T.C.
DOKUZ EYLÜL UNIVERSITY
IZMIR INTERNATIONAL BIOMEDICINE AND GENOME INSTITUTE

NEURON DERIVED EXOSOMES IN DIAGNOSIS AND PROPAGATION OF ALZHEIMER'S DISEASE

DEVRİM YAĞMUR DURUR

DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

MASTER OF SCIENCE THESIS

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AD:	Alzheimer's Disease
Aβ:	Amyloid Beta
AGO:	Arganoute
APOE:	Apoliprotein E
APP:	Amyloid Precursor Protein
AUC:	Area Under the Curve
BACE-1:	Beta Secretase 1
CD171:	Neural Cell Adhesion Molecule L1
CSF:	Cerebrospinal Fluid
CT:	Computed Tomography
DNA:	Deoxyribonucleic Acid
DLS:	Dynamic Light Scattering
DGCR8:	DiGeorge Syndrome Chromosomal Region 8
ELISA:	Enzyme-Linked Immunosorbent Assay
ESCRT:	Endosomal Sorting Complex Required forn Transport
GTP:	Guanosine Triphosphate
HSP:	Heat Shock Protein
IL-α:	Interleukin Alpha
IL-1β:	Interleukin 1 Beta
ILV:	Intraluminal Vesicles
KEGG:	Kyoto Encyclopedia of Genes and Genomes
L1CAM:	Neural Cell Adhesion Molecule L1
MCI:	Mild Cognitive Impairment
miRNA:	MicroRNA
MMP:	Matrix Metalloproteinase 9
mRNA:	Messenger RNA
MRI:	Magnetic Resonance Imaging
MVB:	Multivesicular Bodies
NDE:	Neuron Derived Exosome
Nf-κβ:	Nuclear Factor Kappa β
NTA:	Nanoparticle Tracking Analysis
PBMC:	Periheral Blood Mononuclear Cells

PD:	Parkinson’s Disease
PET:	Positron Emission Tomography
PI:	Propidium Iodine
PI3K/AKT:	Phosphoinositide-3 kinase
PSEN:	Presenilin Protein
pTau:	Hyperphosphorylated Tau
qRT-PCR:	Quantitative Real Time Polymerase Chain Reaction
RISC:	RNA-Induced Silencing Complex
RNA:	Ribonucleic Acid
RNA Pol II:	RNA Polymerase II
ROC:	Receiver-Operating Characteristic Curve
ROS:	Reactive Oxygen Species
SNARE:	Snap Receptor Proteins
snoRNA:	Small Nucleolar RNA
T-Tau:	Total Tau
TLR:	Toll-Like Receptor
TNF-α:	Tumor Necrosis Factor Alpha
TRBP:	Trans-Activator RNA Binding Protein
tRNA:	Transfer RNA
UTR:	Untranslated Region

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NEURON DERIVED EXOSOMES IN DIAGNOSIS AND PROPAGATION OF ALZHEIMER'S DISEASE

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ABSTRACT

Alzheimer's Disease (AD) is the most widespread neurodegenerative disease seen in elderly population over the world. Since the lack of definitive diagnosis, treatment, and also clear understanding of its pathogenesis; AD is very interesting and challenging topic to search.

Exosomes are the smallest class of extracellular vesicles that are nano-sized and secreted from every cell type including central nervous system cells. Exosomes contain nucleic acids, proteins, pathogens, etc. as cargo molecules and they carry them to recipient cells in their environment. Since they have exosome-specific proteins in their membranes, exosomes can be separated from body fluids and disease-specific molecular alterations in exosomes can be determined.

In this thesis, neuron-derived exosomes (NDE) were isolated from plasma samples of AD patients and healthy individuals. Dysregulated miRNAs in those exosomes were determined by small-RNA sequencing. 10 miRNAs were determined as dysregulated and validation of the most dysregulated miRNA, hsa-let-7e-5p, was performed by qRT-PCR to demonstrate its biomarker potential in NDEs of AD patients. Also, human microglial cells were treated with NDEs of patients which contain high level of upregulated hsa-let-7e-5p and miR-96-5p in order to examine their role in cell-to-cell interaction by affecting cytokine levels at protein level and gene expression of IL-1β, IL-6, TNF-α were determined via ELISA and qRT-PCR methods. Results showed that certain miRNAs were altered in NDEs of AD patients and validation of them may confirm their biomarker potential. Also, exosomal miRNAs may be involved in cell-to-cell interactions.

Keywords: Alzheimer's Disease, neuron derived exosomes, miRNA, inflammation

ALZHEIMER HASTALIĞININ TANISINDA VE YAYILIMINDA NÖRON KÖKENLİ EKZOZOMLAR

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ÖZET

Alzheimer hastalığı, yaşlı nüfusta en sık rastlanan nörodejeneratif bir hastalıktır. Kesin tanısı ile tedavisinin olmaması ve patogenezinin tam olarak aydınlatılamaması Alzheimer hastalığını ilgi çekici ve önemli bir araştırma alanı haline getirmektedir.

Ekzozomlar merkezi sinir sistemi hücreleri de dahil olmak üzere her hücre tipinden salgılanaibilen nano boyuttaki ekstraselüler veziküllerdir. Nükleik asit, protein ve patojen gibi kargo molekülleri içerdikleri için hücreden salgılandıklarında çevredeki diğer hedef veya komşu hücrelere bu molekülleri taşıyıp hücreler arası iletişimde rol oynarlar. Membranlarında bulunan ekzozom spesifik proteinler sayesinde kan gibi vücut sıvılarından izole edilebilirler ve böylece ekzozomların içindeki hastalığa özgü moleküler değişimler tespit edilebilir.

Bu tezde, Alzheimer hastalarına ve sağlıklı kontrol grubuna ait plazma örneklerinden nöron kökenli ekzozomlar izole edildi. Small-RNA sekanslaması yapılarak bu ekzozomlardaki miRNA değişimleri belirlendi. Kontrol grubunun ekzozomlarına göre hasta grubunun ekzozomlarında 10 tane miRNA'nın değişim gösterdiği belirlendi ve bunlardan en çok değişen miRNA'nın (hsa-let-7e-5p) biyomarkır potansiyelini analiz etmek için qRT-PCR ile validasyonu gerçekleştirildi. Nöron kökenli ekzozomların hücreler arası iletişimdeki rolünü anlayabilmek amacıyla insan mikroglia hücreleri, hsa-let-7e-5p ve miR-96-5p miRNA'ları yüksek olan nöron kökenli ekzozomlarla muamele edildi. Hücrelerdeki IL-1β, IL-6, TNF-α sitokin seviyesi ve IL-1β, IL-6, TNF-α genlerinin ekspresyon değişimleri ELISA ve qRT-PCR yöntemleriyle belirlendi. Sonuç olarak, Alzheimer hastalarının ekzozomlarında belirli miRNA düzeylerinin değiştiği ve bu miRNA'ların biyomarkır potansiyelinin validasyon ile doğrulandığı görülmüştür. Ayrıca, nöron kökenli ekzozomal miRNA'ların hücresel iletişimde rol alabileceği belirlenmiştir.

Anahtar Sözcükler: Alzheimer hastalığı, nöron kökenli ekzozom, miRNA, inflamasyon

1. INTRODUCTION AND AIM

1.1. Statement and Importance of the Problem

Exosomes are secreted from a variety of cell types and they have a role in cellular communication by carrying its content (protein, miRNA, etc.) to recipient cells. Cargo molecules in exosomes may trigger several cellular events according to molecules that reflect the physiologal situation of cells that they are released from.

To date, the studies about the role of NDEs in Alzheimer's Disease (AD) and AD propagation are limited. AD is a widespread neurodegenerative and neuroinflammatory disease and early diagnosis methods are still not enough of this disease. NDEs of AD patients may reflect the molecular physiology of AD brain because of its content. The studies showed that several proteins and miRNAs are increased and decreased in exosomes according to the characteristics of the cells they are released. Thereby, some altered miRNAs may be a biomarker in NDEs of AD patients and the role of patient NDEs on AD-related genes and cytokine expression of human microglial cells can be searched to understand cellular communication via exosomes.

1.2. Aim of Study

The study aims to determine the potential biomarkers in NDEs of AD patients to diagnose AD and importance of NDEs of AD patients in cell-to-cell interaction via transfering its miRNA content.

1.3. Hypothesis of Study

The hypothesis of the study:

- Expression levels of microRNAs are altered in neuron derived exosomes of AD patients.
- NDEs are internalized by HMC3 microglial cells and changes the microglia functions of recipient cell via exosomal microRNAs.

2. GENERAL INFORMATION

2.1. Alzheimer's Disease

Alzheimer's Disease (AD) is a chronic neurodegenerative disorder which affects over 25 million people worldwide. In 1907, Aloysius Alöis Alzheimer characterized the features of the disease according to his patient's symptoms for the first time. Some of those symptoms were memory loss, speaking and writing problems and being lack of understanding words or questions. After his patient died, he used histological staining techniques to observe brain parts on microscope and he discovered amyloid plaques and neurofibrillary tangles as the hallmarks of the disease (Bondi, Edmonds, & Salmon, 2017).

AD is known as the most common type of dementia, now. It affects cerebral cortex and hippocampus in brain with the specific hallmarks of the disease such as amyloid beta (Aβ) plaques and neurofibrillary tangles containing hyperphosphorylated tau (pTau) (Masters et al., 2015), (Blennow, de Leon, & Zetterberg, 2006). Abnormalities are first detected in frontal and temporal lobes and then some parts of neocortex (Masters et al., 2015). Familial AD is a very rare type of disease caused by amyloid precursor protein (APP) and presenilin gene mutations. Sporadic form is more common type of AD, caused by aging, genetics and life-style factors (Blennow et al., 2006). Mean age of onset of sporadic form is 80 years (Masters et al., 2015).

Although average duration of AD is 10 years, it arises many years before symptoms appear (Blennow et al., 2006). In the first period of the disease, neurons get damaged or destroyed and cognitive problems arise such as memory loss, language problems. Along with the progression of the disease, neurons in the other parts of brain get damaged and destroyed and some psychiatric symptoms appear such as depression, hallucinations, etc. At the end, difficulties with activities in daili living show up such as eating, swallowing, walking, dressing, etc. (Association, 2019), (Scheltens et al., 2016). AD is fatal and there is still no cure or treatment for that.

2.1.1. Clinical Features

AD is very insidious and slowly-progressing disease. Clinical symptoms arise many years after the pathological changes in brain happen. Degeneration is thought to begin 20-30 years before clinical features show up. In the course of degeneration, Aβ plaques and neurofibrillary tangles are increased and at a point, it leads to the first clinical feature. This stage is called as mild cognitive impairment (MCI) which represents the most common feature, impairment of episodic

memory (Blennow et al., 2006). Also, difficulties with multi-tasking, making decision, orientation, and loss of confidence emerge. As cognitive difficulties progress, problems with activities in daily living show up and behavioural changes start. Also, non-memorial symptoms are appeared such as atrophy, aphasia, apraxia, and agnosia which are related with distribution of Aβ plaques and loading of tau protein in wide areas of brain parts (Lane, Hardy, & Schott, 2018).

2.1.2. Risk Factors

AD is a result of combination of several causes. There are multiple factors which they lead to developing of this disease.

The most important and unalterable risk factors are age, e4 form of APOE gene and family history. Age is the greatest risk factor among them. According to the researches, number of people with AD increases upon aging (Association, 2019). The most of people with AD are 65 years old and older. Prevalence of the disease increases 2-fold every 5 years and it approaches 30%-40% when the age is 85 and older (Dalvi, 2012). Another risk factor is APOE-e4 form. APOE is a gene that codes a protein that play a role in cholesterol transport in the blood. Three forms of the APOE gene are present as e2, e3, and e4. When e4 form was compared to e3 form, it was found that e4 form has a higher risk to develop AD. In spite of these results, researches are still continued. Also, family history increases the prevalence of AD. Especially, people having first-degree relative are more likely to develop AD and it increases the risk as 4-fold. At this point, family genetics and same lifestyle with the family members may be important (Association, 2019). Mutations on APP, presenilin-1 and presenilin-2 proteins contribute to emergence of AD as genetic factors (Dalvi, 2012).

Modifiable risk factors are able to be reduced for AD or dementia. At this point, lifestyle related factors play a role such as cardiovascular risk factors (obesity, diabetes, hypertension), smoking, physical and mental inactivity, low level of education, being asocial, diet or traumatic brain injury (head trauma). Heart and blood vessels are very important organs for brain health. Heart pumps blood for brain, whilst vessels supply oxygen and blood for brain's functions. More years of education reduces the risk for AD by leading to form cognitive reserve that means a result of education benefits and mental activities. This makes brain gain flexible synapses (neuron-to-neuron interactions) and it gives resistance to the brain changes pathologically. Mental activity required jobs also help to reduce the risk. Education affects also socioeconomic status, indirectly diet, health care, treatment. Being socially active is very supportive for cognitive reserve. Despite

the reason is unknown yet, it may be because of forming of new connections in brain (Association, 2019).

Some researches have been shown that intake of certain vitamins (such as B12), antioxidants, unsaturated fatty acids, consumption of moderate alcohol, especially red wine, dietary intake of fruits and vegetables, Mediterranean type diet, exercise, and use of nonsteroidal anti-inflammatory drugs could reduce risk for AD (Dalvi, 2012), (Blennow et al., 2006).

2.1.3. Pathogenesis

The specific hallmarks of AD are senile (amyloid) plaques and neurofibrillary tangles in parts of temporal lobe and some parts of cortex in brain. Also, degeneration of synapses, neuronal death, high level of certain neurotransmitters, loading of abnormal protein deposits on neurons, high level of inflammation, and oxidative stress are other neurochemical and pathological signs of AD (Masters et al., 2015), (Dalvi, 2012).

The core hypothesis of AD is the amyloid cascade hypothesis which covers the lack of balance between clearance and overproduction of Aβ that causes neurodegeneration. In amyloid cascade hypothesis, APP is cleaved into Aβ peptides ($A\beta_{40}$ and $A\beta_{42}$) with β-secretase and y-secretase enzymes. $A\beta_{42}$ peptides (the 42 amino acid isoforms of Aβ) are more prone to aggregate than $A\beta_{40}$ form and overproduction of those leads to aggregation of them to form oligomers and finally, fibrils which build amyloid plaques and neurofibrillary tangles (Masters et al., 2015), (Dalvi, 2012). They cause synaptic loss, neuronal dysfunction, inflammation, dysfunction of neurotransmitters such as glutamate, norepinephrine, and serotonin. APP, PSEN1 and PSEN2 mutations contribute to forming of this pathology. APP mutations play a role on cleavage and aggregation of Aβ. PSEN1 and PSEN2 enable catalyctic subunit to y-secretase and that affects APP processing. Mutations on these genes cause problems in APP processing and forming of longer Aβ peptides and it results in high levels of $A\beta_{42}$ peptides (Scheltens et al., 2016).

Tau is a soluble microtubule protein and another hallmark of AD. Studies have indicated elevation of tau and phosphorylated tau level in CSF and brain of AD patients (Masters et al., 2015). Aggregated tau in neurofibrillary tangles is phosphorylated but phosphorylation mechanism is still not clear. Also, insoluble form of tau is aggregated in those tangles during this process, which leads to degeneration of neurons. Alterations in tau protein and neurofibrillary tangles are linked to overproduction of Aβ in amyloid cascade hypothesis (Ballard et al., 2011).

2.1.4. Treatment

Available drugs for treatment of AD were developed due to dysregulation of neurotransmitters. These drugs increase the reduced level of certain neurotransmitters and lead to modify symptoms of AD (Blennow et al., 2006). Food and Drug Administration approved acetyl-cholinesterase inhibitors – rivastigmine, galantamine, donepezil and glutamate antagonists – memantine as two classes of drugs for AD treatment. While memantine (*N*-methyl-D-aspartate antagonist) enhances decreasing of L-glutamate neurotoxicity and avoids some receptors to stimulate nerve cells, other ones increase the level of neurotransmitters in brain (Association, 2019). Acetylcholinesterase inhibitors block catalysis of synaptic acetylcholine and increase the amount of acetylcholine (Lane et al., 2018). Memantine and acetylcholine esterase inhibitors can be used as combined. Developing thereapautic strategies are focused on early detection of Aβ cascade, γ and β secretase inhibitors, and anti amyloid approaches such as Aβ vaccines (Dalvi, 2012).

2.1.5. Biomarkers

Several biofactors changes in pathological conditions and the presence and absence of certain biomarkers show the progression of the disease. Since AD symptoms appear many years after AD arise, biomarkers play a very important role for early diagnosis. Various biomarkers have been determined in AD such as abnormal levels of beta amyloid and tau protein, the change of several microRNAs or proteins in brain, blood and CSF by blood tests and PET imaging (Association, 2019). CT and MRI are also used to detect lesions for clinical diagnosis but biomarkers are early messenger factors for AD.

2.1.5.1. Cerebrospinal Fluid (CSF) Biomarkers

Abnormal levels of CSF biomarkers are though as active for a long time and before the first symptoms, therefore it is a good source for biomarkers in AD (Bekris & Leverenz, 2015). The most significant CSF biomarkers are Aβ ($A\beta_{42}$), total tau (t-tau) and p-tau. $A\beta_{42}$ is related with amyloid deposition in brain, t-tau indicates neurodegeneration and p-tau reflects neurofibrillary pathology. Relation between the level of Aβ and total/hyperphosphorylated tau is very important point which is studied in biomarker studies in AD (Scheltens et al., 2016). Low

concentration of $A\beta_{42}$ that shows Aβ burden and elevated level of tau which is a marker related with neuronal loss were reported in studies (Tan, Yu, & Tan, 2014). The change of these markers has a high sensitivity and specificity for more accurate diagnosis of AD. However, standardisation and validation studies are need to be developed beacuse of variability in patients.

Also, different CSF biomarkers such as Aβ oligomers which are toxic form of Aβ that leads to synaptic dysfunction in brain were also detected in CSF studies but CSF is not a good source to detect these oligomers all the time and it causes a problem to measure these proteins (Scheltens et al., 2016).

Inflammation process is another sign of AD. Inflammatory markers are released into CSF such as matrix metalloproteinase 9 (MMP-9) which has a role in tissue modelling. Elevated level of MMP-9 in CSF indicates the transfer of white blood cells through damaged blood-brain barrier during inflammation (C. C. Tan et al., 2014).

2.1.5.2. Blood Biomarkers

Blood is easier to be accessed than CSF to study biomarkers in AD. Blood tests, ELISAs, proteomics and RNA sequencing studies are performed to specify altered proteins, microRNAs and other biomolecules as biomarkers. Aβ protein, Aβ autoantibodies, and Aβ protein precursor (AβPP) isoforms can be found in plasma or serum. Especially, the level of plasma Aβ can be utilized as marker with other peripheral biomarkers (C. C. Tan et al., 2014).

2.1.5.3. MicroRNAs (miRNAs)

miRNAs are small, endogenous, non-protein coding RNA species composed of one strand, and 20-24 nucleotides which participate in regulation of gene expression by silencing the mRNAs of protein coding genes (Paul et al., 2018). The first miRNA was discovered in gene *lin-4* of *Caenorhabditis elegans* in 1993 (Vishnoi & Rani, 2017). It is found that miRNAs are present in eukaryotes and also some type of viruses. Today, over 2000 miRNAs were identified in the human genome. Almost half of protein-coding genes in mammalians are under control of miRNAs. miRNAs give rise to suppression of a gene by interacting with the 3'- untranslated region (UTR) of a target mRNA. Interestingly, it was found that miRNAs activate gene expression in some circumstances (Reddy et al., 2017). miRNAs participate in processes such as cell proliferation,

growth, differentiation, homeostasis, cell to cell interaction and also human diseases as biomarkers.

Serum, plasma and CSF are enriched in miRNAs and up- or down- regulation of these molecules were reported in several AD studies. miRNAs have a role in pathogenesis of AD by targeting APP processing genes (Bekris & Leverenz, 2015) and formation of Aβ plaques and neurofibrillary tangles. MiRNAs change Aβ pathology and get involved in dysregulation of APP gene and enzymes related with Aβ production such as BACE1. Also, they affect tau pathology. MiR-34a and miR-132/miR-212 cluster are known as regulator of tau expression (Maoz, Garfinkel, & Soreq, 2017). Therefore, alteration of level of some miRNAs might be a signature of AD.

2.1.5.3.1 Canonical Pathway of miRNA Biogenesis

miRNA regions are located in intronic parts of DNA (Paul et al., 2018) and miRNAs are transcribed from the genomic DNA by RNA Pol II (Pol II) in nucleus as primary miRNA (pri-miRNA) with a polyadenylated tail and 7-methylguanosine cap (S. Jiang & Yan, 2016). At this step, transcription factors including p53, MYC, REST, ZEB1 and ZEB2 regulate miRNA biogenesis by upregulating or downregulating the transcription. pri-miRNAs contain several stem-loop structures called as hairpin that each of them containing a different mature miRNA sequences (Treiber, Treiber, & Meister, 2019). Maturation of pri-miRNA is induced via microprocessor complex containing Drosha (RNase III) and DiGeorge syndrome critical region gene 8 (DGCR8, also known as Pasha) that is double-stranded RNA binding protein. Hairpin structures in pri-miRNA are recognized by Microprocessor and DGCR8 enhances Drosha to cleave and release hairpin RNA structures resulting in 60-70 nucleotide in length precursor miRNA (pre-miRNA) (Allmer & Yousef, 2016). This process generates in the forming of a 2 nt 3′overhang on pre-miRNA. Exportin-5, RAN-GTP and export receptor, recognize this overhang and enhances transport of pre-miRNA to the cytoplasm from nucleus (Paul et al., 2018). GTP is hydrolyzes and pre-miRNA is secreted via inducing of Exportin-5 in the cytoplasm (Graves & Zeng, 2012).

Following the transport, released pre-miRNA form is cleaved by cytoplasmic RNase III enzyme Dicer, and protein trans-activator RNA binding protein (TRBP) resulting in ~22 nucletide miRNA duplex. Duplex is unwinded by helicase enzyme and generates mature miRNA strands. One strand of these mature miRNAs is loaded Arganoute (AGO) protein, that selects only one

strand called as guide strand to be mature miRNa, to generate RNA-induced silencing complex (RISC). Other strand called passenger strand is discarded. miRNA loaded into RISC interacts with the 3'-UTR regions of target mRNAs and regulate gene silencing by mRNA cleavage or translational suppression (Reddy et al., 2017).

2.1.5.3.2. Non-Canonical Pathway of miRNA Biogenesis

miRNAs can be produced by alternative mechanisms without key components of canonical pathway such as Drosha and DGCR8, (Microprocessor), or Dicer. Non-canonical pathway was first discovered in mirtrons, which was found in *Drosophila*. pre-miRNAs of mirtrons are generated by splicing in Microprocessor-independent manner (Graves & Zeng, 2012). Also, Smitrons, mirtron-like miRNAs independent of splicing, require Drosha but not splicing, Dicer, or DGCR8 proteins. Small nucleolar RNAs (snoRNAs) and transfer RNAs (tRNA) are another source of pre-miRNAs which are Microprocessor-independent RNA structures (Pauley & Chan, 2008). Maturation of miRNA-like molecules derived from snoRNAs requires Dicer but not Drosha. Another examples is production of pre-miR-451 which is bypassing Dicer and cleaved by Ago2 protein (Graves & Zeng, 2012).

2.2. Alzheimer's Disease and miRNAs

2.2.1. miRNA Change in Brain

miRNAs are found in brain and involved in regulation processes such as neuronal differentation, plasticity, neurite outgrowth, neuroinflammation, and pathological conditions. miR-9, -29a/b, -107, -124, -128, -134, and -137 are some of those brain-enriched miRNAs which are important when their expressions are altered (Van Giau & An, 2016). To understand the dysregulation of miRNAs in AD brain, several miRNA expression studies were performed in brain regions of AD patients. The expression change of miRNAs has been shown that these changes are consistent with AD pathology and related with the disease pathway. In a study of which Cogswell et. al. revealed, 12 miRNAs were altered in hippocampus and frontal gyrus parts. Also, miR-146b was found as continually changing in both hippocampus and medial frontal gyrusin in AD brain (Cogswell et al., 2008). In a study of Lukiw et. al. which was performed in hippocampus of AD brain, it was found that miR-9, -124a, -125b, -128, -132 and -219 were altered in hippocampus of AD brain (Lukiw, 2007). Dysregulation of 5 miRNAs (miR-370, miR-328,

miR-138, miR-132 and miR-15a) was determined via microarray and validated by qRT-PCR in post mortem brain of AD patients (Bekris et al., 2013).

A review of Zhao et. al indicated the miRNAs which are involved in pathology of AD with Aβ deposition and Tau phosphorylation. In summary, the levels of miR-124, -106b, -20a, -17, -106b, -106a, -155, -101, -16, -147, -153, -323-3p, -644, -655, - 485-5p, -29a, -29b-1, -9, -29c, -298, -328, -107, -9, -29a/b-1, -137, -181c, -33, -132, -181c, 212 were decreased, whereas the levels of -26a, -9, -34, -181c, -128a, -139, -206-3p, -218, 125b, 135a were increased in brain (J. Zhao et al., 2017).

2.2.2. miRNAs Change in Cerebrospinal Fluid (CSF)

CSF is a body fluid that is present in the brain and organs of central nervous system. CSF represents the characteristics of the brain in any situation such as neurological disorders, inflammation, or infection (Caplan, 2017). Because of its direct relation with central nervous system, miRNAs in CSF may reflect the pathological changes in AD.

Several studies are performed in CSF samples of AD patients. One of them was next generation small RNA sequencing of miRNAs of AD patients, PD patients and control group. It has been shown that 41 miRNAs were dysregulated of AD patient group (Burgos et al., 2014). In another study, it was reported that the levels of miR-9, -34a, -125b, -155, -28, and -146 were increased in AD patient group (Alexandrov et al., 2012). Lusardi et. al. identified 36 miRNAs with significant difference in CSF of AD patients (Lusardi et al., 2017). In the study of Denk. et. al., elevation of seven miRNAs (miR-146a, -100, -505, -4467, -766, -3622b, and -296) and downregulation of eight miRNAs in AD patients (miR-449, -1274a, -4674, -335, -375, -708, -219, and -103) were reported as potential AD biomarkers in CSF (Denk et al., 2015). Levels of miR-29a and -29b were determined as increased in CSF of AD patients but not plasma of them and the level of miR-125b was downregulated (Kiko et al., 2014). In a seperate study, miR-222 was upregulated in CSF of AD patients as a novel potential biomarker (Dangla-Valls et al., 2017).

2.2.3. miRNAs Change in Plasma

Plasma is an another source of miRNAs for AD diagnosis. Kiko et. al. identified low levels of miR-34a and miR-146a by using qRT-PCR in plasma samples of AD patients (Kiko et al., 2014). In another study, level of miR-34c was reported as increased in plasma components of AD patients' blood (Bhatnagar et al., 2014). Kumar et. al. indicated that elevation of six miRNAs

including miR-563, -600, -545, -323b, -323b, -1274a, and -1975, and decreasing of seven miRNAs including let-7d, -7g, -15b, -142, -301a, -545, and -191were in AD and MCI patients (P. Kumar et al., 2013). Among these miRNAs, miR-34a and miR-545 were validated in another research as potential early biomarkers of AD (Cosin-Tomas et al., 2017). Also, in a new study that covers the link between miRNAs related to synaptic function and AD has shown that the levels of miR-92a, -181c, and -210 were elevated in plasma samples from AD patients (Siedlecki-Wullich et al., 2019).

2.2.4. miRNAs Change in Serum

There are several miRNA studies within serum which is a stable miRNA source as plasma. Burgos et. al. have shown that 20 miRNA were dysregulated in serum of AD patients versus control group using next generation small RNA sequencing: miR-34b, 219-2, 22, 125b, 1307, 34c, 34b, 887, 182, 135a, 184, 30c, 873, 125a, 671, 21, 1285, 375, b3176, 127 (Burgos et al., 2014). 9 miRNAs have been found deregulated in AD serum as novel biomarkers and among them, has-miR-22-3p was the miRNA that has best sensivity and specifity (Guo et al., 2017). In a serum miRNA analysis with NGS and validation with qRT-PCR, difference in level of miR-98, miR-885, miR-483, miR-342, miR-191, and miR-let-7d was found in AD patients (L. Tan et al., 2014). Dong et. al identified four miRNAs (miR-143, miR-146a, miR-31, miR-93) as serum biomarkers for AD diagnosis (Dong et al., 2015). Also, serum miR-501, miR-222, miR-223 were reported as downregulated, and miR-206 was shown as upregulated in studies (Hara et al., 2017), (Zeng et al., 2017), (Jia & Liu, 2016), (Xie et al., 2017).

2.2.5. miRNAs Change in Peripheral Blood Mononuclear Cells (PBMC)

In one of those limited miRNA studies in PBMC of AD patients, miRNA expressions in PBMC of 16 patients and 16 controls were compared to each other and elevation of miR-34a and miR-181b in PBMC of AD patients (Schipper, Maes, Chertkow, & Wang, 2007). Schipper et. al. demonstrated that the change of those miRNAs in PBMC of AD patients may indicate that miR34-a and miR-181b may be potential target to determine AD in patients (S. Kumar & Reddy, 2016). In a study of Villa. et. al., the decreasing of hsa-miR-590-3p was found in PBMC of AD patients (Villa et al., 2011). In a more comprehensive study, upregulation of 2 specific miRNAs (miR-339 and miR-425) in PBMC of AD patients were observed as potential biomarkers in AD. Also, they

indicated that these miRNAs participate in pathogenesis of AD by inhibiting and repression of BACE1 protein (Ren et al., 2016).

2.2.6. miRNAs Change in Exosomes

2.2.6.1. Exosomes

Exosomes are extracellular vesicles between 30-150 nm in size which are originated from endosomes (Y. Zhang, Liu, Liu, & Tang, 2019). Exosomes was first discovered in 1983 as released reticulocytes (Pan & Johnstone, 1983) and the term exosome was first used in the 1980s but it did not take much attention as other extracellular vesicles because that they were thought as cellular waste secreted by cells because of cellular damage or cell homeostasis (Y. Zhang et al., 2019). In recent years, they became popular because of their role in cell-to-cell interaction and as being shuttles transporting cargo molecules between the different/neighbour cells.

Exosomes have lipid bilayer consisting of cholesterol, sphingolipid, and phospholipid (Simons & Raposo, 2009). Also, several membrane proteins which enable inter/intra-cellular communication between cells are localized in lipid bilayer. Exosomes consist of important biomolecules such as proteins, lipids, RNA, DNA, and non-coding RNAs (Liao et al., 2019),. Exosomes exist in almost all type of body fluids such as serum, plasma, urine, saliva, tear, and milk (Liao et al., 2019). They are secreted by almost any kind of cells in several physiologic and pathological situations and they have been implicated in critical functions such as cell-to-cell communication, transfer of biomolecules, immune response, spreading of proteins or other molecules which are characterized by different disease (Xiao et al., 2017). Since the content of exosomes is different depending on the cell type which they are secreted from, and the state of the cells, they and their cargo molecules may give information about biological conditions and diseases, such as neurodegenerative disease, cardiovascular disease, inflammation, metabolic diseases, or tumors (Y. Zhang et al., 2019). Also, they may give clue about the spreading of the disease because of transported molecules to target cells from where they release.

2.2.6.2. Biogenesis of Exosomes

Exosomes are produced via the endosomal pathway. Biogenesis of exosomes starts with inward budding of outer membrane of the cell. This step is called as endocytosis that forms early endosomes. Early endosomes are transformed to late endosomes and cytoplasmic or cargo molecules such as proteins, lipids or mRNA start to accumulate in them (Liao et al., 2019). At the end, invagination of late endosomes results in forming of multivesicular bodies (MVBs) (Xiao et al., 2017). There are two options for MVBs; some of them may sent to lysosomes for degradation processes and some of them fuse with the plasma membrane for releasing its content into the extracellular space and at this point, they are called as exosomes. This process is regulated by Endosomal Sorting Complex Required for Transport (ESCRT) - dependent and ESCRT – independent mechanisms. ESCRT is a protein complex that is present on the membrane of MVBs and is composed of four different cytosolic proteins, (ESCRT-0, ESCRT-I, ESCRT-II, ESCRT-III). This complex is involved in MVB formation, inward budding of endosomes, and protein cargo sorting (Liao et al., 2019), (Y. Zhang et al., 2019). Recent studies have shown that ESCRTs are not necessary for MVB formation. Stuffers et.al. have shown that silencing of subunits of all kinds of ESCRTs does not affect the formation of MVBs. Also, tetraspanins, lipids, exosomal protein Alix could play role in formation of MVBs (Reza-Zaldivar et al., 2018).

2.2.6.3. Composition of Exosomes

Exosomes contain several structural and cargo molecules. Structural molecules are unique to exosomes and required for structure and functions of exosomes. Cargo molecules are proteins, lipids and nucleic acids which are variable according to the cell type from which exosomes are secreted and also the physiological and pathological conditions of the cell (Xiao et al., 2017). This indicates that exosomes reflect characteristics of the cell origin which they are derived.

A lot of proteins such as tetraspanins (CD9, CD63, CD81, CD82), heat shock proteins (HSP70, HS90), proteins for MVB formation and releasing of exosomes such as Alix, TSG101 have been identified in exosomes that are important for cell penetration, fusion, and invasion. Some of these proteins are exosomal marker proteins (Y. Zhang et al., 2019). In different cell origins and conditions, type of proteins released from exosomes may be variable.

Also, exosomal membrane is enriched in some lipids such as cholesterol, sphingomyelin, and phosphatidylserine, phosphatidic acid, prostoglandins, and other fatty acids for their stability

and exosomes have lipolytic enzymes which are able to generate units of lipids (Reza-Zaldivar et al., 2018). Researches have shown that prostoglandins are transfered to recipient cells via exosomes.

Beside these, exosomes contain several RNA molecules. Especially miRNA molecules are abundant in exosomes. These miRNAs could be transfered between cells in exosomes and participate in several processes and give rise to changes in recipient cells. Except miRNAs; long non-coding RNAs, circular RNAs, transfer RNAs are existed in exosomes (Y. Zhang et al., 2019).

2.2.6.4. Functions of Exosomes

Once exosomes were discovered, they were considered as garbage bags that are secreted and not used by cells. Developing studies have shown that exosomes participate in cell-to-cell interactions and cellular communication via carrying cargo molecules to the target/recipient cells (Liao et al., 2019). The involving of exosomes in immune responses, programmed cell death, angiogenesis, inflammation, and coagulation had been showed in several studies. Exosomes get involved in spreading of some pathogens or disease protein, and nucleic acids. Also, they may be carrier for cell growth, division, survival, differentiation, etc.. In central nervous system, exosomes have a role in eliminating of waste molecules, regulating brain activity and processes and also, inhibiting of neurodegenerative diseases such as AD (Xiao et al., 2017). In this conditions, molecular content of exosomes changes and it makes exosomes “biomarker” according to the difference between exosomes of healty and normal cells.

2.2.6.5. AD miRNAs in Exosomes

Several miRNA studies have been carried out in body fluid exosomes. In the study of Cheng et. al. in serum exosomes of AD patients, they showed that 14 miRNAs were upregulated (miR-361, -30e, -93, -15a, -143, -335, -106b, -101-3p, -425, -106a, -18b, -3065, -20a-5p, -582) and 3 miRNAs were downregulated (miR-1306, -342, -15b) (Cheng et al., 2015). Although these miRNAs need to be validated, they have a potential as biomarkers of AD. In another study of Yang et. al. in human serum exosomes of AD patients, elevation of miR-135a and miR-384 and downregulation of miR-193b were determined compared to that of control group. It was found

that combination of these 3 miRNAs gave the better result for diagnosis of AD instead of using only one miRNA as biomarker (T. T. Yang, Liu, Gao, Zhang, & Wang, 2018).

As serum, plasma exosomes are enriched by miRNAs as potential biomarkers. Lugli et. al. studied for determining the difference of miRNA expressions in plasma exosomes of AD patients vs. healthy controls and they found that 20 miRNAs were dysregulated. Among them, especially miR-342-3p was very important because that it is one of the most found miRNA in brain and also, it was downregulated significantly (Lugli et al., 2015).

CSF composition is very reflective of molecular changes in brain and this makes CSF exosomes and their composition a good source for potential biomarkers for determining disorders. A study of McKeever et. al. about miRNA profiling of CSF exosomes from Young-Onset AD patients showed that expression of miR-16-5p, miR-125b-5p, miR-451a, and miR-605-5p was altered in patients compared with control group (McKeever et al., 2018).

2.3. Exosomes in Neuron – Glia Interactions

Exosomes secreted from nervous system cells play a role in brain homeostasis, synaptic remodeling, regeneration and brain functions. Neuronal exosomes participate in synaptic remodeling by eliminating of synapses with communication and activation of microglial cells (Caruso Bavisotto et al., 2019). Astrocyte derived exosomes were found efficient in neuroprotection for blood brain barrier integrity against cultured neuronal cells in hypoxic conditions (Newman, 2003). Astrocyte derived exosomes also carry apoliprotein D to neurons regulate neuronal survival (Pascua-Maestro et al., 2018). Neuron cell signals (neurotransmitters) trigger exosome secretion from oligodendrocytes by increasing calcium levels and when neuronal cells uptake those exosomes, neurons use their cargo content and exosomes can enhance axon protection (Fruhbeis, Frohlich, & Kramer-Albers, 2012). As a conclusion, nervous system cells can communicate with each other via exosomes.

2.4. Exosomal miRNAs in Cell-to-Cell Interactions

Exosomes are released from the cells to the surrounding area and they are uptaken by neighbour cells. Since exosomes are enriched with nucleic acids such as miRNAs, it also leads to transfer of exosomal miRNAs into recipient cells. Functional miRNAs get involved in cellular events of the target cells and may change their state, homeostasis and functions. Also, state-

specific miRNAs in exosomes may be a marker of the situation of the cells which they are released. It causes an interaction between cells (Y. Zhang et al., 2019).

MiRNAs that are in exosomes are carried with Argonuate protein (AGO2). AGO2 is thought as controller of miRNA loading into exosomes. A study showed that amount of certain miRNAs in exosomes were decreased when Ago2 was knockout (Abels & Breakefield, 2016). MiRNA and AGO2 are linked to each other and AGO2 identifies the target mRNA for miRNA.

First of all, exosome secretion takes place with the fusion of multivesicular body membrane with plasma membrane. Rab GTPases (RAB11, RAB35, RAB7, RAB27A-B) are important in this process. Also, different molecules have a role in releasing of exosomes such as SNARE proteins, diacylglycerol kinase alpha, etc. These mechanisms may change the content of exosomes. It means that exosomes could be secreted from different type of late endosomes and by several mechanisms with very altered content.

Secondly, uptake of exosomes is mediated by fusion and different types of endocytosis (clathrin-dependent, caveolin-dependent, lipid-raft dependent, macropinocytosis, and phagocytosis). Uptake mechanism depends on cell type or the state of that cell type. Endocytosis, clathrin-dependent endocytosis, macropinocytosis and phagocytosis pathways were shown in studies (Mulcahy, Pink, & Carter, 2014). Surface proteins on exosomes adhere to the receptors of target cells. Tetraspanins (CD9, CD63, CD81) are most important and highly expressed surface proteins in exosomes that provide antigen recognition and signal transduction. They link to the target cell receptor and this connection leads to internalization by one ore more of those uptake mechanisms.

After that, they delivery their exosomal miRNAs into recipient cells. Several studies showed that exosomal miRNAs contribute to development of cancer metabolism, atherosclerosis, or metabolic diseases (Li, Jiang, & Wang, 2019). Also, biomarker potential of exosomal miRNAs in neruodegenerative diseases is still been searching. NGS studies are performed for detecting altered miRNAs in disease samples compared to healthy samples. At the end, exosomal miRNAs may be potential cellular communicator vehicles.

3. MATERIALS AND METHODS

3.1. Type of Study

The study is a case-control and in vitro type experimental study.

3.2. Time and Place of Study

The study was conducted at İzmir Biomedicine and Genome Institute, between April 2019 and January 2020.

3.3. Population and Sample of Study

The patients with AD diagnosis (n=20) in Dokuz Eylül University Neurology Department Dementia Polyclinic were included as patient group in the study. Diagnosis of AD was established based on "Diagnostic and Statistical Manual of Mental Disorders, 4th edition" (DSM-IV) and "The National Institute of Neurological and Communicative Disorders and Stroke and the AD and Related Disorders Association" (NINCDS-ADRDA) as diagnosis criteria. Control group was chosen from people who do not have any neurological disorders.

3.4. Materials of Study

Plasma samples from control and patient group and HMC3 human microglia cells were used as materials of study.

3.5. Variables of Study

Independent Variable: AD.

Dependent Variable: Level of plasma exosomal miRNAs, level of inflammatory cytokines (IL-1β, IL-6, TNF-α), mRNA level of cytokines (IL-1β, IL-6, TNF- α).

3.6. Tools for Data Collection

3.6.1. Isolation of Total Exosomes from Plasma

Norgen Plasma/Serum Exosome Purification Mini Kit (Norgen, Canada) was used for isolation of plasma exosomes. Plasma samples were centrifuged at 400 x g for 2 minutes at room temperature. Supernatants were transferred to new tubes. Volume of supernatants were brought to 1 ml using nuclease-free water. After that, 3 ml nuclease-free water was added to 1 ml plasma sample and 100 µl of ExoC buffer added into 4 ml sample. Then, 200 µl of slurry E was added

to each sample and samples were mixed by vortexing for 10 seconds. After incubation at room temperature for 5 minutes, samples were mixed by vortexing for 10 seconds again and centrifuged at 2000 x rpm for 2 minutes at room temperature. Supernatants were discarded and 200 µl of ExoR buffer was applied to each pellet. Samples were mixed by vortexing for 10 seconds and incubated at room temperature for 5 minutes. Then, they were mixed by vortexing for 10 seconds and centrifuged at 500 x rpm for 2 minutes. At the end, supernatants were transfered to mini filter spin column in elution tubes and centrifuged at 6000 x rpm for 1 minutes.

3.6.2. Enrichment of Neuron Derived Exosomes

Exosome suspensions were incubated with 8 µg of mouse anti-human CD171 (L1CAM neural adhesion protein) biotinylated antibody (clone 5G3; eBioscience, San Diego, CA) in 100 µL of 3% bovine serum albumin (BSA; 1:3.33 dilution of BSA 10% solution in PBS [Thermo Scientific, Inc., Rockford, IL.]) for 60 minutes at 4 °C. After incubation, 80 µL of Streptavidin-Plus UltraLink resin (Pierce-Thermo Scientific, Inc., Rockford, IL) in 120 µL of 3% BSA was added to mixtures and they were incubated at 4 °C for 30 minutes. Followed by centrifugation at 200 x g at 4 °C for 10 minutes, pellets were resuspended in 200 µL of 0.05 M glycine-HCl (pH 3.0) and incubated at 4 °C for 10 minutes, Then, they were recentrifuged at 4500 x g at 4 °C for 10 minutes. Finally, supernatants were transferred to new tubes containing 30 µL of 1 M Tris-HCl (pH 8.0). x rpm for 1 minute.

3.6.3. Transmission Electron Microscopy (TEM)

Exosome morphologies were determined by TEM. 5 µl of exosome suspension was dropped onto parafilm. 100 mesh Formwar-coated copper grid (Electron Microscopy Sciences, USA) was left onto that exosome suspension. After incubation for 1 hour at room temperature, grid was washed on 30 µl of PBS for 2 minutes long and this washing step was repeated for three times in total. Then, grid was fixed with a drop of 2% paraformaldehyde by incubation for 10 minutes at room temperature and washing steps were repeated as before. Grid was contrasted with a drop of 2% uranyl acetate for 15 minutes at room temperature and in the dark. Grid was left to be dried for 5 minutes. Exosome suspension was diluted 1:5 before adsorption to avoid aggregation. Dried grids were examined with Zeiss Sigma500 transmission electron microscope in the electron microscopy core at the Izmir Biomedicine and Genome Institute at Dokuz Eylül University.

3.6.4. Determination of Protein Level in Exosome Samples by BCA Assay

To determine protein concentration of NDEs, 5 µl of exosome suspension was mixed with 55 µl M-PER Mammalian Protein Extraction Reagent (Thermo Scientfic, Inc., Rockford, IL) and the suspension was vortexed for 10 minutes. Protein concentration was measured with BCA Protein Assay Kit (Takara, Japan).

3.6.5. Determination of Exosomal Markers at Protein Level by Western Blotting

M-PER Mammalian Protein Extraction Reagent (Thermo Scientfic, Inc., Rockford, IL) was used for lysing of NDEs for 10 minutes by vortexing. 10 µg of proteins were separated by using 10% SDS-PAGE and then transferred to a 0.45 µm polyvinylidene fluoride (PVDF) membranes (Sigma-Aldrich, USA). Membranes were blocked with 3% milk powder (Bioshop, Canada) in phosphate buffered saline with tween (TBST) for 1 hour at room temperature and incubated with CD63 and Calnexin primary antibodies at 4 °C overnight. Secondary antibodies were added for 1 hour at room temperature. After washing steps with TBST, membranes were imaged by using chemiluminescence way using Luminata Forte Horseradish Peroxidase Substrate reagent (Millipore, USA). CD63 (SC-5275, anti-mouse) and Calnexin (SC-11397, anti-rabbit) primary antibodies were purchased from Santa Cruz (California, USA), (1:500); HRP-conjugated secondary antibodies were purchased from Cell Signaling (Massachusetts, USA), (1:1000).

3.6.6. RNA Isolation from Neuron Derived Exosomes

Exosomal RNAs were isolated from NDEs suspensions using exoRNeasy Serum/Plasma Midi Kit (Qiagen, Hilden, Germany). RNA was eluted in 18 µL nuclease-free water. Also, miRNeasy Mini Kit (Qiagen, Hilden, Germany) was used for same purpose. Kits were used according to the manufacturer's protocol. Nanodrop was used for RNA quantification.

3.6.7. Next Generation Sequencing (NGS)

Neuron derived exosomal RNAs of AD patients and healthy volunteers were profilled via small RNA-seq on Illumina NextSeq 500 by Norgen Biotek Corp (Canada). Library preparation was performed using Norgen Biotek Small RNA Library Prep Kit (Cat. 63600, Norgen Biotek, Canada). Analysis of raw data was performed by Gökhan Karakülah and his team members.

3.6.8. Bioinformatic Analysis

Bioinformatic analysis of NGS raw data was performed by Gökhan Karakülah and his team members. After determination of altered miRNAs in NDEs of AD patients, potential targets and related pathways of these exosomal miRNAs were analyzed in Kyoto Encyclopedia of Genes and Genomes (KEGG) based on the DIANA-miRPath v3.0.

3.6.9. qRT-PCR Validation

To validate the results of NGS analysis, one of the altered miRNAs, hsa-let-7e-5p, was selected for validation by qRT-PCR in healthy and patient NDEs. Total exosomal RNA (40 ng) was used as input for input for cDNA reaction using miScript II RT Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The cDNA synthesis program consisted of 1 cycle at 37 °C for 60 minutes and 1 cycle at 95 °C for 5 minutes.

miScript SYBR Green PCR Kit (Qiagen, Hilden, Germany) was used to determine expression level of hsa-let-7e-5p. 2 µL of cDNA was used for each reaction. All reactions were performed in 20 µL reaction volume. Amplification steps consisted of 1 cycle at 95 °C for 15 minutes, 45 cycles at 95 °C for 15 seconds, and 1 cycle at 60 °C for 1 minute and they were performed on LightCycler 480 II (Roche, Switzerland). For normalization of miRNAs, *C.elegans* miR-39 primer (Qiagen, Hilden, Germany) was used. The sequences for miRNAs are listed in Table 1.

Table 1. The list of human miRNA primer sequences for validation study.

miRNA	**miRBase Accession**	**Sequence**
ce_miR-39	MIMAT0000010	5'UCACCGGGUGUAAAUCAGCUUG
hsa-let-7e-5p	MIMAT0000066	5'UGAGGUAGGAGGUUGUAUAGUU

3.6.10. Cell Culture

The human microglia cell line HMC3 was purchased from the ATCC (Manassas, VA). HMC3 cells were cultured in EMEM medium (Sigma Aldrich, Saint Louis, MO, USA) contaning 10% exosome-depleted FBS, and 1% Penicillin-Streptomycin (10,000 U/mL) (Thermo

Scientific, Inc., Rockford, IL) in a humidified atmosphere supplemented with 5% CO_2 at 37 °C.

3.6.11. Labelling and Uptake of Exosomes by HMC3 Microglia Cells

Labelling of exosomes waw performed with PKH67 Green Fluorescent Cell Linker Kit for General Cell Membrane Labelling (Sigma Aldrich, Saint Louis, MO, USA). 100 µg protein equivalents of exosomes in 250 µl PBS were added to 250 µL of Diluent C and mixed with 500 µL of PKH67 dye diluted in Diluent C for 5 minutes in dark. The dye reaction was ended by addition of 1000 µL of exosome-depleted FBS (Thermo Scientific, Inc., Rockford, IL). After 1 minute incubation, exosome-dye mixture was centrifuged at 100,000 x g for 70 minutes. The exosome pellet was washed twice with PBS at 100,000 x g for 30 minutes. Finally, exosome pellet was resuspended in 100 µL of PBS.

HMC3 cells were cultured in EMEM medium (Sigma Aldrich, Saint Louis, MO, USA) containing 10% exosome-depleted FBS, and 1% Penicillin-Streptomycin (10,000 U/mL) (Thermo Fisher Scientific, Inc., Rockford, IL). Following by seeding 7500 cell/per well into 96-well plate (Sarstedt, Germany) and incubation for 24 hours, 2 µg of PKH67 labelled exosomes were incubated with HMC3 cells for 4 and 24 hours at 37 °C in incubator. The uptake of exosomes by HMC3 cells was visualised with fluorescence microscope (Olympus IX-71, Japan).

3.6.12. Determination of Non-Toxic Doses of Exosomes by Presto Blue Viability Assay

Presto Blue TM Cell Viability Reagent (Invitrogen, USA) was used to detect cytotoxicity of doses of exosomes. 7500 cells/well were seeded to 96-well plate with 200 µl of final well volume and incubated overnight under 37 °C and 5% CO_2 incubation conditions. After overnight incubation, microglial cells were treated with various concentrations of exosomes (10, 20, 30, 40, 50 µg/ul) for 24 hours. After the experiment, supernatant was collected, and Presto Blue was added according to the given protocol. Absorbance was measured at 535 nm - 615 nm wavelengths with microplate reader Varioskan Flash (Thermo Scientific, USA). When Presto Blue is resazurin based reagent and its non-fluorescent blue color changes to highly fluorescent red color when it enters a living cell and resazurin is reduced to resorufin. Non-viable cells can not transform resazurin to resorufin. Cell viability is calculated by following formula:

$$\text{Cell viability} = (OD_{Sample}) / (- OD_{Control}) * 100.$$

3.6.13. Determination of Cell Death by PI Staining

Propidium iodide (PI) (Sigma-Aldrich, USA) dye was used to detection of cell death. HMC3 cells were seeded into 96-well plate with 7500 cells/well with 200 µl of final well volume and incubated overnight under 37 °C and 5% CO_2 conditions. After overnight incubation, cells were incubated with exosomes for 24 hours. After the experiment, PI (50 µg/ml) was added into the culture media and was incubated for 15 minutes. Cells were observed using fluorescent microscope (Olympus IX-71, Japan). PI dye enters the damaged cell membrane and dead cells could be observed in red color. PI positive and negative cells were quantified in ImageJ 1.51n software and result was indicated as percentage of PI positive cells.

3.6.14. Determination of IL-1 beta, IL-6 and TNF-alpha Cytokine Level by ELISA

Exosomes of AD patients and healthy controls were incubated with HMC3 cells (50.000 cell/per well) for 24 hours in 96 well plate. Culture supernatants were collected and IL-6, IL-1 beta and TNF-alpha production were determined by human ELISA kits (Thermo Fisher Scientific, Inc., Rockford, IL), according to the manufacturer's protocol.

3.6.15. RNA Isolation from HMC3 Microglia Cells Treated with Neuron Derived Exosomes

500.000/well of HMC3 cells were seeded in 6-well plates. After overnight incubation, cells were treated with 20 µg/ml healthy and patient NDEs and for 24 hours. Control group was not treated and also 100 ng/ml LPS treated group was used as positive control. Healthy exosomes were chosen from exosomes containing low amount of hsa-let-7e-5p and miR-96-5p and patient exosomes were chosen from exosomes containing high amount of hsa-let-7e-5p and miR-96-5p according to NGS data analysis. After treatment, cells were collected. Total RNAs were extracted from NDE treated HMC3 cells using miRNeasy Mini Kit (Qiagen, Hilden, Germany). Kit was used according to the manufacturer's protocol. RNA was quantified using the Nanodrop.

3.6.16. cDNA Synthesis

1000 ng of cellular RNA for mRNA quantification were used as input for cDNA reaction using miScript II RT Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The cDNA synthesis program consisted of 1 cycle at 37 °C for 60 minutes and 1 cycle at 95 °C

for 5 minutes.

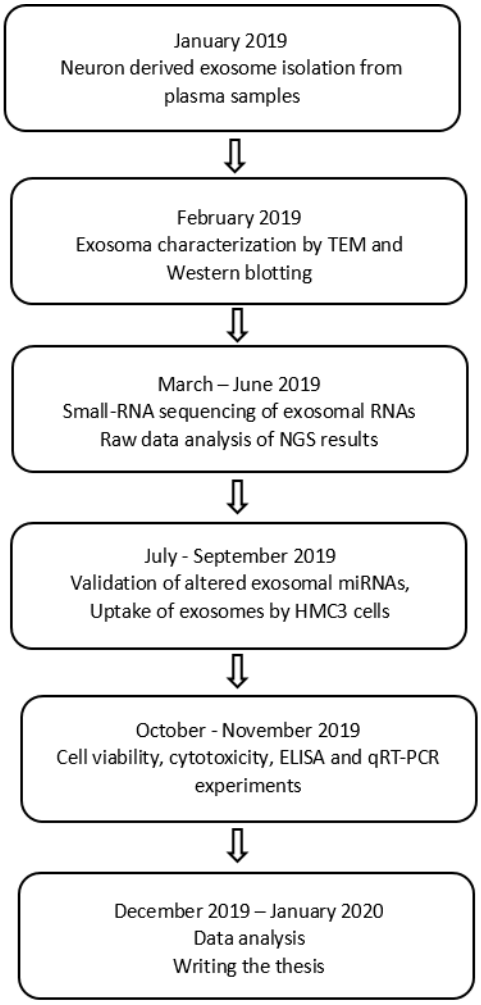
3.6.17. Quantitative PCR (q-PCR)

2 µL of cellular cDNA was used for each reaction for quantification of mRNA levels of IL-1β, IL-6 and TNF-α. All reactions were performed in 20 µL reaction using GoTaq qPCR Master Mix Kit (Promega, Madison, USA). Amplification steps consisted of 1 cycle at 95 °C for 2 minutes, 45 cycles at 95 °C for 10 seconds, and 1 cycle at 60 °C for 1 minute and they were performed on ABI 7500 (Applied Biosystems, California, USA). For normalization of mRNAs, GAPDH (endogenous Glyceraldehyde - 3- phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. The sequences of primers were shown in Table 2.

Table 2. The list of sequences of human mRNA primers.

mRNA Primer	Sequence
IL-1β	F:ATGATGGCTTATTACAGTGGCAA R: GTCGGAGATTCGTAGCTGGA
IL-6	F: AAGAGCTTCCAGCCAGTTGCC R: GTGGTATCCTCTGTGAAGTCT
TNF-α	F: CCTCTCTCTAATCAGCCCTCTG R: GAGGACCTGGGAGTAGATGAG
GAPDH	F: ACCACAGTCCATGCCATCAC R: TCCACCCTGTTGCTGTA

3.7. Study Plan and Calendar



3.8. Data Evaluation

Prism 8.0 (Graphpad, USA) program was used to visualize the data. To compare between groups, Mann-Whitney U test was used. Significance level was accepted as $p<0.05$ in all evaluations.

ROC (receiver-operating characteristics) curve analysis was performed to determine predictive power and diagnostic cut-off value of exosomal miRNA expression level. Significance level was accepted as $p<0.0001$. The area under the curve (AUC) was determined and sensitivity and specifity were calculated to determine diagnostic value of miRNA expression level.

3.9. Limitations of Study

NGS and validation studies were limited to certain number of healthy individuals and patients. Exosomal RNA level was low in exosomes isolated from 1 ml plasma sample and it decreased number of healthy and patient individuals for NGS. Validation of all miRNAs were not performed. Inhibitors of hsa-let-7e-5p miRNA and miR-96-5p were not used for determining the role of exosomal miRNAs of patients on IL-6 gene expression level.

HMC3 microglial cell line was used for cell-to-cell interaction studies via NDEs *in vitro*. HMC3 has low phagocytic activity, low responsiveness to stimulators, and it is unable to produce several inflammatory molecules (TNF-α, IL-1β, caspase).

3.10. Ethics Committee Approval

The study has ethics committee approval with 399-SBKAEK protocol number under date of 21.08.2017. It is shown in Appendix Part of this thesis.

4. RESULTS

4.1. Demographic Data

Demographic data of healthy controls and AD patients was given in Table 3. There was no significant alteration for age and MMSE scores between patient groups of scanning and validation. MMSE scores of patients were very low compared to healthy controls for each groups.

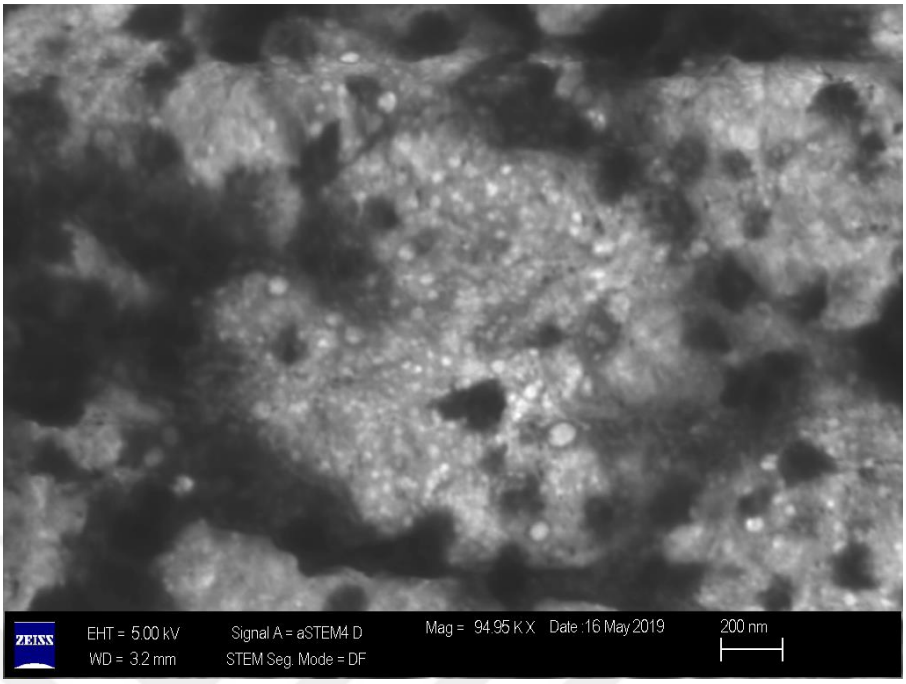
Table 3. Demographic data of healthy controls and AD patients.

	Scanning Group		Validation Group	
	HC (n=8)	**AD (n=8)**	**HC (n=15)**	**AD (n=20)**
Age, Mean (SD)	71.4 (9.4)	61.4 (6.5)	70.3 (7.4)	59.3 (7.8)
Gender (F/M)	4/4	4/4	5/10	13/7
MMSE Score, Mean (SD)	>29	12.6 (8.7)	>29	12.9 (7.6)

4.2. Characterization of Exosomes by TEM

After the isolation of total exosomes from plasma samples, exosome characterization was performed with TEM visualization by detecting size and morphology of exosomes. TEM photographs showed that exosomes are within the expected size and morphology. Size of exosomes was determined in the range of 60-80 nm and their shape was observed as spherical.

A.



B.

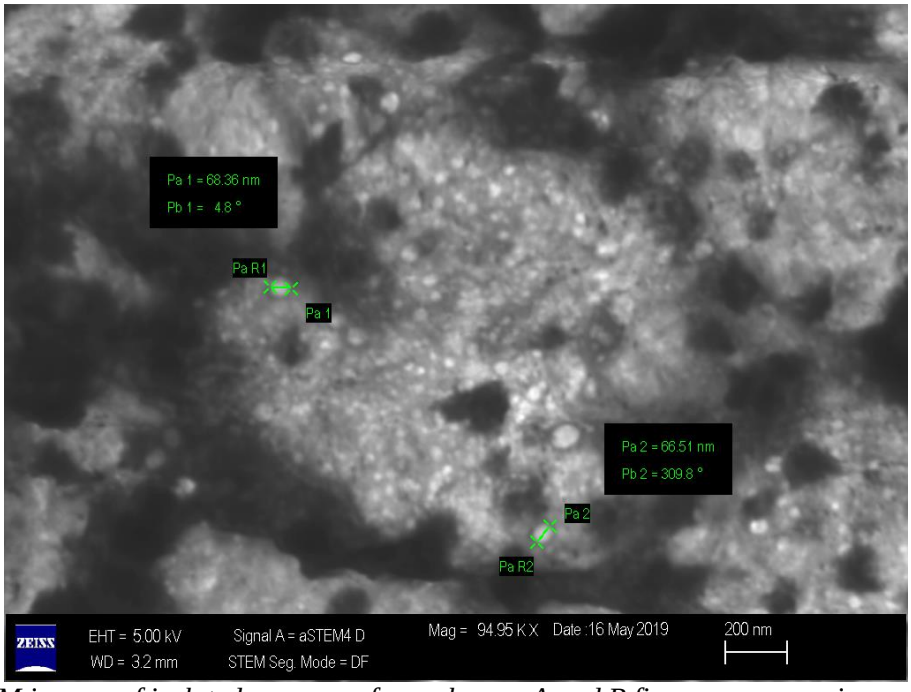


Figure 1. TEM images of isolated exosomes from plasma. A and B figures are same images. Size of exosomes is indicated in figure B.

4.3. Characterization of Exosomes by Western Blotting

Isolated exosomes were characterized via Western blotting by detecting of exosome-specific surface marker CD63 and absence of endoplasmic reticulum protein – as negative exosomal marker. Western blot results showed that CD63 was enriched in both healthy and patient exosome samples and Calnexin was not detected at all.

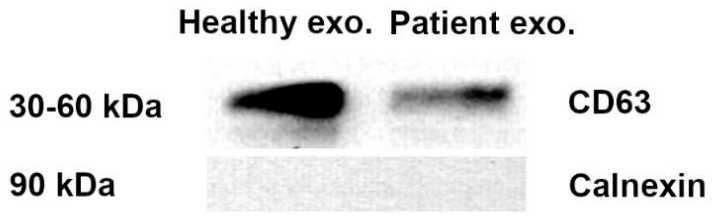


Figure 2. Western blot images showing of presence of exosomal protein CD63 and absence of non-EV protein Calnexin.

4.4. Detection of Altered miRNAs in Neuron Derived Exosomes of Alzheimer's Disease by NGS

NGS analysis was accessed to determine the alteration of miRNA expression levels in NDEs of AD compared to healthy control NDEs. According to the results, 10 miRNAs were identified as changed, the list of altered miRNAs are given in Table 3. Expression patterns of altered miRNAs are given in Figure 3 as a heat-map. Pathway analysis of 10 miRNAs are also presented in Figure 4.

Table 4. The list of altered miRNAs in NDEs of AD patients in NGS.

miRNA	Fold Change	*p* Value
Upregulation		
hsa-let-7e-5p	10.43672348	0.015768001
hsa-miR-96-5p	9.150599406	0.014882893
hsa-miR-484	1.831689473	0.016646117
Downregulation		
hsa-miR-99b-5p	0.80096355	0.025590825
hsa-miR-100-5p	0.780593154	0.011785077
hsa-miR-30e-5p	0.771071036	0.010119012
hsa-miR-378i	0.167696343	0.00455315
hsa-miR-145-5p	0.120377952	0.019664775
hsa-miR-378c	0.260485164	0.037098149
hsa-miR-451a	0.505692067	0.011934314

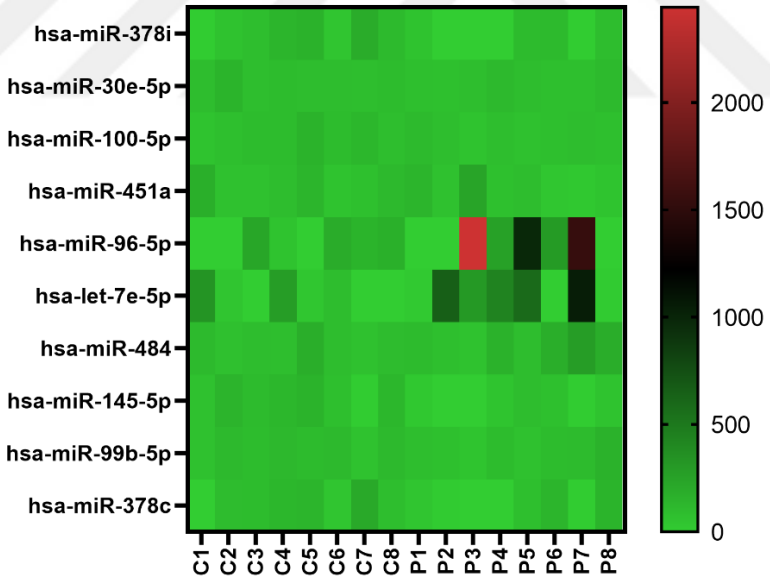


Figure 3. Heat-map of altered miRNAs in NDEs in AD.
Each row demonstrates miRNA names and each column demonstrates control and patient numbers. Dark cells represent high expression, while light cells represent low expression.

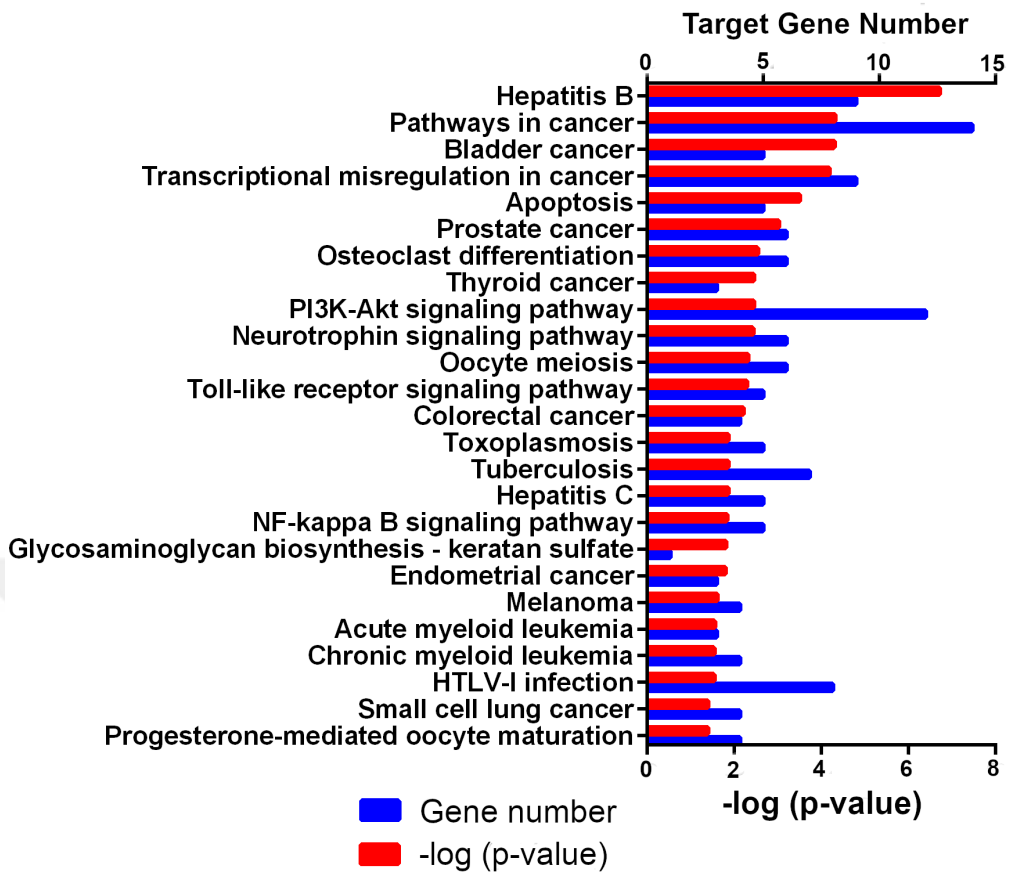


Figure 4. KEGG pathway analysis of targets of 10 altered miRNAs. The analysis revealed the most significant 25 pathways. X-axis shows the probability of the pathway enrichment and X-axis shows tha number of target genes to the given pathway. Significant pathways ($p<0.05$) were selected.

4.5. Validation of Altered miRNAs in NGS by Quantitative PCR

miRNAs to be validated were chosen according to the criteria; p value < 0.05 and 2 fold change. Since hsa-let-7e-5p was most significantly altered miRNA among those 10 miRNAs, it was the first chosen miRNA to be validated in NDEs of a new group of control and patient and quantitative PCR was performed as validation method.

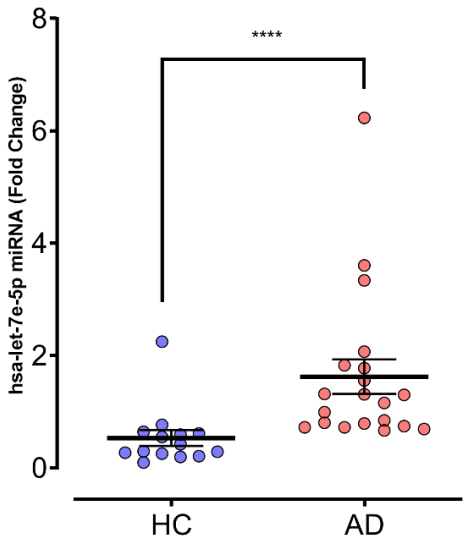


Figure 5. Validation of hsa-let-7e-5p miRNA in NDEs of healthy and patient group by qPCR. HC means healthy control and AD means AD. Results were given as mean ±SD.

According to the qPCR results, hsa-let-7e-5p was compatible with NGS results. hsa-let-7e-5p miRNA fold change was significantly increased as 1,624 ± 0,3053 standard deviation with p<0,0001 in NDEs of AD depending on Mann-Whitney Test.

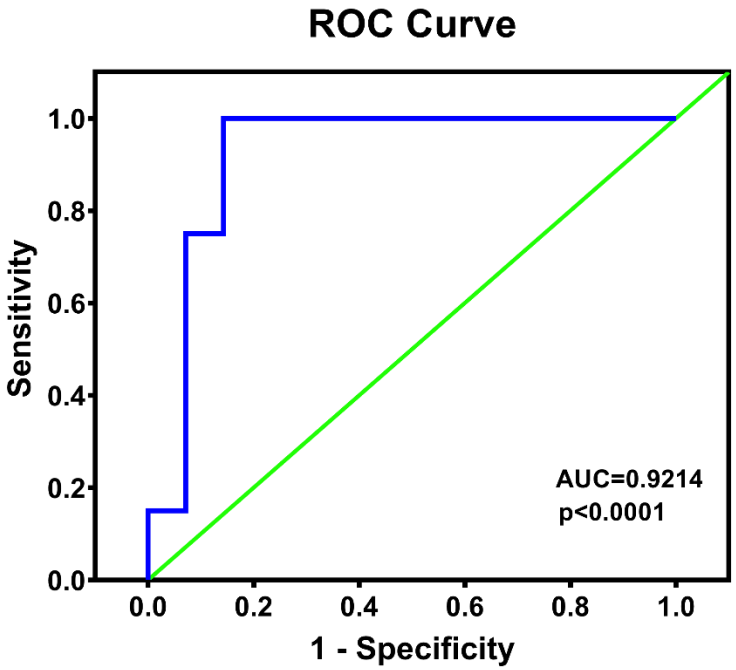


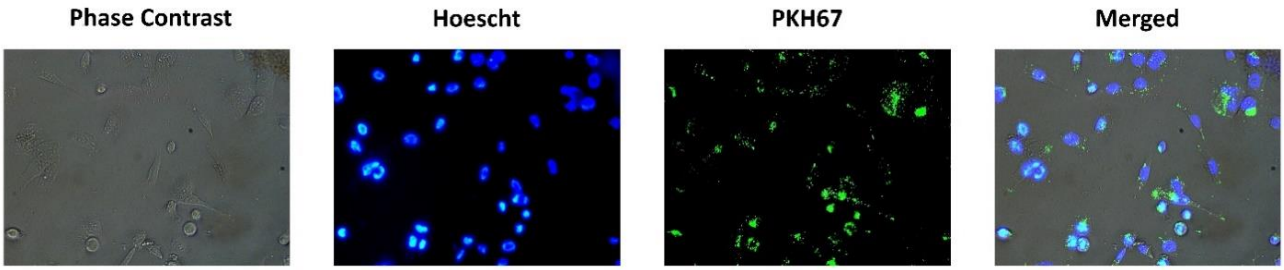
Figure 6. Receiver-operating characteristic curve (ROC) analysis of deregulated hsa-let-7e-5p miRNA. AUC: Area under the curve.

ROC analysis was performed to determine sensitivity and 1-specifity of hsa-let-7e-5p exosomal miRNA in AD patients. Cutoff value of hsa-let-7e-5p was determined as 0.6573. Results showed that AUC=0.9214, %95 CI: 0.8016 to 1.000, specifity: 100%, sensitivity: 85.7% and p<0.0001.

4.6. Uptake of Neuron Derived Exosomes by HMC3 Microglia Cells

Exosomes were labelled with PKH67 dye and incubated with HMC3 cells to visualize the uptake of them by microglial cells. Hoescht dye was added to show nucleus of cells. HMC3 microglial cells were incubated with labeled exosomes for 24 hours. 20X and 40X photos were taken of exosome-internalized cells. Uptake of exosomes into cytoplasm and nucleus of cells was shown in Figure 7.

A.



B.

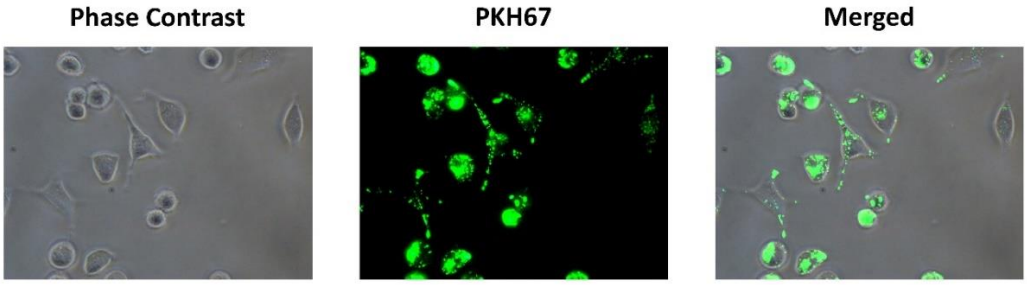


Figure 7. Uptake of PKH67 labeled NDEs by HMC3 microglial cells. Figure A is 20X image of internalization of exosomes with Hoeshct dyed nucleus. Figure B is 40X image of uptake of exosomes by cells.

4.7. Detection of Cytotoxic Doses of Neuron Derived Exosomes by Presto Blue Viability Assay

Varying doses of health and patient NDEs were given to HMC3 microglial cells and also non-treatment group was used as control without addition of NDEs to determine the non-toxic dose of exosome. Firstly, 10 and 50 µg/ml and then 20-30-40 µg/ml of NDE doses were applied.

Figure 8A shows that cell viability was detected to be 80,99 ± 3,632 standard deviation with p<0,0095 and 79,83 ± 0,9542 standard deviation with p<0,0061 in 50 µg/ml healthy and patient exosome treated cells. Figure 8B shows that cell viability was detected to be 87,99 ± 2,205 standard deviation with p<0,0025 and 80,19 ± 1,241 standard deviation with p<0,0016 in 40 µg/ml healthy and patient exosome treated cells depending on Mann-Whitney test. According to this result, 40 and 50 µg/ml doses of NDE treatment decreased cell viability significantly and 10, 20 and 30 µg/ml of NDE doses were appropriate for microglial cells for treatment and their viability.

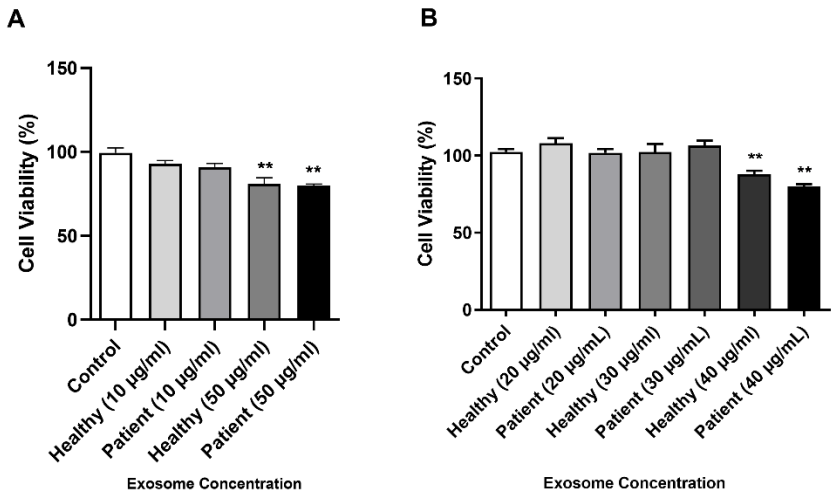


Figure 8. Effect of varying NDE doses on HMC3 cell viability. Results were given as mean ± SD.

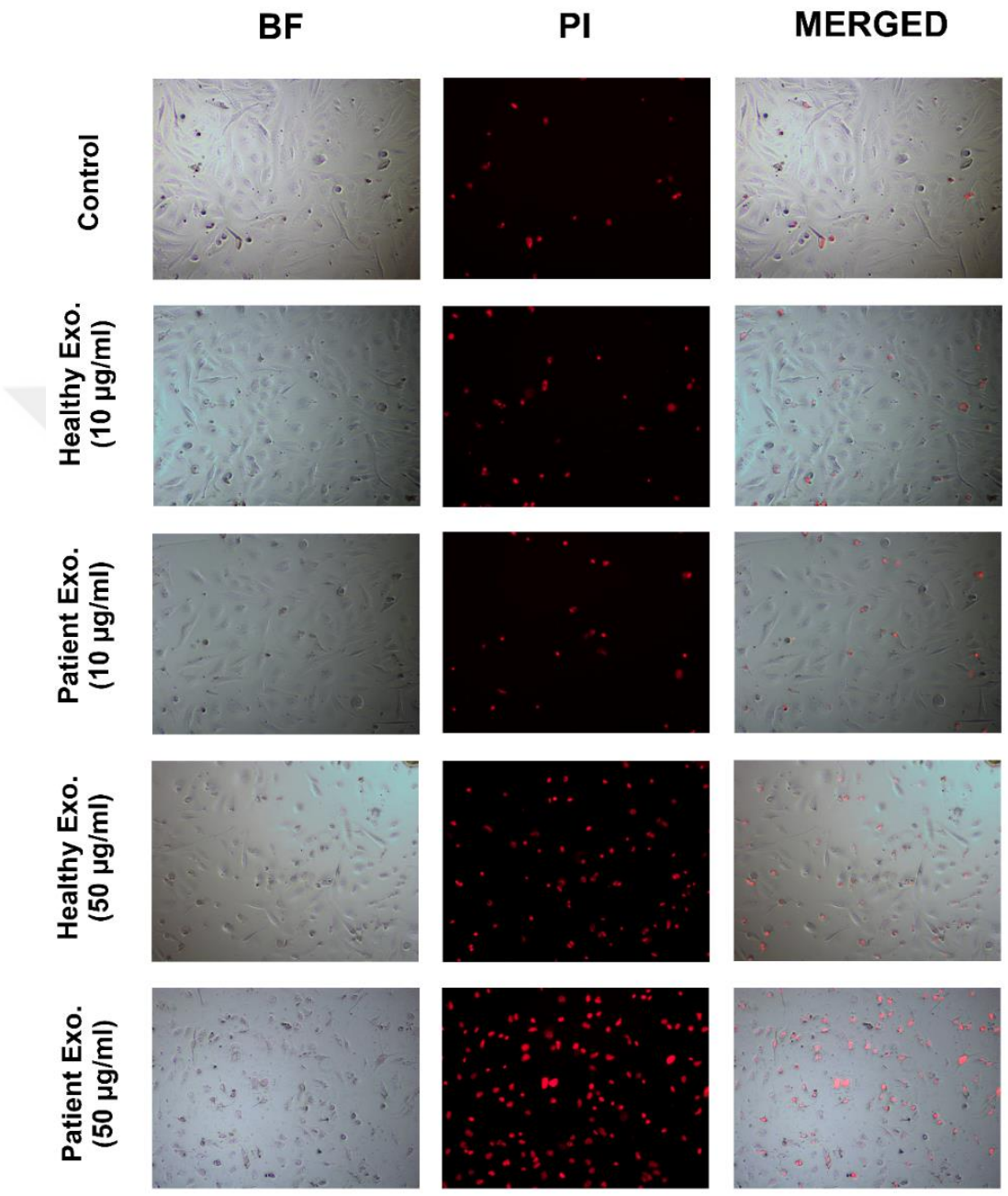
4.8. Detection of Cytotoxic Doses of Neuron Derived Exosomes by PI Staining

HMC3 cell were stained with propidium iodine (PI) dye after treatment with varying doses of NDEs to indicate cell death. Firstly, 10 and 50 µg/ml and then 20-30-40 µg/ml of NDE

doses were applied.

As seen in Figure 9, after 50 μg/ml NDE treatment of healthy and patient samples, HMC3 cell death was significantly increased with 17,75 ± 1,191 standard deviation with $p<0,0101$ and 43,29 ± 1,874 standard deviation with $p<0,0061$. As seen in Figure 10, following the treatment of HMC3 cells with 40 μg/ml healthy and patient NDEs, HMC3 cell death was significantly elevated with 21,20 ± 4,554 standard deviation with $p<0,0025$ and 62,80 ± 1,625 standard deviation with $p<0,0025$. This result showed 40 and 50 μg/ml doses of NDEs significantly increased HMC3 cell death.

A.


BF
PI
MERGED
Control
Healthy Exo. (10 µg/ml)
Patient Exo. (10 µg/ml)
Healthy Exo. (50 µg/ml)
Patient Exo. (50 µg/ml)

B.

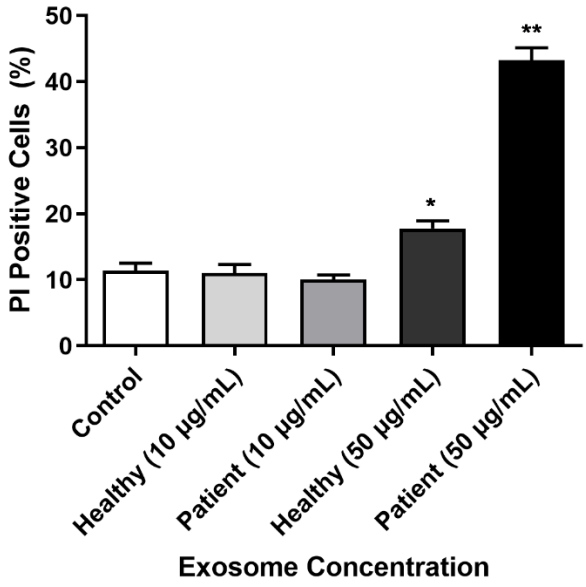
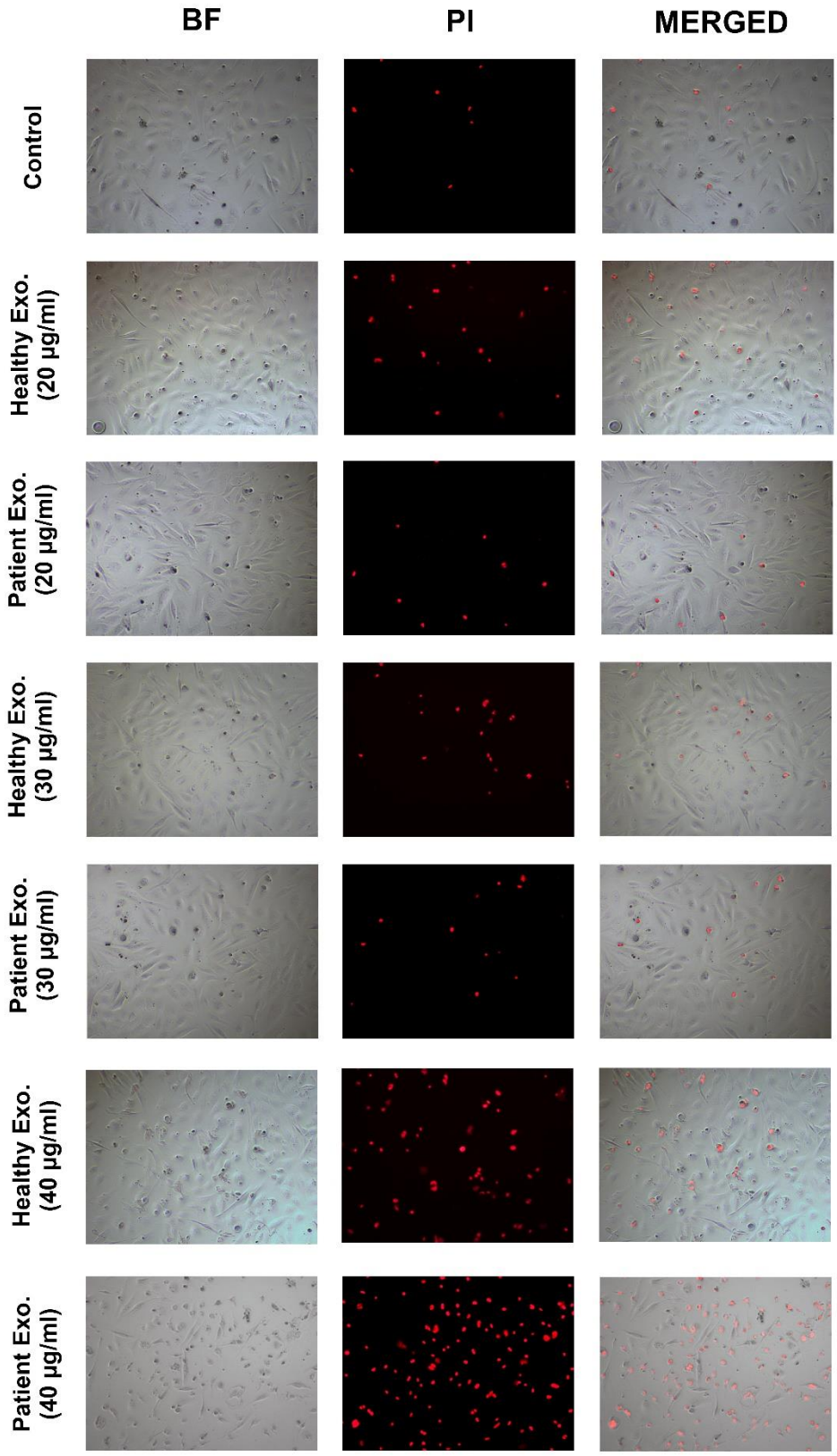


Figure 9. Effect of 10 and 50 µg/ml doses of NDE on HMC3 cell death via PI staining. Results were given as mean ±SD.

A.


BF
PI
MERGED
Control
Healthy Exo. (20 µg/ml)
Patient Exo. (20 µg/ml)
Healthy Exo. (30 µg/ml)
Patient Exo. (30 µg/ml)
Healthy Exo. (40 µg/ml)
Patient Exo. (40 µg/ml)

B.

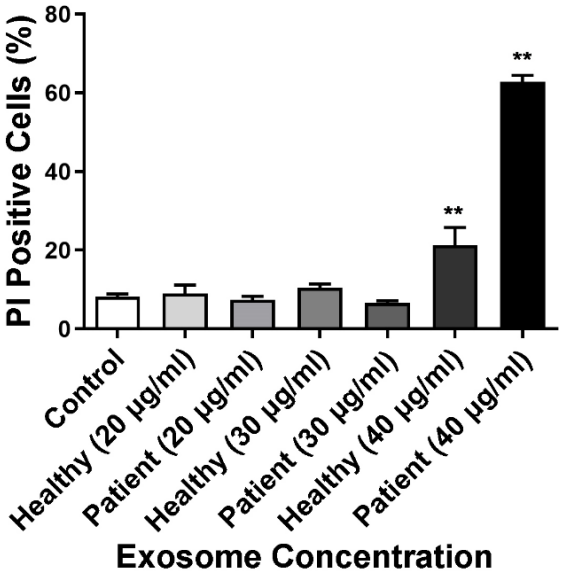


Figure 10. Effect of 20, 30 and 40 µg/ml doses of NDEs on HMC3 cell death via PI staining. Results were given as mean ±SD.

4.9. Quantification of Levels of Inflammatory Cytokines by ELISA

To detect the effect of NDEs in inflammatory cytokine secretion in HMC3 cells, ELISA of IL-1β, IL-6 and TNF-α were performed. LPS treated group was used as positive control.

HMC3 cells treated with 10 µg/ml dose of NDEs of all healthy and patient exosomes for 24 hours to detect alteration of IL-6 cytokine between groups.

Also, cells were incubated with NDEs which contain high level of hsa-let-7e-5p and miR-96-5p for patient group and low level of hsa-let-7e-5p and miR-96-5p for healthy group separately. These are the most upregulated miRNAs in NGS results. Also, two different doses of exosomes (10 and 20 µg/ml) were performed on cells.

In figure 11, there was no significant alteration of IL-6 cytokine level between healthy and patient groups for all samples. Also, as seen in Figure 12 and 13, it was found that there was no significant alteration of IL-6 level between groups at protein level by treating cells with 10 µg/ml and 20 µg/ml doses of NDEs. Level of IL-6 in LPS group was found upregulated (Data was not shown). IL-1β and TNF-α cytokines were not detected (Data was not shown).

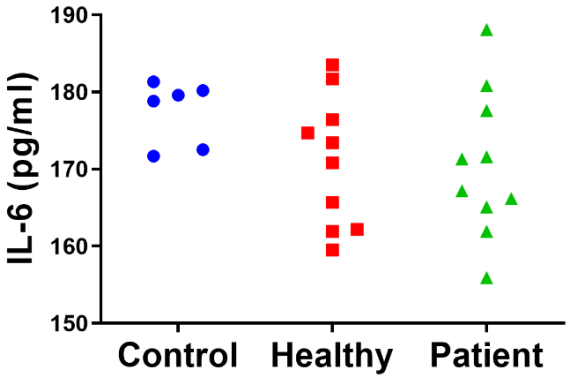


Figure 11. The level of IL-6 cytokine in cells treated with 10 µg/ml NDEs of all healthy controls and patients. Results were given as mean ±SD.

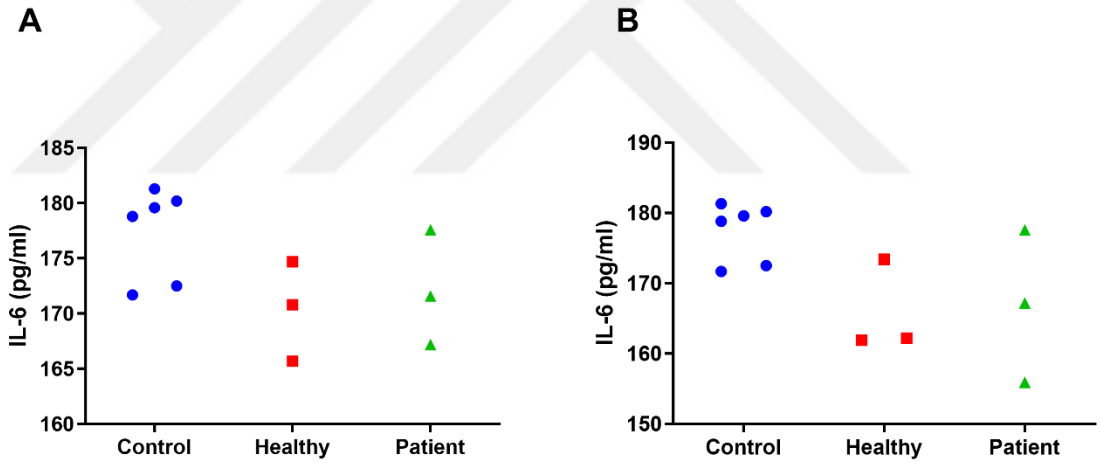


Figure 12. The level of IL-6 cytokine in cells treated with 10 µg/ml NDEs of healthy and patient group with high expression of miRNAs. A figure: Cytokine level depending on hsa-let-7e-5p level, B figure: Cytokine level depending on miR-96-5p level. Results were given as mean ±SD.

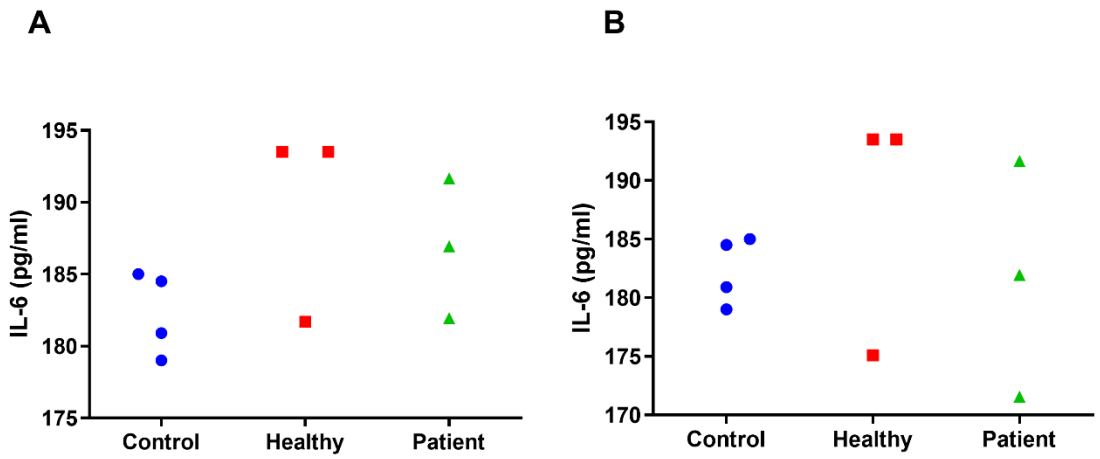


Figure 13. The level of IL-6 cytokine in cells treated with 20 µg/ml NDEs of healthy and patient group with high expression of miRNAs. A figure: Cytokine level depending on hsa-let-7e-5p level, B figure: Cytokine level depending on miR-96-5p level. Results were given as mean ±SD.

4.10. Effect of Neuron Derived Exosomes on mRNA Expression Levels of IL-1β, IL-6 and TNF-α Genes in HMC3 Cells by qPCR

To detect the effect of NDEs in gene expression of cytokines in HMC3 cells, qPCR of IL-1β, IL-6 and TNF-α genes was performed. HMC3 cells treated with NDEs which contain high level of hsa-let-7e-5p and miR-96-5p for patient group and low level of hsa-let-7e-5p and miR-96-5p for healthy group, separately. Only 20 µg/ml exosome dose was applied on cells. LPS treated group was used as positive control.

TNF-α gene expression was not detected in these microglial cells. There was no significant alteration of IL-1β gene between control and patient groups and also, between healthy and patient groups. The most interesting point is that elevation was found in IL-6 mRNA level of patient group versus control and healthy groups but not significantly. As a positive control, gene expression of IL-1β in LPS group was found upregulated as 4 fold change and and IL-6 gene expression was elevated in LPS group as 3 fold change (Data was not shown).

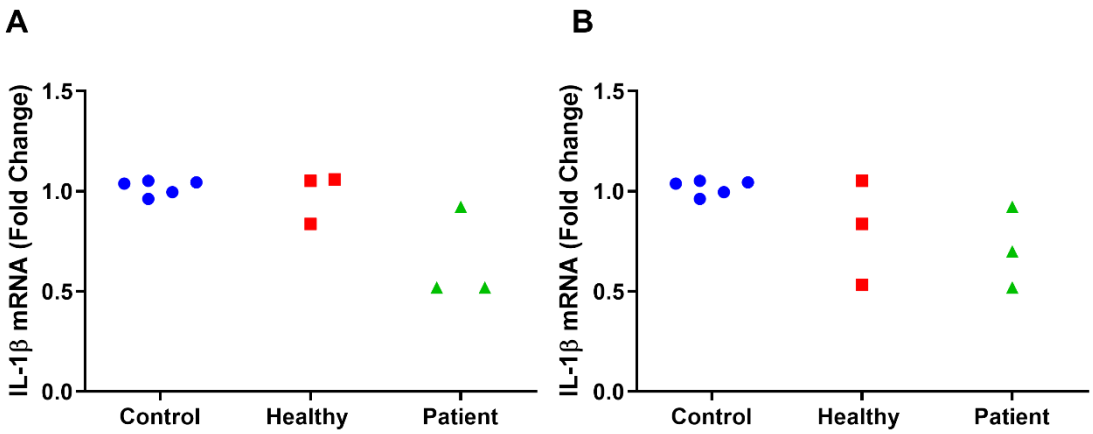


Figure 14. The level of IL-1β mRNA in cells treated with 20 µg/ml NDEs *of healthy and patient group with high expression of miRNAs. A figure: Gene expression depending on hsa-let-7e-5p level, B figure: Gene expression depending on miR-96-5p level. Results were given as mean ±SD.*

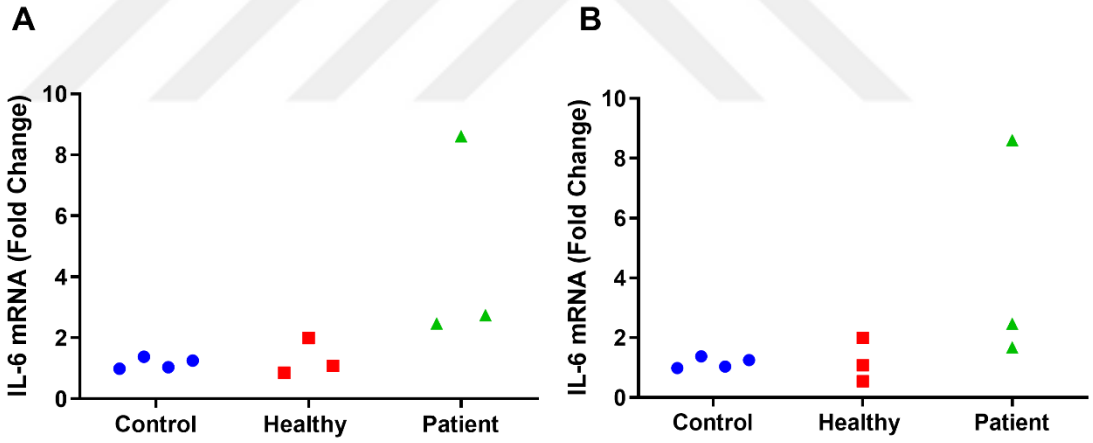


Figure 15. The level of IL-6 mRNA in cells treated with 20 µg/ml NDEs *of healthy and patient group with high expression of miRNAs. A figure: Gene expression depending on hsa-let-7e-5p level, B figure: Gene expression depending on miR-96-5p level. Results were given as mean ±SD.*

5. DISCUSSION

Alzheimer's Disease was discovered by Alois Alzheimer in the first decade of 1900s. Despite its characteristics, symptoms and molecular hallmarks were identified by researchers over a century, there is still not a definitive diagnosis method and treatment. Also, exact reasons that leading to development and progression of AD are still not elucidated. Diagnosis is based on imaging techniques such as PET and detection of AD-associated biomarkers in CSF or other body fluids. Pharmacologic treatments are limited to decelerate the progression of disease and repress the symptoms but they do not provide healing of patient (Weller & Budson, 2018). Since clinical features appear after the years AD arises, early diagnosis is very important as much as treatments. New strategies are focused on developing early diagnosis and precaution methods (Hane et al., 2017).

Also, biomarkers are measurable clinical identifiers of a medical state of patients and they help to determine pathological condition and alterations of a body. Dysregulated miRNAs, Aβ, tau, inflammatory cytokines, proteins, and reactive oxygen species (ROS) are considered as possible biomarkers in blood, urine or CSF for AD (N. Sharma & Singh, 2016). Blood is an easier source to determine biomarkers because it is less invasive and it can be obtained from patients easily. There is an elevation upon blood-based biomarkers in AD researches (Zetterberg & Burnham, 2019), (Zetterberg & Schott, 2019).

Since exosomes were discovered in 1983, researches have been shown that exosomes are considered as mediators in normal physiology and several diseases. They have many roles in cells such as neurogenesis (P. Sharma et al., 2019), inflammation and inflammatory diseases (Chan et al., 2019), cancer (C. Zhang et al., 2018), (Wang et al., 2018), (Huang, Yang, Li, Yang, & Feng, 2018), and cellular communication (Men et al., 2019). When exosomes are released from cells, they can reflect the state of origin cells because they carry cargo molecules including proteins, lipids and nucleic acids which may be up and deregulated in diseases and pathological conditions. Therefore, exosomes are considered as new biomarker source for neurodegenerative diseases such as AD, too.

In the present study, firstly, total exosomes were isolated from plasma samples of AD patient and healthy individual groups. Exosomes were characterized by determining size and exosomal marker. After enrichment of NDEs from total exosome samples, miRNA alterations in NDEs of AD patients were determined by small-RNA sequencing and pathway analysis of

deregulated miRNAs was performed. Validation of the most dysregulated miRNA was assessed by qRT-PCR. Then, the effect of NDEs in cell-to-cell interaction was investigated.

5.1. miRNA Alterations in NDEs of AD Patients

MiRNA alterations in NDEs of AD patients and healthy individuals were examined and compared to determine differences between these groups and potential biomarker miRNAs. Fiandaca et. al. showed that neuronal exosomes enriched from blood samples may reflect neuronal alterations in *vivo* and they developed a method to separate neuronal exosomes (Fiandaca et al., 2015). For this purpose, they used anti-L1CAM (L1 cell adhesion molecule) antibody to precipitate neuronal exosomes. L1CAM is expressed only in neurons in central nervous system. Faure et. al. discovered that this molecule was found in exosomes which were purified from rat primary cortical cultures that consisting of mainly neurons (Faure et al., 2006). Mustapic et. al. showed that L1CAM-exosomes have higher amount of neuron specific proteins versus total plasma exosomes (Mustapic et al., 2017). Therefore, in this thesis, L1CAM immunoprecipitation was used to separate NDEs which may reflect more accurate physiology of neurons that were damaged in AD pathology.

MiRNAs are small RNA species that regulates expression of genes. They are abnormally dysregulated in variable diseases. Analysis of miRNA alteration between healthy and patient groups is performed on several researchs to determine potential biomarkers for disease. Specific miRNAs or combination of a miRNA group may give information about the pathological condition of disease process. Various studies have shown that miRNA alterations in brain, plasma, serum, CSF and exosomes as seen in Chapter 2.3.

Exosomal miRNAs are promising sources that can be used as markers. In this study, 3 miRNAs were found as upregulated (hsa-let-7e-5p, hsa-miR-96-5p, hsa-miR-484) and 7 miRNAs were found as downregulated (hsa-miR-99b-5p, hsa-miR-100-5p, hsa-miR-30e-5p, hsa-miR-378i, hsa-miR-145-5p, hsa-miR-378c, hsa-miR-451a) in NDEs isolated from plasma samples of AD patients.

hsa-let-7e was the most significantly altered and upregulated miRNA. Let-7 family is one of the most expressed miRNAs in microglial cells and neurons. Binding of let-7 members to TLRs triggers the releasing of inflammatory cytokines IL-6 and TNF-α and also activates Nf-κβ signaling pathway and finally leads to neurodegeneration (Fabbri et al., 2012). TLRs are

thought as associated with neurological disorders including AD. In the study of Derkow et. al., they found that level of let-7 members were variable, let-7e was elevated and associated with extracellular vesicles in AD (Derkow et al., 2018). Also, hsa-let-7 family was appeared as the most deregulated miRNA group in AD exosomes (Gámez-Valero et al., 2019). Therefore, hsa-let-7e miRNA was chosen as first miRNA for validation by qPCR in our study. Deregulation of this miRNA has not been showed before in NDEs in AD. According to the results, level of hsa-let-7e-5p was elevated in patient group significantly and AUC was determined as 0.9214 with 100% specifity and 85.7% sensitivity by ROC analysis. When AUC value is near to 1, distinguishing of healthy and patient groups gets better. These results may make let-7e miRNA a signature in NDEs of AD.

miR-96-5p elevation may be important of neuroprotection. In a study of Kinoshita et. al., it was found that EAAC1 molecule that is associated with neuronal glutathione production was downregulated via miR-96-5p. Decreased glutathione level is correlated with AD. Level of EAAC1 was upregulated and neuroprotection was enabled by inhibiton of miR-96-5p (Kinoshita et al., 2014). In our study, elevation of miR-96-5p in neuronal exosomes is similar to AD-related miRNA markers.

miR-484 was detected as upregulated in plasma exosomes of AD patients (Rani et al., 2017). The result of this thesis may support that data after qPCR validation of this miRNA.

miR-30e-5p was found as downregulated in plasma neuronal exosomes in the current study. Alteration of this miRNA was determined in several studies before. In the study of Cheng et.al., level of hsa-miR-30e-5p was upregulated in serum exosomes (Cheng et al., 2015). In another study of McKeever and Schneider, miR-451a was found as diminished in CSF exosomes of young-onset and late-onset AD patients (McKeever et al., 2018) and our study indicated that miR-451a was also downregulated in plasma neuronal exosomes. Decreased level of miR-451a was reported in several studies in hippocampus and CSF in AD (Cogswell et al., 2008). Since miR-451 is one of those enriched miRNAs in neuron cells (Jovicic et al., 2013), decreasing level of that in NDEs may be a signature of AD.

miR-99b-5p was found as correlated with AD (Patrick et al., 2017). Also, the role of miR-99b-5p and miR-100-5p was studied in a research and it was found that these miRNAs were decreased at early stage of AD in APP/PS1 mice. Also it was investigated that mTOR is target of these miRNAs and they induce apoptosis by treating mice with Aβ and it has an effect

on neuronal survival (Ye et al., 2015).

Although upregulation of miR-378h, 378f, 378b, 378d, 378c, 378a, 378e was found in CSF and serum of AD patients (Burgos et al., 2014), miR-378i was not detected before in any research. Also, there was no any study the role of miR-145 in AD.

5.2. Pathway Analysis of Deregulated miRNAs

MiRNA pathway analysis was accessed to examine if these miRNAs have a role in neurodegeneration or AD-related pathways. 25 significant pathways were identified by KEGG pathway analysis and the most remarkable and AD-related ones of them are apoptosis, PI3K-Akt signaling pathway, neurotrophin signaling pathway, toll-like receptor pathway, Nf-κβ signaling pathway, and also cancer-associated pathways.

Apoptosis is one of the most important pathways. Apoptosis is known as programmed cell death that is induced by stimuli and it mediates clearance of damaged cells. Several studies have shown that neuronal cell death in AD was mediated by apoptosis (S. Zhao, Zhao, Zhang, & Guo, 2016). Oxidative stress is reasonable for Aβ aggregation in neurons and leads to accumulation of ROS which induces cell death via apoptosis (Daulatzai, 2017). Also, recent studies have shown that Nf-κβ signaling pathway mediates apoptosis of neuron cells (Chen et al., 2012). In the study of Cui et.al., they indicated that downregulation of endosplasmic reticulum stress through activation of PI3K/AKT signalling pathway in mice with AD model led to neuroprotection of cells (Cui et al., 2017).

Nf-κβ signaling mediates inflammation, apoptosis, oxidative stress and Nf-κβ is stimulated by ROS, cytokines, growth factors, or glutamate in neurons (Jones & Kounatidis, 2017). Neurotrophin signaling that includes nerve growth factor, neurotrophins, brain-derived neurotrophic factors, is another activator of Nf-κβ and it is correlated with AD. Neurotrophins are proteins beneficial for growth, differentiation and survival of neuron cells and they have potential for treatment of AD. This signaling pathway is occurred by two ways and one of them is PI3K-AKT (phosphoinositide 3-kinase or protein kinase B). In study of Kazim et.al, mimetic molecules of neurotrophic factors enhanced regeneration and repair of neurons (Kazim & Iqbal, 2016).

Toll-like receptors (TLRs) are recognition receptors. Activation of signal cascade is

induced by binding of a ligand onto TLRs. This signaling triggers cytokine, chemokine and NOS production and it is linked to activation of Nf-κβ or MAP kinase pathways (Han & Ulevitch, 2005).

As a conclusion, these pathways are related with each other and play a role in AD. However, our study is limited with validation of only hsa-let-7e-5p miRNA. Other miRNAs are required to be validated and combined analysis of them will be more accurate for pathway analysis and also diagnosis of AD via NDEs.

5.3. The Role of NDEs in Cellular Interactions

Next objective of this thesis was examining cell-to-cell interaction via exosomes and determining the effect on microglial cells treated with NDEs of AD patients and healthy controls. This represents neuron-glia interaction via exosomes in a sense. First step for this purpose was to visualize internalization of labeled NDEs to HMC3 microglial cells. PKH67 fluorescent dye was used for staining. PKH dyes enhance strong and stable signal power for imaging of exosomes or EVs and they are less prone to leak from exosomes to cell membrane (Horan, Melnicoff, Jensen, & Slezak, 1990). Uptake of exosomes by microglial cells was visualized in various studies (Yuyama, Sun, Mitsutake, & Igarashi, 2012).

After dose determination, firstly 10 and 20 μg/ml NDE doses were used for treatment with HMC3 cells to measure cytokines by ELISA. We hypothesized that the most upregulated exosomal miRNAs in NGS, hsa-let-7e-5p and miR-96-5p, were going to make an alteration in cytokine level of HMC3 cells via miRNAs. Therefore, NDEs of patients with AD which have high amount of hsa-let-7e-5p and miR-96-5p; and NDEs of healthy controls that have low amount of these miRNAs were used for treatments. We did not prove this suggestion at protein level. IL-1β and TNF-α cytokines were not detected in control group and NDE-treated HMC3 cells, as similar as previous studies (Ambrosius et al., 2017), (Rajalakshmy, Malathi, & Madhavan, 2015), (Hjorth et al., 2013). There was no significant alteration of IL-6 at protein level between control and patient exosome groups and also between healthy and patient exosome groups in this thesis.

These cytokines were also quantified by qPCR. Only 20 μg/ml NDE dose was used for qPCR experiments because that protein level of IL-6 was not altered with treatment of lower dose of NDEs. Therefore, it was suggested that higher dose treatment may be more effective

for gene expression. Expression of TNF-α was not detected as what it was at protein level. This result is investigatable because that TNF-α was expressed in these cells in a study of Jadhav et.al. (Jadhav, Krause, & Singh, 2014). The most important finding is that IL-6 gene expression level was elevated in patient group insignificantly for 2 miRNAs. Content of isolated NDEs from patients may differ from each other because of gender, age, progress of AD, etc and they affect cell in different levels. Although elevation was not significant, it was shown that higher level of IL-6 mRNA expression compared to control and healthy exosome treated cells. In various studies, it was shown that cytokine levels were altered in different cell types including microglia via exosomal miRNAs (Ramanathan et al., 2019), (Salvi et al., 2018), (Y. Yang et al., 2019). Inhibitors of these miRNAs need to be used in cells for understanding that this effect was occurred via these specific exosomal miRNAs.

5.4. Characterization of Exosomes

Exosome characterization is very important and required step to confirm exosome characteristics. Since electron microscopy is considered as gold standard imaging method for morphological characterization and size determination of extracellular vesicles because of its high resolution, TEM imaging was performed to examine their size and shapes (Chuo, Chien, & Lai, 2018), (X. Li et al., 2019). In first samples, exosomes were observed as aggregated and in bigger size than normal range. A study of Martins et. al. indicates that high level of exosomes is more prone to be aggregated (Soares Martins, Catita, Martins Rosa, O, & Henriques, 2018). Thereby, dilution was applied in exosome samples to overcome this problem. Also, samples were vortexed to disturb clumped exosomes. Characteristic shape of exosomes is considered as spherical. Chernyshev et.al. confirmed this hypothesis via TEM imaging of both normal hydrated and desiccated exosomes. When exosomes are exposed to different conditions (drying, thawing, etc.), their morphology can be changed and that affects their shape and size. It was found that hydrated exosomes are prone to be spherical shape (Chernyshev et al., 2015). In this study, it was shown that isolated exosomes from plasma samples have characteristic spherical shape and size (~60-80 nm).

Another method was detection of exosomal markers by Western blotting. Exosomes have several specific proteins which are considered as markers. Tetraspanins are the most common marker group among them. CD63, CD9, CD81 are most common tetraspanins that are

enriched in exosome membrane and CD63 is one of the most found ten proteins (Yakimchuk, 2015). Western blotting results showed that CD63 protein was detected in both healthy and patient exosomes and as a negative control, Calnexin, which is non-exosomal endoplasmic reticulum protein was not detected in them.

5.5. Cytotoxic Effect of High Dose NDEs in HMC3 Cells

Since various doses of exosomes were treated with microglial cells in reference studies such as 10 μg/ml (M. Jiang et al., 2018), 30 μg/ml (Ding et al., 2018) and 50 μg/ml (Feng, Jia, & Huang, 2019), we decided to determine non-toxic and optimum dose of NDEs. As a result of viability and cytotoxicity assay, 40 and 50 μg/ml doses of healthy and patient groups' NDEs were found cytotoxic for HMC3 microglial cells.

5.6. Limitations

Since IL-6 mRNA level was found upregulated in cells treated with NDEs of patients which have high amount of hsa-let-7e-5p and miR-96-5p, inhibitors of these miRNAs must be used and reverse effect of IL-6 level in patient group must be determined to confirm upregulation of IL-6 occurred via exosomal miRNAs. Also, level of hsa-let-7e-5p and miR-96-5p must be determined after treatment with NDEs of patients. At this point, after determining the level of these miRNAs in recipient cells, exosome uptake inhibitors can be used to examine whether this effect is because of exosomes. Alexander et.al. demonstrated that exosomal miRNAs miR-155 and miR-146 regulates inflammatory responses to endotoxin by using GW4869, an exosome uptake inhibitor (Alexander et al., 2015). Inhibitors or mimics of selected miRNAs are often used to understand that whether exosomal miRNAs participate in that regulation by reversing those miRNAs effect (Kanlikilicer et al., 2018), (X. Zhang et al., 2019), (Naseri, Oskuee, Jaafari, & Forouzandeh Moghadam, 2018).

Except characterization methods which had been used in this study, exosome characterization can be performed by several techniques. Nanoparticle Tracking Analysis (NTA) and Dynamic Light Scattering (DLS) are very common methods for this purpose and they are thought to be performed for supporting data of this study. NTA is a method based on tracking Brownian motion of nanoparticles in a suspension, it means NTA tracks the movement

of exosomes and it can measure size and concentration of exosomes based on this movement. Also, it allows particle phenotype to be observed. It is easy method and also makes possible to use the sample after measurement (Szatanek et al., 2017). Therefore, after TEM imaging, NTA is the most popular characterization method and it is used in almost every research (Takov, Yellon, & Davidson, 2019).

DLS is the technique based on scattering of laser beam. Laser beam passes through suspension and when it hits a particle, light is dispersed and scattered. Intensity of light is correlated with size of exosomes but this method is not used for visualizing of sample (Lawrie, Albanyan, Cardigan, Mackie, & Harrison, 2009), and it requires long running time. Also, the most disadvantage of this method is that suspension must consist of one type of particle. Varying in size of particles prevent the detection of smaller molecules (Hoo, Starostin, West, & Mecartney, 2008). DLS was performed to measure size and size distribution of extracellular vesicles isolated from red blood cells in the studies (Lawrie et al., 2009)

These techniques may be used for supporting characterization data of this study as a future aspect.

6.CONCLUSION AND SUGGESTIONS

- 10 miRNAs were found to be deregulated in NDEs of patients with AD compared to healthy control group. The most significant alteration was upregulation of hsa-let-7e-5p. Validation of hsa-let-7e-5p was performed in exosomal RNAs by qPCR successfully. Level of hsa-let-7e-5p was upregulated and ROC analysis showed that it has high sensitivity and specifity to diagnose AD in patients. Promising signature potential of hsa-let-7e-5p in NDEs of patients with AD was demonstrated in this study. Validation of all miRNAs in NDEs may be performed in more comprehensive healthy control and AD patient population to impower of reliability and enhance confidence interval.
- Gene expression of IL-6 mRNA was upregulated insignificantly in HMC3 cells treated with patient NDEs (20 μg/ml) which contain high level of hsa-let-7e-5p and miR-96-5p miRNAs. To evaluate that if this effect was occurred via upregulated miRNAs, firstly gene expression of hsa-let-7e-5p and miR-96-5p in HMC3 cells must be assessed after treatment with NDEs by qRT-PCR. Then, inhibitors of hsa-let-7e-5p and miR-96-5p must be used and reverse effect must be confirmed as down-regulation of IL-6 mRNA level.
- Cytokine protein levels in HMC3 cells were not changed after treatment with 10 and 20 μg/ml doses of NDEs. 30 μg/ml dose of NDE treatment may be used since it is nontoxic dose as 10 and 20 μg/ml.
- Cytotoxic effect of NDEs in HMC3 cells was assessed by viability and toxicity assays. 40 and 50 μg/ml doses were found to be cytotoxic for cells.
- Exosome characterization was assessed by TEM imaging and determination of exosomal marker (CD63) with Western blotting. Other exosomal markers (CD9, TSG101, Alix, etc.) may be detected by Western blotting and also NTA and DLS may be performed for size characterization of exosomes to support the data.
- Exosome uptake inhibitors (Dynasore, Cytoclasin D, EIPA, etc.) may be used for determining the uptake route of exosomes by HMC3 cells.
- Phagocytosis function and migration ability of microglial cells will be examined with exosome treatment.

7. REFERENCES

Abels, E. R., & Breakefield, X. O. (2016). Introduction to Extracellular Vesicles: Biogenesis, RNA Cargo Selection, Content, Release, and Uptake. *Cell Mol Neurobiol, 36*(3), 301-312. doi:10.1007/s10571-016-0366-z

Alexander, M., Hu, R., Runtsch, M. C., Kagele, D. A., Mosbruger, T. L., Tolmachova, T., . . . O'Connell, R. M. (2015). Exosome-delivered microRNAs modulate the inflammatory response to endotoxin. *Nat Commun, 6*, 7321. doi:10.1038/ncomms8321

Alexandrov, P. N., Dua, P., Hill, J. M., Bhattacharjee, S., Zhao, Y., & Lukiw, W. J. (2012). microRNA (miRNA) speciation in Alzheimer's disease (AD) cerebrospinal fluid (CSF) and extracellular fluid (ECF). *Int J Biochem Mol Biol, 3*(4), 365-373.

Allmer, J., & Yousef, M. (2016). Computational miRNomics. *J Integr Bioinform, 13*(5), 1-2. doi:10.1515/jib-2016-302

Ambrosius, B., Faissner, S., Guse, K., von Lehe, M., Grunwald, T., Gold, R., . . . Chan, A. (2017). Teriflunomide and monomethylfumarate target HIV-induced neuroinflammation and neurotoxicity. *J Neuroinflammation, 14*(1), 51. doi:10.1186/s12974-017-0829-2

Association, A. s. (2019). 2019 Alzheimer's Disease Facts and Figures. *Alzheimer's and Dementia, 15*(3). doi:10.1016/j.jalz.2019.01.010

Ballard, C., Gauthier, S., Corbett, A., Brayne, C., Aarsland, D., & Jones, E. (2011). Alzheimer's disease. *Lancet, 377*(9770), 1019-1031. doi:10.1016/s0140-6736(10)61349-9

Bekris, L. M., & Leverenz, J. B. (2015). The biomarker and therapeutic potential of miRNA in Alzheimer's disease. *Neurodegener Dis Manag, 5*(1), 61-74. doi:10.2217/nmt.14.52

Bekris, L. M., Lutz, F., Montine, T. J., Yu, C. E., Tsuang, D., Peskind, E. R., & Leverenz, J. B. (2013). MicroRNA in Alzheimer's disease: an exploratory study in brain, cerebrospinal fluid and plasma. *Biomarkers, 18*(5), 455-466. doi:10.3109/1354750x.2013.814073

Bhatnagar, S., Chertkow, H., Schipper, H. M., Yuan, Z., Shetty, V., Jenkins, S., . . . Wang, E. (2014). Increased microRNA-34c abundance in Alzheimer's disease circulating blood plasma. *Front Mol Neurosci, 7*, 2. doi:10.3389/fnmol.2014.00002

Blennow, K., de Leon, M. J., & Zetterberg, H. (2006). Alzheimer's disease. *Lancet, 368*(9533), 387-403. doi:10.1016/s0140-6736(06)69113-7

Bondi, M. W., Edmonds, E. C., & Salmon, D. P. (2017). Alzheimer's Disease: Past, Present, and Future. *J Int Neuropsychol Soc, 23*(9-10), 818-831. doi:10.1017/s135561771700100x

Burgos, K., Malenica, I., Metpally, R., Courtright, A., Rakela, B., Beach, T., . . . Van Keuren-Jensen, K. (2014). Profiles of extracellular miRNA in cerebrospinal fluid and serum from patients with Alzheimer's and Parkinson's diseases correlate with disease status and features of pathology. *PLoS One, 9*(5), e94839. doi:10.1371/journal.pone.0094839

Caplan, L. R. (2017). *Primer on cerebrovascular diseases* (Second edition. ed.). London: Elsevier, Academic Press.

Caruso Bavisotto, C., Scalia, F., Marino Gammazza, A., Carlisi, D., Bucchieri, F., Conway de Macario, E., . . . Campanella, C. (2019). Extracellular Vesicle-Mediated Cell(-)Cell Communication in the Nervous System: Focus on Neurological Diseases. *Int J Mol Sci, 20*(2). doi:10.3390/ijms20020434

Chan, B. D., Wong, W. Y., Lee, M. M., Cho, W. C., Yee, B. K., Kwan, Y. W., & Tai, W. C. (2019). Exosomes in Inflammation and Inflammatory Disease. *Proteomics, 19*(8), e1800149. doi:10.1002/pmic.201800149

Chen, C.-H., Zhou, W., Liu, S., Deng, Y., Cai, F., Tone, M., . . . Song, W. (2012). Increased NF-κB signalling up-regulates BACE1 expression and its therapeutic potential in Alzheimer's disease. *International Journal of Neuropsychopharmacology, 15*(1), 77-90. doi:10.1017/s1461145711000149

Cheng, L., Doecke, J. D., Sharples, R. A., Villemagne, V. L., Fowler, C. J., Rembach, A., . . . Hill, A. F. (2015). Prognostic serum miRNA biomarkers associated with Alzheimer's disease shows concordance with neuropsychological and neuroimaging assessment. *Mol Psychiatry, 20*(10),

1188-1196. doi:10.1038/mp.2014.127
Chernyshev, V. S., Rachamadugu, R., Tseng, Y. H., Belnap, D. M., Jia, Y., Branch, K. J., . . . Skliar, M. (2015). Size and shape characterization of hydrated and desiccated exosomes. *Anal Bioanal Chem, 407*(12), 3285-3301. doi:10.1007/s00216-015-8535-3
Chuo, S. T., Chien, J. C., & Lai, C. P. (2018). Imaging extracellular vesicles: current and emerging methods. *J Biomed Sci, 25*(1), 91. doi:10.1186/s12929-018-0494-5
Cogswell, J. P., Ward, J., Taylor, I. A., Waters, M., Shi, Y., Cannon, B., . . . Richards, C. A. (2008). Identification of miRNA changes in Alzheimer's disease brain and CSF yields putative biomarkers and insights into disease pathways. *J Alzheimers Dis, 14*(1), 27-41.
Cosin-Tomas, M., Antonell, A., Llado, A., Alcolea, D., Fortea, J., Ezquerra, M., . . . Kaliman, P. (2017). Plasma miR-34a-5p and miR-545-3p as Early Biomarkers of Alzheimer's Disease: Potential and Limitations. *Mol Neurobiol, 54*(7), 5550-5562. doi:10.1007/s12035-016-0088-8
Cui, W., Wang, S., Wang, Z., Wang, Z., Sun, C., & Zhang, Y. (2017). Inhibition of PTEN Attenuates Endoplasmic Reticulum Stress and Apoptosis via Activation of PI3K/AKT Pathway in Alzheimer's Disease. *Neurochem Res, 42*(11), 3052-3060. doi:10.1007/s11064-017-2338-1
Dalvi, A. (2012). Alzheimer's disease. *Dis Mon, 58*(12), 666-677. doi:10.1016/j.disamonth.2012.08.008
Dangla-Valls, A., Molinuevo, J. L., Altirriba, J., Sanchez-Valle, R., Alcolea, D., Fortea, J., . . . Antonell, A. (2017). CSF microRNA Profiling in Alzheimer's Disease: a Screening and Validation Study. *Mol Neurobiol, 54*(9), 6647-6654. doi:10.1007/s12035-016-0106-x
Daulatzai, M. A. (2017). Cerebral hypoperfusion and glucose hypometabolism: Key pathophysiological modulators promote neurodegeneration, cognitive impairment, and Alzheimer's disease. *J Neurosci Res, 95*(4), 943-972. doi:10.1002/jnr.23777
Denk, J., Boelmans, K., Siegismund, C., Lassner, D., Arlt, S., & Jahn, H. (2015). MicroRNA Profiling of CSF Reveals Potential Biomarkers to Detect Alzheimer`s Disease. *PLoS One, 10*(5), e0126423. doi:10.1371/journal.pone.0126423
Derkow, K., Rossling, R., Schipke, C., Kruger, C., Bauer, J., Fahling, M., . . . Lehnardt, S. (2018). Distinct expression of the neurotoxic microRNA family let-7 in the cerebrospinal fluid of patients with Alzheimer's disease. *PLoS One, 13*(7), e0200602. doi:10.1371/journal.pone.0200602
Ding, M., Shen, Y., Wang, P., Xie, Z., Xu, S., Zhu, Z., . . . Yang, H. (2018). Exosomes Isolated From Human Umbilical Cord Mesenchymal Stem Cells Alleviate Neuroinflammation and Reduce Amyloid-Beta Deposition by Modulating Microglial Activation in Alzheimer's Disease. *Neurochem Res, 43*(11), 2165-2177. doi:10.1007/s11064-018-2641-5
Dong, H., Li, J., Huang, L., Chen, X., Li, D., Wang, T., . . . Zhang, C. Y. (2015). Serum MicroRNA Profiles Serve as Novel Biomarkers for the Diagnosis of Alzheimer's Disease. *Dis Markers, 2015*, 625659. doi:10.1155/2015/625659
Fabbri, M., Paone, A., Calore, F., Galli, R., Gaudio, E., Santhanam, R., . . . Croce, C. M. (2012). MicroRNAs bind to Toll-like receptors to induce prometastatic inflammatory response. *Proc Natl Acad Sci U S A, 109*(31), E2110-2116. doi:10.1073/pnas.1209414109
Faure, J., Lachenal, G., Court, M., Hirrlinger, J., Chatellard-Causse, C., Blot, B., . . . Sadoul, R. (2006). Exosomes are released by cultured cortical neurones. *Mol Cell Neurosci, 31*(4), 642-648. doi:10.1016/j.mcn.2005.12.003
Feng, N., Jia, Y., & Huang, X. (2019). Exosomes from adipose-derived stem cells alleviate neural injury caused by microglia activation via suppressing NF-kB and MAPK pathway. *J Neuroimmunol, 334*, 576996. doi:10.1016/j.jneuroim.2019.576996
Fiandaca, M. S., Kapogiannis, D., Mapstone, M., Boxer, A., Eitan, E., Schwartz, J. B., . . . Goetzl, E. J. (2015). Identification of preclinical Alzheimer's disease by a profile of pathogenic proteins in neurally derived blood exosomes: A case-control study. *Alzheimers Dement, 11*(6), 600-607.e601. doi:10.1016/j.jalz.2014.06.008
Fruhbeis, C., Frohlich, D., & Kramer-Albers, E. M. (2012). Emerging roles of exosomes in neuron-glia communication. *Front Physiol, 3*, 119. doi:10.3389/fphys.2012.00119
Gámez-Valero, A., Campdelacreu, J., Vilas, D., Ispierto, L., Reñé, R., Álvarez, R., . . . Beyer, K. (2019). Exploratory study on microRNA profiles from plasma-derived extracellular vesicles in

Alzheimer’s disease and dementia with Lewy bodies. *Transl Neurodegener, 8*(1), 31. doi:10.1186/s40035-019-0169-5
Graves, P., & Zeng, Y. (2012). Biogenesis of mammalian microRNAs: a global view. *Genomics Proteomics Bioinformatics, 10*(5), 239-245. doi:10.1016/j.gpb.2012.06.004
Guo, R., Fan, G., Zhang, J., Wu, C., Du, Y., Ye, H., . . . Lu, Z. (2017). A 9-microRNA Signature in Serum Serves as a Noninvasive Biomarker in Early Diagnosis of Alzheimer's Disease. *J Alzheimers Dis, 60*(4), 1365-1377. doi:10.3233/jad-170343
Han, J., & Ulevitch, R. J. (2005). Limiting inflammatory responses during activation of innate immunity. *Nat Immunol, 6*(12), 1198-1205. doi:10.1038/ni1274
Hane, F. T., Robinson, M., Lee, B. Y., Bai, O., Leonenko, Z., & Albert, M. S. (2017). Recent Progress in Alzheimer's Disease Research, Part 3: Diagnosis and Treatment. *J Alzheimers Dis, 57*(3), 645-665. doi:10.3233/jad-160907
Hara, N., Kikuchi, M., Miyashita, A., Hatsuta, H., Saito, Y., Kasuga, K., . . . Kuwano, R. (2017). Serum microRNA miR-501-3p as a potential biomarker related to the progression of Alzheimer's disease. *Acta Neuropathol Commun, 5*(1), 10. doi:10.1186/s40478-017-0414-z
Hjorth, E., Zhu, M., Toro, V. C., Vedin, I., Palmblad, J., Cederholm, T., . . . Schultzberg, M. (2013). Omega-3 fatty acids enhance phagocytosis of Alzheimer's disease-related amyloid-beta42 by human microglia and decrease inflammatory markers. *J Alzheimers Dis, 35*(4), 697-713. doi:10.3233/jad-130131
Hoo, C. M., Starostin, N., West, P., & Mecartney, M. L. (2008). A comparison of atomic force microscopy (AFM) and dynamic light scattering (DLS) methods to characterize nanoparticle size distributions. *Journal of Nanoparticle Research, 10*(1), 89-96. doi:10.1007/s11051-008-9435-7
Horan, P. K., Melnicoff, M. J., Jensen, B. D., & Slezak, S. E. (1990). Fluorescent cell labeling for in vivo and in vitro cell tracking. *Methods Cell Biol, 33*, 469-490. doi:10.1016/s0091-679x(08)60547-6
Huang, Z., Yang, M., Li, Y., Yang, F., & Feng, Y. (2018). Exosomes Derived from Hypoxic Colorectal Cancer Cells Transfer Wnt4 to Normoxic Cells to Elicit a Prometastatic Phenotype. *Int J Biol Sci, 14*(14), 2094-2102. doi:10.7150/ijbs.28288
Jadhav, V. S., Krause, K. H., & Singh, S. K. (2014). HIV-1 Tat C modulates NOX2 and NOX4 expressions through miR-17 in a human microglial cell line. *J Neurochem, 131*(6), 803-815. doi:10.1111/jnc.12933
Jia, L. H., & Liu, Y. N. (2016). Downregulated serum miR-223 servers as biomarker in Alzheimer's disease. *Cell Biochem Funct, 34*(4), 233-237. doi:10.1002/cbf.3184
Jiang, M., Wang, H., Jin, M., Yang, X., Ji, H., Jiang, Y., . . . Li, L. (2018). Exosomes from MiR-30d-5p-ADSCs Reverse Acute Ischemic Stroke-Induced, Autophagy-Mediated Brain Injury by Promoting M2 Microglial/Macrophage Polarization. *Cell Physiol Biochem, 47*(2), 864-878. doi:10.1159/000490078
Jiang, S., & Yan, W. (2016). Current View of microRNA Processing. *Signal Transduction Insights, 5*, STI.S12317. doi:10.4137/STI.S12317
Jones, S. V., & Kounatidis, I. (2017). Nuclear Factor-Kappa B and Alzheimer Disease, Unifying Genetic and Environmental Risk Factors from Cell to Humans. *Front Immunol, 8*, 1805. doi:10.3389/fimmu.2017.01805
Jovicic, A., Roshan, R., Moisoi, N., Pradervand, S., Moser, R., Pillai, B., & Luthi-Carter, R. (2013). Comprehensive expression analyses of neural cell-type-specific miRNAs identify new determinants of the specification and maintenance of neuronal phenotypes. *J Neurosci, 33*(12), 5127-5137. doi:10.1523/jneurosci.0600-12.2013
Kanlikilicer, P., Bayraktar, R., Denizli, M., Rashed, M. H., Ivan, C., Aslan, B., . . . Lopez-Berestein, G. (2018). Exosomal miRNA confers chemo resistance via targeting Cav1/p-gp/M2-type macrophage axis in ovarian cancer. *EBioMedicine, 38*, 100-112. doi:10.1016/j.ebiom.2018.11.004
Kazim, S. F., & Iqbal, K. (2016). Neurotrophic factor small-molecule mimetics mediated neuroregeneration and synaptic repair: emerging therapeutic modality for Alzheimer’s disease. *Mol Neurodegener, 11*(1), 50. doi:10.1186/s13024-016-0119-y
Kiko, T., Nakagawa, K., Tsuduki, T., Furukawa, K., Arai, H., & Miyazawa, T. (2014). MicroRNAs in

plasma and cerebrospinal fluid as potential markers for Alzheimer's disease. *J Alzheimers Dis, 39*(2), 253-259. doi:10.3233/jad-130932
Kinoshita, C., Aoyama, K., Matsumura, N., Kikuchi-Utsumi, K., Watabe, M., & Nakaki, T. (2014). Rhythmic oscillations of the microRNA miR-96-5p play a neuroprotective role by indirectly regulating glutathione levels. *Nature Communications, 5*(1), 3823. doi:10.1038/ncomms4823
Kumar, P., Dezso, Z., MacKenzie, C., Oestreicher, J., Agoulnik, S., Byrne, M., . . . Oda, Y. (2013). Circulating miRNA biomarkers for Alzheimer's disease. *PLoS One, 8*(7), e69807. doi:10.1371/journal.pone.0069807
Kumar, S., & Reddy, P. H. (2016). Are circulating microRNAs peripheral biomarkers for Alzheimer's disease? *Biochim Biophys Acta, 1862*(9), 1617-1627. doi:10.1016/j.bbadis.2016.06.001
Lane, C. A., Hardy, J., & Schott, J. M. (2018). Alzheimer's disease. *Eur J Neurol, 25*(1), 59-70. doi:10.1111/ene.13439
Lawrie, A. S., Albanyan, A., Cardigan, R. A., Mackie, I. J., & Harrison, P. (2009). Microparticle sizing by dynamic light scattering in fresh-frozen plasma. *Vox Sang, 96*(3), 206-212. doi:10.1111/j.1423-0410.2008.01151.x
Li, J., Jiang, X., & Wang, K. (2019). Exosomal miRNA: an alternative mediator of cell-to-cell communication. *ExRNA, 1*(1), 31. doi:10.1186/s41544-019-0025-x
Li, X., Corbett, A. L., Taatizadeh, E., Tasnim, N., Little, J. P., Garnis, C., . . . Li, I. T. S. (2019). Challenges and opportunities in exosome research-Perspectives from biology, engineering, and cancer therapy. *APL Bioeng, 3*(1), 011503. doi:10.1063/1.5087122
Liao, W., Du, Y., Zhang, C., Pan, F., Yao, Y., Zhang, T., & Peng, Q. (2019). Exosomes: The next generation of endogenous nanomaterials for advanced drug delivery and therapy. *Acta Biomater, 86*, 1-14. doi:10.1016/j.actbio.2018.12.045
Lugli, G., Cohen, A. M., Bennett, D. A., Shah, R. C., Fields, C. J., Hernandez, A. G., & Smalheiser, N. R. (2015). Plasma Exosomal miRNAs in Persons with and without Alzheimer Disease: Altered Expression and Prospects for Biomarkers. *PLoS One, 10*(10), e0139233. doi:10.1371/journal.pone.0139233
Lukiw, W. J. (2007). Micro-RNA speciation in fetal, adult and Alzheimer's disease hippocampus. *Neuroreport, 18*(3), 297-300. doi:10.1097/WNR.0b013e3280148e8b
Lusardi, T. A., Phillips, J. I., Wiedrick, J. T., Harrington, C. A., Lind, B., Lapidus, J. A., . . . Saugstad, J. A. (2017). MicroRNAs in Human Cerebrospinal Fluid as Biomarkers for Alzheimer's Disease. *J Alzheimers Dis, 55*(3), 1223-1233. doi:10.3233/jad-160835
Maoz, R., Garfinkel, B. P., & Soreq, H. (2017). Alzheimer's Disease and ncRNAs. In R. Delgado-Morales (Ed.), *Neuroepigenomics in Aging and Disease* (pp. 337-361). Cham: Springer International Publishing.
Masters, C. L., Bateman, R., Blennow, K., Rowe, C. C., Sperling, R. A., & Cummings, J. L. (2015). Alzheimer's disease. *Nat Rev Dis Primers, 1*, 15056. doi:10.1038/nrdp.2015.56
McKeever, P. M., Schneider, R., Taghdiri, F., Weichert, A., Multani, N., Brown, R. A., . . . Tartaglia, M. C. (2018). MicroRNA Expression Levels Are Altered in the Cerebrospinal Fluid of Patients with Young-Onset Alzheimer's Disease. *Mol Neurobiol, 55*(12), 8826-8841. doi:10.1007/s12035-018-1032-x
Men, Y., Yelick, J., Jin, S., Tian, Y., Chiang, M. S. R., Higashimori, H., . . . Yang, Y. (2019). Exosome reporter mice reveal the involvement of exosomes in mediating neuron to astroglia communication in the CNS. *Nature Communications, 10*(1), 4136. doi:10.1038/s41467-019-11534-w
Mulcahy, L. A., Pink, R. C., & Carter, D. R. (2014). Routes and mechanisms of extracellular vesicle uptake. *J Extracell Vesicles, 3*. doi:10.3402/jev.v3.24641
Mustapic, M., Eitan, E., Werner, J. K., Jr., Berkowitz, S. T., Lazaropoulos, M. P., Tran, J., . . . Kapogiannis, D. (2017). Plasma Extracellular Vesicles Enriched for Neuronal Origin: A Potential Window into Brain Pathologic Processes. *Front Neurosci, 11*, 278. doi:10.3389/fnins.2017.00278
Naseri, Z., Oskuee, R. K., Jaafari, M. R., & Forouzandeh Moghadam, M. (2018). Exosome-mediated delivery of functionally active miRNA-142-3p inhibitor reduces tumorigenicity of breast cancer

in vitro and in vivo. *Int J Nanomedicine, 13*, 7727-7747. doi:10.2147/ijn.s182384
Newman, E. A. (2003). New roles for astrocytes: regulation of synaptic transmission. *Trends Neurosci, 26*(10), 536-542. doi:10.1016/s0166-2236(03)00237-6
Pan, B. T., & Johnstone, R. M. (1983). Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: selective externalization of the receptor. *Cell, 33*(3), 967-978.
Pascua-Maestro, R., Gonzalez, E., Lillo, C., Ganfornina, M. D., Falcon-Perez, J. M., & Sanchez, D. (2018). Extracellular Vesicles Secreted by Astroglial Cells Transport Apolipoprotein D to Neurons and Mediate Neuronal Survival Upon Oxidative Stress. *Front Cell Neurosci, 12*, 526. doi:10.3389/fncel.2018.00526
Patrick, E., Rajagopal, S., Wong, H. A., McCabe, C., Xu, J., Tang, A., . . . De Jager, P. L. (2017). Dissecting the role of non-coding RNAs in the accumulation of amyloid and tau neuropathologies in Alzheimer's disease. *Mol Neurodegener, 12*(1), 51. doi:10.1186/s13024-017-0191-y
Paul, P., Chakraborty, A., Sarkar, D., Langthasa, M., Rahman, M., Bari, M., . . . Chakraborty, S. (2018). Interplay between miRNAs and human diseases. *J Cell Physiol, 233*(3), 2007-2018. doi:10.1002/jcp.25854
Pauley, K. M., & Chan, E. K. (2008). MicroRNAs and their emerging roles in immunology. *Ann N Y Acad Sci, 1143*, 226-239. doi:10.1196/annals.1443.009
Rajalakshmy, A. R., Malathi, J., & Madhavan, H. N. (2015). Hepatitis C Virus NS3 Mediated Microglial Inflammation via TLR2/TLR6 MyD88/NF-kappaB Pathway and Toll Like Receptor Ligand Treatment Furnished Immune Tolerance. *PLoS One, 10*(5), e0125419. doi:10.1371/journal.pone.0125419
Ramanathan, S., Douglas, S. R., Alexander, G. M., Shenoda, B. B., Barrett, J. E., Aradillas, E., . . . Ajit, S. K. (2019). Exosome microRNA signatures in patients with complex regional pain syndrome undergoing plasma exchange. *Journal of Translational Medicine, 17*(1), 81. doi:10.1186/s12967-019-1833-3
Rani, A., O'Shea, A., Ianov, L., Cohen, R. A., Woods, A. J., & Foster, T. C. (2017). miRNA in Circulating Microvesicles as Biomarkers for Age-Related Cognitive Decline. *Front Aging Neurosci, 9*(323). doi:10.3389/fnagi.2017.00323
Reddy, P. H., Williams, J., Smith, F., Bhatti, J. S., Kumar, S., Vijayan, M., . . . Reddy, A. P. (2017). MicroRNAs, Aging, Cellular Senescence, and Alzheimer's Disease. *Prog Mol Biol Transl Sci, 146*, 127-171. doi:10.1016/bs.pmbts.2016.12.009
Ren, R. J., Zhang, Y. F., Dammer, E. B., Zhou, Y., Wang, L. L., Liu, X. H., . . . Cheng, Q. (2016). Peripheral Blood MicroRNA Expression Profiles in Alzheimer's Disease: Screening, Validation, Association with Clinical Phenotype and Implications for Molecular Mechanism. *Mol Neurobiol, 53*(8), 5772-5781. doi:10.1007/s12035-015-9484-8
Reza-Zaldivar, E. E., Hernandez-Sapiens, M. A., Minjarez, B., Gutierrez-Mercado, Y. K., Marquez-Aguirre, A. L., & Canales-Aguirre, A. A. (2018). Potential Effects of MSC-Derived Exosomes in Neuroplasticity in Alzheimer's Disease. *Front Cell Neurosci, 12*, 317. doi:10.3389/fncel.2018.00317
Salvi, V., Gianello, V., Busatto, S., Bergese, P., Andreoli, L., D'Oro, U., . . . Bosisio, D. (2018). Exosome-delivered microRNAs promote IFN-alpha secretion by human plasmacytoid DCs via TLR7. *JCI Insight, 3*(10). doi:10.1172/jci.insight.98204
Scheltens, P., Blennow, K., Breteler, M. M., de Strooper, B., Frisoni, G. B., Salloway, S., & Van der Flier, W. M. (2016). Alzheimer's disease. *Lancet, 388*(10043), 505-517. doi:10.1016/s0140-6736(15)01124-1
Schipper, H. M., Maes, O. C., Chertkow, H. M., & Wang, E. (2007). MicroRNA expression in Alzheimer blood mononuclear cells. *Gene Regul Syst Bio, 1*, 263-274.
Sharma, N., & Singh, A. N. (2016). Exploring Biomarkers for Alzheimer's Disease. *J Clin Diagn Res, 10*(7), Ke01-06. doi:10.7860/jcdr/2016/18828.8166
Sharma, P., Mesci, P., Carromeu, C., McClatchy, D. R., Schiapparelli, L., Yates, J. R., . . . Cline, H. T. (2019). Exosomes regulate neurogenesis and circuit assembly. *Proceedings of the National Academy of Sciences, 116*(32), 16086-16094. doi:10.1073/pnas.1902513116

Siedlecki-Wullich, D., Catala-Solsona, J., Fabregas, C., Hernandez, I., Clarimon, J., Lleo, A., . . . Minano-Molina, A. J. (2019). Altered microRNAs related to synaptic function as potential plasma biomarkers for Alzheimer's disease. *Alzheimers Res Ther, 11*(1), 46. doi:10.1186/s13195-019-0501-4

Simons, M., & Raposo, G. (2009). Exosomes--vesicular carriers for intercellular communication. *Curr Opin Cell Biol, 21*(4), 575-581. doi:10.1016/j.ceb.2009.03.007

Soares Martins, T., Catita, J., Martins Rosa, I., O, A. B. d. C. E. S., & Henriques, A. G. (2018). Exosome isolation from distinct biofluids using precipitation and column-based approaches. *PLoS One, 13*(6), e0198820. doi:10.1371/journal.pone.0198820

Szatanek, R., Baj-Krzyworzeka, M., Zimoch, J., Lekka, M., Siedlar, M., & Baran, J. (2017). The Methods of Choice for Extracellular Vesicles (EVs) Characterization. *Int J Mol Sci, 18*(6). doi:10.3390/ijms18061153

Takov, K., Yellon, D. M., & Davidson, S. M. (2019). Comparison of small extracellular vesicles isolated from plasma by ultracentrifugation or size-exclusion chromatography: yield, purity and functional potential. *J Extracell Vesicles, 8*(1), 1560809. doi:10.1080/20013078.2018.1560809

Tan, C. C., Yu, J. T., & Tan, L. (2014). Biomarkers for preclinical Alzheimer's disease. *J Alzheimers Dis, 42*(4), 1051-1069. doi:10.3233/jad-140843

Tan, L., Yu, J. T., Tan, M. S., Liu, Q. Y., Wang, H. F., Zhang, W., . . . Tan, L. (2014). Genome-wide serum microRNA expression profiling identifies serum biomarkers for Alzheimer's disease. *J Alzheimers Dis, 40*(4), 1017-1027. doi:10.3233/jad-132144

Treiber, T., Treiber, N., & Meister, G. (2019). Regulation of microRNA biogenesis and its crosstalk with other cellular pathways. *Nat Rev Mol Cell Biol, 20*(1), 5-20. doi:10.1038/s41580-018-0059-1

Van Giau, V., & An, S. S. (2016). Emergence of exosomal miRNAs as a diagnostic biomarker for Alzheimer's disease. *J Neurol Sci, 360*, 141-152. doi:10.1016/j.jns.2015.12.005

Villa, C., Fenoglio, C., De Riz, M., Clerici, F., Marcone, A., Benussi, L., . . . Galimberti, D. (2011). Role of hnRNP-A1 and miR-590-3p in neuronal death: genetics and expression analysis in patients with Alzheimer disease and frontotemporal lobar degeneration. *Rejuvenation Res, 14*(3), 275-281. doi:10.1089/rej.2010.1123

Vishnoi, A., & Rani, S. (2017). MiRNA Biogenesis and Regulation of Diseases: An Overview. *Methods Mol Biol, 1509*, 1-10. doi:10.1007/978-1-4939-6524-3_1

Wang, S., Xu, M., Li, X., Su, X., Xiao, X., Keating, A., & Zhao, R. C. (2018). Exosomes released by hepatocarcinoma cells endow adipocytes with tumor-promoting properties. *J Hematol Oncol, 11*(1), 82. doi:10.1186/s13045-018-0625-1

Weller, J., & Budson, A. (2018). Current understanding of Alzheimer's disease diagnosis and treatment. *F1000Res, 7*. doi:10.12688/f1000research.14506.1

Xiao, T., Zhang, W., Jiao, B., Pan, C. Z., Liu, X., & Shen, L. (2017). The role of exosomes in the pathogenesis of Alzheimer' disease. *Transl Neurodegener, 6*, 3. doi:10.1186/s40035-017-0072-x

Xie, B., Liu, Z., Jiang, L., Liu, W., Song, M., Zhang, Q., . . . Xu, S. (2017). Increased Serum miR-206 Level Predicts Conversion from Amnestic Mild Cognitive Impairment to Alzheimer's Disease: A 5-Year Follow-up Study. *J Alzheimers Dis, 55*(2), 509-520. doi:10.3233/jad-160468

Yakimchuk, K. (2015). Exosomes: isolation methods and specific markers. *Materials and Methods, 5*. doi:10.13070/mm.en.5.1450

Yang, T. T., Liu, C. G., Gao, S. C., Zhang, Y., & Wang, P. C. (2018). The Serum Exosome Derived MicroRNA-135a, -193b, and -384 Were Potential Alzheimer's Disease Biomarkers. *Biomed Environ Sci, 31*(2), 87-96. doi:10.3967/bes2018.011

Yang, Y., Ye, Y., Kong, C., Su, X., Zhang, X., Bai, W., & He, X. (2019). MiR-124 Enriched Exosomes Promoted the M2 Polarization of Microglia and Enhanced Hippocampus Neurogenesis After Traumatic Brain Injury by Inhibiting TLR4 Pathway. *Neurochem Res, 44*(4), 811-828. doi:10.1007/s11064-018-02714-z

Ye, X., Luo, H., Chen, Y., Wu, Q., Xiong, Y., Zhu, J., . . . Wan, J. (2015). MicroRNAs 99b-5p/100-5p Regulated by Endoplasmic Reticulum Stress are Involved in Abeta-Induced Pathologies. *Front Aging Neurosci, 7*, 210. doi:10.3389/fnagi.2015.00210

Yuyama, K., Sun, H., Mitsutake, S., & Igarashi, Y. (2012). Sphingolipid-modulated exosome secretion

promotes clearance of amyloid-beta by microglia. *J Biol Chem, 287*(14), 10977-10989. doi:10.1074/jbc.M111.324616

Zeng, Q., Zou, L., Qian, L., Zhou, F., Nie, H., Yu, S., . . . Zhang, H. (2017). Expression of microRNA222 in serum of patients with Alzheimer's disease. *Mol Med Rep, 16*(4), 5575-5579. doi:10.3892/mmr.2017.7301

Zetterberg, H., & Burnham, S. C. (2019). Blood-based molecular biomarkers for Alzheimer's disease. *Molecular Brain, 12*(1), 26. doi:10.1186/s13041-019-0448-1

Zetterberg, H., & Schott, J. M. (2019). Biomarkers for Alzheimer's disease beyond amyloid and tau. *Nature Medicine, 25*(2), 201-203. doi:10.1038/s41591-019-0348-z

Zhang, C., Xiao, X., Chen, M., Aldharee, H., Chen, Y., & Long, W. (2018). Liver kinase B1 restoration promotes exosome secretion and motility of lung cancer cells. *Oncol Rep, 39*(1), 376-382. doi:10.3892/or.2017.6085

Zhang, X., Sai, B., Wang, F., Wang, L., Wang, Y., Zheng, L., . . . Xiang, J. (2019). Hypoxic BMSC-derived exosomal miRNAs promote metastasis of lung cancer cells via STAT3-induced EMT. *Molecular Cancer, 18*(1), 40. doi:10.1186/s12943-019-0959-5

Zhang, Y., Liu, Y., Liu, H., & Tang, W. H. (2019). Exosomes: biogenesis, biologic function and clinical potential. *Cell Biosci, 9*, 19. doi:10.1186/s13578-019-0282-2

Zhao, J., Yue, D., Zhou, Y., Jia, L., Wang, H., Guo, M., . . . Xu, L. (2017). The Role of MicroRNAs in Abeta Deposition and Tau Phosphorylation in Alzheimer's Disease. *Front Neurol, 8*, 342. doi:10.3389/fneur.2017.00342

Zhao, S., Zhao, J., Zhang, T., & Guo, C. (2016). Increased apoptosis in the platelets of patients with Alzheimer's disease and amnestic mild cognitive impairment. *Clin Neurol Neurosurg, 143*, 46-50. doi:10.1016/j.clineuro.2016.02.015

8. APPENDIX

8.1. Ethics Committee Approval

KLİNİK ARAŞTIRMALAR ETİK KURULU KARAR FORMU

ARAŞTIRMANIN AÇIK ADI	**Alzheimer Hastalığının Tanısında ve Yayılımında Nöron Kökenli Ekzozomlar**
VARSA ARAŞTIRMANIN PROTOKOL KODU	-
ETİK KURUL PROTOKOL NUMARASI	399-SBKAEK

ETİK KURUL BİLGİLERİ	ETİK KURULUN ADI	Dokuz Eylül Üniversitesi Klinik Araştırmalar Etik Kurulu
	AÇIK ADRESİ	Dokuz Eylül Üniversitesi Sağlık Yerleşkesi Dekanlık Binası Kat:2 İnciraltı 35340 İZMİR-TÜRKİYE
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	E-POSTA	etikkurul@deu.edu.tr

BAŞVURU BİLGİLERİ	KOORDİNATÖR/SORUMLU ARAŞTIRMACI UNVANI/ADI/SOYADI	**Prof.Dr.Şermin GENÇ**			
	KOORDİNATÖR/SORUMLU ARAŞTIRMACININ UZMANLIK ALANI	Nöroloji/sinirbilimler			
	KOORDİNATÖR/SORUMLU ARAŞTIRMACININ BULUNDUĞU MERKEZ	Sağlık Bilimleri Enstitüsü, Sinirbilimler A.D			
	VARSA İDARİ SORUMLU UNVANI/ADI/SOYADI	-			
	DESTEKLEYİCİ	-			
	PROJE YÜRÜTÜCÜSÜ UNVANI/ADI/SOYADI (TÜBİTAK vb. gibi kaynaklardan destek alanlar için)	**Prof.Dr.Şermin GENÇ**			
	DESTEKLEYİCİNİN YASAL TEMSİLCİSİ	-			
	ARAŞTIRMANIN FAZI VE TÜRÜ	FAZ 1	☐		
		FAZ 2	☐		
		FAZ 3	☐		
		FAZ 4	☐		
		Gözlemsel ilaç çalışması	☐		
		Tıbbi cihaz klinik araştırması	☐		
		In vitro tıbbi tanı cihazları ile yapılan performans değerlendirme çalışmaları	☐		
		İlaç dışı klinik araştırma	☒		
		Diğer ise belirtiniz			
	ARAŞTIRMAYA KATILAN MERKEZLER	TEK MERKEZ ☒	ÇOK MERKEZLİ ☐	ULUSAL ☒	ULUSLARARASI ☐

	Belge Adı	Tarihi	Versiyon Numarası	Dili		
DEĞERLENDİRİLEN BELGELER	ARAŞTIRMA PROTOKOLÜ			Türkçe ☐	İngilizce ☐	Diğer ☐
	Bilgilendirilmiş 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	☒	mevcut			
	BİYOLOJİK MATERYEL TRANSFER FORMU	☐				
	İLAN	☐				
	YILLIK BİLDİRİM	☐				
	SONUÇ RAPORU	☐				
	GÜVENLİLİK BİLDİRİMLERİ	☐				
	DİĞER	☒	-Araştırma akış şeması			

Etik Kurul Başkanının
Unvanı/Adı/Soyadı: Prof.Dr.Ayşegül Yıldız
İmza:

Not: Etik kurul başkanı, imzasının yer almadığı her sayfaya imza atmalıdır.

KLİNİK ARAŞTIRMALAR ETİK KURULU KARAR FORMU

ARAŞTIRMANIN AÇIK ADI	**Alzheimer Hastalığının Tanısında ve Yayılımında Nöron Kökenli Ekzozomlar**
VARSA ARAŞTIRMANIN PROTOKOL KODU	-
ETİK KURUL PROTOKOL NUMARASI	399-SBKAEK

KARAR BİLGİLERİ	**Karar No:2017/15-05**	**Tarih:21.08.2017**
	Yukarıda bilgileri verilen başvuru dosyası ile ilgili belgeler araştırmanın/çalışmanın gerekçe, amaç, yaklaşım ve yöntemleri dikkate alınarak incelenmiş ve söz konusu yazılımın prototipinin geliştirilmesi açısından 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.	

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

Unvanı/Adı/Soyadı	Uzmanlık Alanı	Kurumu	Cinsiyet		Araştırma ile ilişki		Katılım *		İmza
Prof.Dr Ayşegül YILDIZ	Psikiyatri	DEU Tıp Fakültesi Psikiyatri Anabilim Dalı	E☐	K☒	E☐	H☒	E☐	H☒	katıldı
Prof.Dr Hülya ELLİDOKUZ	Halk Sağlığı	DEU Onkoloji Enstitüsü Prevantif Onkoloji A.D	E☐	K☒	E☐	H☒	E☐	H☐	
Prof.Dr Nuray DUMAN	Çocuk Sağlığı ve Hastalıkları (Yeni Doğan)	DEU Tıp Fakültesi Çocuk Sağlığı ve Hastalıkları Anabilim Dalı	E☐	K☒	E☐	H☒	E☐	H☐	
Prof.Dr.Bahar KUVAKİ BALKAN	Anesteziyoloji ve Reanimasyon	DEU Tıp Fakültesi Anesteziyoloji ve Reanimasyon A.D	E☐	K☒	E☐	H☒	E☐	H☐	
Prof.Dr Taner DAĞCI	Fizyoloji	Ege Üniversitesi Tıp Fakültesi Fizyoloji A.D	E☒	K☐	E☐	H☒	E☐	H☒	katıldı
Prof.Dr Pembe KESKİNOĞLU	Biyoistatistik	DEU Tıp Fakültesi Biyoistatistik ve Tıbbi Bilişim A.D	E☐	K☒	E☒	H☐	E☐	H☒	*Toplantıda bulunmadı
Prof.Dr Erdem YAKA	Nöroloji	DEU Tıp Fakültesi Nöroloji A.D	E☒	K☐	E☐	H☒	E☐	H☐	
Prof.Dr Cenk ECEVİT	KBB	DEU Tıp Fakültesi KBB A.D	E☒	K☐	E☐	H☒	E☐	H☐	
Prof.Dr Uluç YIŞ	Çocuk Sağlığı ve Hastalıkları (Çocuk Nöroloji)	DEU Tıp Fakültesi Çocuk Sağlığı ve Hastalıkları Anabilim Dalı	E☒	K☐	E☐	H☒	E☐	H☐	
Doç.Dr Uğur Önsel TÜRK	Kardiyoloji	Batı Anadolu Mersinli Tıp Merkezi	E☒	K☐	E☐	H☒	E☐	H☒	katıldı
Doç.Dr Yasemin ERAÇ	Farmakoloji	Ege Üniversitesi Eczacılık Fakültesi Farmakoloji Anabilim Dalı	E☐	K☒	E☐	H☒	E☐	H☐	
Öğr.Gör.Dr Cemal Hüsevin GÜVERCİN	Tıp Tarihi ve Etik	DEU Tıp Fakültesi Tıp Tarihi ve Etik A.D	E☐	K☒	E☐	H☒	E☐	H☐	
Av.Fulya TANTAŞ	Hukuk	Dokuz Eylül Üniversitesi Rektörlüğü Hukuk Müşavirliği	E☐	K☒	E☐	H☒	E☐	H☒	katıldı
Av.İrem TUNCER TIRAŞ	Hukuk	Dokuz Eylül Üniversitesi Rektörlüğü Hukuk Müşavirliği	E☐	K☒	E☐	H☒	E☐	H☐	
Hayat ALBAYRAK	Sağlık Mesleği Mensubu Olmayan Üye	Emekli	E☐	K☒	E☐	H☒	E☐	H☐	

*:Toplantıda Bulunma

Etik Kurul Başkanının
Unvanı/Adı/Soyadı: Prof.Dr.Ayşegül Yıldız
İmza

Not: Etik kurul başkanı, imzasının yer almadığı her sayfaya imza atmalıdır.

8.2. Curriculum Vitae

ÖZGEÇMİŞ

DEVRİM YAĞMUR DURUR

Doğum Tarihi:	1995
Yazışma Adresi :	Dokuz Eylül Üniversitesi, İzmir Uluslararası Biyotıp ve Genom Enstitüsü, Sağlık Yerleşkesi, Mithatpaşa Caddesi, 58/5, 35340, Balçova, İzmir / Türkiye.
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EĞİTİM BİLGİLERİ					
Ülke	**Üniversite**	**Fakülte/Enstitü**	**Öğrenim Alanı**	**Derece**	**Mezuniyet Yılı**
Türkiye	Dokuz Eylül	İzmir Uluslararası Biyotıp ve Genom Enstitüsü	Moleküler Biyoloji ve Genetik	Yüksek Lisans	2020
Türkiye	Ege	Fen Fakültesi	Biyokimya	Lisans	2017

UZMANLIK ALANLARI
Ekzozom, MikroRNA, Moleküler Biyoloji ve Genetik, Proteomik

GÖREV ALDIĞI PROJELER			
Proje Adı	**Kurum**	**Proje Dönemi**	**Projedeki Görevi**
1. Neuron Derived Exosomes in Diagnosis and Propagation of Alzheimer's Disease	TÜBİTAK	2018 – Devam ediyor	**Araştırmacı**
2. Etil Pirüvatın Mikroglial Hücrelerde HMGB1 Aracılığıyla İnflamazom Aktivasyonuna Etkisi	Dokuz Eylül Üniversitesi BAP	2018 – Devam ediyor	**Araştırmacı**
3. İnfantil Kolik ile Migren ve Biyoritim Düzensizliklerinin İlişkisinin Değerlendirilmesi	Dokuz Eylül Üniversitesi BAP	2017 - 2019	**Araştırmacı**

ULUSAL ve ULUSLARARASI KONGRE/KONFERANS/SEMPOZYUM BİLDİRİLERİ
ULUSAL
1. Egeli, T., Tüzün, F., **Durur, D.Y.**, Tüfekci, K.U., Ural, C.'' İnfantil Kolik Bir Biyoritim Bozukluğu Mudur?'' 27. Ulusal Neonatoloji Kongresi, 3-7 Nisan 2019, Antalya. (Sözlü Bildiri)

EĞİTİM, KURS ve DİĞER ETKİNLİKLER
1. Therapeutic mAb Engineering and Production Workshop, 11 Kasım 2017, İzmir Uluslararası Biyotıp ve Genom Merkezi, İzmir.
2. Gaz Kromatografisi Sertifika Eğitimi, 13-14 Ağustos 2016, ETC Danışmanlık, İzmir.
3. Yüksek Performanslı Sıvı Kromatografisi (HPLC) Sertifika Eğitimi, 13-14 Ağustos 2016, ETC Danışmanlık, İzmir.
4. Uluslararası Genomik ve Biyoinformatik Konferansı, 7 Mayıs 2016, İzmir Uluslararası Biyotıp ve Genom Merkezi, İzmir.
5. 8. Geleneksel Bilim ve Teknoloji Sempozyumu: Omik Bilimleri ve Teknolojileri, 24 Nisan 2016, Ege Üniversitesi, İzmir.
6. 1. Bilim Okulu Günleri, 20-21 Nisan 2016, Ege Üniversitesi, İzmir.
7. TÜRKKÖK Kök Hücre Günleri, 16 Nisan 2016, Ege Üniversitesi, İzmir.
8. Ege Bilim Günleri, 25-26 Mart 2016, Ege Üniversitesi, İzmir.
9. Temel Kök Hücre Eğitimi Semineri, 15 Aralık 2015, Ege Üniversitesi, İzmir.
10. 5. Ulusal Kimya Öğrenci Kongresi, 17-19 Mayıs 2014, İstanbul Üniversitesi, İstanbul.