specifically, for up-regulated cDEGs, their predicted miRNA targets obtained from databases will be considered as down-regulated miRNAs. These down-regulated predicted miRNAs will then be compared to the down-regulated miRNAs identified in the serum, parietal lobe, and temporal lobe sample datasets. First of all, to construct the mRNA-miRNA networks, we obtained the predicted miRNAs that serve as targets for the cDEGs from miRNet and Network Analyst databases. These predicted miRNAs were then compared to the up-regulated DEMs derived from serum (GSE120584),

parietal lobe (GSE16759), and temporal lobe (GSE157239) samples. The Venn diagram in Figure 16 illustrates the overlap between the up-regulated data on the left and the down-regulated data on the right. Nevertheless, no overlap was detected among the miRNAs present in serum, brain, and the databases. Instead, we identified a common miRNAs between serum miRNAs and those in the databases. This guided our focus towards studying this overlap, as our objective was to unravel the brain's molecular alterations in Alzheimer's Disease and pinpoint corresponding blood-based biomarkers for diagnosis. Among the findings, we identified 67 miRNAs as up-regulated common DEMs (cDEMs) that are shared between the predicted targets of down-regulated cDEGs obtained from miRNet, Network Analyst tools, and the DEMs obtained from serum (GSE120584). Moreover, we selected 21 miRNAs as down-regulated cDEMs shared between the predicted targets of up-regulated cDEGs obtained from miRNet, Network Analyst tools, and the down-regulated DEMs from serum (GSE120584). When constructing mRNA-miRNA networks, the limited number of miRNAs obtained from the temporal and parietal lobes could explain the absence of overlapping miRNAs between the brain, serum, and predicted miRNAs.

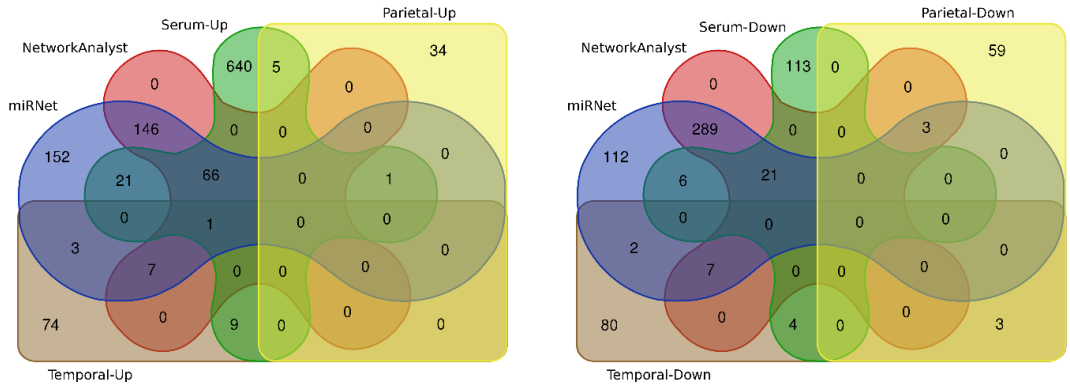


Figure 16. Venn diagram shows overlapped common miRNAs between predicted miRNAs of DEGs and DEMs from serum and brain regions of Alzheimer's Disease patients.

Through computational prediction, we identified a set of DEMs, consisting of 67 up-regulated and 21 down-regulated miRNAs, based on the analysis of cDEGs in the brain samples. These cDEMs provided initial insights into potential dysregulated miRNAs relevant to the condition of interest. Furthermore, we ranked the FC of cDEMs based

on the serum dataset (GSE120584), which provided microarray data as seen in Figure 17. Green bars indicate up-regulated DEMs, red bars indicate down-regulated DEMs.

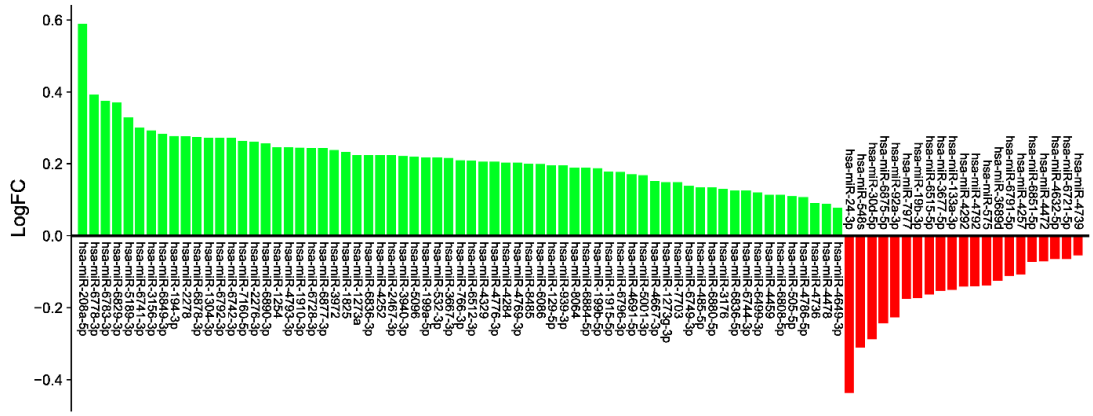


Figure 17. Overlapped miRNAs between predicted miRNAs of DEGs and DEMs from serum of patients with Alzheimer's Disease are presented according to their logFC value.

To assess the binding probabilities between the identified cDEMs and their target cDEGs, we utilized the StarMir (156) which is based on Clip based prediction method (155). The results are listed according to logicpro value (156), delta g (157). Alluvial plot was plotted by https://www.bioinformatics.com.cn/en, which is an online web-based tool for data visualization seen in Figure 18 and Figure 19. By calculating these probabilities, we constructed an mRNA-miRNA network, revealing strong interactions between the cDEGs and cDEMs as seen in Figure 18.

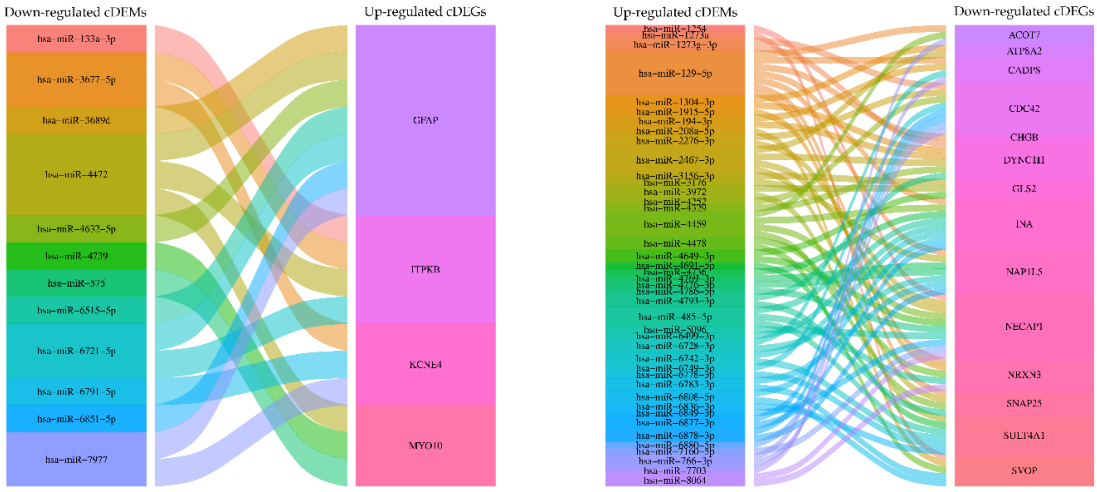


Figure 18. Alluvial plot visualization of STarMir interaction results of down-regulated cDEMs-up-regulated cDEGs on the left and up-regulated cDEMs-down-regulated cDEGs on the right.

From this network, we selected the highest interacting pairs for both up-regulated and down-regulated miRNAs as seen in Figure 19. For the up-regulated cDEGs, we identified GFAP as a potential target, which interacted with miR-7977. Conversely, for the down-regulated cDEGs, SNAP-25 emerged as a candidate, showing interaction with miR-129-5p. While the microarray analysis provided valuable insights into miRNA expression, we acknowledge the need for additional validation to strengthen the reliability of our findings. Therefore, we plan to conduct qRT-PCR experiments. qRT-PCR offers a more targeted and accurate quantification of specific miRNAs, allowing us to confirm and validate the dysregulation observed in the microarray data.

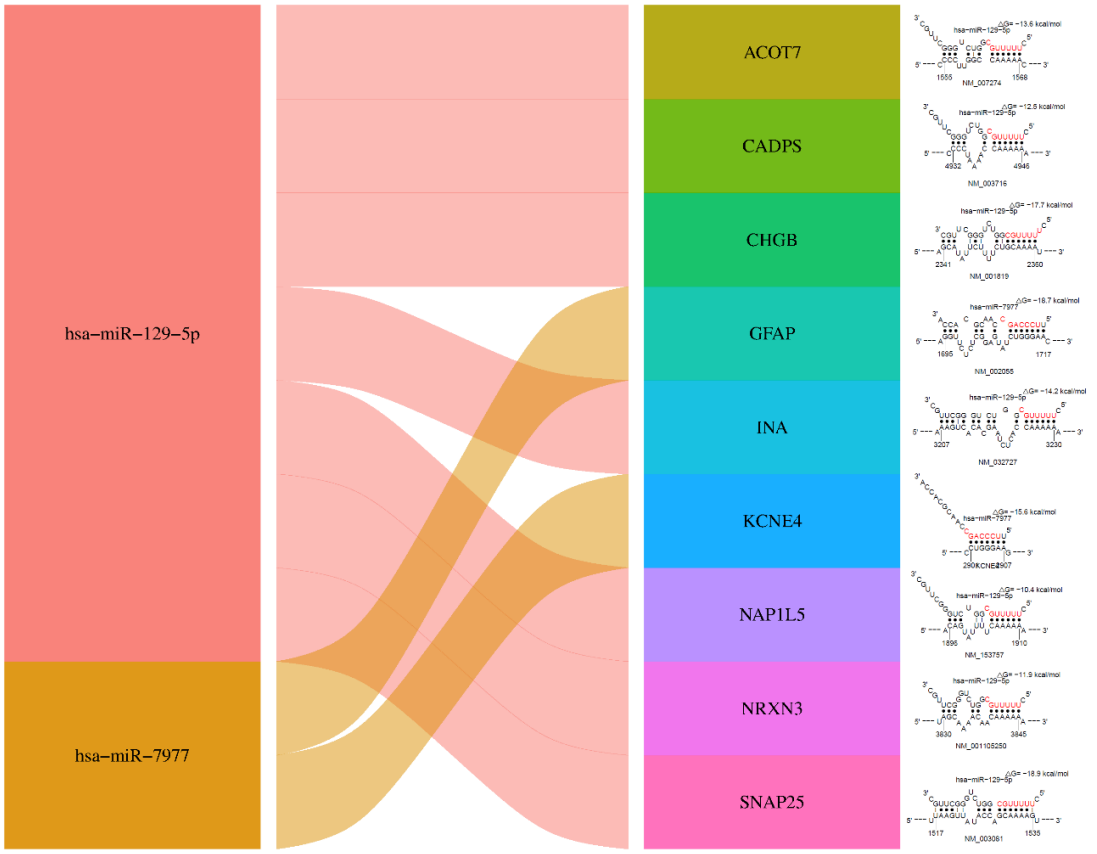


Figure 19. STarMir results of miR-129-5p-down-regulated cDEGs and miR-7977-up-regulated cDEGs and their binding illustations on the right with ΔG of each interaction.

To summarize, we identified potential miRNA biomarkers for Alzheimer's disease by overlapping cDEMs and miRNA sets from brain and serum samples. The construction of an mRNA-miRNA network highlighted promising interactions, including GFAP with miR-7977 and SNAP-25 with miR-129-5p. While computational predictions provide insights into potential miRNA-gene interactions, experimental validation is crucial to confirm these relationships. Techniques such as luciferase reporter assays, qRT-PCR, and western blotting can be used to validate the regulatory effect of miRNAs on target gene expression.

3.6 qRT-PCR Results and Statistical Tests

This thesis demonstrated a comprehensive approach to investigating miRNA expression profiles, combining computational prediction with biological validation. The results obtained from the intersection of cDEMs with the serum dataset provided

initial insights into potential miRNA biomarkers, while the inclusion of qRT-PCR validation will further strengthen the robustness of our findings and enhance their potential for clinical relevance. In this section, we aimed to test the expression levels of selected miRNAs (miR-7977 and miR-129-5p) as well as mRNAs (GFAP and SNAP-25) in plasma samples advanced Alzheimer's Disease patients and healthy controls using qRT-PCR. To analyse the expression levels of the target miRNAs (miR-7977 and miR-129-5p) as well as GFAP and SNAP-25 relative to suitable reference RNA and gene, the $2^{-\Delta\Delta Ct}$ method (158) was employed. U6 was used as the endogenous control for miRNAs, while β-actin served as the endogenous control for mRNAs. Statistical analysis was conducted using the student's t-test, and p-values were calculated using GraphPad PRISM software (Version 9.5.1 (Trial), GraphPad Software, San Diego, CA). Significance levels were designated as *p<0.0332, **p<0.0021 ***p<0.0002 and ****p<0.0001). We examined the expression levels of cDEGs (GFAP and SNAP-25) and their target cDEMs (miR-7977 and miR-129-5p) in plasma samples of advanced Alzheimer's Disease patients compared to healthy controls. Based on the results obtained, significant differences in the expression levels of miR-7977, miR-129-5p, GFAP, and SNAP-25 were observed in in Alzheimer's Disease patients compared to healthy controls. Figure 20 shows that miR-7977 was down-regulated whereas Figure 21 represents that GFAP is up-regulated in Alzheimer's Disease patients compared to healthy controls. These findings suggest a potential dysregulated network where down-regulated miR-7977 is associated with the up-regulation of GFAP in Alzheimer's Disease. Moreover, Figure 22 indicates that SNAP-25 was down-regulated whereas Figure 23 points that miR-129-5p was up-regulated in Alzheimer's Disease patients compared to healthy controls. These results suggest a potential dysregulated network where down-regulated SNAP-25 is associated with the up-regulation of miR-129-5p in Alzheimer's Disease. However, it is important to note that these findings are preliminary, and further tests and validation are needed to confirm the dysregulated networks involving these miRNAs (miR-7977 and miR-129-5p) and their respective target genes (GFAP and SNAP-25) in Alzheimer's Disease. To validate and further characterize the dysregulated networks associated with Alzheimer's Disease, it is essential to conduct additional experiments such as luciferase assays, western blotting, and is necessary to increase the sample

size, and investigating other sample types such as CSF or serum. These additional experiments will provide more comprehensive insights into the molecular mechanisms underlying Alzheimer's Disease and the potential biomarkers involved.

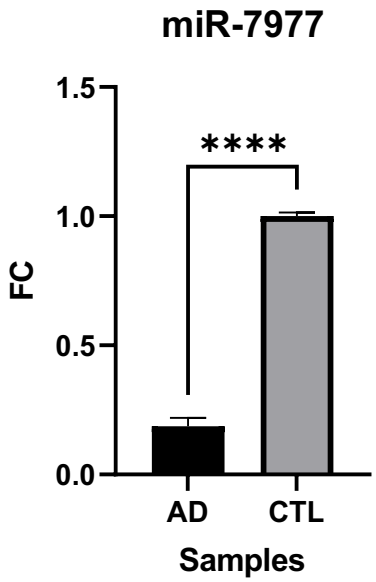


Figure 20. Fold change levels of miR-7977 in advanced Alzheimer's Disease and healthy control groups. (**p<0.0001).**

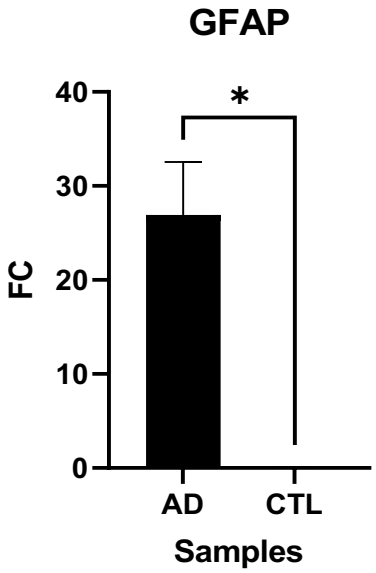


Figure 21. Fold change levels of GFAP in advanced Alzheimer's Disease and healthy control groups. (*p<0.0332).

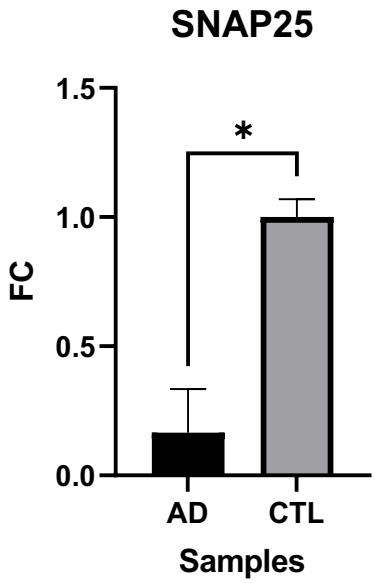


Figure 22. Fold change levels of SNAP-25 in advanced Alzheimer's Disease and healthy control groups. (*p<0.0332).

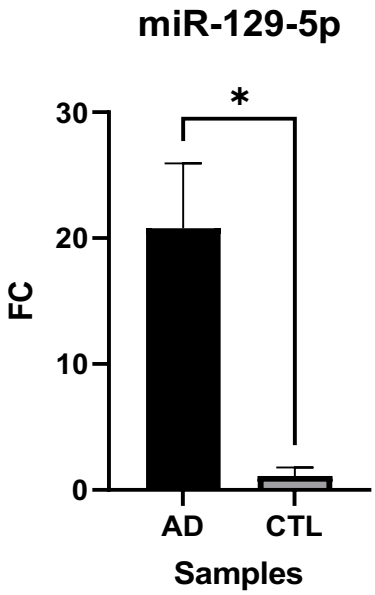


Figure 23. Fold change levels of miR-129-5p in advanced Alzheimer's Disease and healthy control groups. Significance level is designated as *p<0.0332.

4. DISCUSSION

Alzheimer's Disease, a progressive neurodegenerative condition, impacts substantial number of individuals globally. As per the World Health Organization (WHO), around 50 million individuals are affected by dementia worldwide, with Alzheimer's Disease being the primary contributor, particularly evident in individuals over 65 years. The data might be an underrepresentation due to potential underdiagnosis and underreporting, particularly in less affluent countries, where the reported prevalence is comparatively lower(5). The disease is characterized by the presence of abnormal protein deposits in the brain that cause nerve cells to degenerate and die, leading to cognitive decline and memory loss (164). At present, there is no cure for Alzheimer's Disease; however, extensive research is ongoing to understand the molecular mechanisms behind its development to search for new potential treatments. Biomarkers are essential for disease diagnosis, progression, and development of treatments for many diseases and are very rapid in the evaluation of disease progression, including Alzheimer's Disease (73). Extensive research has been conducted with the goal of locating Alzheimer's Disease biomarkers due to the fact that early detection is essential for preventing the illness from progressing further. In particular, bioinformatics-based research have been carried out to undertake an analysis of big data sets containing genetic and molecular information with the goal of locating possible therapeutic targets for the treatment of many diseases including Alzheimer's Disease (102, 116). Studying miRNAs as biomarkers were proved to be essential for Alzheimer's Disease research. This thesis represents a detailed investigation of the molecular foundations of Alzheimer's Disease, by employing bioinformatics to identify new diagnostic biomarkers and targets for treatment. In this thesis, we implemented a comprehensive bioinformatics framework to explore the molecular intricacies of Alzheimer's Disease Alzheimer's Disease and develop potential diagnostic and therapeutic avenues.

Our methodology involved several key steps, starting with differential gene expression analyses in Alzheimer's Disease-affected brain regions, including the hippocampus, entorhinal cortex, temporal cortex, and frontal lobe. In the discussion section of our thesis, the selection of specific brain regions for the analysis was based on the presence

of a higher number of common genes among different datasets. Firstly, data includes brain and blood samples of patients of Alzheimer's Disease and healthy controls are collected for differential gene expression analysis and differential miRNA analysis from NCBI GEO database (104). We applied a crucial criterion by excluding samples from individuals younger than 70 years from the analysis. This selection was made to enhance the homogeneity of the study population, as older individuals are more likely to exhibit symptoms and characteristics associated with Alzheimer's Disease. By focusing on individuals above the age of 70, we aimed to ensure a more consistent and representative study cohort, reducing potential age-related variations in gene expression and miRNA profiles. We identified the overlapped differentially expressed genes (DEGs) within the same brain region, including entorhinal cortex, hippocampus, frontal cortex, and temporal cortex, from various datasets. These shared molecular signatures across the brain regions provided valuable insights into the common pathological mechanisms underlying Alzheimer's Disease. To enhance the robustness and reliability of our findings, specific combinations of brain regions were selected based on the number of intersected common DEGs (cDEGs). We focused on the intersection between frontal cortex and temporal cortex for up-regulated cDEGs, and the intersection of hippocampus, frontal cortex, and temporal cortex for down-regulated cDEGs, as they exhibited the highest overlap of DEGs. Notably, the entorhinal cortex was excluded from the combined analysis due to a lower number of DEGs. By strategically focusing on brain regions with a substantial overlap of DEGs, we identified a subset of genes consistently dysregulated across multiple regions in Alzheimer's Disease, thereby strengthening the significance and relevance of our findings. According to this, GFAP, LOC100131541, ITPKB, AEBP1, KCNE4, and NACC2 were detected as common up-regulated DEGs; whereas NRXN3, SNAP-25, CDC42, ACOT7, CHGB, SULT4A1, CADPS, SVOP, NAP1L5, NECAP1, INA, DYNC1I1, GLS2, ATP8A2 were identified as common down-regulated DEGs in brain samples (Table 11). Studying the differential expression of genes in different brain regions affected by Alzheimer's Disease offers several advantages in understanding the disease. It provides insights into the regional specificity of the disease, identifies molecular mechanisms, potential biomarkers, and therapeutic targets, facilitating personalized medicine approaches for more effective interventions.

Then, we conducted functional enrichment analyses to explore the implications of cDEGs in Alzheimer's Disease. Using DAVID (132) and EnrichR (134) tools, we identified enriched BP, CC, and MF (illustrated in Table 12), tissues, KEGG pathways and Reactome (showed in Figure 10) associated with cDEGs. Results illustrate that the functional enrichment analysis of cDEGs in Alzheimer's Disease reveals important insights into the underlying biological processes, cellular components, and molecular functions implicated in the disease. The results revealed disruptions in neurotransmitter regulation, organelle localization, cytoskeleton organization, vesicle transport, synaptic signalling, and neuronal development in Alzheimer's Disease pathology. Enriched components included synaptic structures, neuronal compartments, cytoplasmic vesicles, and organelles, emphasizing their relevance in the disease. MF analysis highlighted protein binding, calcium regulation, motor activity, and ion channel function as significant in Alzheimer's Disease. The analysis of cDEG enrichment in specific tissues and pathways provided further insights, implicating multiple brain regions and pathways related to phagocytosis, neurotransmitter metabolism, immune responses, cytoskeletal dynamics, and mitochondrial function in Alzheimer's Disease. The results of tissue and pathway enrichment analyses of cDEGs highlighted the involvement of specific brain regions such as the caudate nucleus, medulla oblongata, cingulate cortex, thalamus, and others in Alzheimer's Disease pathology (Figure 10-b). KEGG pathway analysis revealed cDEGs enrichment in various pathways, including Fc gamma R-mediated phagocytosis, D-Glutamine and D-glutamate metabolism, and pathogenic Escherichia coli infection (Figure 10-b). Reactome analysis further identified several enriched pathways, such as glutamate neurotransmitter release cycle, neuronal system, autophagy, and RHO GTPases activation (Figure 10-c). Overall, our enrichment analyses provide insightful information into the diverse molecular landscape of Alzheimer's disease that will help direct ongoing research and future therapy options.

Additionally, we constructed a PPI network to gain insights into the potential interactions and functional relationships among cDEGs in Alzheimer's Disease to observe the potential interactions and functional relationships among these proteins. In the PPI network constructed using the STRING database (137), several disconnected nodes are observed, including ACOT7, ATP8A2, KCNE4, NACC2,

NECAP1, GLS2, NRXN3, AEBP1, ITPKB, and NAP1LK5 (Figure 11). These disconnected nodes suggest that these proteins may have limited direct interactions with the other proteins in the network, potentially indicating their unique roles or involvement in distinct pathways related to Alzheimer's Disease. On the other hand, a cluster of interconnected proteins is also observed in the PPI network through Cytoscape (135) and Cytohubba (138) (Figure 12). This cluster includes hub proteins such as SNAP-25, INA, CDC42, GFAP, CADPS, CHGB, DYNC1I1, NRXN3, MYO10, and SULT4A1. Hub proteins are highly connected and play essential roles in mediating protein interactions and cellular processes (165). The identification of these hub proteins highlights their potential importance in Alzheimer's Disease pathogenesis. Among the top-ranked hub proteins, SNAP-25, a protein involved in synaptic vesicle exocytosis (166) and neurotransmitter release (167), stands out as a critical player in synaptic function. In the literature, there are findings suggesting that abnormalities in SNAP-25 may contribute to the pathogenesis of Alzheimer's Disease (168) and in one study, it is shown that presynaptic vesicle proteins are reduced in Alzheimer's Disease (169). In another studies, SNAP-25 is also proposed as a promising novel biomarker for synapse degeneration in Alzheimer's Disease (170, 171). GFAP is also among the highly connected hub protein, which is up-regulated cDEGs. Differential regulation of GFAP, an intermediate filament protein predominantly found in astrocytes (172), suggests gliosis and reactive astrogliosis, which are commonly observed in Alzheimer's Disease (173). As demonstrated in a particular study, plasma GFAP emerges as an early indicator for amyloid-β pathology in Alzheimer's Disease, yet doesn't exhibit a correlation with tau pathology (174). In another study, serum GFAP not only discriminates between Alzheimer's Disease and frontotemporal dementia but also forecasts the transition from mild cognitive impairment to dementia (175). A recently conducted study found that elevated blood GFAP levels were associated with Aβ-positive individuals, those with Alzheimer's disease or mild cognitive impairment, suggesting that measuring blood GFAP could enhance Alzheimer's Disease diagnosis and prognosis (176). The findings of differential expression in genes SNAP25 and GFAP within the context of Alzheimer's Disease holds substantial promise for advancing our understanding of this complex neurodegenerative condition. With existing literature providing validation for these

findings, these genes emerge as crucial players in the disease's molecular landscape. However, the current lack of knowledge regarding associated drugs and regulatory mechanisms underscores the intricate nature of Alzheimer's Disease. By shedding light on these specific genes, our work not only reinforces established knowledge but also paves the way for deeper understanding of Alzheimer's disease research and treatment.

To identify potential therapeutic candidates, subsequently, we implemented a drug repositioning strategy, utilizing molecular docking to assess drug-protein binding probabilities for Alzheimer's Disease treatment**.** This approach offer several advantages, including cost-effectiveness, faster turnaround times, and the ability to screen a large number of compounds in a relatively short period. To identify potential drug-protein interactions of target proteins that are GFAP and SNAP-25, we utilized the DGIdb (140) and the L1000CDS2 databases (141). In this study, we obtained a set of drugs (Sorbitol, Permethrin, Oprelvekin, Dronabinol, Amphetamine) associated with GFAP, and another set of drugs (Onabotulinumtoxina, Incobotulinumtoxina, Abobotulinumtoxina, Diazoxide, Vorinostat, Trichostatin A, Scriptaid, Lestaurtinib, Alvocidib, Pracinostat, Nintedanib, Mocetinostat) associated with SNAP-25. Our focus is to perform molecular docking of these drugs to their respective protein targets (GFAP and SNAP-25). By conducting molecular docking analyses, we aim to understand the potential interactions and binding affinities of these drugs with GFAP and SNAP-25, which could provide valuable insights into their therapeutic potentials for Alzheimer's Disease or related conditions. It is important to utilize appropriate molecular docking tools and validate the results through complementary experimental studies to strengthen the findings of this research. For this, we obtained the three-dimensional structure of the drug molecules from DrugBank (144), and GFAP from the PDB (142). We exclude drugs that are Onabotulinumtoxina, Incobotulinumtoxina, Abobotulinumtoxina for molecular docking due to the substantial size difference between the drugs and the target proteins. Because molecular docking analysis using AutoDock Vina was deemed impractical and excluded from the experiments to avoid potential inaccuracies and lack of meaningful results. We specifically selected a suitable GFAP structure from the PDB to serve as the basis for our molecular docking simulations. Regarding SNAP-25, we encountered challenges in finding an isolated structure of SNAP-25 without other associated proteins or ligands in the available

databases. To overcome this limitation, we obtained the predicted structure of SNAP-25 generated by the AlphaFold protein structure prediction method (143), is a well-developed and trustworthy system that predicts protein structures with impressive precision using deep learning techniques. By employing the predicted structure of SNAP-25 from AlphaFold (143), we were able to proceed with our molecular docking simulations and investigate its potential interactions with candidate drug molecules. For GFAP, we selected three distinct binding sites. These binding sites were chosen for further investigation based on their potential interactions with the selected drug candidates. Similarly, for SNAP-25, two binding sites were selected. These binding sites were visually represented in Figure 13, with different colors assigned to each site to facilitate clear visualization and analysis. To evaluate the binding affinities of specific drugs to GFAP and SNAP-25, we employed AutoDock Vina (163), a widely used software tool for molecular docking simulations. The results demonstrated varying binding affinities of different drugs to the selected binding sites of GFAP is shown in Figure 14 and for SNAP-25 is shown in Figure 15. For GFAP, drugs such as amphetamine, sorbitol, dronabinol, and permethrin exhibited distinct binding affinities across the chosen binding sites. Similarly, for SNAP-25, drugs such as scriptaid, vorinostat, alvocidib, pracinostat, mocetinostat, nintedanib, lestaurtinib, diazoxide, and trichostatin A displayed diverse binding affinities for the selected binding sites. To aid in visualizing the docking results, we utilized PyMol to visualize the interaction of GFAP in Figure 14, of SNAP-25 in Figure 15. These figures depicted the predicted binding sites of each gene and highlighted the lowest binding affinities of specific drugs within those binding sites. The outcomes of our molecular docking simulations provide valuable insights into the potential drug candidates and their interactions with the target proteins GFAP and SNAP-25. Molecular docking studies indicated that dronabinol and permethrin exhibited a relatively low ΔG to GFAP compared to other tested drugs. This statement suggests that dronabinol and permethrin have a higher binding interaction with GFAP in the molecular docking study. Dronabinol is a medication that is classified as a cannabinoid receptor agonist. It is an FDA approved synthetic form of delta-9-tetrahydrocannabinol (THC), which is the main psychoactive compound found in the Cannabis sativa plant (144). Dronabinol has shown potential in the treatment of Alzheimer's Disease in different studies (177, 178, 179, 180).

Permethrin works by affecting the nervous system of insects and parasites, leading to paralysis and eventual death. It acts as a neurotoxin by binding to sodium channels, which are essential for the normal functioning of the insects' nerve cells (144). Its potential to Alzheimer's Disease is suggested in one study (181). On the other hand, scriptaid, alvocidib, and lestaurtinib exhibited lower ΔG values, indicating a stronger binding affinity to SNAP-25 compared to the other tested drugs. These findings suggest that scriptaid, alvocidib, and lestaurtinib have the potential to form robust interactions with SNAP-25, making them promising candidates for further investigation as potential therapeutics for Alzheimer's Disease. Scriptaid is a small molecule compound that is known as a histone deacetylase (HDAC) inhibitor. HDAC inhibitors are substances that can modify the activity of enzymes called histone deacetylases, which are involved in regulating gene expression and chromatin structure (144). One of the study shows the interaction of scriptaid with tau acetylation in Alzheimer's Disease (182). Alvocidib, also known as flavopiridol, is a small molecule compound that has been studied as a potential anticancer agent. It belongs to the class of cyclin-dependent kinase (CDK) inhibitors. CDKs are enzymes involved in cell cycle regulation and are often dysregulated in cancer cells, leading to uncontrolled cell growth and division (144). Alvocidib is used in Alzheimer's mouse model to inhibit CDKs (183). Lestaurtinib, also known as CEP-701, is a small molecule inhibitor that targets multiple tyrosine kinases, including the FMS-like tyrosine kinase 3 (FLT3) and Janus kinase 2 (JAK2). It has been primarily studied for its potential therapeutic application in hematological malignancies, particularly acute myeloid leukemia (AML) and myeloproliferative neoplasms (MPNs) (144). Lestaurtinib is proposed in one research paper as protein kinase inhibitors in Alzheimer's Disease through drug repositioning (184). As seen from the results, some of the drugs are identified as cancer-related drugs. Although the side effects and expenses associated with these cancer drugs present obstacles, they also offer valuable lessons and opportunities. Through meticulous dose adjustments, personalized applications, and rigorous clinical trials, it might be possible to harness the benefits of these drugs while mitigating their drawbacks (185, 186). To establish the effectiveness and safety of these prospective medications, more research and experimental validation are necessary. While these drugs have shown potential in preclinical or early-phase

studies, further research is important to comprehensively evaluate their efficacy and safety in the context of Alzheimer's Disease. Nevertheless, the results obtained from our *in silico* approach serve as a valuable foundation for guiding future experimental investigations and potentially accelerating the drug discovery process for Alzheimer's Disease and other related conditions.

Next, we aim to construct such a network by integrating predicted miRNA targets of cDEGs with DEMs obtained from serum samples and brain regions of Alzheimer's Disease patients. These miRNAs hold promise as non-invasive biomarkers for early Alzheimer's Disease detection and monitoring. To elucidate miRNA-mediated regulatory interactions, we predicted target binding sites on differentially expressed genes using computational tools. To begin with, the predicted miRNA targets of up-regulated cDEGs were also obtained from miRNet and Network Analyst, and their overlap with the down-regulated DEMs from serum samples, parietal lobe, and temporal lobe datasets was examined. The Venn diagram analysis identified the miRNAs that were consistently down-regulated across the datasets (Figure 16). However, we did not observe an intersection between the miRNAs found in serum, brain, and the databases. Instead, we found an intersection between the miRNAs in serum and those in the databases. We decided to continue focusing on this intersection because we aimed to understand the molecular changes occurring in the brain during Alzheimer's Disease and identify corresponding biomarkers in the blood for diagnostic purposes. Based on these analyses, a total of 67 up-regulated cDEMs and 21 down-regulated cDEMs between databases and serum samples were selected as potential candidates for the mRNA-miRNA network. The fold change values of these selected miRNAs were further ranked according to their differential expression levels in the GSE120584 dataset (Figure 17). In conclusion, by constructing mRNA-miRNA network, we aimed to construct an mRNA-miRNA network, which would facilitate the identification of potential biomarkers in blood and offer insights into the molecular alterations occurring in the brain related to Alzheimer's Disease.

Furthermore, the computational prediction was done by STarMir tool (156) to predict the binding affinity of miRNAs to 3' UTR region of target mRNAs of cDEGs. The results are filtered according to determined cutoffs. The miRNA-mRNA interaction that has lowest ΔG, and highest logicpro is selected in Figure 18. Among the numerous

interactions identified, particular focus was given to hsa-miR-129-5p and hsa-miR-7977 (Figure 19), according to their highest number of targets and lowest ΔG values. By investigating the predicted interactions of hsa-miR-129-5p with SNAP-25 and hsa-miR-7977 with GFAP, it is possible to gain insights into their potential roles in Alzheimer's Disease. In conclusion, the use of STarMir for predicting mRNA-miRNA interactions and the subsequent selection of hsa-miR-129-5p and hsa-miR-7977 as target miRNAs provide a foundation for further investigation into their roles in Alzheimer's Disease. The results obtained from STarMir and the subsequent qRT-PCR analysis can help identify specific miRNA-mRNA interactions that may play crucial roles in disease pathogenesis as pre-liminary data.

Finally, we validated miRNA expression through pre-liminary data from qRT-PCR experiments, confirming their potential as blood-based biomarkers in Alzheimer's Disease. Based on network analysis, we hypothesized that the down-regulation of miR-7977 could lead to the up-regulation of GFAP, while the up-regulation of miR-129-5p might result in the down-regulation of SNAP-25. Understanding these interconnected networks is essential in utilizing these miRNAs as biomarkers for the early detection of changes in gene expression levels of their target genes in Alzheimer's disease. This knowledge holds significant promise in developing non-invasive diagnostic tools for timely detection and monitoring of Alzheimer's disease, ultimately leading to improved clinical outcomes and treatment strategies. To check our hypothesis with preliminary experiments, we conducted qRT-PCR analysis to examine the expression levels of these target molecules in plasma samples of Alzheimer's Disease patients and healthy controls. First, we observed a significant down-regulation of miR-7977 in Alzheimer's Disease patients compared to healthy controls (Figure 20). In contrast, we found a significant up-regulation of GFAP (Figure 21), a marker for astrocyte activation (159, 173), in Alzheimer's Disease patients compared to healthy controls. These results suggest that the dysregulation of miR-7977 may contribute to the up-regulation of GFAP, highlighting the importance of miR-7977 in the pathogenesis of Alzheimer's Disease and its potential role as blood-based biomarkers in Alzheimer's Disease. Additionally, miR-129-5p showed a significant up-regulation in the plasma samples of Alzheimer's Disease patients (Figure 23). Conversely, SNAP-25, a gene involved in synaptic vesicle exocytosis (166) and

neurotransmitter release (167), exhibited a significant down-regulation in Alzheimer's Disease patients (Figure 22). These findings highlight the significance of miR-129-5p in the pathophysiology of Alzheimer's Disease and its potential use as a blood-based biomarker in Alzheimer's Disease and indicates that dysregulation of miR-129-5p may contribute to the down-regulation of SNAP-25. The observed up-regulation of miR-129-5p in the serum of individuals with Alzheimer's Disease, despite previous literature reporting its down-regulation in the brain, suggests a potential discrepancy between miRNA expression profiles in different tissues (187). This inconsistency may be attributed to the complex interplay between various factors, such as tissue-specific regulation, cellular heterogeneity, and the dynamic nature of miRNA expression in different stages of the disease. Further investigations are needed to elucidate the underlying mechanisms contributing to the differential expression of miR-129-5p between the brain and plasma samples in Alzheimer's Disease. To sum up, further experiment based on luciferase assay will be necessary to confirm the interaction between the miR-7977 and GFAP-25 3′ UTR region and the miR-129-5p and SNAP-25 3′ UTR region and effects of such interaction on GFAP and SNAP-25 expression, respectively.

By integrating these methodologies, our study aimed to unravel the complex molecular landscape of Alzheimer's Disease, providing insights for future diagnostic and therapeutic advancements. In summary, our results reveal miRNA-mRNA networks through integrated bioinformatics and demonstrate distinct expression pattern of miR-7977, miR-129-5p, GFAP, and SNAP-25 in Alzheimer's Disease, providing valuable insights into their potential roles in the disease. The findings from this study may have important implications for the development of diagnostic markers and therapeutic targets, as well as providing valuable insights into the molecular pathways underlying the disease.

Studying advanced Alzheimer's disease through qRT-PCR for testing biomarkers is important, despite the importance of assessing these biomarkers in early stages (188). Investigating advanced stages provides crucial insights into the established disease pathology, offering a comprehensive understanding of the molecular changes that have occurred over time. This knowledge can help validate the relevance of biomarkers found in earlier stages and establish their consistency across disease progression.

Furthermore, studying advanced Alzheimer's aids in identifying biomarkers that might exhibit enhanced specificity for advanced stages, aiding in accurate disease classification and prognosis. However, future studies needs to include early and mild stages of Alzheimer’s Disease, that improves the ability to identify markers with high sensitivity for detecting early signs of Alzheimer's, paving the way for more effective treatment strategies.

While this study on bioinformatics analysis and biomarker discovery in Alzheimer’s Disease provides valuable insights, there are certain limitations and potential future directions that should be considered. Firstly, the analysis is based on differential gene expression in different brain regions, which might not fully capture the complex and heterogeneous nature of Alzheimer’s Disease. It would be beneficial to incorporate additional brain regions or include more diverse samples to obtain a comprehensive understanding of the disease. The statistical power and reproducibility of the results can be improved by include a larger cohort of patients, allowing for stronger conclusions regarding the found differentially expressed genes, miRNAs, and possible biomarkers linked with Alzheimer's Disease. Furthermore, while online databases provide a valuable resource for identifying target miRNAs, experimental validation of these miRNA-target interactions is essential to confirm their functional relevance. Future studies should include functional experiments such as luciferase assays or RNA interference to validate the predicted miRNA-target interactions. Additionally, the use of plasma samples for miRNA analysis provides a non-invasive approach, but it is important to acknowledge that miRNA expression in plasma may not always reflect the changes occurring specifically in the brain, other biofluids need to be investigated (189). Therefore, further investigations incorporating miRNA expression analysis in brain tissue samples would enhance the understanding of their role in Alzheimer’s Disease. Moreover, the proposed drug repositioning strategy is based on *in silico* molecular docking, and further in vitro and *in vivo* experiments are necessary to confirm the efficacy and safety of the identified drugs in Alzheimer’s Disease models. In conclusion, while this study provides promising findings, it highlights the need for additional experimental validations and expanded approaches to address the limitations and improve the understanding and treatment of Alzheimer’s Disease.

5. CONCLUSION

In conclusion, our comprehensive bioinformatics study delved into the molecular complexities of Alzheimer's Disease. Analysing gene expression data from affected brain regions, we pinpointed GFAP and SNAP-25 as key hub genes associated with the disease. Employing a drug repositioning strategy and molecular docking, we identified potential therapeutic candidates such as dronabinol and permethrin for GFAP, and lestaurdinib, scriptaid, and alvocidib for SNAP-25.

Furthermore, our focus extended to identifying differentially expressed miRNAs in blood samples that correlated with the target genes in the brain. Constructing an mRNA-miRNA network revealed specific miRNAs, including down-regulated miR-7977 and up-regulated miR-129-5p, with associations to GFAP and SNAP-25, respectively. Validating these findings through quantitative PCR in plasma samples indicated their potential as blood-based biomarkers for Alzheimer's Disease, but further molecular validations are needed to strengthen these results.

Overall, our study advances the understanding of Alzheimer's Disease by providing potential biomarkers and drug candidates for further exploration. While shedding light on the complex molecular mechanisms i1nvolved in the disease, we emphasize the necessity for additional experimental validations to confirm these discoveries and pave the way for personalized diagnosis and treatment strategies. This comprehensive bioinformatics framework holds significant promise for future advancements in Alzheimer's Disease research and clinical practice.

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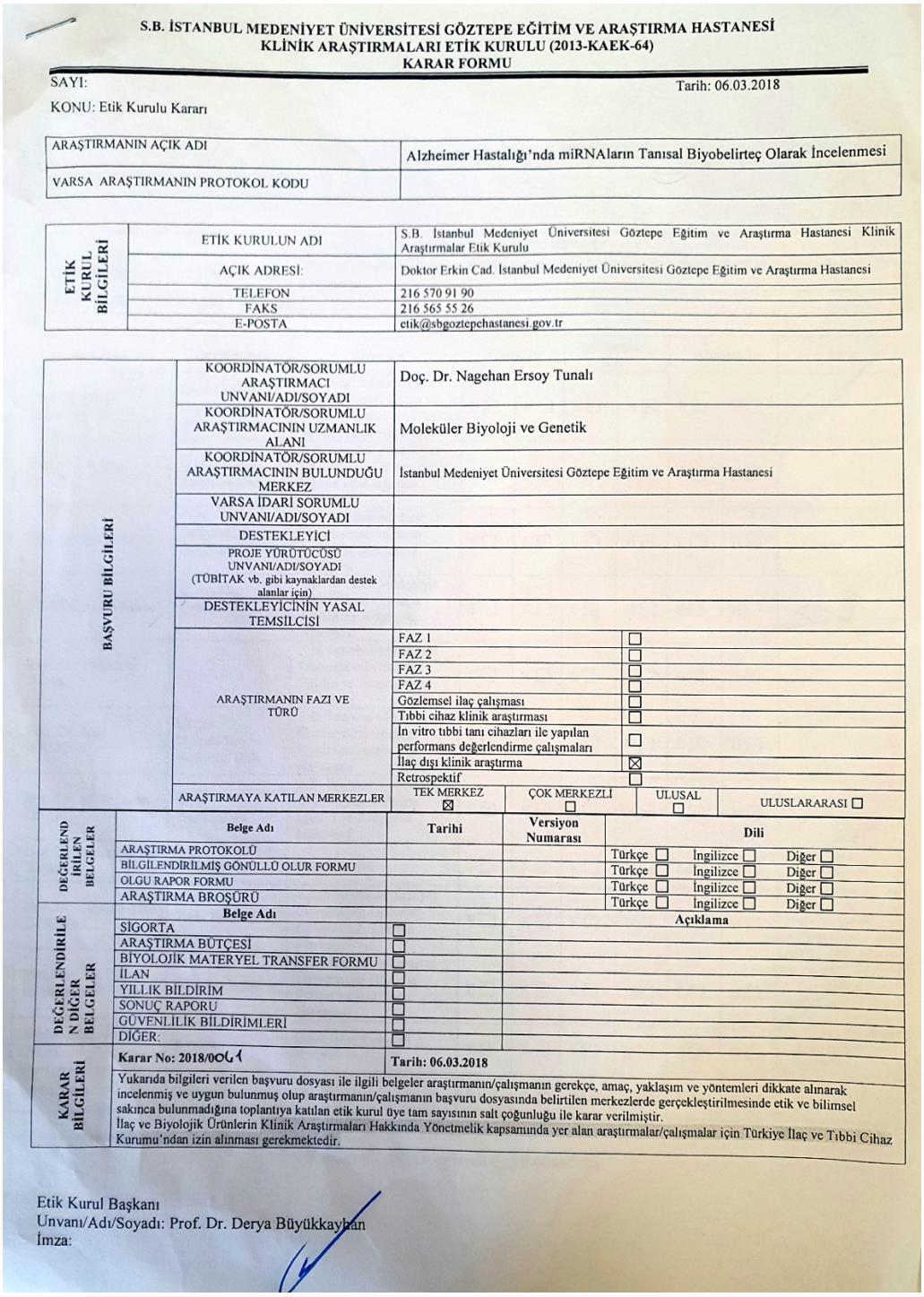
7. ETHICAL REPORT



S.B. İSTANBUL MEDENİYET ÜNİVERSİTESİ GÖZTEPE EĞİTİM VE ARAŞTIRMA HASTANESİ
KLİNİK ARAŞTIRMALARI ETİK KURULU (2013-KAEK-64)
KARAR FORMU

SAYI: Tarih: 06.03.2018

KONU: Etik Kurulu Kararı

ARAŞTIRMANIN AÇIK ADI	Alzheimer Hastalığı'nda miRNAların Tanısal Biyobelirteç Olarak İncelenmesi
VARSA ARAŞTIRMANIN PROTOKOL KODU	

ETİK KURUL BİLGİLERİ		
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	KOORDİNATÖR/SORUMLU ARAŞTIRMACI UNVANI/ADI/SOYADI	Doç. Dr. Nagehan Ersoy Tunalı			
	KOORDİNATÖR/SORUMLU ARAŞTIRMACININ UZMANLIK ALANI	Moleküler Biyoloji ve Genetik			
	KOORDİNATÖR/SORUMLU ARAŞTIRMACININ BULUNDUĞU MERKEZ	İstanbul Medeniyet Üniversitesi Göztepe Eğitim ve Araştırma Hastanesi			
	VARSA İDARİ SORUMLU UNVANI/ADI/SOYADI				
	DESTEKLEYİCİ				
	PROJE YÜRÜTÜCÜSÜ UNVANI/ADI/SOYADI (TÜBİTAK vb. gibi kaynaklardan destek alanlar için)				
	DESTEKLEYİCİNİN YASAL TEMSİLCİSİ				
	ARAŞTIRMANIN FAZI VE TÜRÜ	FAZ 1 ☐	FAZ 2 ☐	FAZ 3 ☐	FAZ 4 ☐
		Gözlemsel ilaç çalışması ☐	Tıbbi cihaz klinik araştırması ☐	İn vitro tıbbi tanı cihazları ile yapılan performans değerlendirme çalışmaları ☐	
		İlaç dışı klinik araştırma ☒	Retrospektif ☐		
	ARAŞTIRMAYA KATILAN MERKEZLER	TEK MERKEZ ☒	ÇOK MERKEZLİ ☐	ULUSAL ☐	ULUSLARARASI ☐

DEĞERLENDİRİLEN BELGELER	Belge Adı	Tarihi	Versiyon Numarası	Dili
	ARAŞTIRMA PROTOKOLÜ			Türkçe ☐ İngilizce ☐ Diğer ☐
	BİLGİLENDİRİLMİŞ GÖNÜLLÜ OLUR FORMU			Türkçe ☐ İngilizce ☐ Diğer ☐
	OLGU RAPOR FORMU			Türkçe ☐ İngilizce ☐ Diğer ☐
	ARAŞTIRMA BROŞÜRÜ			Türkçe ☐ İngilizce ☐ Diğer ☐

DEĞERLENDİRİLEN DİĞER BELGELER	Belge Adı		Açıklama
	SİGORTA	☐	
	ARAŞTIRMA BÜTÇESİ	☐	
	BİYOLOJİK MATERYEL TRANSFER FORMU	☐	
	İLAN	☐	
	YILLIK BİLDİRİM	☐	
	SONUÇ RAPORU	☐	
	GÜVENLİLİK BİLDİRİMLERİ	☐	
	DİĞER:	☐	

KARAR BİLGİLERİ	
Karar No: 2018/0061	**Tarih: 06.03.2018**
Yukarıda bilgileri verilen başvuru dosyası ile ilgili belgeler araştırmanın/çalışmanın gerekçe, amaç, yaklaşım ve yöntemleri dikkate alınarak incelenmiş ve uygun bulunmuş olup araştırmanın/çalışmanın başvuru dosyasında belirtilen merkezlerde gerçekleştirilmesinde etik ve bilimsel sakınca bulunmadığına toplantıya katılan etik kurul üye tam sayısının salt çoğunluğu ile karar verilmiştir. İlaç ve Biyolojik Ürünlerin Klinik Araştırmaları Hakkında Yönetmelik kapsamında yer alan araştırmalar/çalışmalar için Türkiye İlaç ve Tıbbi Cihaz Kurumu'ndan izin alınması gerekmektedir.	

Etik Kurul Başkanı
Unvanı/Adı/Soyadı: Prof. Dr. Derya Büyükkaya
İmza:

S.B. İSTANBUL MEDENİYET ÜNİVERSİTESİ GÖZTEPE EĞİTİM VE ARAŞTIRMA HASTANESİ
KLİNİK ARAŞTIRMALARI ETİK KURULU (2013-KAEK-64)
KARAR FORMU

SAYI: Tarih: 06.03.2018

KONU: Etik Kurulu Kararı

ARAŞTIRMANIN AÇIK ADI	Alzheimer Hastalığı'nda miRNAların Tanısal Biyobelirteç Olarak İncelenmesi
VARSA ARAŞTIRMANIN PROTOKOL KODU	

KLİNİK ARAŞTIRMALAR ETİK KURULU	
ETİK KURULUN ÇALIŞMA ESASI	İlaç ve Biyolojik Ürünlerin Klinik Araştırmaları Hakkında Yönetmelik, İyi Klinik Uygulamaları Kılavuzu
BAŞKANIN UNVANI / ADI / SOYADI:	

Unvanı/Adı/Soyadı	Uzmanlık Alanı	Kurumu	Cinsiyet		Araştırma ile ilişki		Katılım *		İmza
Prof. Dr. Derya Büyükkayhan	Çocuk Sağlığı ve Hastalıkları Anabilim Dalı	T.C. Sağlık Bakanlığı Zeynep Kamil Kadın ve Çocuk Hastalıkları Eğitim ve Araştırma Hastanesi	E ☐	K ☒	E ☐	H ☒	E ☒	H ☐	
Prof. Dr. Aytekin OĞUZ	İç Hastalıkları Anabilim Dalı	S.B. İstanbul Medeniyet Üniversitesi Göztepe Eğitim ve Araştırma Hastanesi	E ☒	K ☐	E ☐	H ☒	E ☐	H ☒	
Prof. Dr. Işıl MARAL	Halk Sağlığı Anabilim Dalı	S.B. İstanbul Medeniyet Üniversitesi Göztepe Eğitim ve Araştırma Hastanesi	E ☐	K ☒	E ☐	H ☒	E ☐	H ☒	
Prof. Dr. Asıf Yıldırım	Üroloji	S.B. İstanbul Medeniyet Üniversitesi Göztepe Eğitim ve Araştırma Hastanesi	E ☒	K ☐	E ☐	H ☒	E ☒	H ☐	
Prof. Dr. Süleyman Daşdağ	Biyofizik	S.B. İstanbul Medeniyet Üniversitesi Göztepe Eğitim ve Araştırma Hastanesi	E ☒	K ☐	E ☐	H ☒	E ☐	H ☒	
Doç. Dr. Asiye KANBAY	Göğüs Hastalıkları Anabilim Dalı	S.B. İstanbul Medeniyet Üniversitesi Göztepe Eğitim ve Araştırma Hastanesi	E ☐	K ☒	E ☐	H ☒	E ☒	H ☐	
Doç. Dr. Şükrü Sadık ÖNER	Tıbbi Farmakoloji	S.B. İstanbul Medeniyet Üniversitesi Göztepe Eğitim ve Araştırma Hastanesi	E ☒	K ☐	E ☐	H ☒	E ☒	H ☐	
Doç. Dr. Sebahat Dilek Torun	Halk Sağlığı	Özel Kuruluş	E ☐	K ☒	E ☐	H ☒	E ☒	H ☐	
Doç. Dr. Sıdıka Şeyma ÖZKANLI	Tıbbi Patoloji	S.B. İstanbul Medeniyet Üniversitesi Göztepe Eğitim ve Araştırma Hastanesi	E ☐	K ☒	E ☐	H ☒	E ☐	H ☐	
Yrd. Doç. Dr. Hacer Hicran Mutlu	Aile Hekimliği	S.B. İstanbul Medeniyet Üniversitesi Göztepe Eğitim ve Araştırma Hastanesi	E ☐	K ☒	E ☐	H ☐	E ☒	H ☐	
Avukat Mahmut ÇELİK	Avukat	Çelik Gönen Hukuk Bürosu	E ☒	K ☐	E ☐	H ☒	E ☐	H ☐	
Saliha Şahin	İşçi		E ☐	K ☒	E ☐	H ☐	E ☒	H ☐	

*:Toplantıda Bulunma

Karar: ☒ Onaylandı ☐ Reddedildi

Etik Kurul Başkanı
Unvanı/Adı/Soyadı: Prof. Dr. Derya Büyükkayhan
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