CHARACTERIZATION OF BLOOD-DERIVED EXOSOMES FOR DIAGNOSTICS INSIGHTS IN NEUROLOGICAL DISEASES

By

Javaria Tahir

(Registration No: 00000399843)

Department of Biomedical Engineering and Sciences

School of Mechanical and Manufacturing Engineering

National University of Sciences & Technology (NUST)

Islamabad, Pakistan

(2025)

Characterization of Blood-Derived Exosomes for Diagnostics Insights in Neurological Diseases

By

Javaria Tahir

(Registration No: 00000399843)

A thesis submitted to the National University of Sciences and Technology, Islamabad,

in partial fulfillment of the requirements for the degree of

Master of Science in
Biomedical Sciences

Supervisor: Dr. Saima Zafar

School of Mechanical and Manufacturing Engineering

National University of Sciences & Technology (NUST)

Islamabad, Pakistan

(2025)

THESIS ACCEPTANCE CERTIFICATE

Certified that final copy of MS/MPhil thesis written by **Regn No. 00000399843 Javaria Tahir** of **School of Mechanical & Manufacturing Engineering (SMME)** has been vetted by undersigned, found complete in all respects as per NUST Statues/Regulations, is free of plagiarism, errors, and mistakes and is accepted as partial fulfillment for award of MS/MPhil degree. It is further certified that necessary amendments as pointed out by GEC members of the scholar have also been incorporated in the said thesis titled: **Characterization of Blood-Derived Exosomes for Diagnostics Insights in Neurological Diseases**

Dr. Saima Zafar
Professor
Department of Biomedical Engg & Sciences
School of Mechanical & Manufacturing
Engineering (SMME), NUST, Islamabad

Signature:

Name (Supervisor): Dr Saima Zafar

Date: 14/02/2025

Signature (HOD):

Date: 14/02/2025

Signature (DEAN):

Date: 14-2-25

FORM TH-4

National University of Sciences & Technology

MASTER THESIS WORK

We hereby recommend that the dissertation prepared under our supervision by: (Student Name & Regn No.) **Javaria Tahir** Regn No: **00000399843**

Titled: **Characterization of Blood-Derived Exosomes for Diagnostics Insights in Neurological Diseases.** be accepted in partial fulfillment of the requirements for the award of Master in Biomedical Sciences degree.

Examination Committee Members

1. Name: Dr. Muhammad Asim Waris Signature: _____ Date: 14/02/2025

2. Name: Dr. Aneeqa Noor Signature: _____ Date: 14/02/2025

3. Name: Dr.Ahmed Fuwad Signature: _____ Date: 14/02/2025

Supervisor's name: Dr. Saima Zafar Signature: _____ Date: 14/02/2025

Dr. Saima Zafar
Professor
Department of Biomedical Engineering & Sciences
School of Mechanical & Manufacturing Engineering (SMME), NUST, Islamabad

_____ 14/02/2025
Head of Department Date

COUNTERSINGED

Date: 14-2-25

_____
Dean/Principal

CERTIFICATE OF APPROVAL

This is to certify that the research work presented in this thesis, entitled **"Characterization of Blood-Derived Exosomes for Diagnostics Insights in Neurological Diseases"** was conducted by Ms. Javaria Tahir under the supervision of Professor Dr. Saima Zafar. No part of this thesis has been submitted anywhere else for any other degree. This thesis is submitted to the Department of Biomedical Engineering and Sciences in partial fulfillment of the requirements for the degree of Master of Science in the Field of Biomedical Sciences, Department of National University of Sciences and Technology, Islamabad.

Student Name: Javaria Tahir Signature: 

Supervisor Name: Dr. Saima Zafar Signature: 

Name of Dean/HOD: Dr. Muhammad Asim Waris Signature: 

AUTHOR'S DECLARATION

I **JAVARIA TAHIR** hereby state that my MS thesis titled **"Characterization of Blood-Derived Exosomes for Diagnostics Insights in Neurological Diseases"** is my own work and has not been submitted previously by me for taking any degree from the National University of Sciences and Technology, Islamabad, or anywhere else in the country/ world.

At any time if my statement is found to be incorrect even after I graduate, the university has the right to withdraw my MS degree.

Name of Student: Javaria Tahir

Date: 10-02-2025

PLAGIARISM UNDERTAKING

I solemnly declare that research work presented in the thesis titled "Characterization of Blood-Derived Exosomes for Diagnostics Insights in Neurological Diseases" is solely my research work with no significant contribution from any other person. Small contribution/help wherever taken has been duly acknowledged and that complete thesis has been written by me.

I understand the zero-tolerance policy of the HEC and National University of Sciences and Technology (NUST), Islamabad towards plagiarism. Therefore, I as an author of the above titled thesis declare that no portion of my thesis has been plagiarized and any material used as reference is properly referred/cited.

I undertake that if I am found guilty of any formal plagiarism in the above titled thesis even after award of MS degree, the University reserves the rights to withdraw/revoke my MS degree and that HEC and NUST, Islamabad has the right to publish my name on the HEC/University website on which names of students are placed who submitted plagiarized thesis.

Student Signature: 

Name: Javaria Tahir

I lovingly dedicate this MS thesis to my late Grandfather

Mr. Abdul Rasheed Saddozai,

whose blessings and memories continue to inspire me, and

to my Parents, Mr. Muhammad Tahir Saddozai & Mrs. Sumera Badar, the true architects of my dreams and the pillars of my strength. You gave me wings to soar and roots to keep me grounded. Your support, endless sacrifices, and ceaseless prayers have been the foundation of my every success.

Thank You for believing in me and nurturing my dreams.

ACKNOWLEDGEMENTS

All praise and gratitude to Almighty Allah, the Most Gracious and the Most Merciful, whose countless blessings have enabled me to complete this research journey. Without His divine help, this accomplishment would not have been possible.

I extend my deepest and sincere gratitude to my supervisor, Professor Dr. Saima Zafar, for her invaluable guidance, patience, and constant encouragement throughout this journey. Her belief in me and my potential gave me the confidence to push my limits and strive for excellence. I am truly grateful for her mentorship.

A heartfelt thanks to my Parents Mr. Muhammad Tahir Saddozai and Mrs. Sumera Badar, and my little sister Yashfa Tahir, whose endless love, prayers, and sacrifices have been my greatest strength. Their patience in bearing with me through my stress, frustration, and endless academic rants is something I can never repay. A warm thanks to my family and a special mention and deepest gratitude to my uncles, Muhammad Owais Saddozai & Muhammad Taimur Saddozai, for their immense care, support, and selfless services. Their kindness and generosity have made a significant difference in my journey, and I am truly fortunate to have them by my side.

To my friends and roommates, thank you for being my source of comfort and motivation. Your constant encouragement, conversations, and shared laughter made even the hardest days easier to bear. This journey would have been far more difficult without you all by my side. A special thanks to my lab mates for their collaboration, technical assistance, and valuable discussions, which have enriched this research experience.

Finally, I extend my appreciation to everyone who has contributed to this research in any way whether through their guidance, support, or simply by believing in me. This journey has been challenging, but the incredible people around me have made it one of growth, learning, and resilience.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMS

Aβ peptide	Amyloid beta (Abeta or beta-amyloid)
α-syn	Alpha-synuclein
AD	Alzheimer's disease
APP	Amyloid precursor protein
dNTPs	Deoxynucleotide triphosphates
EDTA	Ethylenediaminetetraacetic acid
ESCRT	Endosomal sorting complex required for transport
HSPs	Heat shock proteins
ILVs	Intraluminal vesicles
MS	Multiple Sclerosis
MVBs	Multivesicular bodies
NCV	Nerve conduction velocity
PBS	Phosphate buffer saline
PD	Parkinson's disease
PEG	Polyethylene glycol
PET	Positron emission tomography
RT-PCR	Reverse transcription polymerase chain reaction
SEM	Scanning electron microscope
tau protein	Tubulin associated unit
TRIzol	Total RNA isolation

ABSTRACT

Exosomes, nanosized extracellular vesicles, are emerging as potential non-invasive biomarkers for diagnosing neurological disorders. In this study, we explored the isolation and characterization of blood-derived exosomes to assess their diagnostic relevance. Blood samples were collected from two groups: a control group of individuals without neurological disorders and a patient group diagnosed with various neurological conditions. Plasma was extracted using differential centrifugation to remove cellular debris, apoptotic bodies, and larger vesicles. Exosomes were isolated from the plasma using a polymer-based precipitation method optimized with PEG-6000 to achieve high yield and purity. The isolated exosomes were then characterized using Scanning Electron Microscopy (SEM) to verify their morphology and methylene blue staining to confirm their extracellular vesicular nature. After staining, RNA was extracted from the exosomes using TRIzol, followed by cDNA synthesis to analyze the molecular profile. Gel electrophoresis was performed to assess the quality of the cDNA, and RT-PCR was conducted to detect biomarkers associated with neurological disorders. These molecular analyses provided insights into the differences between the control and diseased groups, highlighting potential biomarkers for disease diagnosis. The results of this study promise valuable insights into the potential properties of blood-derived exosomes in advancing non-invasive diagnostics for neurological diseases by contributing to early detection strategies and paving the way for improved patient care through innovative diagnostic tools.

Keywords: α-synuclein, Blood-brain barrier, Exosomes, Neurological diseases, Precipitation.

CHAPTER 1: INTRODUCTION

1.1 Background of Study

1.1.1 Overview of Neurological Diseases and their Diagnostic Challenges

Neurological diseases are medically defined as disorders that affect the brain, the nerves found throughout the human body, and the spinal cord (Chandra et al., 2006). Structural, biochemical, or electrical abnormalities in the brain, spinal cord, or other nerves can result in a range of symptoms. Examples of symptoms include paralysis, muscle weakness, poor coordination, loss of sensation, seizures, confusion, pain, and altered levels of consciousness (Hirtz et al., 2007). The specific causes of neurological problems vary but can include genetic disorders, congenital abnormalities or disorders, infections, lifestyle or environmental health problems including malnutrition, brain injury, spinal cord injury, or nerve injury (Farooqui et al., 2012). There are many recognized neurological disorders, as can be seen in the Figure 1.1, some relatively common, but many rare. Mental disorders, on the other hand, are 'Psychiatric Illnesses' or diseases that appear primarily as abnormalities of thought, feeling, or behavior, producing either distress or impairment of function (Najjar et al., 2013). According to the U.S. National Library of Medicine, there are more than 600 neurologic diseases. Neurological disabilities include a wide range of disorders, such as epilepsy, learning disabilities, neuromuscular disorders, autism, brain tumours, and cerebral palsy, just to name a few (Sá-Caputo et al., 2021). Some neurological conditions are congenital, emerging before birth. Other conditions may be caused by tumours, degeneration, trauma, infections or structural defects. Regardless of the cause, all neurological disabilities result from damage to the nervous system (Mukherjee et al., 2023).

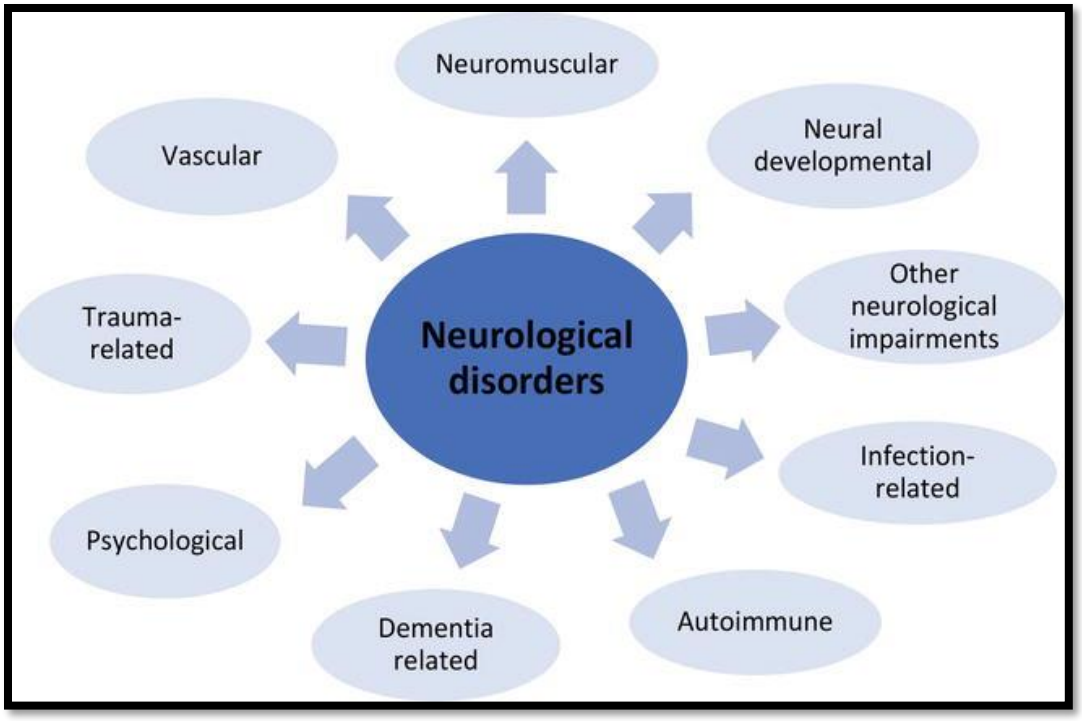


Figure 1.1 Approaches linked to neurological disorders (Sá-Caputo et al., 2021)

Diagnosing nervous system disorders is complex due to overlapping symptoms, varying combinations of signs, and the absence of definitive causes, markers, or tests for many conditions. Healthcare providers begin with a thorough medical history and physical examination before employing advanced diagnostic tools to gain more insight (Lima et al., 2022). Imaging tests play a crucial role in identifying nervous system issues. **CT** scans (computed tomography scans) and **MRIs** (magnetic resonance imaging) provide detailed images of the brain, spine, and nervous system, with MRIs offering superior detail without radiation exposure. **PET scans** (positron emission tomography) detect early disease activity by evaluating organ and tissue function. **Arteriograms** and **Neurosonography** assess blood flow and identify vascular abnormalities like blockages or narrowing. Additionally, **Ultrasounds** visualize blood vessels and organs in real-time. Electrical activity tests help assess brain and nerve function. An **EEG** (electroencephalography) records continuous brain activity through scalp electrodes, while **Electrodiagnostic Tests** like **EMG** (electromyography) and **NCV** (nerve conduction velocity) evaluate muscle and nerve responses. **Evoked Potential Tests**

measure the brain's reaction to visual, auditory, and sensory stimuli, further aiding diagnosis (National Academies of Sciences and Medicine, 2023). Specialized procedures like the **Spinal Tap** analyze cerebrospinal fluid to detect infections or other problems. At the same time, a **Myelogram** uses dye to enhance spinal canal imaging, although it is less common due to widespread MRI use (Espay et al., 2018). Together, these diagnostic methods provide essential information to understand and address complex nervous system disorders.

Neurological disease diagnosis is particularly challenging due to its complexity and intricate nature, overlapping symptoms, and the absence of reliable early-stage biomarkers (Mavroudis et al., 2024). While advancements in neuroimaging and molecular diagnostics have improved accuracy, many conditions, like Alzheimer's and multiple sclerosis, remain difficult to identify in their early stages. Emerging biomarkers, such as tau proteins, amyloid-beta, and exosome-based markers, hold promise but face hurdles like invasive collection methods and variability. Similarly, tools like genetic testing and advanced imaging improve diagnosis but are often inaccessible due to high costs or lack of infrastructure, leaving many patients undiagnosed or misdiagnosed (Ningrum & Kung, 2023). New technologies like artificial intelligence and machine learning are helping to analyze complex data, enhancing diagnostic precision (Raghavendra et al., 2020). However, these tools are still limited by issues like standardization and integration into everyday clinical practice. The lack of access to advanced tools and specialists in resource-poor settings further compounds the problem (Lima et al., 2022). Efforts like telemedicine and affordable diagnostics aim to address these gaps, but more work is needed. A combined focus on validating biomarkers, improving access to technology, and creating equitable diagnostic systems is critical for making progress in the early and accurate detection of neurological diseases.

1.1.2 Introduction to Exosomes and their Biological Significance

Exosomes, with a diameter of about 30-150 nm, are biological nano-sized small extracellular spherical lipid bilayer vesicles secreted by cells, carrying nucleic acids, proteins, lipids, and other bioactive substances to play a role in the body's physiological

and pathological processes (Chen et al., 2024). These are small extracellular vesicles, that encapsulate various biomolecules, playing a vital role in intercellular communication and the regulation of numerous physiological and pathological processes (Miron & Zhang, 2024).

Exosomes serve a wide range of functions, both major and minor, in cellular communication and biological processes. At their core, they facilitate the transfer of proteins, lipids, and nucleic acids between cells, enabling intercellular communication (Vlassov et al., 2012a). This process is crucial for regulating immune responses, as exosomes can influence the activity of immune cells by presenting antigens and modulating immune signalling (Zhou et al., 2020a). They also play a role in tissue repair and regeneration by transporting growth factors and enzymes to target cells. Additionally, exosomes contribute to the removal of waste from cells, acting as a mechanism for the disposal of unwanted proteins and RNA (Stoorvogel et al., 2002). In disease contexts, such as cancer or neurological disorders, exosomes can carry disease-related biomarkers, making them valuable for diagnostics and as potential therapeutic targets. Overall, exosomes are essential for maintaining cellular homeostasis, coordinating tissue functions, and influencing disease progression.

Based on these extensive properties, exosomes hold immense potential as diagnostic tools. Their ability to encapsulate and transport specific biomolecules reflects the physiological state of their parent cells. This makes them reliable biomarkers for various diseases, including neurological disorders, cancers, and cardiovascular conditions (Rashed et al., 2017). Their small size, stability in biological fluids, and non-invasive collection methods, such as from blood, urine, or saliva, further enhance their practicality for diagnostic applications. Additionally, the molecular cargo of exosomes can be analyzed using advanced techniques like PCR, sequencing, and proteomics, providing critical insights into disease mechanisms and progression (Ashique et al., 2024).

1.1.3 Sources of Exosomes

Exosomes can be found in various biological fluids, originating from different cell types. They are released into fluids like blood, urine, and saliva, reflecting the state of the cells

they come from (Yuyama & Igarashi, 2016). Table 1.1 provides an overview of the sources of exosomes in these fluids, highlighting where they are commonly found and their potential roles in research.

Table 1.1 Exosomes origin across various Biological Systems

Fluid	**Exosomes Source**	**Advantages**	**Challenges / Disadvantages**	**Use in Research**	**Collection Method**	**Exosomes Yield**
Blood (Plasma/ Serum)	Blood cells, platelets, endothelial cells.	Minimal/ Non-invasive collection (for plasma/serum). Widely available. Large volumes for study.	Complex isolation due to contaminants. Variability in exosome concentration across individuals.	Cancer Neurological Diseases Cardiovascular Diseases	Venipuncture	Moderate to High
Cerebrospinal Fluid (CSF)	Brain and spinal cord cells.	A direct reflection of CNS activity. Useful for neurologica	Invasive collection (lumbar puncture). Limited sample	Alzheimer's disease Parkinson's disease Multiple	Lumbar puncture	Low to Moderate

		l diseases.	volume. Low exosome yield compared to blood.	Sclerosis etc		
Urine	Renal and urological systems.	Non-invasive collection. Suitable for repeated sampling. Easy to access.	Low exosome concentration. Contaminants such as proteins and cellular debris.	Kidney diseases Urinary tract cancers	Midstream urine sample	Low
Saliva	Oral epithelial cells, immune cells.	Non-invasive. Easy collection. Suitable for frequent sampling.	Low exosome concentration. Contaminants such as mucins. Variability in sample quality.	Oral cancers Periodontal diseases	Spitting or swabbing	Low
Breast Milk	Mammary gland	Abundant exosome	Variability between	Neonatal immunity	Milk expression	High

	epithelial cells.	content. Non-invasive. Ideal for early-life health studies.	individuals. Need for specialized protocols to avoid contamination.	Breast cancer		

1.2 Problem-Solution Perspective

- Traditional methods of neurodiagnostic, such as imaging techniques and cerebrospinal fluid analysis, are often invasive, costly, and may not provide timely or comprehensive results.
- Blood-derived exosomes offer minimal/non-invasive, accessible alternative for diagnosing neurological diseases, as they carry molecular signatures reflective of the disease state.
- Current methods for isolating and characterizing blood-derived exosomes face challenges in sensitivity, yield, and subtype specificity.
- This study aims to optimize the isolation of blood-derived exosomes, providing a more efficient, non-invasive diagnostic tool for neurological diseases compared to conventional methods.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction to the Chapter

This chapter provides an overview of the existing research on exosomes and their potential use in diagnosing neurological diseases. It explains what exosomes are, how they work in the body, and why they are important in understanding diseases. It also looks at how they can be used as minimally invasive to non-invasive biomarkers for diagnosing neurological conditions that help set the stage for the study, showing why it's important to explore and better understand blood-derived exosomes for diagnosing diseases early and accurately.

2.2 Exosomes: An Overview

2.2.1 Structure and Composition

Exosomes are tiny, flattened spheres (Chernyshev et al., 2015), membrane-bound vesicles released by a wide variety of cell types into the extracellular compartment by exocytosis (Kalluri & LeBleu, 2020). They are 30-150 nanometers in size (Pegtel & Gould, 2019) and can be observed in many cell types, such as platelets, mastocytes, B cells, T cells, dendritic cells, etc (Vlassov et al., 2012a). They are found in various biological fluids, including serum, blood, cerebrospinal fluids, urine, breast milk, and saliva (Yuyama & Igarashi, 2016). These nano-sized extracellular functional vesicles carry a complex cargo of proteins, lipids, and nucleic acids, as shown in the Figure 2.1, and are capable of delivering these cargos to the target cells they encounter, which may ultimately reprogram the recipient cells distal from their release (Lau & Yam, 2023). As exosomes are small extracellular vesicles, they encapsulate various biomolecules, playing a vital role in intercellular communication and the regulation of numerous physiological and pathological processes (Agarwal et al., 2024).

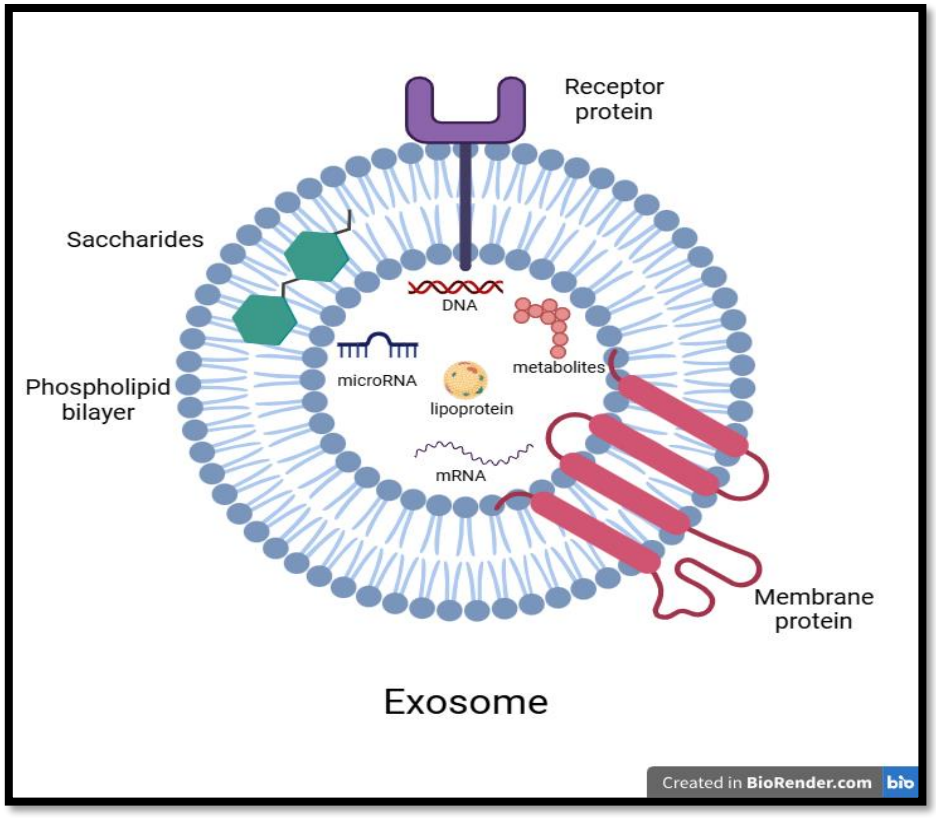


Figure 2.1 General Structure of an Exosome. (The figure was created using Biorender.com)

Exosomes possess a unique contented makeup of biomolecules having distinct duties to perform and are equally important in compiling to give them an eccentric appearance and roles (Cao et al., 2019). They comprise an assortment of membrane-associated, high-order oligomeric protein complexes and are formed by budding at both the plasma and endosomal membranes (Keller et al., 2006). The composition of exosomes includes proteins, lipids, saccharides, and genetic materials, each contributing to their structure and function. They contain a diverse range of proteins critical to their formation, release, and biological activity. **Fusion proteins**, such as annexins, GTPases, and flotillin, facilitate the fusion and transport of membranes, ensuring the biogenesis and proper release of exosomes (Zeng et al., 2023). **Tetraspanins**, including CD9, CD63, CD81, and CD82, are transmembrane proteins integral to exosomal structure. They mediate protein trafficking, signaling pathways, and membrane fusion, which are essential for exosome-

target cell interactions (Andreu & Yáñez-Mó, 2014). **Heat-shock proteins** (Hsp70 and Hsp90) are abundant in exosomes and contribute to their stress-response mechanisms. These proteins assist in membrane deformation and ensure the efficient release of exosomes from cells under physiological stress (Lauwers et al., 2018). Another significant protein group includes multivesicular body (MVB) biogenesis proteins, such as **Alix** and **TSG101**. These proteins are involved in the formation of MVBs, precursors to exosomes, and aid in the engulfment of intraluminal vesicles (ILVs) (Yang et al., 2021). Additionally, **lipid-related proteins**, like phosphatidylserine and ceramide are associated with cell-to-cell communication. They support the transfer of bioactive molecules between cells, emphasizing their functional importance (Mathivanan & Simpson, 2009).

Saccharides in exosomes play a key role in modulating interactions with recipient cells. These carbohydrate molecules, such as polylactosamine, α-2,6 sialic acid, and complex N-linked glycans, enhance the exosome's ability to bind and be internalized by target cells (Della Rosa et al., 2021). The presence of saccharides ensures the specificity and efficiency of exosome-mediated cellular communication. Their involvement in recipient cell absorption is critical for processes such as signal transduction, immune response modulation, and disease progression (Zhou et al., 2020b). **Lipids** are fundamental to the structural stability and functionality of exosomes. Sphingomyelin, phosphatidylcholine, cholesterol, and its derivatives are key lipid components of exosomes. These molecules provide a stable bilayer membrane that protects exosomes from enzymatic degradation in the extracellular environment. Beyond structural roles, lipids have regulatory functions, modulating cellular signaling and maintaining the bioavailability of exosomes during intercellular communication. Their involvement also contributes to the exosome's resistance to physical and chemical stressors (Syed Haroon, 2014). Exosomes are carriers of genetic material, making them critical players in the transfer of biological information between cells. They encapsulate **DNA**, **miRNA**, **mRNA**, and other **non-coding RNAs**, which regulate gene expression and influence cellular behavior in recipient cells (Valadi et al., 2007). This genetic content enables exosomes to participate in diverse processes, including the modulation of immune responses, the promotion of tissue repair, and the progression of diseases. Their role as vehicles of genetic information highlights their

potential as biomarkers and therapeutic agents in various clinical applications (Vlassov et al., 2012b).

2.2.2 Biogenesis and Secretion

The biosynthesis process starts when a molecular cargo is endocytosed and enters the cell (Krylova & Feng, 2023), the initial checkpoint is the early endosome that can be seen in the Figure 2.2, which undergoes maturation and results in the late endosome formation (Gurung et al., 2021). Exosome formation starts with the invagination of the multi-vesicular bodies (MVBs) or late endosomes to generate intraluminal vesicles (ILVs) (Lau & Yam, 2023). There are various proposed mechanisms for the formation of MVBs, vesicle budding, and sorting. The most studied and well-known is the endosomal sorting complex required for transport (ESCRT) dependent pathway. **ESCRT-dependent** machinery mediates the ubiquitinated pathway consisting of protein complexes; ESCRT-0, -I, -II, -III, and associated ATPase Vps4 (Scourfield & Martin-Serrano, 2017). ESCRT 0 recognizes and retains ubiquitinated proteins marked for packaging in the late endosomal membrane (Schmidt & Teis, 2012). ESCRT I/II recognizes ESCRT-0 and starts creating the involution of the membrane into the MVB (Hierro et al., 2004). ESCRT-III forms a spiral-shaped structure constricting the neck (McCullough et al., 2018). ATPase VPS4 protein drives the membrane scission. Late endosomes have two possible configurations: they can fuse with lysosomes and then dissolve, or they can merge with the plasma membrane and release exosomes (Lau & Yam, 2023). The syndecan-syntenin-ALIX exosome biogenesis pathway is one of the **ESCRT-independent** or non-canonical pathways for exosome biogenesis (Baietti et al., 2012). The MVBs once formed are trafficked to the internal side of the plasma membrane. These MVBs are transported to the plasma membrane leading to fusion. Many studies have shown that MVBs having higher cholesterol content fuse with the plasma membrane thus releasing exosomes (Babst, 2011). The Rab family of proteins acts as molecular switches, cycling between an inactive state (GDP-loaded) and an active state (GTP-loaded), especially Rab7 attached to the MVB, recognizing its effector receptor (Homma et al., 2021). The SNARE complex (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) from the MVB and the plasma membrane interact and

mediate fusion (Pegtel & Gould, 2019). MVBs fuse with the plasma membrane to release exosomes (Rastogi et al., 2021b).

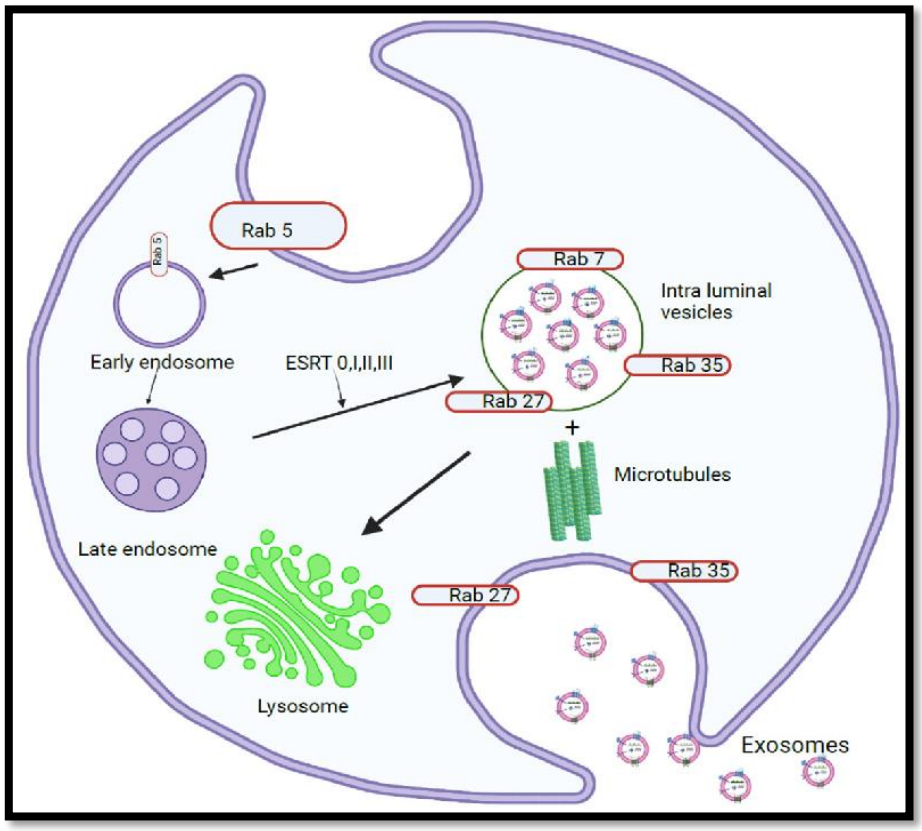


Figure 2.2 Exosome Biogenesis. This figure shows the biogenesis from the endosome, its association with ESRT and Rab proteins, and its release into the extracellular environment (Aseervatham, 2023).

Specific targeting by exosomes is an active area of research. The exact mechanisms of exosome targeting are limited to a few general mechanisms like docking of the exosomes with specific proteins, sugars, and lipids, or micropinocytosis (Gurung et al., 2021). The internalized exosomes are targeted to the endosomes which release their content in the recipient cell.

2.2.3 Biological Roles

Exosomes perform an extensive range of biological tasks, which is why they are essential to many physiological and pathological processes (Agarwal et al., 2024). They are involved in transporting proteins, lipids, and nucleic acids between cells, thereby influencing numerous biological functions. By transferring bioactive molecules, they facilitate communication between cells (Meldolesi, 2018) and this transfer can alter the behavior of recipient cells, influencing processes like immune responses, tissue repair, homeostasis, etc (Isola & Chen, 2017). They can modulate immune responses by presenting antigens to immune cells or by carrying immunosuppressive factors (Zhou et al., 2020b). They play a role in both promoting and inhibiting immune responses, which is significant in contexts such as cancer progression and autoimmune diseases (Gangadaran et al., 2023). They are involved in tissue repair processes by delivering growth factors and signaling molecules that promote healing (Wan et al., 2022). This function is crucial for maintaining homeostasis after injury, ensuring tissues can recover and restore their normal function (Desdín-Micó & Mittelbrunn, 2017).

Exosomes assist in the removal of cellular waste products and damaged proteins (Rashed et al., 2017). By packaging these materials into vesicles, cells can efficiently eliminate potentially harmful components, thereby contributing to cellular health and overall homeostasis. They can influence metabolic processes by transporting metabolic enzymes and regulatory molecules between cells (Isaac et al., 2021). This transport helps coordinate metabolic activities across different tissues, contributing to overall metabolic homeostasis.

Exosomes are involved in the formation of new blood vessels (angiogenesis) through several mechanisms such as delivering the pro-angiogenic factors (vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF)) that stimulate endothelial cell proliferation and migration, essential for new blood vessel formation (Tian et al., 2019). They also have a protective role in the nervous system as they can carry neurotrophic factors such as brain-derived neurotrophic factor (BDNF), which support neuronal survival and function (Oyarce et al., 2022) and they can also modulate inflammatory responses in the brain, potentially reducing neuroinflammation (Liu et al., 2019a).

Due to their composition reflecting the physiological state of their cell of origin, exosomes are being investigated as potential biomarkers for various diseases, including cancer, neurodegenerative disorders, and cardiovascular diseases (Mathew et al., 2021). Exosomes can be engineered to deliver therapeutic agents directly to target cells (Mishra et al., 2021). Their natural ability to fuse with cellular membranes enhances the efficiency of drug delivery systems, making them a promising tool in targeted therapy (Abdelsalam et al., 2023).

In summary, exosomes serve essential functions in cell communication, immune modulation, tissue repair, cellular homeostasis, disease biomarker discovery, drug delivery systems, etc. Their versatility makes them a significant focus of research across various biomedical fields. Thus, exosomes are tiny messengers with big roles, in shaping health, disease, and future therapies.

2.3 Blood-Derived Exosomes

2.3.1 Advantages of using Blood as a source of Exosomes

Blood-derived exosomes offer several advantages over exosomes from other bodily fluids, making them a preferred source for many studies (Jin et al., 2024). Blood is easily accessible through routine venipuncture, allowing for the collection of sufficient volumes with minimal patient discomfort (Shi et al., 2014). In contrast, other fluids like cerebrospinal fluid (CSF) or synovial fluid require invasive procedures, such as lumbar punctures or joint aspirations, which can be risky and less patient-friendly (Evans, 1998). Fluids like saliva and urine are easier to collect but may provide limited exosome yields (Cho et al., 2020), necessitating larger volumes for analysis. The diversity of exosome content in blood is another significant advantage. Blood contains exosomes derived from various cell types throughout the body, offering a comprehensive representation of systemic physiological and pathological states (Tutanov et al., 2022). In comparison, exosomes from other fluids are often more specific to their organ of origin, CSF for neurological conditions, urine for kidney and bladder diseases, and saliva for oral or systemic diseases. While this specificity is beneficial for targeted studies, it limits the broader applicability of findings compared to blood-derived exosomes.

From a diagnostic perspective, blood-derived exosomes are highly versatile and have been extensively studied for a range of systemic diseases, including cancers, cardiovascular disorders, and metabolic syndromes (Hornung et al., 2020). Other fluids like CSF are highly valuable for neurological conditions but are limited to certain applications due to the invasive nature of sample collection (Tumani et al., 2008). Moreover, the high yield of exosomes from blood samples (Baranyai et al., 2015) ensures sufficient material for analysis, whereas fluids like urine and saliva often have lower exosome concentrations, requiring more sensitive isolation techniques. Lastly, the processing and storage of blood samples are well-standardized (Vaught, 2006), though they require immediate handling with anticoagulants to maintain exosome integrity. Other fluids, such as urine and saliva, are prone to enzymatic degradation and require rapid stabilization, while CSF samples are generally stable but require sterile conditions during collection. These factors highlight the practical and logistical advantages of blood as a source of exosomes, while other fluids remain valuable for specific, localized investigations.

2.3.2 Isolation and Characterization Techniques

Exosome isolation and characterization are pivotal in understanding their roles in intercellular communication and their potential applications in diagnostics and therapeutics. Several methods have been developed to isolate and characterize exosomes, each offering distinct advantages and limitations depending on the specific requirements of the research (Willms et al., 2016). The choice of technique largely depends on factors such as the desired purity, yield, complexity, and the type of biological sample being used (Lai et al., 2022).

Differential Centrifugation is one of the most commonly employed methods for isolating exosomes (Livshits et al., 2015). It involves a series of centrifugation steps at increasing speeds, typically starting at 300 × g to remove cells and cell debris and progressing to higher speeds (up to 100,000 × g) to pellet exosomes. This approach is favored for its cost-effectiveness and ability to isolate large quantities of exosomes from biological fluids, such as blood, urine, and cell culture media. However, while it yields

substantial quantities of exosomes, the method may not provide the highest purity, as it can co-isolate other vesicles and proteins of similar size (Yu et al., 2018). For higher purity, **Density-Gradient Centrifugation** is often employed. This method uses a density gradient medium, such as sucrose or iodixanol, to further separate exosomes based on their buoyant density. This technique offers superior purity, as it effectively isolates exosomes from other contaminants, but it is more time-consuming, and complex compared to differential centrifugation. The gradient allows for the careful separation of exosomes, resulting in a highly purified sample, though the method is less suited for high-throughput applications due to its extended duration and need for careful monitoring (Greening et al., 2015). **Ultrafiltration**, another widely used method, relies on membranes with specific pore sizes to separate exosomes based on size exclusion (Diaz et al., 2018). This technique is efficient and straightforward, allowing for rapid isolation of exosomes from larger particles and proteins. However, ultrafiltration may not be sufficient for achieving the highest purity on its own, and it often requires subsequent purification steps, such as additional filtration or centrifugation, to further clean the exosome preparation. **Precipitation Methods** (Nila et al., 2022), including those using polyethylene glycol (PEG), provide a simple and quick approach to exosome isolation. In this method, a precipitating agent is added to the sample, causing exosomes to aggregate and form a pellet upon subsequent centrifugation. Precipitation is a highly accessible technique that can yield relatively high quantities of exosomes. **Immuno-isolation** is a more specific technique that leverages antibodies directed against exosomal surface markers, such as CD9, CD63, and CD81. These antibodies are conjugated to magnetic beads or other solid supports (Taha & Bogoniewski, 2023), allowing for selective isolation of exosomes. Immuno-isolation provides high specificity and can isolate exosomes with a high degree of purity, especially when specific markers are targeted. However, this method often results in lower yields, as the binding process may not capture all exosomes present in the sample. Recent advancements in nanotechnology (Agnihotram et al., 2024) have introduced novel methods for exosome isolation, such as **Resistive Pulse Sensing (RPS)**, **Surface Plasmon Resonance (SPR)-based nanosensors**, and **Deterministic Lateral Displacement (DLD)**. These techniques leverage nanoscale technologies to offer sensitive, precise, and often high-throughput

isolation of exosomes (Yu et al., 2018). They hold promises for overcoming some of the limitations of traditional methods, such as purity, yield, and sensitivity, and may represent the future of exosome isolation in both research and clinical applications.

For characterization, several techniques are available to analyze the size, concentration, and molecular content of exosomes (Miron & Zhang, 2024). **Transmission Electron Microscopy (TEM)** provides high-resolution images of exosome morphology and size, allowing researchers to visually confirm the presence and structural integrity of exosomes. **Nanoparticle Tracking Analysis (NTA)** measures exosome size distribution and concentration by tracking their Brownian motion in solution. NTA uses laser light scattering to detect the movement of individual particles, providing valuable data on exosome size profiles and particle concentration (Bagrov et al., 2021). This method is widely used for its ability to characterize large numbers of exosomes in a relatively short time. **Flow Cytometry** is a powerful technique for analyzing exosomal surface markers, providing quantitative data on exosome populations based on the presence of specific proteins (Lannigan & Erdbruegger, 2017). **Western Blotting** is commonly employed to confirm the identity of exosomes by detecting specific proteins associated with their membrane, such as tetraspanins (e.g., CD9, CD63, and CD81). This method is useful for verifying the presence of exosomes in a sample and assessing their molecular composition. It is often used in combination with other methods to provide a comprehensive understanding of exosomal content (Zhang et al., 2018). **Mass Spectrometry** offers a detailed analysis of the protein composition of exosomes, enabling researchers to explore the molecular cargo of exosomes in greater depth. This technique provides insights into the functional roles of exosomes by identifying proteins, lipids, and RNA content, which is crucial for understanding their involvement in disease processes and cellular functions (Pietrowska et al., 2017).

In summary, the methods employed for isolating and characterizing exosomes each have distinct strengths and weaknesses. The choice of technique depends on the specific requirements of the research, including the need for purity, yield, and the nature of the biological samples being analyzed. As the field progresses, the development of more advanced and efficient methods, particularly those based on nanotechnology (Jang et al.,

2024), holds the potential to further enhance the isolation and characterization of exosomes, opening new avenues for their clinical and therapeutic applications.

2.4 Exosomes in Neurological Diseases

Exosomes contribute significantly to the pathology of neurodegenerative diseases through mechanisms involving protein aggregation, neuroinflammation, and intercellular communication (Soria et al., 2017). They are increasingly recognized as significant players in the progression of neurodegenerative diseases. These small vesicles, serve as carriers of biomolecules. Their ability to cross the blood-brain barrier and mediate intercellular communication positions them as key facilitators in the pathogenesis of neurodegeneration (Liu et al., 2019b). Exosomes have been implicated in transporting misfolded or toxic proteins, that can be seen in the Figure 2.3, which contributes to the spread of pathological molecules and cellular dysfunction across different brain regions (Kalani et al., 2014). This characteristic is particularly evident in several major neurodegenerative disorders, where they play roles in both facilitating and aggravating disease processes.

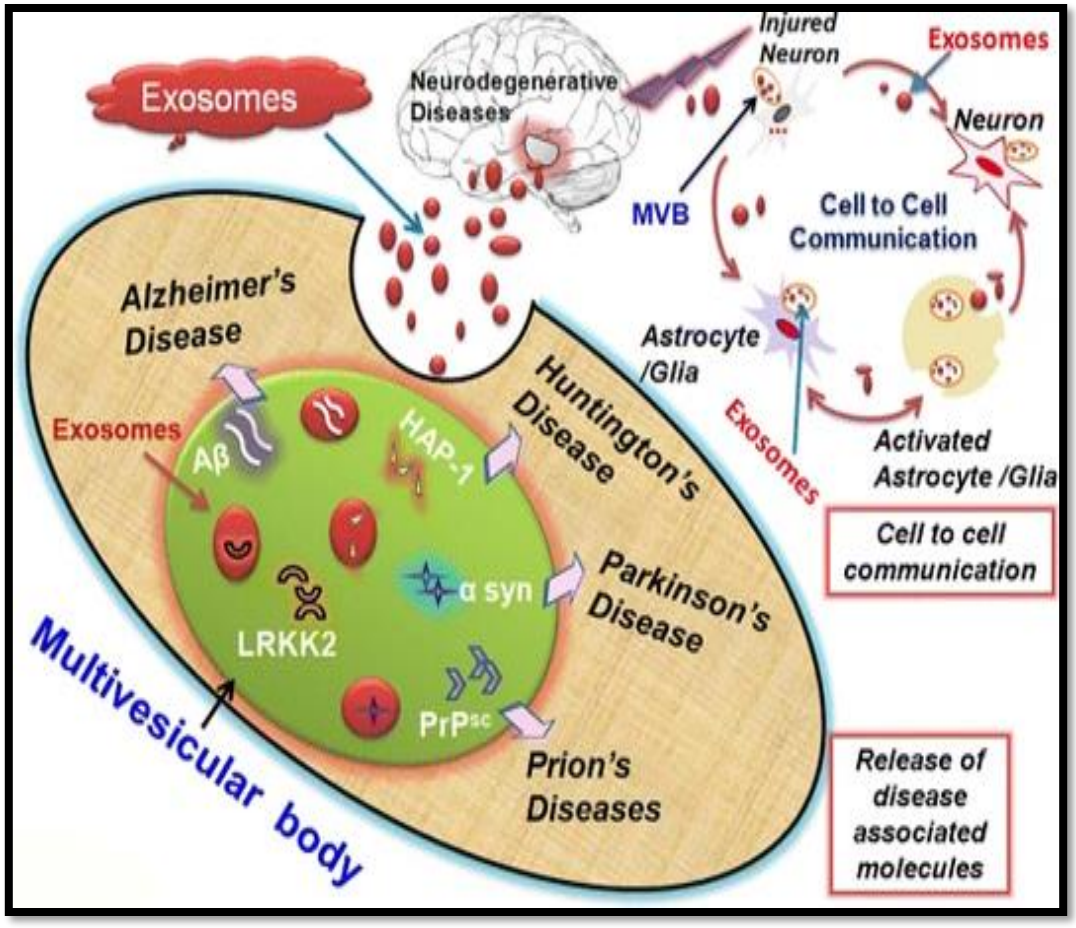


Figure 2.3 Cell-to-Cell communication through Exosomes and processing of Neurologic Toxin Proteins via Exosomes (Kalani et al., 2014)

In **Alzheimer's disease**, exosomes are associated with the transport and accumulation of amyloid-beta (Aβ), a neurotoxic peptide that aggregates to form plaques characteristic of the disease (Malm et al., 2016). These vesicles encapsulate amyloid precursor protein (APP) and facilitate its processing within multivesicular bodies before releasing it into the extracellular space. This process contributes to the dissemination of Aβ, promoting neuronal damage and cognitive decline (Rajendran et al., 2006). Although exosomes also participate in clearing toxic amyloid peptides under certain conditions, an overwhelmed clearance pathway exacerbates the pathological accumulation, highlighting their dual role in this context (Jiang et al., 2019). Similarly, exosomes contribute to the progression of **Parkinson's disease** through their involvement in the intercellular transfer of toxic

alpha-synuclein (α-syn) aggregates (Porro et al., 2019). These aggregates, which are central to Parkinson's pathology, are released by neurons and transported via exosomes to neighboring cells, where they induce further aggregation and cytotoxicity. Exosomes are also influenced by LRRK2 mutations, which enhance their secretion and the release of α-syn, thereby amplifying disease propagation (Taha & Bogoniewski, 2023). Furthermore, these toxic aggregates trigger inflammatory responses in glial cells, contributing to neuronal degeneration. The role of exosomes extends to **prion diseases**, where they facilitate the spread of infectious prion proteins (PrPsc) (Hartmann et al., 2017). These misfolded proteins are encapsulated within exosomes, which protect them from degradation and enable their transmission between cells. This mechanism supports the spatiotemporal progression of prion diseases, marked by severe neuronal damage and spongiform vacuoles in brain tissue (Mohammadinasr et al., 2024). Additionally, **Huntington's disease** involves the incorporation of mutant huntingtin protein within exosomes, which disrupts cellular trafficking pathways and contributes to neuronal dysfunction (Ananbeh et al., 2021).

Collectively, the evidence underscores the pivotal role of exosomes in mediating the pathogenesis of neurodegenerative diseases. They act as vehicles for the transfer of toxic molecules, facilitating the spread of pathology across the nervous system (Mathew et al., 2021). Understanding the molecular mechanisms underlying their involvement offers potential avenues for therapeutic intervention, including strategies to modulate exosome production or target their cargo to mitigate disease progression.

2.5 Diagnostic and Therapeutic Potential of Exosomes

Exosomes aid in diagnostics by serving as carriers of disease-specific biomarkers, offering insights into the pathological processes of neurological disorders (Younas et al., 2022). Molecular cargo such as lipids, proteins, miRNAs, and mRNAs allow exosomes to act as a snapshot of cellular activity, enabling the identification of specific biomarkers linked to neurological conditions (Kaur et al., 2021). For example, in Alzheimer's disease, exosomes derived from neurons carry amyloid-beta peptides and phosphorylated tau proteins, which are hallmark indicators of the disease (Jiang et al., 2019). Similarly,

in Parkinson’s disease, exosomes contain alpha-synuclein aggregates, which are implicated in disease pathology (Haney et al., 2015). Additionally, exosomal miRNAs, such as miR-21, miR-124, etc provide diagnostic information by highlighting processes like neuroinflammation, synaptic dysfunction, and apoptosis (Zhou et al., 2020a). Since exosomes can be isolated from biofluids like CSF, blood, and urine, they offer a minimally invasive and non-invasive means of accessing the brain's biochemical environment, bypassing the challenges posed by the blood-brain barrier. Their stability in circulation and the ability to enrich disease-specific cargo further enhance their utility as reliable diagnostic tools (Kanninen et al., 2016).

Exosomes also hold significant therapeutic potential. They act as natural delivery vehicles, capable of crossing biological barriers like the blood-brain barrier, making them particularly valuable for neurological disorders (Zeng et al., 2023). Exosomes derived from stem cells have shown promise in promoting tissue repair and regeneration by delivering growth factors and anti-inflammatory agents to damaged tissues. Additionally, engineered exosomes can be loaded with therapeutic agents, such as drugs or RNA molecules, to target specific cells or disease pathways with high precision, reducing off-target effects (Krylova & Feng, 2023). Their biocompatibility, low immunogenicity, and stability further enhance their therapeutic applicability (Iyaswamy et al., 2023). Despite these advantages, challenges such as large-scale production, targeted delivery, and potential long-term effects need to be addressed before exosome-based therapies can be widely implemented in clinical practice.

2.6 Justification for the Current Study

Neurological diseases represent a significant healthcare burden worldwide, with their early and accurate diagnosis being a persistent challenge. Despite advancements in diagnostic tools, many existing methods are invasive, expensive, or lack the sensitivity and specificity needed for early detection. Exosomes, particularly blood-derived exosomes, have emerged as promising carriers of disease-specific biomarkers, offering a minimally invasive to non-invasive and accessible approach to diagnostics. Their unique ability to cross the blood-brain barrier makes them invaluable in reflecting brain-specific

pathologies through peripheral blood samples. However, while the potential of exosomes in diagnostics is well-recognized, significant gaps remain in their isolation, characterization, and biomarker validation, particularly for neurological diseases. This study seeks to address these gaps by focusing on the characterization of blood-derived exosomes to uncover diagnostic insights for neurological disorders, thereby contributing to the development of innovative, minimal/non-invasive diagnostic strategies. This research not only aligns with the growing need for biomarker discovery but also has the potential to transform current diagnostic paradigms into neurology.

2.7 Aims and Objectives

This research aims to explore the potential of blood-derived exosomes as diagnostic tools for neurological diseases. By focusing on their isolation, characterization, and unique molecular features, this study seeks to bridge the gap in minimal/non-invasive diagnostic approaches for conditions where early detection remains challenging. The research's objectives are as follows:

i. To isolate and characterize blood-derived exosomes from healthy individuals and patients with neurological diseases.
ii. To identify specific biomarkers in exosomes that are indicative of the disease.
iii. To evaluate the potential of these biomarkers for use in diagnostic strategies.

CHAPTER 3: MATERIALS AND METHODOLOGY

In this study, we focused on isolating, characterizing, and analyzing exosomes derived from human blood samples to explore their role in neurological disorders. We selected a polymer-based precipitation method for exosome isolation due to its efficiency in providing high yield and purity (Ansari et al., 2024). The techniques used for characterization and analysis were chosen to get the best possible results in understanding the molecular processes involved in neurological diseases. Figure 3.1 highlights the experimental approach used in this study.

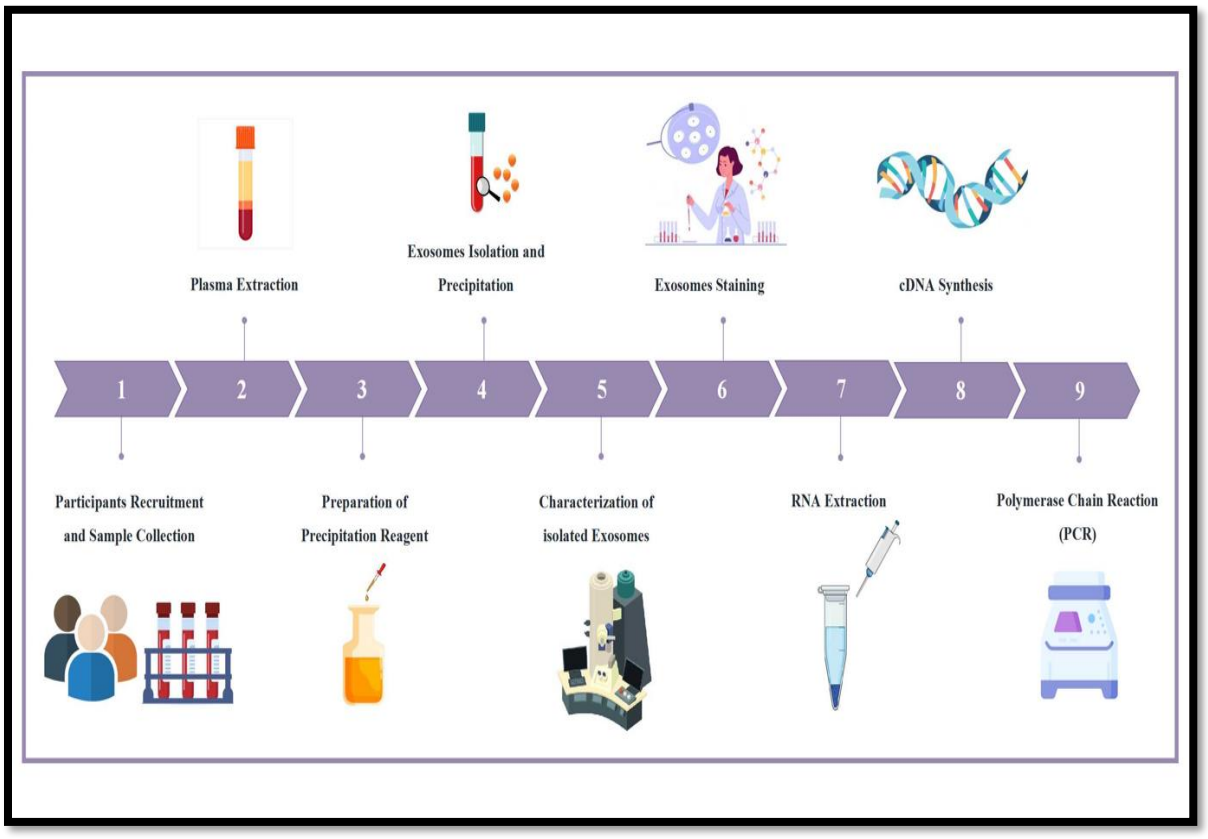


Figure 3.1 Overview of the Methodological Approach of the Study. The figure shows the sequence of steps performed in the study.

3.1 Participants Recruitment and Sample Collection

The first step in the study was the collection of human blood samples, which were essential for isolating and analyzing exosomes. A total of 2 mL of blood was collected from each participant using EDTA tubes, as shown in Figure 3.2, to prevent blood clotting.

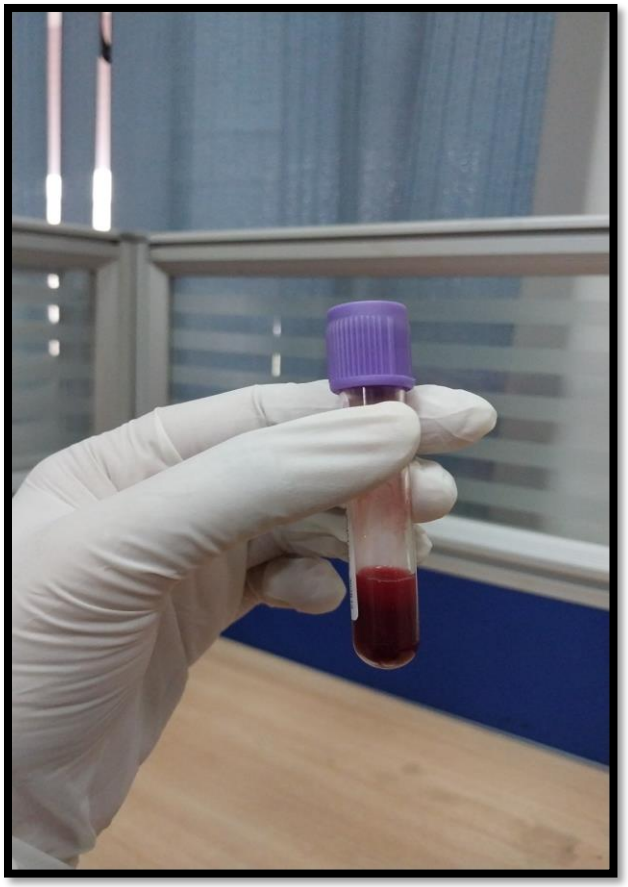


Figure 3.2 Blood sample in EDTA tube

The participants were divided into two groups:

i. **Control Group:** This group consisted of individuals without neurological diseases and with normal CBC (Complete Blood Count) results.
ii. **Diseased Group:** This group consisted of patients diagnosed with various neurological diseases.

Blood samples were collected from two hospitals:

- PIMS Hospital Islamabad.
- Saeed International Hospital, G-8 Markaz, Islamabad.

All samples were collected with informed consent from the participants, ensuring they were fully aware of the study's purpose and procedures. The collection process was carried out with great care, using proper phlebotomy techniques to ensure the safety and comfort of the participants. Ethical considerations were strictly followed, and the study was approved by the relevant ethical review boards. Once collected, the samples were properly labeled and transported under controlled conditions to the laboratory for further processing.

3.2 Plasma Extraction

To extract plasma, the collected blood samples were processed using a centrifugation technique. The samples were spun at 2,000 x g for 15 minutes at 4°C to separate the plasma from the blood cells (Kim et al., 2013). After centrifugation, the plasma was carefully collected, that can be seen in the Figure 3.3, and stored at -80°C to preserve it for exosome isolation and further analysis. This process was done with attention to detail to ensure the plasma remained intact for the next steps.

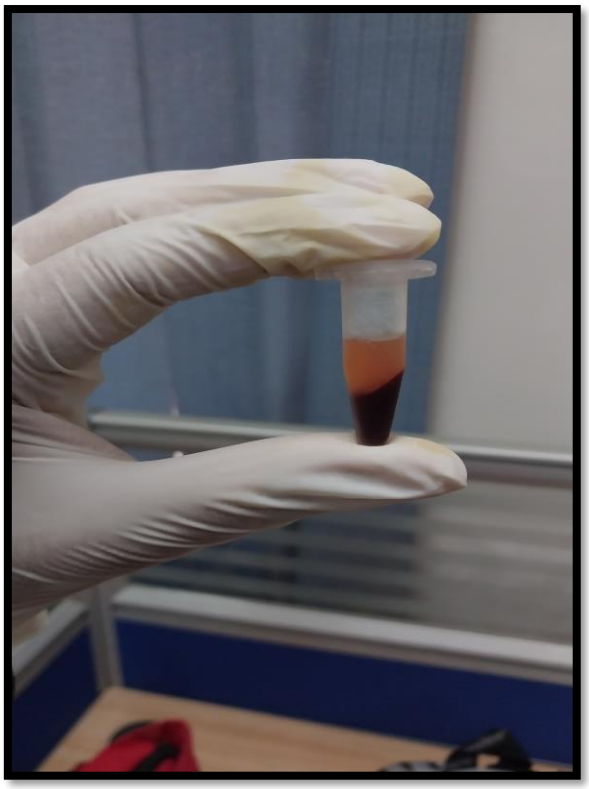


Figure 3.3 Plasma extracted from the whole blood through Centrifugation

3.3 Preparation of Precipitation Reagent

3.3.1 Materials Required

- PEG-6000 (Polyethylene glycol)
- Sodium Chloride (NaCl)
- PBS solution
- Magnetic Stirrer
- Sterile Filter (0.22 µm)

3.3.2 Preparation of PBS Solution

To prepare the PBS solution, 1 tablet of PBS was dissolved in 100 mL of distilled water. The solution was mixed thoroughly until the tablet was completely dissolved, ensuring the correct pH and ionic strength for the precipitation process.

3.3.3 Reagent Preparation

The precipitation reagent was prepared by dissolving the following components in 40 mL of the PBS solution:

- 7 g of PEG-6000
- 2.92 g of NaCl

This resulted in a 25% (w/v) solution of the precipitation reagent. The PEG-6000 and NaCl were thoroughly dissolved in the PBS solution, followed by magnetic stirring for 15 minutes to ensure complete dissolution and uniformity.

3.3.4 Filtration

After stirring, the solution was filtered through 0.22 µm syringe filters to remove any particulate matter and ensure the purity of the solution. The final volume of the precipitation reagent obtained was 30 mL, ready for use in the exosome precipitation process.

3.4 Exosome Isolation and Precipitation

3.4.1 1st Centrifugation: Removal of Cellular Debris

The plasma samples were first subjected to centrifugation at 3,000 x g for 15 minutes at 4°C to remove any remaining cellular debris. This step ensured the separation of larger cellular components from the plasma. The plasma was then filtered using 0.22 µm syringe filters to eliminate any residual particles, providing a cleaner sample for subsequent steps.

3.4.2 2nd Centrifugation: Removal of Larger Micro-Vesicles and Apoptotic Bodies

The filtered plasma was further centrifuged at a higher speed of 10,000 x g for 30 minutes at 4°C. This centrifugation step helped to remove larger micro-vesicles and apoptotic bodies, which were pelleted, while the smaller vesicles, including exosomes, remained in the supernatant.

3.4.3 3rd Centrifugation: Concentration of Exosomes

To concentrate the smaller vesicles, including exosomes, the supernatant was subjected to centrifugation at 20,000 x g for 30 minutes at 4°C. This step allowed the exosomes to pellet while the supernatant, containing larger particles, was discarded.

3.4.4 Exosome Precipitation: Addition of Precipitation Reagent

The supernatant was then mixed with the precipitation reagent in a 1:1 ratio i.e. 0.5ml each, that can be visualized in the Figure 3.4. The mixture was incubated at 4°C overnight to maximize the yield of exosomes through precipitation. This incubation period was essential to allow the exosomes to aggregate and settle.

Figure 3.4 Addition of Precipitation Reagent. Reagent enabling exosome precipitation in the supernatant.

3.4.5 Final Centrifugation: Exosome Pelleting

After the overnight incubation, the mixture was centrifuged at 10,000 x g for 10 minutes at 4°C to pellet the exosomes. The supernatant was discarded, and the exosomal pellet

was carefully resuspended in 200 µL of PBS to ensure proper stabilization for further analysis.

3.5 Characterization of Isolated Exosomes

For the characterization of isolated exosomes, Scanning Electron Microscopy (SEM) was employed. This technique provided a detailed visualization of the surface morphology and size of the isolated exosomes. The analysis was performed using the **JSM-6490LA Analytical Low Vacuum SEM** machine. The exosomal pellet, which had been resuspended in 200 µL of PBS after isolation, was used for SEM analysis. A small drop of the exosome suspension was placed onto a clean glass microscope slide. The glass slide with the exosome suspension was allowed to air-dry completely at room temperature to ensure the removal of excess PBS and moisture, preparing the sample for gold coating. The dried sample was then subjected to gold coating using a sputter coater (model: **JFC-1500**). A thin layer of gold (~10 nm) was applied to the exosome sample to enhance conductivity and improve the imaging quality. The gold coating is essential for obtaining clear and sharp images in SEM by preventing the charging of the sample during imaging.

After gold coating, the glass slide with the exosome sample was carefully mounted onto the SEM sample holder and placed into the JSM-6490LA Analytical Low Vacuum SEM machine. The SEM was set with an accelerating voltage of 15 kV, a working distance of 10 mm, and a magnification range of 10,000x to 100,000x, depending on the required resolution. Imaging was performed by scanning the sample with the electron beam. The exosomes were visualized based on the interaction between the electron beam and the gold-coated surface. Images were captured using the SEM’s built-in imaging system at different magnifications to assess the exosomes' size and morphology. The captured images were analyzed to confirm the presence of exosomes, their size distribution, and surface features.

3.6 Staining of Exosomes

For the characterization and verification of the isolated exosomes, Methylene Blue dye was employed. Methylene blue is a general dye used for staining extracellular vesicles (EVs), which include exosomes as a subclass. Although it is not specific for exosomes (Gray et al., 2015), it is widely utilized due to its ability to bind with the structural components of EVs, thereby enhancing their visibility under a microscope (Harris & Peters, 1953). This staining process was crucial for the qualitative verification of exosome isolation and involved the preparation of methylene blue solution, the staining of exosomes, incubation, washing, centrifugation, resuspension, and final microscopic visualization.

3.6.1 Preparation of Methylene Blue Solution

The preparation of methylene blue solution followed a standardized procedure to ensure consistent staining results. A 1% (w/v) stock solution was prepared by dissolving 1 g of methylene blue powder in 100 mL of distilled water. The solution was stirred thoroughly until all the dye was completely dissolved, ensuring a uniform and homogenous mixture. To eliminate any impurities or undissolved particles that might interfere with the staining process, the solution was filtered using a 0.22 µm syringe filter. The filtered solution was stored at room temperature in a clean, light-protected container to maintain its integrity. This prepared methylene blue solution was used for staining the exosome samples throughout the experiment.

3.6.2 Staining Process

The staining process began by adding 1 part of methylene blue dye to 3 parts of the exosome suspension, maintaining an accurate 1:3 ratio to ensure optimal staining. Specifically, 25 µL of methylene blue solution was mixed with 75 µL of the exosomal pellet, that can be seen in the Figure 3.5. The mixture was gently and repeatedly pipetted up and down for 10-15 seconds to allow the dye to evenly distribute and bind to the surface of the exosomes.

Figure 3.5 Addition of Methylene Blue solution to the Exosomal pellet

3.6.3 Incubation

The stained exosome suspension was incubated at room temperature for 10 minutes. This incubation period was essential for ensuring sufficient interaction time between the dye and the exosomes. The binding of the methylene blue to the exosome surface during this step enhanced the visibility of the vesicles for subsequent analysis. The incubation was performed under controlled conditions to avoid any contamination or evaporation of the sample.

3.6.4 Washing

500 µL of PBS was carefully added to the stained exosome suspension to remove any excess, unbound methylene blue dye suspension to minimize background staining. The samples were gently mixed to ensure thorough washing of the exosomes. This step was critical for improving the clarity of the sample, allowing only the dye bound to the exosomes to remain, thus enhancing the quality of the microscopic visualization.

3.6.5 Centrifugation

To pellet the stained exosomes and separate them from the unbound dye, the suspension was centrifuged at 10,000 x g for 10 minutes at 4°C. The centrifugation step concentrated the exosomes at the bottom of the centrifuge tube, forming a visible pellet. The supernatant, which contained the unbound dye and washing solution, was discarded without disturbing the pellet.

3.6.6 Resuspension

The stained exosomal pellet was then resuspended in 100 µL of PBS. This step was carried out to prepare a uniform suspension of stained exosomes for microscopy. The resuspension was performed carefully by gently pipetting up and down to maintain the structural integrity of the exosomes while ensuring an even distribution within the PBS.

3.6.7 Microscopy for Visualization

A small drop of the stained exosome suspension was placed onto a clean and dry glass microscope slide using a pipette. The slide was carefully covered with a coverslip to prevent drying of the sample and to provide a stable viewing area. The sample was examined under a light microscope at a magnification of 40x, ensuring optimal visualization of the stained exosomes. The microscope settings, including light intensity and focus, were adjusted to achieve clear and sharp images. The stained exosomes were observed as distinct structures against the background, and the results were documented by capturing high-resolution images.

3.7 RNA Extraction from Isolated Exosomes

3.7.1 Materials and Chemicals

The materials needed for the extraction of RNA from exosomal pellet are mentioned in the Table 3.1.

Table 3.1 Ingredients for RNA Extraction

Materials	Quantity (µL)
TRIzol	500
Chloroform	200
Isopropanol	500
Ethanol	100
RNase-free water	50

3.7.2 Homogenization with TRIzol

To initiate the RNA extraction process, 500 µL of TRIzol reagent was added to the exosomal pellet. The mixture was homogenized thoroughly to ensure complete lysis of the exosomal membranes, releasing the RNA into the solution. This homogenized sample was incubated at room temperature for 5 minutes to allow complete dissociation of nucleoprotein complexes.

3.7.3 Phase Separation

Following homogenization, the sample was centrifuged at 12,000 rpm for 10 minutes at 4°C to separate cellular debris. The supernatant was carefully transferred to a new tube to avoid contamination with the pellet. To this, 200 µL of chloroform was added, and the mixture was shaken vigorously for 30 seconds to enable the separation of RNA into the aqueous phase. The sample was then centrifuged again at 12,000 rpm for 10 minutes at 4°C, resulting in three distinct layers: an aqueous phase (containing RNA), an interphase, and an organic phase. The aqueous phase was carefully pipetted into a fresh tube for subsequent steps.

3.7.4 RNA Precipitation

To precipitate RNA, 500 µL of isopropanol was added to the aqueous phase. The mixture was then incubated at room temperature for 10 minutes to facilitate RNA precipitation. Following incubation, the sample was centrifuged at 12,000 x g for 10 minutes at 4°C. The RNA formed a visible pellet at the bottom of the tube, and the supernatant was carefully discarded.

3.7.5 RNA Washing, Drying, and Resuspension

To remove impurities, the RNA pellet was washed with 100 µL of 75% ethanol. The sample was vortexed briefly to ensure the ethanol reached all parts of the pellet. It was then centrifuged at 12,000 x g for 2 minutes at 4°C, and the supernatant (wash buffer) was discarded. The RNA pellet was then air-dried for 5-10 minutes to remove residual ethanol, ensuring the RNA was not over-dried, which could make resuspension difficult. Once dry, the pellet was resuspended in 20-50 µL of RNase-free water and stored at -80 until further use.

3.7.6 Evaluating RNA Concentration and Quality

The concentration and quality of the extracted RNA were assessed using a Colibri NanoDrop (TitertekBerthold, Germany). Both the concentration and purity were within acceptable ranges.

3.8 cDNA Synthesis

3.8.1 Materials and Chemicals

The following reagents, mentioned in the Table 3.2, were used to synthesized complementary DNA (cDNA). The mentioned volumes are for one RNA sample.

Table 3.2 Ingredients for cDNA Synthesis

Materials	Quantity (µL)
RNA Sample	04
Oligo(dT) primer	01
dNTP mix	02
Reaction Buffer	04
Nuclease-free water	14
Reverse Transcriptase Enzyme	01

3.8.2 Preparation of Reaction Mixture

A total reaction volume of 25 µL was prepared for each RNA sample. In a PCR tube, 4 µL of RNA was added, followed by 1 µL of Oligo(dT) primer, 2 µL of dNTP mix, and 4 µL of reaction buffer. 14 µL of nuclease-free water was added to achieve the required volume.

3.8.3 Incubation

The mixture was incubated at 55°C for 5 minutes to facilitate primer binding and remove any secondary structures in the RNA.

3.8.4 Reverse Transcription

After incubation, 1 µL of reverse transcriptase enzyme was added to the reaction mixture. The sample was then subjected to thermal cycling under the required conditions to complete cDNA synthesis. The synthesized cDNA was stored at -4°C for subsequent experiments.

3.9 Gel Electrophoresis

3.9.1 Materials and Chemicals

- 2% Agarose gel
- TBE (Tris-borate-EDTA) buffer
- Ethidium bromide (EtBr)
- DNA ladder
- cDNA samples
- Electrophoresis unit
- UV transilluminator

3.9.2 Preparation of Agarose Gel

To prepare the gel, 2g of agarose was dissolved in 98 mL TBE buffer and heated until fully dissolved. Once the solution cooled to approximately 50°C, a staining agent (e.g., ethidium bromide) was added to visualize the bands under UV light. The solution was

then poured into a gel casting tray with a comb to create wells. The gel was left at room temperature until it solidified.

3.9.3 Sample Preparation and Loading

Each cDNA sample was mixed with loading dye to enhance visibility and improve sample loading. A DNA ladder was also prepared to serve as a molecular size marker. The gel was placed in an electrophoresis tank filled with TBE buffer, and the prepared samples were loaded into the wells using a micropipette.

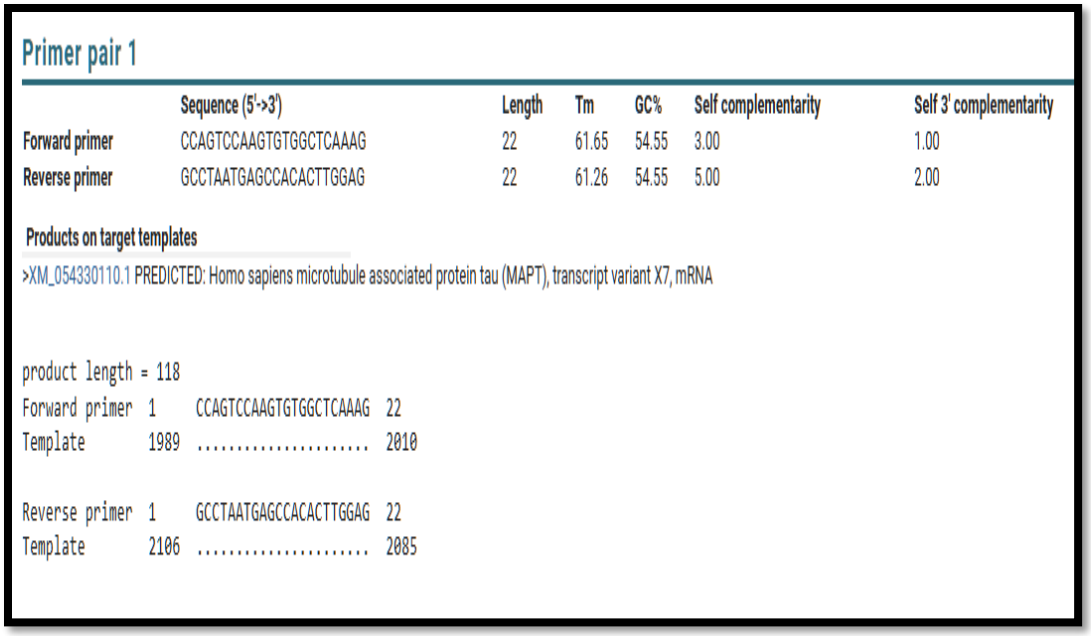
3.9.4 Electrophoresis Run

The electrophoresis unit was set to 100V for approximately 40 minutes, allowing the DNA fragments to migrate through the gel. The negatively charged DNA moved towards the positive electrode, with smaller fragments traveling faster than larger ones. After the run, the gel was placed under a UV transilluminator, and the bands were observed and documented.

3.10 Polymerase Chain Reaction (PCR)

3.10.1 Designing of Primer

The primer selection process involved consulting published literature. Subsequently, a Primer-BLAST was performed in the National Center for Biotechnology and Information (NCBI) to confirm the specificity and accuracy of the selected primers with the target as indicated in Figure 3.6 and Figure 3.7 before their application in Polymerase Chain Reaction (PCR). The chosen primers were tau, abeta (Amyloid-beta), and β-actin.



Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	CCAGTCCAAGTGTGGCTCAAAG	22	61.65	54.55	3.00	1.00
Reverse primer	GCCTAATGAGCCACACTTGGAG	22	61.26	54.55	5.00	2.00

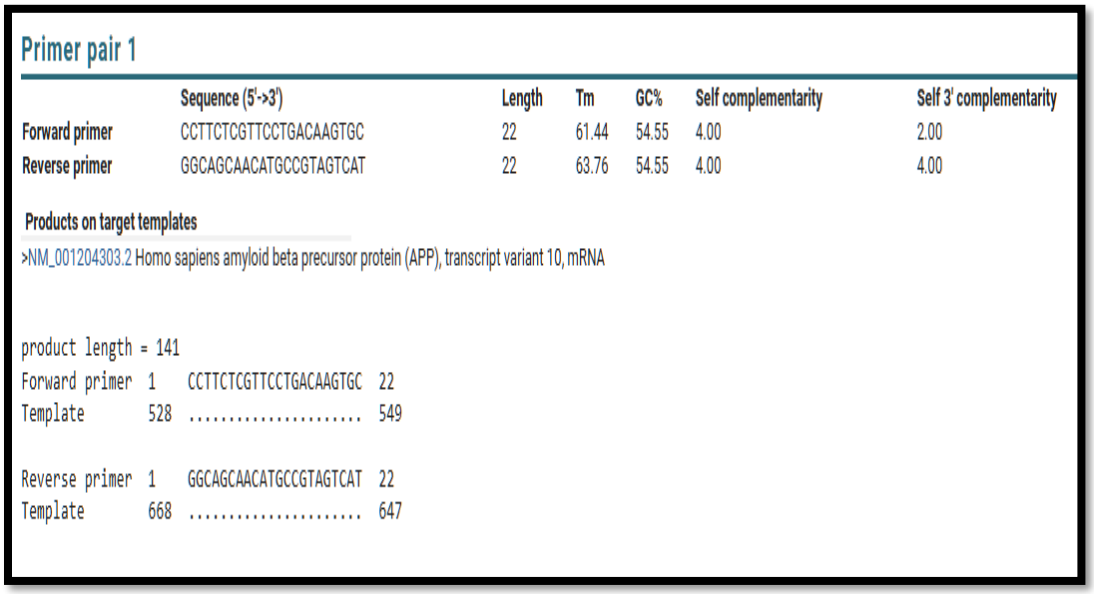
Products on target templates

>XM_054330110.1 PREDICTED: Homo sapiens microtubule associated protein tau (MAPT), transcript variant X7, mRNA

```
product length = 118
Forward primer  1     CCAGTCCAAGTGTGGCTCAAAG  22
Template        1989  ......................  2010

Reverse primer  1     GCCTAATGAGCCACACTTGGAG  22
Template        2106  ......................  2085
```

Figure 3.6 TAU Primer's NCBI BLAST Analysis. The TAU primer's specificity was verified by doing a primer BLAST analysis in NCBI.



Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	CCTTCTCGTTCCTGACAAGTGC	22	61.44	54.55	4.00	2.00
Reverse primer	GGCAGCAACATGCCGTAGTCAT	22	63.76	54.55	4.00	4.00

Products on target templates

>NM_001204303.2 Homo sapiens amyloid beta precursor protein (APP), transcript variant 10, mRNA

```
product length = 141
Forward primer  1     CCTTCTCGTTCCTGACAAGTGC  22
Template        528   ......................  549

Reverse primer  1     GGCAGCAACATGCCGTAGTCAT  22
Template        668   ......................  647
```

Figure 3.7 Amyloid-Beta Primer's NCBI BLAST Analysis. The Amyloid-Beta primer's specificity was verified by doing a primer BLAST analysis in NCBI.

Table 3.3 illustrates the optimal temperature for our selected primers.

Table 3.3 Characteristics of Primers

Gene	Sequences' Direction	Sequence	Product Length	Temperature °C
β-actin	Forward	CATCCCCCAAAGATTCTAC	347	61.5
β-actin	Reverse	CAAAGCCTTCATACATC	347	61.5
Tau	Forward	CCAGTCCAAGTGTGGCTCAAAG	118	61.65
Tau	Reverse	GCCTAATGAGCCACACTTGGAG	118	61.26
Amyloid-beta	Forward	CCTTCTCGTTCCTGACAAGTGC	141	61.44
Amyloid-beta	Reverse	GGCAGCAACATGCCGTAGTCAT	141	63.76

3.10.2 qPCR Master Mix Preparation

The Table 3.4 shows the ingredients along with their quantities to make a total of 20 µL PCR mix.

Table 3.4 Components of qPCR Master Mix Preparation and their Quantities

	Components	Quantity (µL)
1.	Forward Primer	01
2.	Reverse Primer	01
3.	cDNA Template	01
4.	SYBR Green Master Mix	04
5.	Nuclease Free Water	13
	Total Reaction Volume	20

3.10.3 Real-time PCR

Real-time polymerase chain reaction was executed utilizing the CFX96 TouchTM Real Time PCR Systems (C1000, BIO-RAD, USA). Following the preparation of a reaction mixture comprising 4ul of WizPureTM qPCR Master (SYBR) (Wizbio Solutions, catalog no: W1711, South Korea) 1ul of specific forward and reverse primers, and 1ul of cDNA template, the volume was increased to 20µl using Nuclease free water. The thermocycling settings consisted of initial denaturation for 05 minutes at 95°C, second denaturation for thirty seconds at 95°C, 35 cycles of thirty seconds at 61.65°C for TAU and 61.44 °C for AMYLOID-BETA, thirty seconds at 72°C, can be seen in the Figure 3.8. The values obtained were analyzed about gene expression using their ΔCt values after all values were normalized to those obtained for ß-actin.

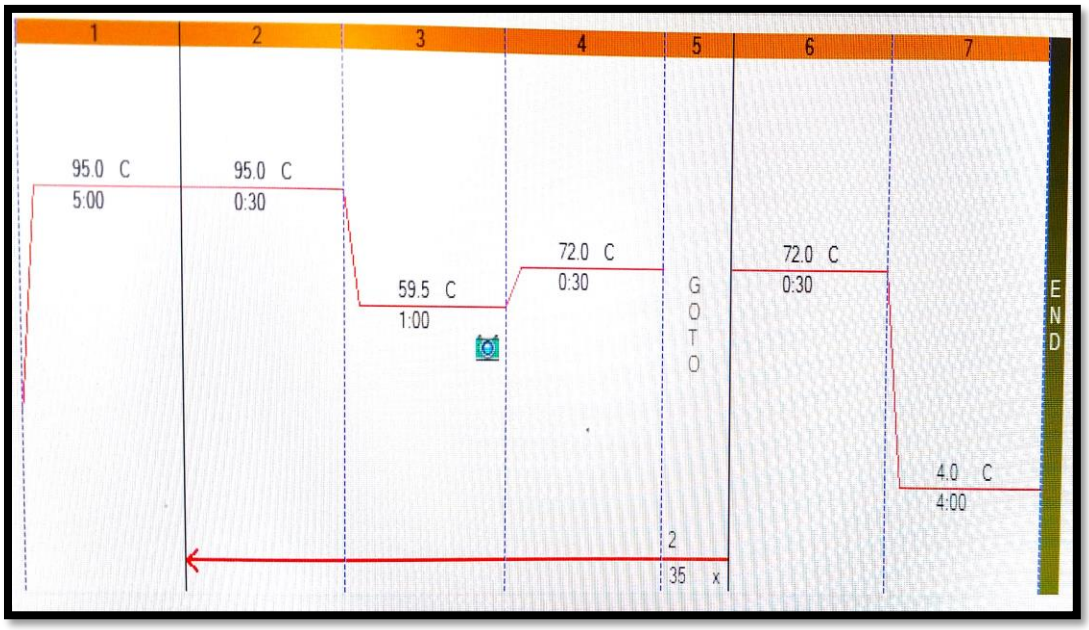


Figure 3.8 qPCR Cycling Parameters. The figure shows the thermal cycling profile. PCR circumstances: 35 cycles of denaturation at 95°C (5 min), annealing at 59.5°C (1 min), and elongation at 72°C (30 sec).

3.10.4 Statistical Analysis

Before statistical analysis, normality was assessed for all data sets. Comparative analyses between healthy control and diseased group were performed using the T-test. Graphs were created using GraphPad Prism version 10.0, and a significance threshold of $P < 0.05$ was applied. Data and results were presented with the standard error of the mean (SEM).

CHAPTER 4: RESULTS

4.1 Morphological Characterization of Exosomes via SEM

The SEM images provide a comparative analysis of exosomes isolated from healthy and diseased blood samples. In the healthy control group, Figure 4.1 & Figure 4.2, exosomes are observed to have a uniform, spherical shape with smooth surfaces, indicative of well-preserved and functionally stable extracellular vesicles. These morphological characteristics are consistent with previous findings in the literature. In contrast, exosomes isolated from diseased samples, Figure 4.3 & Figure 4.4, show significant morphological alterations. The observed size heterogeneity, irregular shapes, and rough or aggregated surfaces suggest potential disruptions in exosome formation and release mechanisms. These structural abnormalities may be attributed to disease-associated stress, altered cellular metabolism, or pathological modifications affecting membrane composition. Such morphological changes have been previously linked to variations in exosomal cargo and functionality, which may serve as potential diagnostic markers for neurological disorders.

4.1.1 Healthy Group Images

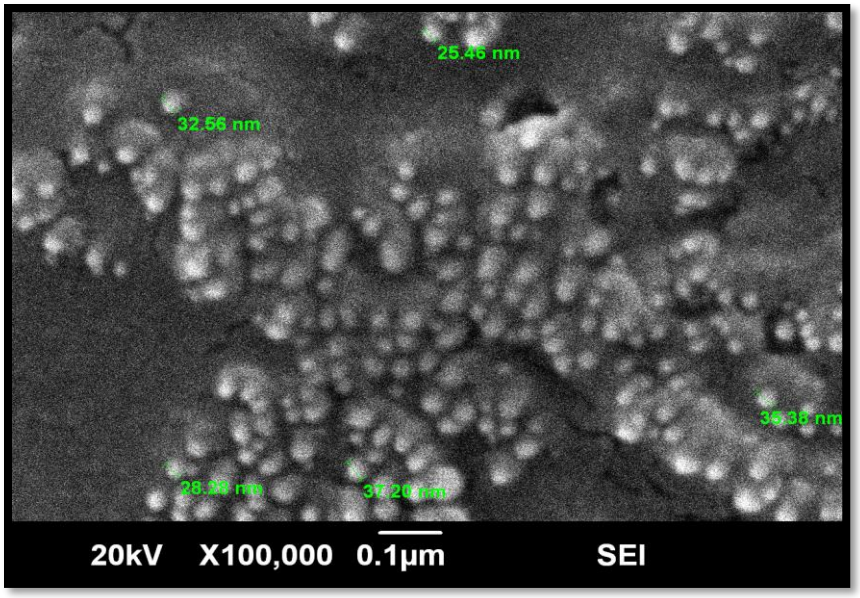


Figure 4.1 SEM Image of Exosomes from healthy control sample at a higher magnification of 100,000X. The image shows well-defined, spherical exosomes with intact membrane structures and minimal aggregation in the healthy control sample.

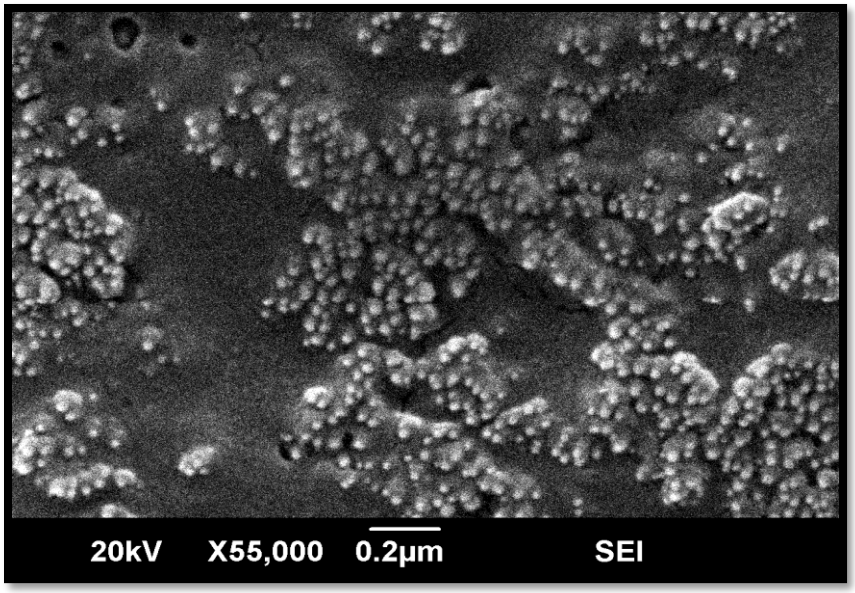


Figure 4.2 SEM Image of Exosomes from healthy control sample at a magnification of 55,000X. The image shows well-defined, spherical exosomes with intact membrane structures and minimal aggregation in the healthy control sample.

4.1.2 Diseased Group Images

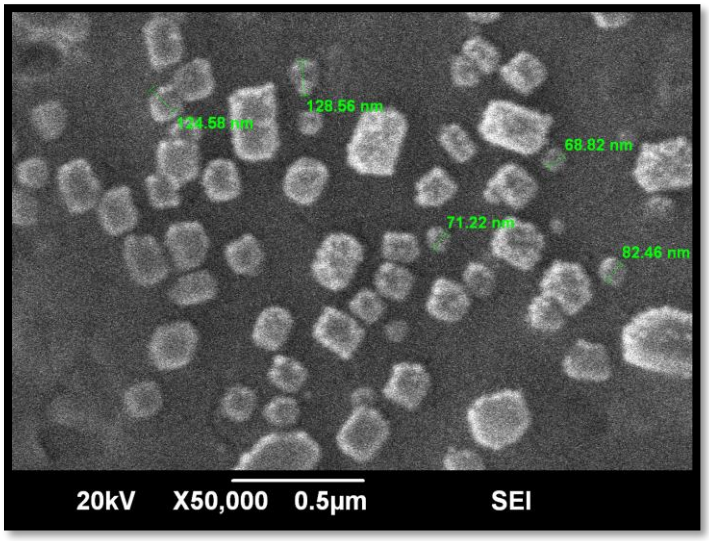


Figure 4.3 SEM Image of Exosomes from the diseased sample at a magnification of 50,000X. The image shows exosomes from the diseased sample with irregular morphology, increased aggregation, and potential structural alterations compared to healthy controls.

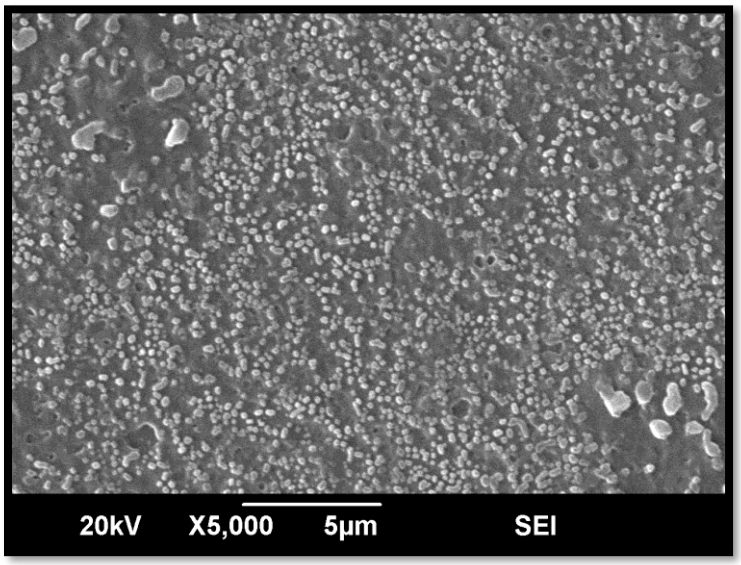


Figure 4.4 SEM Image of Exosomes from the diseased sample at a magnification of 5,000X. The image shows exosomes from the diseased sample with irregular

morphology, increased aggregation, and potential structural alterations compared to healthy controls.

4.1.3 Comparison

Exosomes from healthy samples, exhibit a spherical shape, uniform size, and moderate density and exosomes from diseased samples appear larger, irregularly shaped, have blebs, and are more densely packed, with signs of surface roughness and aggregation. The observed differences in exosome morphology between healthy and diseased samples support the hypothesis that disease conditions impact extracellular vesicle biogenesis, leading to structural and functional alterations. These findings reinforce the potential of exosome-based biomarkers for early disease detection and mechanistic studies in neurological disorders.

4.2 Methylene Blue Staining Results (Visualization)

The staining results serve as a preliminary confirmation of exosome isolation. The extracellular vesicles successfully retained the dye, appeared as grouped structures, and were clearly visible under the microscope, which can be seen in the Figure 4.5. The clustering effect is the characteristic of exosomes due to their natural tendency to aggregate in solution. The uniform staining pattern indicates the effective vesicular nature of exosomes.

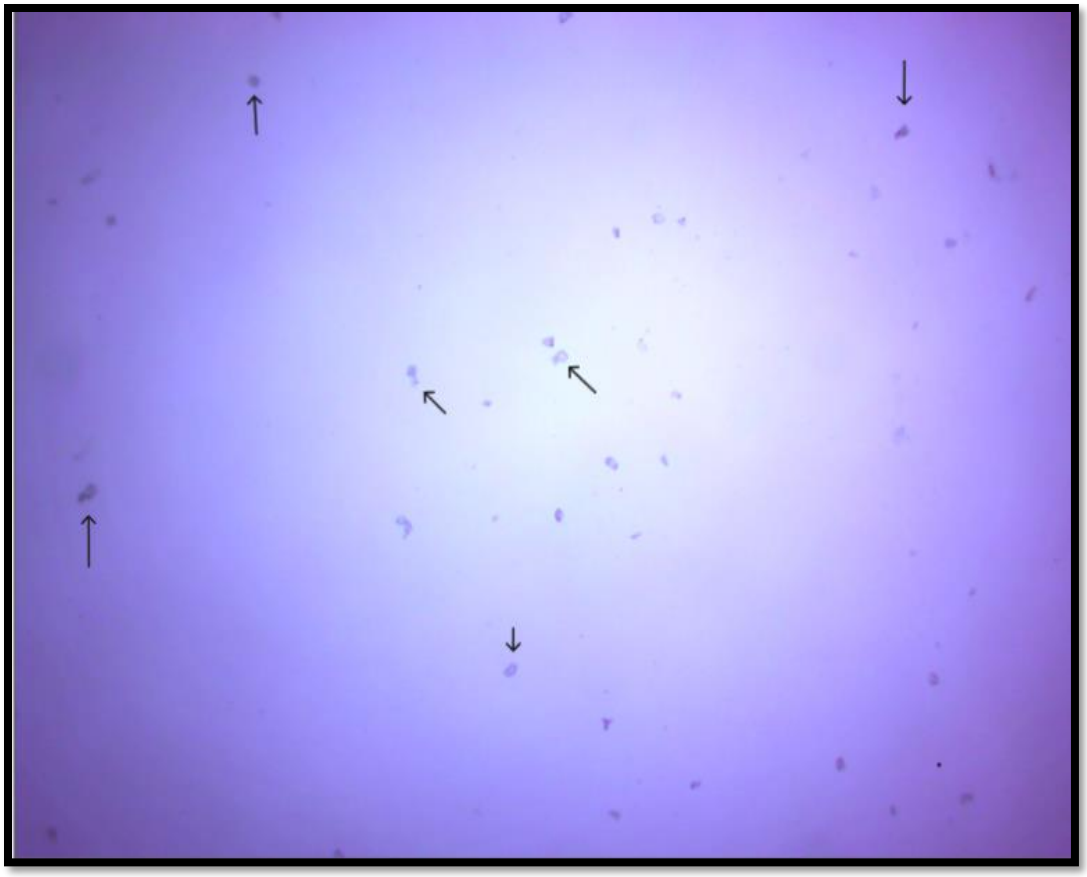


Figure 4.5 Stained Exosomes. Exosomes were successfully stained in group form with Methylene blue dye solution, confirming their vesicular nature and presence in the isolated sample.

4.3 RNA Quality Assessment via Nanodrop Spectrophotometry

Nanodrop spectrophotometry gives us information about the purity of our extracted RNA samples. This is typically measured using two ratios: 260/280 and 260/230, which correspond to absorbance at the wavelengths of 230, 260, and 280 nm. The 260/280 ratio is used to assess the purity of the sample. For RNA samples, an optimal 260/280 ratio ranges from 1.80 to 2.01. As far as exosomes are concerned, a typical acceptable 260/280 ratio for exosomal RNA is considered to be around 1.6, due to the often-low concentration and potential for contaminants in exosome isolation. And a typical concentration range for exosomal RNA is considered to be very low, usually between 50-100 pg/µL due to the inherently small amount of RNA present in exosomes, and

concentration within this range is considered as ‘NORMAL’ for exosomal RNA analysis. Our results of Nanodrop spectrophotometry confirmed the successful extraction of RNA from exosomes, with concentration and purity ratios falling within acceptable ranges. Table 4.1 illustrates that both the concentration and the purity of our extracted RNA samples were reliable; thus, we were able to use them in qPCR.

Table 4.1 Purity of Extracted RNA from Isolated Exosomes. This table provides a detailed overview of the concentration and purity of the RNA extracted from exosomes, which are intended for use in subsequent qPCR analysis. This information is crucial for ensuring the accuracy and reliability of the qPCR results.

Nanodrop Spectrophotometer Analysis		
Sample No.	**A260/A280 Ratio**	**Concentration (ng/ µL)**
01	**1.59**	**22.24**
02	**1.90**	**31.09**
03	**2.02**	**23.02**
04	**1.70**	**59.37**
05	**1.79**	**33.69**
06	**1.63**	**51.09**

07	**2.00**	**60.31**
08	**2.03**	**57.76**

The following figures, Figure 4.6 & Figure 4.7 represent the Nanodrop spectrophotometry visual results of few exosomal RNA samples.

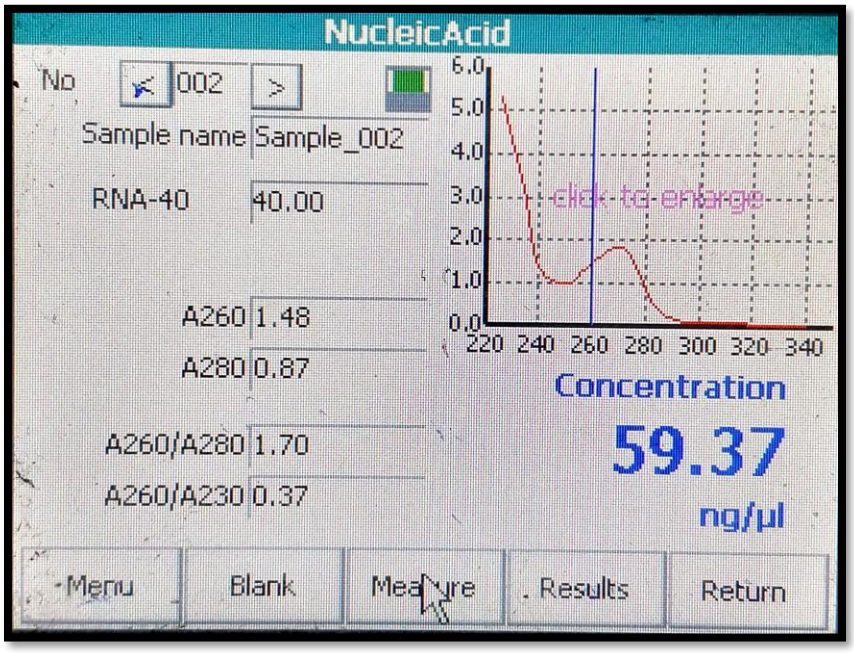


Figure 4.6 Absorbance Spectra and Purity Ratios of an Exosomal RNA Sample (01).

The A260/A280 ratio of the sample is 1.70 and the concentration is 59.37 ng/µL.

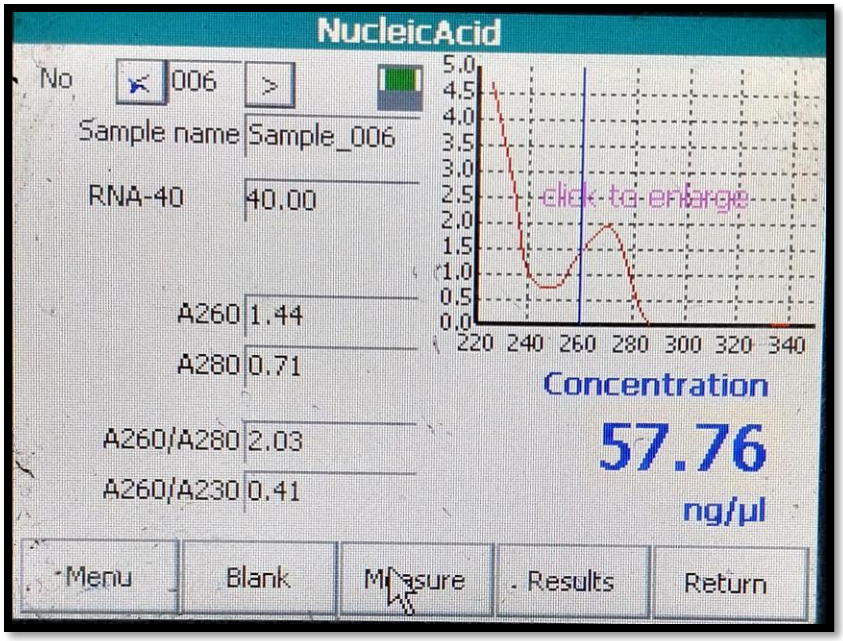


Figure 4.7 Absorbance Spectra and Purity Ratios of an Exosomal RNA Sample (02).

The A260/A280 ratio of the sample is 2.03 and the concentration is 57.76 ng/µL.

4.4 Real-Time PCR Results and Findings

To examine the mRNA expression levels of amyloid-beta and tau protein, which are the key protein players associated with neurological and neurodegenerative disorders, we used real-time PCR analysis. The results obtained revealed distinct modulation in the expression levels, highlighting variations between the healthy and diseased samples.

4.4.1 Tau Protein Relative Expression Analysis

The graph in Figure 4.8 illustrates the relative expression levels of tau protein in two groups: a healthy group and a diseased group. The X-axis distinguishes between the two groups, while the Y-axis measures the relative expression of tau protein, with values ranging from 0 to 4. The healthy group exhibits a significantly higher expression of tau protein, with a relative expression value around 3.0, suggesting substantial expression in healthy individuals. In contrast, the diseased group shows a marked reduction in tau protein expression, with a value close to 1.0, indicating downregulation. The error bars reflect the variability within each group, with the diseased group showing slightly larger

variability, possibly due to individual factors such as disease stage or severity. A star (*) symbol between the two bars indicates a statistically significant difference in tau protein expression, likely with a p-value less than 0.05. This suggests that tau protein downregulation in the diseased group is statistically significant, potentially playing a role in the pathophysiology of the disease. The findings point to tau protein as a possible biomarker for the condition, with its expression levels differing between healthy and diseased individuals.

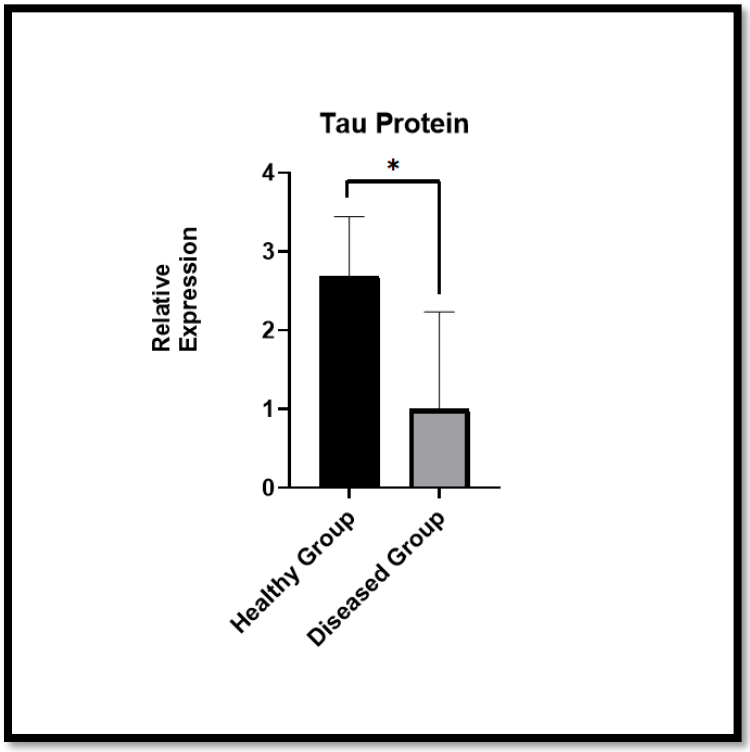


Figure 4.8 Tau Protein relative mRNA Expression results (normalized to β-actin). The graph shows the effects of the disease condition on the relative expression of tau protein in the healthy and diseased groups. A t-test comparison with the healthy group indicated a statistically significant decrease in tau protein expression in the diseased group ($p < 0.05$). Values are presented as mean ± SEM.

4.4.2 Amyloid-Beta Relative Expression Analysis

The Figure 4.9 illustrates the relative expression levels of amyloid beta in two groups: the healthy group and the diseased group. The healthy group shows minimal expression, with values close to 0, indicating a significant downregulation of amyloid beta in normal conditions. In contrast, the diseased group exhibits markedly higher expression levels, with a relative expression value of approximately 1.5, reflecting an upregulation of amyloid beta. The error bars indicate minimal variability in the healthy group, while the diseased group displays greater variability, suggesting more heterogeneous expression levels within this group. A statistical comparison between the two groups is marked by a star (*), signifying a significant difference in amyloid beta expression, likely with a p-value of less than 0.05. This indicates that the observed upregulation of amyloid beta in the diseased group is not due to random variation but is likely linked to disease pathology, highlighting its potential role as a biomarker or contributor to the condition.

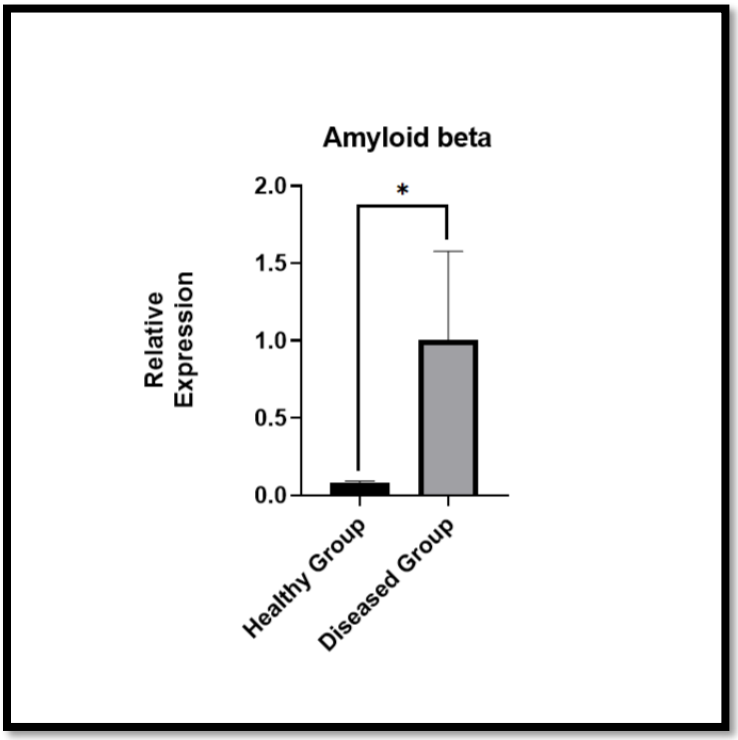


Figure 4.9 Amyloid-Beta relative mRNA Expression results (normalized to β-actin).

The graph shows the effects of the disease condition on the relative expression of

amyloid-beta in the healthy and diseased groups. A t-test comparison with the healthy group indicated a statistically significant increase in amyloid beta expression in the diseased group ($p < 0.05$). Values are presented as mean ± SEM.

CHAPTER 5: DISCUSSION

Exosomes, a subset of extracellular vesicles (EVs), have emerged as crucial mediators of intercellular communication, facilitating the exchange of biomolecules such as proteins, lipids, and nucleic acids between cells. These nanosized vesicles are secreted by almost all cell types and play a pivotal role in maintaining cellular homeostasis by modulating immune responses, removing cellular waste, and participating in signal transduction pathways (Agarwal et al., 2024). In the central nervous system (CNS), exosomes are particularly significant due to their ability to cross the blood-brain barrier (BBB), enabling bidirectional communication between the brain and peripheral systems. This unique property has positioned exosomes as potential biomarkers and therapeutic agents for neurodegenerative diseases (Kalani et al., 2014). In the context of neurological disorders, exosomes contribute to both neuroprotection and disease progression. They can facilitate the clearance of toxic proteins, such as tau and amyloid-beta, by shuttling them for degradation. However, they may also propagate pathological proteins, promoting the spread of neurodegenerative processes. For instance, in Alzheimer's disease (AD), exosomes have been implicated in the dissemination of hyperphosphorylated tau and amyloid-beta aggregates, exacerbating disease pathology. Similarly, in Parkinson's disease and amyotrophic lateral sclerosis (ALS), exosomes have been shown to mediate the transmission of misfolded α-synuclein and superoxide dismutase-1 (SOD1), respectively, leading to neuronal dysfunction and disease progression (Rastogi et al., 2021a).

Exosomes can be extracted using a variety of methods, each with its own set of advantages. Differential ultracentrifugation is often considered the gold standard for exosome isolation due to its ability to separate particles based on size and density, resulting in relatively pure populations of exosomes. Density gradient centrifugation offers high purity by separating vesicles from non-vesicular particles but is time-consuming and may not distinguish between similar densities. Size-exclusion chromatography (SEC) provides a quick, easy, and cost-effective method that preserves the structure and uniformity of exosomes. Microfluidics combines small sample volume

requirements with rapid detection capabilities, offering a promising platform for integrating isolation and analysis processes (Yu et al., 2018).

Given their dual role in neurodegeneration, exosomes are now being extensively investigated for their diagnostic and therapeutic potential. Engineered exosomes are being explored as delivery vehicles for neuroprotective agents, presenting a promising avenue for targeted therapeutic interventions. Understanding the precise mechanisms governing exosome-mediated communication in the CNS is essential for harnessing their potential in combating neurodegenerative diseases (Tao & Gao, 2024). The presence of disease-associated biomarkers, such as tau and amyloid-beta, within exosomes suggests their involvement in neurodegenerative processes. The association between tau protein and exosomes has been widely studied in neurodegenerative diseases, particularly Alzheimer's disease. Tau is a microtubule-associated protein that becomes hyperphosphorylated and aggregates in neurons, leading to neurodegeneration. The presence of tau within exosomes suggests that these vesicles may facilitate its spread across neurons, contributing to disease progression. In this study, tau was detected in exosomes isolated from blood samples, supporting previous research indicating that tau-containing exosomes can serve as potential biomarkers. The ability of exosomes to cross the blood-brain barrier enhances their significance in liquid biopsy-based diagnostics, allowing for the detection of neurological disease-associated tau levels in peripheral blood. However, the variation in tau levels between healthy and diseased samples suggests that further studies are required to establish clear diagnostic thresholds. Tau which is a microtubule-associated protein, plays a crucial role in both normal neuronal function and in the development of neurodegenerative diseases known as tauopathies. In a healthy brain, tau regulates microtubule structure and dynamics, facilitates axonal transport, maintains genomic DNA integrity, and modulates neuronal activity, neurogenesis, iron export, and long-term depression. However, in tauopathies like Alzheimer's disease (AD), frontotemporal dementia with parkinsonism-17 (FTDP-17), Pick disease (PiD), progressive supranuclear palsy (PSP), and corticobasal degeneration (CBD), tau becomes hyperphosphorylated and aggregated, leading to the formation of neurofibrillary tangles (NFTs). This aggregation disrupts normal neuronal function by reducing functional tau levels, sequestering cellular components, and impeding axonal

transport, ultimately causing neurodegeneration. Hyperphosphorylation weakens tau's binding affinity to microtubules, promoting instability and self-aggregation, while also inducing tau missorting, altering its degradation, and affecting interactions with other proteins. Soluble tau oligomers, rather than NFTs, are implicated in neuronal toxicity and propagate through endocytosis and trans-synaptic pathways, triggering aggregation in postsynaptic neurons (Gao et al., 2018).

Amyloid-beta, another hallmark of neurodegenerative disorders, is known for its role in amyloid plaque formation, which leads to neuronal damage. Exosomes have been implicated in beta-amyloid aggregation and clearance, with studies suggesting that they may act as carriers for the extracellular transport of beta-amyloid peptides. The presence of beta-amyloid within exosomes in this study supports existing evidence that exosomes contribute to the dissemination of toxic protein aggregates. Elevated beta-amyloid levels in diseased samples highlight the potential of exosomal beta-amyloid as a diagnostic marker. However, the extent to which exosomes promote or mitigate amyloid toxicity remains a subject of ongoing research. Further studies are needed to differentiate whether exosome-associated amyloid-beta plays a protective role in clearing toxic peptides or if it exacerbates neurodegeneration by facilitating their spread. In AD, amyloid-beta (Aβ) plaques interact with tau, initiating tau-mediated neurotoxicity by facilitating tau phosphorylation and directing pathogenic tau species into dendritic spines, leading to spine collapse and dendritic degeneration. Tau reduction can protect against Aβ toxicity by inhibiting neuronal over-excitation, highlighting the complex interplay between Aβ and tau in neurodegenerative processes (Maltsev et al., 2011).

The findings of this study align with previous research demonstrating the role of exosomes in neurodegenerative diseases. Several studies have reported the presence of tau and amyloid-beta in exosomes derived from cerebrospinal fluid and blood, reinforcing the notion that exosomes serve as vehicles for these pathogenic proteins. However, differences in exosomal content, isolation techniques, and disease stages across studies contribute to variability in results. Some studies suggest that exosome-associated tau and amyloid-beta levels are significantly higher in patients with advanced disease, while others indicate that early-stage detection remains challenging due to overlapping

biomarker levels with healthy controls. The inconsistencies in findings emphasize the need for standardized protocols in exosome isolation and biomarker quantification to enhance diagnostic reliability (Ningrum & Kung, 2023). If validated in larger cohorts, exosomal tau and amyloid-beta detection could provide an early and accessible diagnostic tool, reducing the reliance on invasive cerebrospinal fluid analysis. Additionally, exosomes hold promise for therapeutic applications, including targeted drug delivery and gene therapy. Understanding their role in disease pathology may pave the way for exosome-based interventions aimed at modulating neurodegenerative processes.

Moving forward, larger-scale studies are essential to validate these findings and refine exosome-based diagnostic approaches. Standardizing isolation and quantification methods will be critical in ensuring reproducibility across different research settings. Additionally, exploring the mechanistic role of exosomes in disease progression through in-vitro and in-vivo studies could provide deeper insights into their pathological contributions. Clinical trials evaluating the feasibility of exosome-based biomarkers in real-world diagnostic settings will be crucial in translating these findings into clinical applications.

Limitations

- The limited number of both healthy and diseased samples reduces the statistical power of the findings and may not fully represent the broader population.
- Differences in isolation efficiency and purity may introduce variability in exosome yield, affecting biomarker quantification and reproducibility across studies.
- While tau and amyloid-beta were detected in exosomes, their functional role in disease pathology was not assessed through in-vitro or in-vivo experiments.
- The study focused on tau and amyloid-beta, but other relevant biomarkers were not analyzed, which could provide a more comprehensive understanding of disease mechanisms.

- The presence of co-isolated non-exosomal vesicles or protein aggregates may influence the specificity of biomarker detection.
- While exosome-based diagnostics hold promise, further validation in larger, clinically diverse cohorts is needed before integration into routine clinical practice.

CHAPTER 6: CONCLUSION AND FUTURE RECOMMENDATIONS

Blood-derived exosomes have emerged as promising biomarkers for neurological disorders due to their ability to carry disease-specific molecular signatures. Their presence in biofluids and stability make them valuable tools for non-invasive diagnostics. In this research, exosomes were successfully isolated using the precipitation method and characterized through Scanning Electron Microscopy (SEM), confirming their expected morphology and size. The study provided important insights into their diagnostic potential, but challenges such as variations in exosome purity and yield, as well as a limited number of diseased samples, highlighted the need for further refinement in experimental approaches. Despite these limitations, the findings reinforce the role of exosomes as potential biomarkers and contribute to the growing body of research in the field.

Future research should focus on increasing the sample size and including a more diverse patient population to enhance statistical significance and improve the reliability of exosome-based biomarkers. Standardizing isolation and characterization protocols is essential to ensure consistency and reproducibility across different studies. Exploring alternative isolation methods such as ultracentrifugation and size-exclusion chromatography could help improve the purity and efficiency of exosome recovery. Additionally, expanding molecular profiling to include proteomics, transcriptomics, and lipidomics will provide a deeper understanding of the molecular composition of exosomes and their relevance in neurological diseases.

Functional validation of exosomal biomarkers should also be a priority, as understanding how exosomal cargo contributes to disease mechanisms will strengthen their clinical application. Furthermore, integrating exosome-based diagnostics into longitudinal studies could help monitor disease progression and assess treatment responses over time. Beyond diagnostics, exosomes hold significant potential in therapeutic applications, particularly in targeted drug delivery and neuroprotection. Future research should explore strategies for engineering exosomes to carry therapeutic molecules, which could open new avenues for treatment.

The potential of exosome-based diagnostics in neurological disorders is promising, but several challenges must be addressed before they can be widely implemented in clinical practice. Advancements in isolation techniques, molecular characterization, and functional validation will be crucial in translating exosome research from the laboratory to real-world medical applications. With continuous progress in the field, exosomes could revolutionize early disease detection and personalized medicine, ultimately improving patient outcomes in neurological disorders.

REFERENCES

1. Abdelsalam, M., Ahmed, M., Osaid, Z., Hamoudi, R., & Harati, R. (2023). Insights into exosome transport through the blood–brain barrier and the potential therapeutical applications in brain diseases. *Pharmaceuticals*, *16*(4), 571.
2. Agarwal, P., Anees, A., Harsiddharay, R. K., Kumar, P., & Tripathi, P. K. (2024). A Comprehensive Review on Exosome: Recent Progress and Outlook. *Pharmaceutical Nanotechnology*, *12*(1), 2–13.
3. Agnihotram, R., Dhar, R., Dhar, D., Purushothaman, K., Narasimhan, A. K., & Devi, A. (2024). Fusion of Exosomes and Nanotechnology: Cutting-Edge Cancer Theranostics. *ACS Applied Nano Materials*, *7*(8), 8489–8506.
4. Ananbeh, H., Vodicka, P., & Kupcova Skalnikova, H. (2021). Emerging roles of exosomes in Huntington's disease. *International Journal of Molecular Sciences*, *22*(8), 4085.
5. Andreu, Z., & Yáñez-Mó, M. (2014). Tetraspanins in extracellular vesicle formation and function. *Frontiers in Immunology*, *5*, 442.
6. Ansari, F. J., Tafti, H. A., Amanzadeh, A., Rabbani, S., Shokrgozar, M. A., Heidari, R., Behroozi, J., Eyni, H., Uversky, V. N., & Ghanbari, H. (2024). Comparison of the efficiency of ultrafiltration, precipitation, and ultracentrifugation methods for exosome isolation. *Biochemistry and Biophysics Reports*, *38*(October 2023), 101668. https://doi.org/10.1016/j.bbrep.2024.101668
7. Aseervatham, J. (2023). Dynamic Role of Exosome microRNAs in Cancer Cell Signaling and Their Emerging Role as Noninvasive Biomarkers. *Biology*, *12*(5), 1–32. https://doi.org/10.3390/biology12050710
8. Ashique, S., Kumar, N., Mishra, N., Muthu, S., Rajendran, R. L., Balakumar, C., Obeng, B. F., Hong, C. M., Krishnan, A., & Ahn, B.-C. (2024). Unveiling the role of exosomes as cellular messengers in neurodegenerative diseases and their potential therapeutic implications. *Pathology-Research and Practice*, 155451.
9. Babst, M. (2011). MVB vesicle formation: ESCRT-dependent, ESCRT-independent and everything in between. *Current Opinion in Cell Biology*, *23*(4), 452–457.

10. Bagrov, D. V, Senkovenko, A. M., Nikishin, I. I., Skryabin, G. O., Kopnin, P. B., & Tchevkina, E. M. (2021). Application of AFM, TEM, and NTA for characterization of exosomes produced by placenta-derived mesenchymal cells. *Journal of Physics: Conference Series*, *1942*(1), 12013.
11. Baietti, M. F., Zhang, Z., Mortier, E., Melchior, A., Degeest, G., Geeraerts, A., Ivarsson, Y., Depoortere, F., Coomans, C., & Vermeiren, E. (2012). Syndecan–syntenin–ALIX regulates the biogenesis of exosomes. *Nature Cell Biology*, *14*(7), 677–685.
12. Baranyai, T., Herczeg, K., Onódi, Z., Voszka, I., Módos, K., Marton, N., Nagy, G., Mäger, I., Wood, M. J., El Andaloussi, S., Pálinkás, Z., Kumar, V., Nagy, P., Kittel, Á., Buzás, E. I., Ferdinandy, P., & Giricz, Z. (2015). Isolation of exosomes from blood plasma: Qualitative and quantitative comparison of ultracentrifugation and size exclusion chromatography methods. *PLoS ONE*, *10*(12), 1–13. https://doi.org/10.1371/journal.pone.0145686
13. Cao, F., Gao, Y., Chu, Q., Wu, Q., Zhao, L., Lan, T., & Zhao, L. (2019). Proteomics comparison of exosomes from serum and plasma between ultracentrifugation and polymer-based precipitation kit methods. *Electrophoresis*, *40*(23–24), 3092–3098. https://doi.org/10.1002/elps.201900295
14. Chandra, V., Pandav, R., Laxminarayan, R., Tanner, C., Manyam, B., Rajkumar, S., Silberberg, D., Brayne, C., Chow, J., & Herman, S. (2006). Neurological disorders. *Disease Control Priorities*, 21.
15. Chen, Y.-F., Luh, F., Ho, Y.-S., & Yen, Y. (2024). Exosomes: a review of biologic function, diagnostic and targeted therapy applications, and clinical trials. *Journal of Biomedical Science*, *31*(1), 67.
16. Chernyshev, V. S., Rachamadugu, R., Tseng, Y. H., Belnap, D. M., Jia, Y., Branch, K. J., Butterfield, A. E., Pease, L. F., Bernard, P. S., & Skliar, M. (2015). Size and shape characterization of hydrated and desiccated exosomes. *Analytical and Bioanalytical Chemistry*, *407*, 3285–3301.
17. Cho, S., Yang, H. C., & Rhee, W. J. (2020). Development and comparative analysis of human urine exosome isolation strategies. *Process Biochemistry*, *88*, 197–203.

18. Della Rosa, G., Ruggeri, C., & Aloisi, A. (2021). From Exosome Glycobiology to Exosome Glycotechnology, the Role of Natural Occurring Polysaccharides. *Polysaccharides 2021, Vol. 2, Pages 311-338*, *2*(2), 311–338. https://doi.org/10.3390/POLYSACCHARIDES2020021
19. Desdín-Micó, G., & Mittelbrunn, M. (2017). Role of exosomes in the protection of cellular homeostasis. *Cell Adhesion & Migration*, *11*(2), 127–134.
20. Diaz, G., Bridges, C., Lucas, M., Cheng, Y., Schorey, J. S., Dobos, K. M., & Kruh-Garcia, N. A. (2018). Protein digestion, ultrafiltration, and size exclusion chromatography to optimize the isolation of exosomes from human blood plasma and serum. *Journal of Visualized Experiments: JoVE*, *134*.
21. Espay, A. J., Aybek, S., Carson, A., Edwards, M. J., Goldstein, L. H., Hallett, M., LaFaver, K., LaFrance, W. C., Lang, A. E., & Nicholson, T. (2018). Current concepts in diagnosis and treatment of functional neurological disorders. *JAMA Neurology*, *75*(9), 1132–1141.
22. Evans, R. W. (1998). Complications of lumbar puncture. *Neurologic Clinics*, *16*(1), 83–105.
23. Farooqui, A. A., Farooqui, T., Panza, F., & Frisardi, V. (2012). Metabolic syndrome as a risk factor for neurological disorders. *Cellular and Molecular Life Sciences*, *69*, 741–762.
24. Gangadaran, P., Madhyastha, H., Madhyastha, R., Rajendran, R. L., Nakajima, Y., Watanabe, N., Velikkakath, A. K. G., Hong, C. M., Gopi, R. V., Muthukalianan, G. K., Valsala Gopalakrishnan, A., Jeyaraman, M., & Ahn, B. C. (2023). The emerging role of exosomes in innate immunity, diagnosis and therapy. *Frontiers in Immunology*, *13*(January), 1–20. https://doi.org/10.3389/fimmu.2022.1085057
25. Gao, Y.-L., Wang, N., Sun, F.-R., Cao, X.-P., Zhang, W., & Yu, J.-T. (2018). Review Article on Translational Neurodegeneration Tau in neurodegenerative disease. *Annals of Translational Medicine*, *6*(10), 175. https://doi.org/10.21037/atm.2018.04.23
26. Gray, W. D., Mitchell, A. J., & Searles, C. D. (2015). An accurate, precise method for general labeling of extracellular vesicles. *MethodsX*, *2*, 360–367.

27. Greening, D. W., Xu, R., Ji, H., Tauro, B. J., & Simpson, R. J. (2015). A protocol for exosome isolation and characterization: evaluation of ultracentrifugation, density-gradient separation, and immunoaffinity capture methods. *Proteomic Profiling: Methods and Protocols*, 179–209.
28. Gurung, S., Perocheau, D., Touramanidou, L., & Baruteau, J. (2021). The exosome journey: from biogenesis to uptake and intracellular signalling. *Cell Communication and Signaling*, *19*(1), 47.
29. Haney, M. J., Klyachko, N. L., Zhao, Y., Gupta, R., Plotnikova, E. G., He, Z., Patel, T., Piroyan, A., Sokolsky, M., & Kabanov, A. V. (2015). Exosomes as drug delivery vehicles for Parkinson's disease therapy. *Journal of Controlled Release*, *207*, 18–30.
30. Harris, J. E., & Peters, A. (1953). Experiments on vital staining with methylene blue. *Journal of Cell Science*, *3*(26), 113–124.
31. Hartmann, A., Muth, C., Dabrowski, O., Krasemann, S., & Glatzel, M. (2017). Exosomes and the prion protein: more than one truth. *Frontiers in Neuroscience*, *11*, 194.
32. Hierro, A., Sun, J., Rusnak, A. S., Kim, J., Prag, G., Emr, S. D., & Hurley, J. H. (2004). Structure of the ESCRT-II endosomal trafficking complex. *Nature*, *431*(7005), 221–225.
33. Hirtz, D., Thurman, D. J., Gwinn-Hardy, K., Mohamed, M., Chaudhuri, A. R., & Zalutsky, R. (2007). How common are the "common" neurologic disorders? *Neurology*, *68*(5), 326–337.
34. Homma, Y., Hiragi, S., & Fukuda, M. (2021). Rab family of small GTPases: an updated view on their regulation and functions. *The FEBS Journal*, *288*(1), 36–55.
35. Hornung, S., Dutta, S., & Bitan, G. (2020). CNS-Derived Blood Exosomes as a Promising Source of Biomarkers: Opportunities and Challenges. *Frontiers in Molecular Neuroscience*, *13*(March), 1–16. https://doi.org/10.3389/fnmol.2020.00038
36. Isaac, R., Reis, F. C. G., Ying, W., & Olefsky, J. M. (2021). Exosomes as mediators of intercellular crosstalk in metabolism. *Cell Metabolism*, *33*(9), 1744–

1762.

37. Isola, A. L., & Chen, S. (2017). Exosomes: The Messengers of Health and Disease. *Current Neuropharmacology*, *15*(1), 157–165. https://doi.org/10.2174/1570159X14666160825160421
38. Iyaswamy, A., Thakur, A., Guan, X.-J., Krishnamoorthi, S., Fung, T. Y., Lu, K., Gaurav, I., Yang, Z., Su, C.-F., & Lau, K.-F. (2023). Fe65-engineered neuronal exosomes encapsulating corynoxine-B ameliorate cognition and pathology of Alzheimer's disease. *Signal Transduction and Targeted Therapy*, *8*(1), 404.
39. Jang, Y. O., Roh, Y., Shin, W., Jo, S., Koo, B., Liu, H., Kim, M. G., Lee, H. J., Qiao, Z., & Lee, E. Y. (2024). Transferrin-conjugated magnetic nanoparticles for the isolation of brain-derived blood exosomal MicroRNAs: A novel approach for Parkinson's disease diagnosis. *Analytica Chimica Acta*, *1306*, 342623.
40. Jiang, L., Dong, H., Cao, H., Ji, X., Luan, S., & Liu, J. (2019). Exosomes in pathogenesis, diagnosis, and treatment of Alzheimer's disease. *Medical Science Monitor: International Medical Journal of Experimental and Clinical Research*, *25*, 3329.
41. Jin, X., Zhang, J., Zhang, Y., He, J., Wang, M., Hei, Y., Guo, S., Xu, X., & Liu, Y. (2024). Different origin-derived exosomes and their clinical advantages in cancer therapy. *Frontiers in Immunology*, *15*, 1401852.
42. Kalani, A., Tyagi, A., & Tyagi, N. (2014). Exosomes: Mediators of neurodegeneration, neuroprotection and therapeutics. *Molecular Neurobiology*, *49*(1), 590–600. https://doi.org/10.1007/s12035-013-8544-1
43. Kalluri, R., & LeBleu, V. S. (2020). The biology, function, and biomedical applications of exosomes. *Science*, *367*(6478), eaau6977.
44. Kanninen, K. M., Bister, N., Koistinaho, J., & Malm, T. (2016). Biochimica et Biophysica Acta Exosomes as new diagnostic tools in CNS diseases ☆. *BBA - Molecular Basis of Disease*, *1862*(3), 403–410. https://doi.org/10.1016/j.bbadis.2015.09.020
45. Kaur, S., Verma, H., Dhiman, M., Tell, G., Gigli, G. L., Janes, F., & Mantha, A. K. (2021). Brain exosomes: friend or foe in Alzheimer's disease? *Molecular Neurobiology*, 1–15.

46. Keller, S., Sanderson, M. P., Stoeck, A., & Altevogt, P. (2006). Exosomes: from biogenesis and secretion to biological function. *Immunology Letters*, *107*(2), 102–108.
47. Kim, J.-H., Woenker, T., Adamec, J., & Regnier, F. E. (2013). Simple, miniaturized blood plasma extraction method. *Analytical Chemistry*, *85*(23), 11501–11508.
48. Krylova, S. V., & Feng, D. (2023). The Machinery of Exosomes: Biogenesis, Release, and Uptake. *International Journal of Molecular Sciences*, *24*(2). https://doi.org/10.3390/ijms24021337
49. Lai, J. J., Chau, Z. L., Chen, S. Y., Hill, J. J., Korpany, K. V., Liang, N. W., Lin, L. H., Lin, Y. H., Liu, J. K., Liu, Y. C., Lunde, R., & Shen, W. T. (2022). Exosome Processing and Characterization Approaches for Research and Technology Development. *Advanced Science*, *9*(15), 1–29. https://doi.org/10.1002/advs.202103222
50. Lannigan, J., & Erdbruegger, U. (2017). Imaging flow cytometry for the characterization of extracellular vesicles. *Methods*, *112*, 55–67.
51. Lau, N. C. H., & Yam, J. W. P. (2023). From exosome biogenesis to absorption: key takeaways for cancer research. *Cancers*, *15*(7), 1992.
52. Lauwers, E., Wang, Y.-C., Gallardo, R., Van der Kant, R., Michiels, E., Swerts, J., Baatsen, P., Zaiter, S. S., McAlpine, S. R., & Gounko, N. V. (2018). Hsp90 mediates membrane deformation and exosome release. *Molecular Cell*, *71*(5), 689–702.
53. Lima, A. A., Mridha, M. F., Das, S. C., Kabir, M. M., Islam, M. R., & Watanobe, Y. (2022). A comprehensive survey on the detection, classification, and challenges of neurological disorders. *Biology*, *11*(3), 469.
54. Liu, W., Bai, X., Zhang, A., Huang, J., Xu, S., & Zhang, J. (2019a). Role of Exosomes in Central Nervous System Diseases. *Frontiers in Molecular Neuroscience*, *12*(October), 1–13. https://doi.org/10.3389/fnmol.2019.00240
55. Liu, W., Bai, X., Zhang, A., Huang, J., Xu, S., & Zhang, J. (2019b). Role of Exosomes in Central Nervous System Diseases. *Frontiers in Molecular Neuroscience*, *12*, 240. https://doi.org/10.3389/FNMOL.2019.00240

56. Livshits, M. A., Khomyakova, E., Evtushenko, E. G., Lazarev, V. N., Kulemin, N. A., Semina, S. E., Generozov, E. V, & Govorun, V. M. (2015). Isolation of exosomes by differential centrifugation: Theoretical analysis of a commonly used protocol. *Scientific Reports*, *5*(1), 1–14.
57. Malm, T., Loppi, S., & Kanninen, K. M. (2016). Exosomes in Alzheimer's disease. *Neurochemistry International*, *97*, 193–199.
58. Maltsev, A. V, Bystryak, S., & Galzitskaya, O. V. (2011). The role of β-amyloid peptide in neurodegenerative diseases. *Ageing Research Reviews*, *10*(4), 440–452. https://doi.org/https://doi.org/10.1016/j.arr.2011.03.002
59. Mathew, B., Mansuri, M. S., Williams, K. R., & Nairn, A. C. (2021). Exosomes as emerging biomarker tools in neurodegenerative and neuropsychiatric disorders—a proteomics perspective. *Brain Sciences*, *11*(2), 1–18. https://doi.org/10.3390/brainsci11020258
60. Mathivanan, S., & Simpson, R. J. (2009). ExoCarta: A compendium of exosomal proteins and RNA. *PROTEOMICS*, *9*(21), 4997–5000. https://doi.org/10.1002/PMIC.200900351
61. Mavroudis, I., Kazis, D., Kamal, F. Z., Gurzu, I.-L., Ciobica, A., Pădurariu, M., Novac, B., & Iordache, A. (2024). Understanding Functional Neurological Disorder: Recent Insights and Diagnostic Challenges. *International Journal of Molecular Sciences*, *25*(8), 4470.
62. McCullough, J., Frost, A., & Sundquist, W. I. (2018). Structures, functions, and dynamics of ESCRT-III/Vps4 membrane remodeling and fission complexes. *Annual Review of Cell and Developmental Biology*, *34*(1), 85–109.
63. Meldolesi, J. (2018). Exosomes and ectosomes in intercellular communication. *Current Biology*, *28*(8), R435–R444.
64. Miron, R. J., & Zhang, Y. (2024). Understanding exosomes: Part 1—Characterization, quantification and isolation techniques. *Periodontology 2000*, *94*(1), 231–256. https://doi.org/10.1111/prd.12520
65. Mishra, A., Singh, P., Qayoom, I., Prasad, A., & Kumar, A. (2021). Current strategies in tailoring methods for engineered exosomes and future avenues in biomedical applications. *Journal of Materials Chemistry B*, *9*(32), 6281–6309.

66. Mohammadinasr, M., Montazersaheb, S., Ayromlou, H., Hosseini, V., Molavi, O., & Hejazi, M. S. (2024). Exosome content–mediated signaling pathways in multiple sclerosis. *Molecular Neurobiology*, 1–14.
67. Mukherjee, S., Ali, S., Hashmi, S., & Jahan, S. (2023). History, Origin and Types of Neurological Disorders. In *Applications of Stem Cells and derived Exosomes in Neurodegenerative Disorders* (pp. 1–32). Springer.
68. Najjar, S., Pearlman, D. M., Alper, K., Najjar, A., & Devinsky, O. (2013). Neuroinflammation and psychiatric illness. *Journal of Neuroinflammation*, *10*, 1–24.
69. National Academies of Sciences and Medicine, E. (2023). Techniques for Neurological Disorders. In *Advances in the Diagnosis and Evaluation of Disabling Physical Health Conditions*. National Academies Press (US).
70. Nila, I. S., Sumsuzzman, D. M., Khan, Z. A., Jung, J. H., Kazema, A. S., Kim, S. J., & Hong, Y. (2022). Identification of exosomal biomarkers and its optimal isolation and detection method for the diagnosis of Parkinson's disease: A systematic review and meta-analysis. *Ageing Research Reviews*, *82*, 101764.
71. Ningrum, D. N. A., & Kung, W.-M. (2023). Challenges and perspectives of neurological disorders. *Brain Sciences*, *13*(4), 676.
72. Oyarce, K., Cepeda, M. Y., Lagos, R., Garrido, C., Vega-Letter, A. M., Garcia-Robles, M., Luz-Crawford, P., & Elizondo-Vega, R. (2022). Neuroprotective and Neurotoxic Effects of Glial-Derived Exosomes. *Frontiers in Cellular Neuroscience*, *16*(June), 1–12. https://doi.org/10.3389/fncel.2022.920686
73. Pegtel, D. M., & Gould, S. J. (2019). Exosomes. *Annual Review of Biochemistry*, *88*, 487–514.
74. Pietrowska, M., Funk, S., Gawin, M., Marczak, Ł., Abramowicz, A., Widłak, P., & Whiteside, T. (2017). Isolation of exosomes for the purpose of protein cargo analysis with the use of mass spectrometry. *Functional Genomics: Methods and Protocols*, 291–307.
75. Porro, C., Panaro, M. A., Lofrumento, D. D., Hasalla, E., & Trotta, T. (2019). The multiple roles of exosomes in Parkinson's disease: an overview. *Immunopharmacology and Immunotoxicology*, *41*(4), 469–476.

76. Raghavendra, U., Acharya, U. R., & Adeli, H. (2020). Artificial intelligence techniques for automated diagnosis of neurological disorders. *European Neurology*, *82*(1–3), 41–64.
77. Rajendran, L., Honsho, M., Zahn, T. R., Keller, P., Geiger, K. D., Verkade, P., & Simons, K. (2006). Alzheimer's disease β-amyloid peptides are released in association with exosomes. *Proceedings of the National Academy of Sciences*, *103*(30), 11172–11177.
78. Rashed, M. H., Bayraktar, E., Helal, G. K., Abd-Ellah, M. F., Amero, P., Chavez-Reyes, A., & Rodriguez-Aguayo, C. (2017). Exosomes: From garbage bins to promising therapeutic targets. *International Journal of Molecular Sciences*, *18*(3). https://doi.org/10.3390/ijms18030538
79. Rastogi, S., Sharma, V., Bharti, P. S., Rani, K., Modi, G. P., Nikolajeff, F., & Kumar, S. (2021a). The evolving landscape of exosomes in neurodegenerative diseases: exosomes characteristics and a promising role in early diagnosis. *International Journal of Molecular Sciences*, *22*(1), 440.
80. Rastogi, S., Sharma, V., Bharti, P. S., Rani, K., Modi, G. P., Nikolajeff, F., & Kumar, S. (2021b). The evolving landscape of exosomes in neurodegenerative diseases: Exosomes characteristics and a promising role in early diagnosis. *International Journal of Molecular Sciences*, *22*(1), 1–31. https://doi.org/10.3390/ijms22010440
81. Sá-Caputo, D. da C. de, Bernardo-Filho, M., Seixas, A., & Taiar, R. (2021). *Introductory Chapter: Neurological Disorders - Therapy Approaches* (M. Bernardo-Filho, R. Taiar, D. da C. de Sá-Caputo, & A. Seixas (Eds.); p. Ch. 1). IntechOpen. https://doi.org/10.5772/intechopen.97178
82. Schmidt, O., & Teis, D. (2012). The ESCRT machinery. *Current Biology*, *22*(4), R116–R120.
83. Scourfield, E. J., & Martin-Serrano, J. (2017). Growing functions of the ESCRT machinery in cell biology and viral replication. *Biochemical Society Transactions*, *45*(3), 613–634.
84. Shi, L., Wang, J., Stevens, L., Ness, P., & Shan, H. (2014). Blood safety and availability: continuing challenges in C hina's blood banking system. *Transfusion*,

54(2), 471–482.

85. Soria, F. N., Pampliega, O., Bourdenx, M., Meissner, W. G., Bezard, E., & Dehay, B. (2017). Exosomes, an unmasked culprit in neurodegenerative diseases. *Frontiers in Neuroscience*, *11*(JAN), 1–12. https://doi.org/10.3389/fnins.2017.00026
86. Stoorvogel, W., Kleijmeer, M. J., Geuze, H. J., & Raposo, G. (2002). The biogenesis and functions of exosomes. *Traffic*, *3*(5), 321–330. https://doi.org/10.1034/j.1600-0854.2002.30502.x
87. Syed Haroon, P. K. (2014). Acta poloniae pharmaceutica. *Acta Poloniae Pharmaceutica - Drug Research*, *71*(2), 301–309.
88. Taha, H. B., & Bogoniewski, A. (2023). Extracellular vesicles from bodily fluids for the accurate diagnosis of Parkinson's disease and related disorders: A systematic review and diagnostic meta-analysis. *Journal of Extracellular Biology*, *2*(11), e121.
89. Tao, H., & Gao, B. (2024). Exosomes for neurodegenerative diseases: diagnosis and targeted therapy. *Journal of Neurology*, 1–13.
90. Tian, W., Liu, S., & Li, B. (2019). Potential Role of Exosomes in Cancer Metastasis. *BioMed Research International*, *2019*, 4649705. https://doi.org/10.1155/2019/4649705
91. Tumani, H., Teunissen, C., Süssmuth, S., Otto, M., Ludolph, A. C., & Brettschneider, J. (2008). Cerebrospinal fluid biomarkers of neurodegeneration in chronic neurological diseases. *Expert Review of Molecular Diagnostics*, *8*(4), 479–494.
92. Tutanov, O., Shtam, T., Grigor'eva, A., Tupikin, A., Tsentalovich, Y., & Tamkovich, S. (2022). Blood plasma exosomes contain circulating DNA in their crown. *Diagnostics*, *12*(4), 854.
93. Valadi, H., Ekström, K., Bossios, A., Sjöstrand, M., Lee, J. J., & Lötvall, J. O. (2007). Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nature Cell Biology*, *9*(6), 654–659. https://doi.org/10.1038/NCB1596
94. Vaught, J. B. (2006). Blood collection, shipment, processing, and storage. *Cancer*

Epidemiology Biomarkers & Prevention, *15*(9), 1582–1584.

95. Vlassov, A. V., Magdaleno, S., Setterquist, R., & Conrad, R. (2012a). Exosomes: Current knowledge of their composition, biological functions, and diagnostic and therapeutic potentials. *Biochimica et Biophysica Acta - General Subjects*, *1820*(7), 940–948. https://doi.org/10.1016/j.bbagen.2012.03.017
96. Vlassov, A. V, Magdaleno, S., Setterquist, R., & Conrad, R. (2012b). Exosomes: current knowledge of their composition, biological functions, and diagnostic and therapeutic potentials. *Biochimica et Biophysica Acta (BBA)-General Subjects*, *1820*(7), 940–948.
97. Wan, R., Hussain, A., Behfar, A., Moran, S. L., & Zhao, C. (2022). The Therapeutic Potential of Exosomes in Soft Tissue Repair and Regeneration. *International Journal of Molecular Sciences*, *23*(7). https://doi.org/10.3390/ijms23073869
98. Willms, E., Johansson, H. J., Mäger, I., Lee, Y., Blomberg, K. E. M., Sadik, M., Alaarg, A., Smith, C. I. E., Lehtiö, J., & El Andaloussi, S. (2016). Cells release subpopulations of exosomes with distinct molecular and biological properties. *Scientific Reports*, *6*(1), 22519.
99. Yang, J., Zhang, Y., Gao, X., Yuan, Y., Zhao, J., Zhou, S., & Wang, H. (2021). *Plasma-Derived Exosomal ALIX as a Novel Biomarker for Diagnosis and Classi fi cation of Pancreatic Cancer*. *11*(May), 1–11. https://doi.org/10.3389/fonc.2021.628346
100. Younas, N., Fernandez Flores, L. C., Hopfner, F., Höglinger, G. U., & Zerr, I. (2022). A new paradigm for diagnosis of neurodegenerative diseases: peripheral exosomes of brain origin. *Translational Neurodegeneration*, *11*(1), 28.
101. Yu, L.-L., Zhu, J., Liu, J.-X., Jiang, F., Ni, W.-K., Qu, L.-S., Ni, R.-Z., Lu, C.-H., & Xiao, M.-B. (2018). A comparison of traditional and novel methods for the separation of exosomes from human samples. *BioMed Research International*, *2018*(1), 3634563.
102. Yuyama, K., & Igarashi, Y. (2016). Physiological and pathological roles of exosomes in the nervous system. *Biomolecular Concepts*, *7*(1), 53–68.
103. Zeng, H., Guo, S., Ren, X., Wu, Z., Liu, S., & Yao, X. (2023). Current

Strategies for Exosome Cargo Loading and Targeting Delivery. *Cells*, *12*(10). https://doi.org/10.3390/cells12101416

104. Zhang, M., Jin, K., Gao, L., Zhang, Z., Li, F., Zhou, F., & Zhang, L. (2018). Methods and Technologies for Exosome Isolation and Characterization. *Small Methods*, *2*(9), 1–10. https://doi.org/10.1002/smtd.201800021

105. Zhou, X., Xie, F., Wang, L., Zhang, L., Zhang, S., Fang, M., & Zhou, F. (2020a). The function and clinical application of extracellular vesicles in innate immune regulation. *Cellular and Molecular Immunology*, *17*(4), 323–334. https://doi.org/10.1038/s41423-020-0391-1

106. Zhou, X., Xie, F., Wang, L., Zhang, L., Zhang, S., Fang, M., & Zhou, F. (2020b). The function and clinical application of extracellular vesicles in innate immune regulation. *Cellular & Molecular Immunology 2020 17:4*, *17*(4), 323–334. https://doi.org/10.1038/s41423-020-0391-1