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NEURONAL EXOSOMAL MIRNAS AS POTENTIAL BIOMARKERS FOR PARKINSON'S DISEASE

A Thesis in

Neuroscience

by

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ABSTRACT

Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimer's. PD is characterized clinically by motor dysfunction, and pathologically by dopamine neuron loss in the substantia nigra of the basal ganglia and the presence of Lewy bodies. The exact pathoetiology and pathogenesis of PD is unknown. The clinical diagnosis of PD relies entirely on neurological findings and the medical history of the patient. Currently, one of the major challenges in studying this progressive neurodegenerative disorder is to identify and develop biomarkers that will aid in the understanding and development of a PD therapy. This will aid clinicians in early stage disease diagnosis for disease management and monitoring of the disease progression. Exosomes, which are nanoscale extracellular vesicles secreted by most cell types, have shown potential to identify biochemical changes in the brain. They encapsulate various biomolecules including microRNA (miRNA), a class of small, non- coding RNAs that regulate protein levels post-transcriptionally. miRNA expression has become a prominent biomarker for a wide variety of diseases including PD and other neurodegenerative disorders. Previous studies only focused on expression profiling of miRNA in whole blood or circulating (cell-free) serum or plasma. This may not be the optimal miRNA source due to baseline contributions of irrelevant cells such as platelets or red/white blood cells or possible degradation of miRNA.

Therefore, we hypothesized that the immunoenrichment of exosomes by a neuronal cell adhesion molecule, CD171, might be beneficial in enriching exosomes of neuronal origin which will allow us to get a more efficacious measure of miRNA biomarkers for PD patients compared to controls. We aimed to develop a robust exosome-miRNA profiling method by enriching neuronal origin exosomes using neuronal marker, CD171. Differentially expressed miRNAs

were analyzed in 60 PD patients and 40 age- and gender-matched healthy volunteers to develop a candidate biomarker panel.

Seventeen (17) miRNAs were significantly up regulated (q-value<0.05) and many were consistent with previous studies. Identification of these non-invasive biomarkers could potentially enhance PD diagnosis and help clinicians with early treatment intervention.

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LIST OF ABBREVIATIONS

BBB, Blood brain barrier

CMA, Chaperone mediated autophagy

CNS, Central nervous system

CSF, Cerebrospinal Fluid

GAPDH, Glyceraldehyde-3-phosphate dehydrogenase

hNPCs, Human neural progenitor cells

HSP, Heat Shock Protein

MHC, Major histocompatibility complexes

miRNA, microRNA

MoCA, Montreal Cognitive Assessment

MPP^+, 1-methyl-4-phenylpyridinium

MVB, Multivesicular body

PD, Parkinson’s disease

PET, Positron Emission Imaging

SNCA, alpha-synuclein

SNpc, Substantia nigra pars compacta

TFR2, Transferrin Receptor 2

TSG, Tumor susceptibility

UPDRS, Unified Parkinson’s Disease Rating Scale

UPSIT, University of Pennsylvania Smell Identification Test

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Chapter 1
INTRODUCTION

1.1 Introduction to Parkinson's disease

Parkinson's disease (PD) is a progressive neurodegenerative disorder with motor symptoms that affects ~1% of the population over 65 years of age (Pringsheim et al. 2014). It was first described by Dr. James Parkinson in the year 1817. Several genes like alpha-synuclein, PTEN induced putative kinase 1 (PINK1), leucine-rich repeat kinase 2 (LRRK2), Parkin and ATP13A2 are dysregulated in PD (Moore et al. 2005). More studies are needed in this area to resolve the involvement of these genes in the molecular pathogenesis of PD. The pathogenesis of PD involves the degeneration of the dopaminergic neurons in the substantia nigra of the midbrain (Braak et al. 2003) along with the presence of Lewy bodies/neuritis in various brain regions (Savitt, Dawson and Dawson 2006). Lewy bodies are composed of a protein called alpha synuclein, and a small amount of ubiquitin, tubulin and calbindin (Spillantini et al. 1998). Alpha-synuclein is a small, 140 amino acid protein that is found in relatively high abundance at the presynaptic terminal of neurons, it represents up to 1% of the cytosolic protein found in the brain and has the ability to generate beta-sheet structures under specific physiological conditions creating amyloid-like fibrils (Stefanis 2012). Soluble, oligomeric alpha-synuclein can transform into spherical protofibrils that eventually assemble into insoluble neurotoxic fibrils (Stefanis 2012). Braak observed a predictable topography of pathological changes in the central nervous beginning in the olfactory bulb and the medulla oblongata which then progresses rostrally from the medulla to the pons then the substantia nigra and midbrain, limbic system and the neocortical structures (Braak et al. 2003).

The mechanisms of preferential death of dopamine neurons are likely caused by a multifactorial interaction of both genes and the environment. Oligomeric alpha synuclein may lead to dopamine release from their vesicles into the cytosol which can contribute to oxidative stress-induced cell death (Mosharov et al. 2006) through disruption of glutathione homeostasis,

which may cause enhance calcium influx and increase calcium-mediated calpain activation and free radical production (Mosharov et al. 2009). On the other hand, neurotoxins such as 6-hydroxydopamine (6-OHDA), the pesticide paraquat and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) have also been shown to destroy dopamine neurons, where some individuals exposed to these chemicals display Parkinsonism (Betarbet et al. 2000). These neurotoxins cause oxidative stress by interfering with complex I of the electron transport chain, that then causes an increase in free radicals, leading to mitochondrial dysfunction and eventually cell death (Jenner 2003). Chronic inflammation from dying cells may also play a role in the degenerative process (Dexter and Jenner 2013).

The degeneration of the nigro-striatal pathways can cause specific symptoms associated with dopamine deficiency that include both motor and non-motors issues. The clinical diagnosis is based on four (4) cardinal motor symptoms; these are resting tremor, bradykinesia, postural instability and muscle rigidity, which are usually delayed in manifestation until late in the progression (Dickson et al. 2009). On the other hand, the non-motor symptoms usually include anxiety, fatigue, constipation, depression, sleep disturbance, gastrointestinal, and sexual dysfunctions.(Schneider and Obeso 2015).

1.2 Need for PD Biomarkers

The key pathology of PD is in the brain and is difficult to assess in living subjects; therefore, it is difficult to monitor PD progression in an unbiased and objective manner. Despite many advances in both medical and surgical symptomatic treatments, PD patients continue to experience progressive motor and non-motor disabilities that ultimately impair their quality of life as the disease advances. At the time of diagnosis, usually when patients presented with motor

systems, approximately 70-80% of dopamine neurons have already been lost. Therefore, a low cost, non-invasive biomarker is required to help us understand the early underlying biological and pathological processes in PD, early intervention and clinical practice. A molecular biomarker is a biological molecule found in the blood body fluids and tissues that can be measured as an indicator for abnormal underlying biological processes and can be used as a diagnostic or prognostic tool for a specific disease. An efficient biomarker for PD may guide the development of potential neuroprotective therapies for pharmaceutical companies and clinicians, and enhance basic understanding of PD-associated pathological processes for neuroscientists (Shtilbans and Henchcliffe 2012).

PD has a subtle long preclinical phase; as such this makes it difficult to identify patients earlier when they may benefit from a therapeutic intervention. Three phases have been proposed, these are: preclinical, premotor and motor phases. In the preclinical phase, no symptoms are present. Non-motor symptoms occur in the premotor phase. Markers for this phase are referred to as premotor or prodromal markers. These include: olfactory dysfunction (Doty, Deems and Stellar 1988) measured using University of Pennsylvania Smell Identification Test (UPSIT), neuropsychiatric, and autosomal changes such as gastrointestinal complications (Abbott et al. 2001), or sleep abnormalities (excessive sleepiness (Abbott et al. 2005), and idiopathic REM sleep behavior disorder (Boeve 2013)). The clinical measures of PD progression of motor symptoms and cognitive decline can be assessed by the Unified Parkinson's Disease Rating Scale (UPDRS), that measures cognition, motor involvement and level of independence where severity of symptoms is measures by the Hoehn and Yahr scale (Disease 2003). These measures are used for diagnosis and monitoring of PD. Limitations of the clinical measures may include subjective fluctuations and influences of drugs. Dopamine loss precedes the motor symptoms

associated with PD as detected by radioligands for dopamine transporter using positron emission imaging (PET) and it has been used to assess the integrity of the dopaminergic severity and in some cases successful in differentiating PD severity (Miller and O'Callaghan 2015). Alpha synuclein accumulation has also been detected in gastrointestinal tract neurons using histopathology (Shannon et al. 2012). These early pathological changes in PD are usually asymptomatic; therefore, biomarkers rather than clinical manifestations are needed for identification and longitudinal follow-up (Kalia and Lang 2016). Lewy body related protein biomarker screening has also been performed in peripheral fluids such as cerebrospinal fluid (CSF). Messenger RNA (mRNA) biomarkers in the blood may also be informative for identifying PD related genes, but due to their variability in each individual, other markers such as miRNA may be more representative of other functional genomic changes and also more biochemically stable. Recently, studies have been profiling miRNA and other; small non-coding RNA in serving as indicator to the biological changes underlying several diseases.

1.3 Role of miRNA in Neurodegenerative Disease

miRNAs are a class of small (22 nucleotides) non-coding RNAs that regulate gene expression through post-transcriptional regulation (Khoo et al. 2012). They are generally known as negative regulators of gene expression and to regulate approximately 30% of genes in the human genome (Rajewsky 2006).

Biogenesis of mature miRNA occurs through a multi-step process that starts in the nucleus with endonucleolytic cleavage of the primary miRNA (pri-microRNA) transcript and ends with a ~20–25 nucleotide long single stranded mature miRNA (miRNA) in the cytosol. The binding of miRNA with imperfect complementarity to target mRNAs leads to a reduced protein

expression by either degradation of the RNA or translational arrest (De Smaele, Ferretti and Gulino 2010).

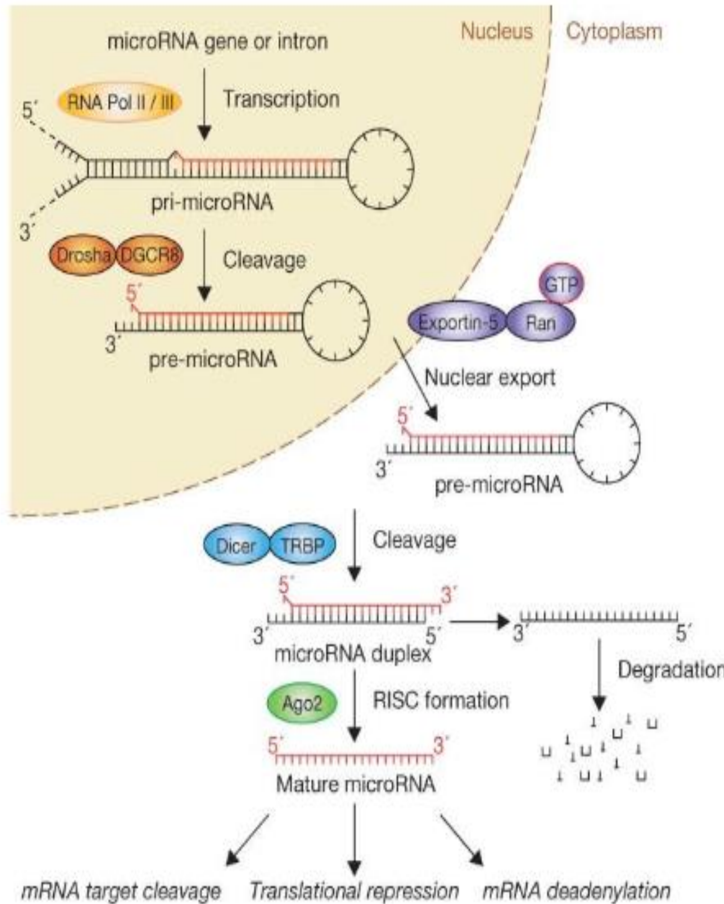


Figure 1: The 'linear' canonical pathway of microRNA processing. This canonical maturation includes the production of the primary miRNA transcript (pri-miRNA) is first produced by RNA polymerase II or III. This is then cleaved to pre-microRNA by the microprocessor complex Drosha–DGCR8 (Pasha) in the nucleus. The pre-miRNA is then exported from the nucleus by Exportin-5–Ran-GTP where it is cleaved by the RNase Dicer in complex with the double-stranded RNA-binding protein TRBP to its mature length. The functional strand of the mature miRNA is loaded together with Argonaute (Ago2) proteins into the RNA-induced silencing complex (RISC), where it guides RISC to silence target mRNAs through mRNA cleavage, translational repression or deadenylation. Image Adapted from Many roads to maturity: microRNA biogenesis pathways and their regulation. (Winter et al. 2009).

Many miRNAs can target multiple mRNAs by recognizing short, specific complementary sequences in the mRNAs and control the expression of hundreds to thousands of genes (Ambros 2004), (Bartel 2004). miRNA expression profiling has been used frequently to identify biomarkers and/or molecular pathways associated with specific human diseases including PD (Khoo et al. 2012). miRNAs are also known to be highly abundant, tissue-specific, quantifiable, highly stable with non-post process modification, which are “ideal” characteristics of biomarkers. In neurodegeneration, although the pathogenic mechanisms are complex and not

fully defined, deregulation of miRNAs has been increasingly recognized to play important roles in the several processes (Lim et al. 2005), such as neuronal differentiation and neuroplasticity.

1.4 Current miRNA Biomarkers Studies in Parkinson's Disease

Many studies have attempted to identify miRNA biomarkers of PD, but the results are highly inconsistent. Even though it is feasible to detect miRNA biomarkers in the early stages of PD, it has been challenging for clinical diagnosis because the peripheral tissues excrete large amounts of different miRNAs that are not organ specific and the miRNA concentrations become diluted when they pass from the CSF through the blood-brain barrier.

In neurodegeneration, although the pathogenic mechanisms are complex and not fully defined, deregulation of miRNAs has been increasingly recognized to play important roles in these pathologic processes (Cheng et al. 2014). Expression profiling of peripheral miRNA has been explored where majority of studies have used whole blood (Vaz et al. 2010) cell-free serum or plasma (Sheinerman et al. 2012, Tsujiura et al. 2010). Although these sources are rich in miRNAs, it can be difficult to differentiate disease-specific miRNA biomarkers as the miRNAs will be derived from any organs that come in contact with blood (Cheng et al. 2014). As summarized in Table 1, a number of miRNAs have been thought to be potential candidates for biomarker for PD.

Studies	Sources	PD, *n*	Ctrl, *n*	Method	Increased miRNA	Decreased miRNA
Margis et al., 2011	Blood	8[1]	8	qPCR		miR-1, miR-22*, miR-29a
Serafin et al., 2015	Blood	36	36	qPCR	miR-29a, miR-30b, miR-103a	
Soreq et al., 2013	Leukocytes	7	6	NGS	miR-18b*, miR-20a, miR-21, miR-150, miR-199b, miR-378c, miR-671, miR-1249, miR-1274b, miR-4293	miR-16, miR-92b, miR-320a, miR-320b, miR-320c, miR-769
Martins et al., 2011	PBMCs	19	13	M-array		miR-19b, miR-26a, miR-28-5p, miR-29b, miR-29c, miR-30b, miR-30c, miR-126, miR-126*, miR-147, miR-151-3p, miR-151-5p, miR-199a-5p, miR-199a/b-3p, miR-301a, miR-335, miR-374a, miR-374b
Cardo et al., 2013	Plasma	31	25	qPCR/TLDA[2]	miR-331-5p	
Khoo et al., 2012	Plasma	42	30	qPCR/M-array[2]	miR-222, miR-505, miR-626	
Li et al., 2017	Plasma	60	60	qPCR	miR-137	miR-124
Current study, 2017	Plasma	46	49	qPCR		miR-133b, miR-433
Burgos et al., 2014	Serum	50	62	NGS	miR-30a-3p, miR-30e-3p, miR-338-3p	miR-16-2-3p, miR-1294
Botta-Orfila et al., 2014	Serum	65	65	TLDA		miR-19b, miR-29a, miR-29c
Cao et al., 2017	Serum	109	40	qPCR	miR-24, miR-195	miR-19b
Ding et al., 2016	Serum	106	91	qPCR/S-seq[2]	miR-195	miR-15b, miR-181a, miR-185, miR-221
Dong et al., 2016	Serum	122	104	qPCR/S-seq[2]		miR-141, miR-146b-5p, miR-193a-3p, miR-214
Ma et al., 2016	Serum	138	112	qPCR		miR-29c, miR-146a, miR-214, miR-221
Vallelunga et al., 2014	Serum	25	25	qPCR/TLDA[2]	miR-24, miR-223*, miR-324-3p	miR-30c, miR-148b
Zhao et al., 2014	Serum	46	46	qPCR		miR-133b
Burgos et al., 2014	CSF	57	65	NGS	miR-19a-3p, miR-19b-3p, let-7g-3p	miR-10a-5p, miR-127-3p, miR-128, miR-132-5p, miR-136-3p, miR-212-3p, miR-370, miR-409-3p, miR-431-3p, miR-433, miR-485-5p, miR-873-3p, miR-1224-5p, miR-4448
Gui et al., 2015	CSF	78	35	qPCR/TLDA[2]	miR-10a-5p, miR-136-3p, miR-153, miR-409-3p, miR-433, let-7g-3p	miR-1, miR-19b-3p
Marques et al., 2016	CSF	28	28	qPCR	miR-205	miR-24

CSF, cerebrospinal fluid; Ctrl, controls; M-array, microarray; NGS, next generation sequencing; PBMCs, peripheral blood mononuclear cells; PD, Parkinson's disease; S-seq, Solexa sequencing; TLDA, TaqMan Low Density Array; [1]Untreated PD patients; [2]qPCR as validation after initial screening using array or sequencing.

Table 1 Showing aberrant expression of miRNAs in fluids or blood cells of PD patients. Image copied from paper entitled "Reduced Circulating Levels of miR-433 and miR-133b Are Potential Biomarkers for Parkinson's Disease" by (Zhang et al. 2017).

These previous studies only focused on expression profiling of miRNA in whole blood or circulating (cell-free) serum or plasma. Recent evidence has shown that miRNA can be stably packaged into nanometer vesicles, called exosomes that are able to travel across the blood brain barrier and aid in understanding the brain pathological processes from non-invasive blood or serum specimens. Gui et al. showed that *miR-1*, *miR-19b-3p*, *miR-153*, *miR-409-3p*, *miR-10a-5p*, and *let-7g-3p* in CSF exosomes are reliable biomarkers of PD, showing good specificity and sensitivity and having the ability to distinguish PD from healthy individuals and patients with Alzheimer's disease (Gui et al. 2015).

1.5 Exosomes

Exosomes are lipid bilayer enclosed vesicles of 30–120 nm in diameter that can be found in physiological fluids such as blood, urine, saliva, breast milk and semen (Looze et al. 2009, Michael et al. 2010, Gonzales et al. 2009, Admyre et al. 2007). They are released into the extracellular environment by most cell types and play an important role in inter-cellular communication (Raposo and Stoorvogel 2013). Exosomes contain various biomolecules, including proteins and nucleic acids, that are secreted from host cells and transmitted to the surrounding or distant body parts (Raposo and Stoorvogel 2013), even bypassing the blood brain barrier (BBB) (Alvarez-Erviti et al. 2011). Various forms of exosomal action are suggested to function in mediating neuron-glia communication, neuronal repair and growth (Frühbeis et al. 2013) (Xiong, Mahmood and Chopp 2017). Exosomal contents are suggested to be specific for a disease state. Accumulating evidence has shown that exosomal miRNAs regulate a wide spectrum of brain functions not only in normal brain development but also in pathogenic conditions including traumatic injury, glioblastoma and neurodegenerative disease (Rajendran et al. 2014). This was shown for example by Shi and colleagues who observed increased alpha-synuclein concentrations in plasma exosomes from Parkinson's disease patients compared to healthy controls. (Shi et al. 2014)

1.6 Exosomes' Biogenesis

Exosomes are produced through the endosomal pathway derived from intraluminal vesicles of late endosomal compartments named multivesicular bodies (MVB). They are first formed by endocytosis, where the cell membrane is internalized to produce endosomes. Many small vesicles are then formed inside the endosome by invaginating parts of the endosome membranes. These endosomes are called multivesicular bodies (MVBs). The MVBs then fuse with the cell membrane

and release the intraluminal endosomal vesicles into the extracellular space to become exosomes (Gruenberg and van der Goot 2006).

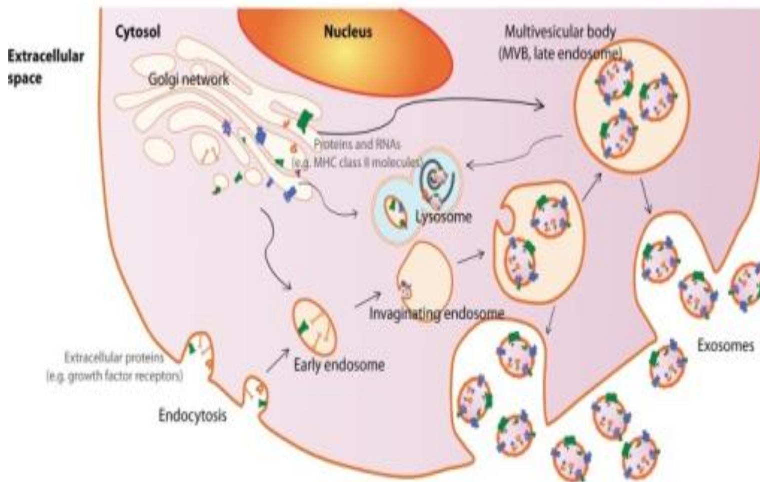


Figure 2. Exosome biogenesis and secretion. Exosomes are released by cells when intracellular organelles called multivesicular bodies (MVBs) fuse with the plasma membrane. MVBs are formed by invaginations of late endosomes, which contained molecules from the Golgi (e.g., MHC class II molecules) or the cell surface (e.g., growth factor receptors). Consequently, exosomes contain cytosolic materials and are enriched in endosome-associated protein markers such as the Rab proteins, ALIX, TSG101, and MHC class II molecules or endocytic proteins, such as transferrin receptors and clathrins. This figure was modified from Lai et al (Lai et al. 2013).

1.7 Molecular Composition of Exosomes

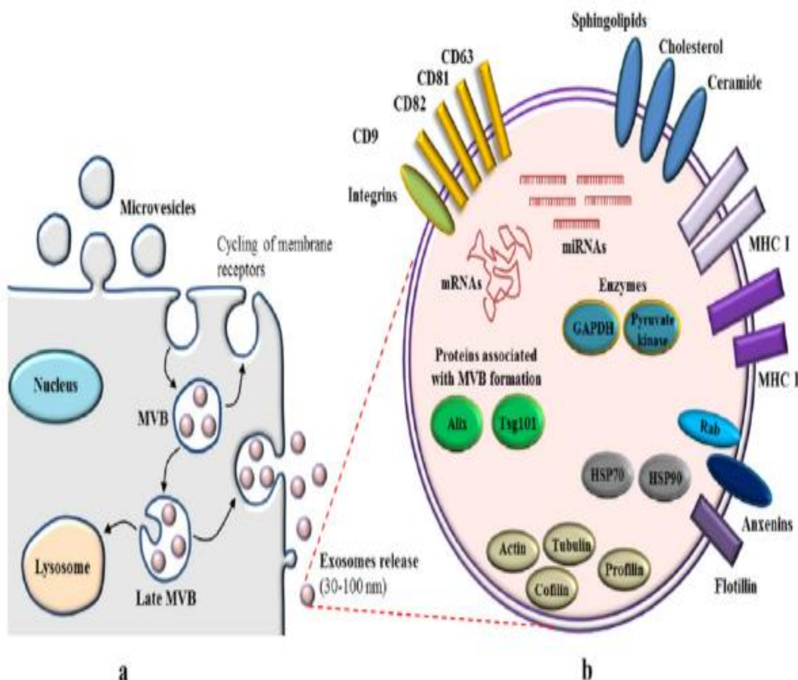


Figure 3. Biogenesis, secretion and molecular composition of exosomes. A: Intracellular machinery of exosome biogenesis and secretion. The exosomes can be secreted into the extracellular environment through either fusion of multivesicular bodies (MVB) with the cell surface or budding pathway.
B. Exosomes are membrane-bound phospholipid nanovesicles (30 – 100 nm in diameter) are secreted from various cell types. They packed with a variety of cellular components including mRNAs, miRNAs, USP70, HSP90, with various proteins involved in antigen presentation as integrins and tetraspanin (CD9, CD63, CD81 and CD82) as well as MHC I and II (Van Giau and An 2016).

Exosomes contain molecules such as TSG101 and Alix that participate in MVB biogenesis. They also contain proteins such as heat shock proteins (HSP70 and HSP90), cytoplasmic proteins such as tubulin, actin, Annexin and Rab family proteins, and tetraspanins (CD9, CD63, and CD81), major histocompatibility complex class I (MHC-I) molecules, and various transmembrane proteins that are involved in signal transduction (De Toro et al. 2015).

1.8 The sorting mechanism for exosomal microRNAs

Studies have shown that parent cells possess a sorting mechanism that guides specific intracellular miRNAs to enter exosomes. Mature miRNAs are sorted into exosomes via four potential modes: (1) The neural sphingomyelinase 2 (nSMase2)-dependent pathway. nSMase2 is the first molecule reported to be related to miRNA secretion into exosomes. Kosaka et al. found that overexpression of nSMase2 increased the number of exosomal miRNAs, while inhibition of nSMase2 expression reduced the number of exosomal miRNAs (Kosaka et al. 2013); (2) The miRNA motif and sumoylated heterogeneous nuclear ribonucleoproteins (hnRNPs)-dependent pathway. Villarroya-Beltri et al. discovered that sumoylated hnRNPA2B1 recognized the GGAG motif in the 3′ portion of miRNA sequences and cause specific miRNAs to be packed into exosomes. Two hnRNP family proteins, hnRNPA1 and hnRNPC have also been found to bind to exosomal miRNAs, these suggests that they may be candidates for miRNA sorting even though no binding motifs have been discovered as yet. (Villarroya-Beltri et al. 2013). (3) The 3′ miRNA sequence-dependent pathway; miRNAs that are preferentially sorted into exosomes have more poly(U) than poly(A) at the 3′ end. Koppers-Lalic et al. found that the 3′ ends of uridylated endogenous miRNAs were mainly presented in exosomes derived from B cells or urine, whereas the 3′ ends of adenylated endogenous miRNAs were mainly presented in B cells (Koppers-Lalic

et al. 2014) (4) The miRNA induced silencing complex (miRISC)-related pathway. Mature miRNAs can interact with assembly proteins to form a complex called miRISC. miRISCs co-localize with the sites of exosome biogenesis (multivesicular bodies) and their components, such as AGO2 protein and miRNA-targeted mRNA, are correlated with sorting of miRNAs into exosomes. The main components of miRISC include miRNA, miRNA-repressible mRNA, GW182, and AGO2. The AGO2 protein in humans, which prefers to bind to U or A at the 5′ end of miRNAs, plays an important role in mediating mRNA:miRNA formation and the consequent translational repression or degradation of the mRNA molecule. (Frank, Sonenberg and Nagar 2010).

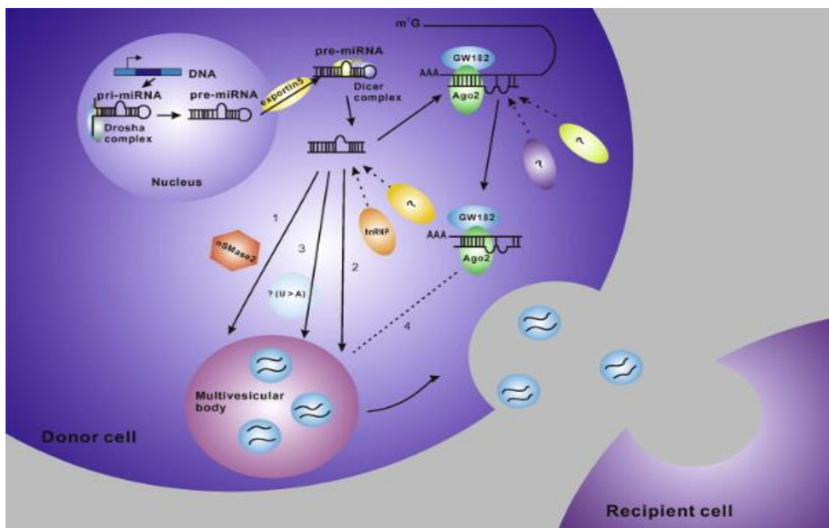


Figure 4. The sorting mechanism for exosomal microRNAs. Mature miRNAs are sorted into exosomes via four potential modes: (1) nSMase2-dependent pathway; (2) miRNA motif and sumoylated hnRNPs-dependent pathway; (3) 3′ miRNA sequence-dependent pathway; miRNAs that are preferentially sorted into exosomes have more poly(U) than poly(A) at the 3′ end and the (4) The miRISC-related pathway. Image Adapted from Exosome and Exosomal MicroRNA: Trafficking, Sorting, and Function (Zhang et al. 2015).

1.9 Importance of Exosomes

Extracellular vesicles (EVs) are gaining importance as a mediator of important physiological and pathological activities within cells. This is made possible through the transfer of their cargo of protein and RNA between cells. Currently, exosomes are the best characterized EVs. They have been notable for their in vitro and in vivo immunomodulatory activities (Zhang

et al. 2014). Exosomes are advantageous for diagnostic purposes and also for biomarker discovery for central nervous system (CNS) disorders. This is because of their ability to cross the blood brain barrier (BBB) into the bloodstream where they express markers that allow their tracking to the cell of origin (Kanninen et al. 2016).

A limitation with current biomarker studies in the peripheral blood has been the inability to link biomarker levels to brain pathology or the uncertainty of their tissue of origin. Recently, a new method of isolation of extracellular vesicles (EVs) enriched for neuronal origin from peripheral blood provides a new diagnostic platform that may reflect and track neuropathological changes in vivo. This method was developed by Edward J. Goetzl and has been published in the field of Alzheimer's disease (AD) with a few variations [(Fiandaca et al. 2015), (Goetzl et al. 2015), (Goetzl et al. 2016), (Kapogiannis et al. 2015)]. This method efficiently isolates total EVs from plasma or serum sample using particle precipitation methods such as ExoQuick (Sáenz-Cuesta et al. 2015), this isolation is then followed by immunoprecipitation with biotinylated antibodies against neuronal surface markers to isolate a sub-population of $NCAM^{+}$ $L1CAM^{+}$ EVs. Neural cell adhesion molecules L1CAM (CD171) have a relatively specific expression in neural tissue and early research has demonstrated high expression on exosomes derived from cultured neurons (Fauré et al. 2006).

1.10 .Study Hypothesis and Aim

We hypothesize that miRNAs are altered in Parkinson's disease neuronal exosomes found in the serum. As a key post-transcriptional regulator, miRNAs associated with the disease may also help describe neuropathology or disease features, such as the age of motor onset. Due to their stability and extracellular transport, neuronal origin miRNA changes may be observed in the

serum, and therefore function as potential disease classifiers or predictors of disease phenotypes. We therefore sought to enrich for neuronal origin exosomes in the serum by using CD171 (Figure 3). Our analysis included a total of 60 patients with PD and 40 healthy age and gender matched controls (Figure 3).

1.11 Study Design

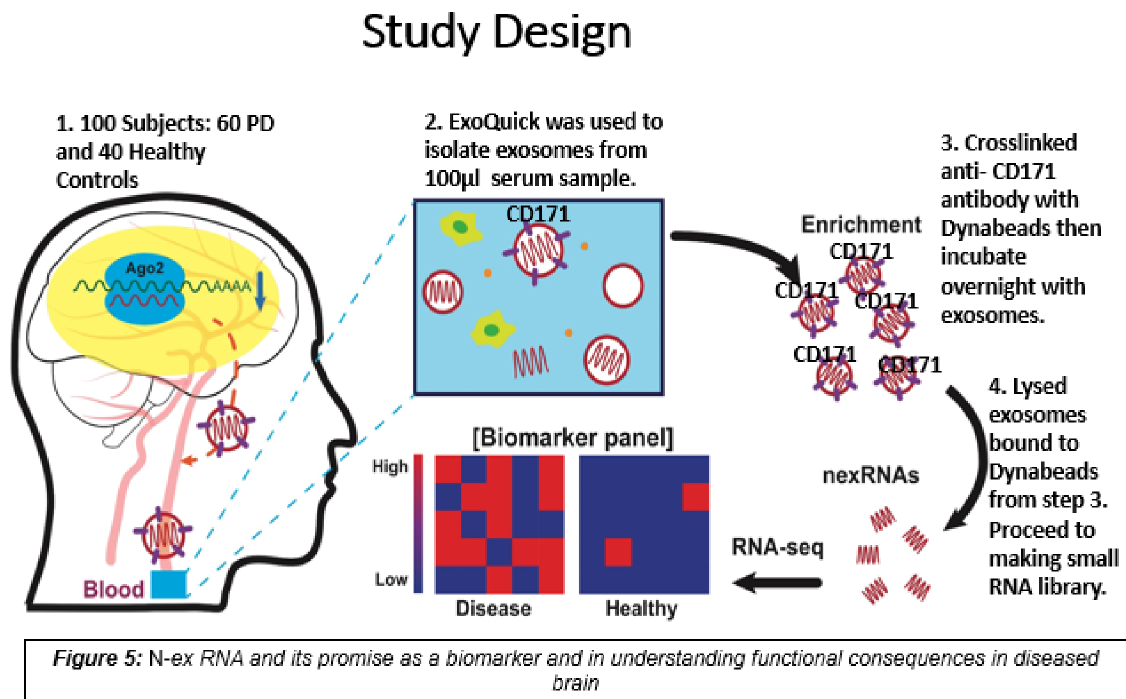


Figure 5: N-ex *RNA and its promise as a biomarker and in understanding functional consequences in diseased brain*

Chapter 2

MATERIALS AND METHODS

2.1 Subjects

A total of 60 PD and 40 control subjects were involved in this study. Subjects were recruited either from a tertiary movement disorders clinic or via an IRB-approved recruitment material posted in the local community. PDs and Controls were matched for age, gender, and education (see Table 3 for detailed demographic information). At baseline, PD diagnosis was confirmed according to published criteria (Hughes et al. 1992) by movement disorder specialists. Disease duration was obtained from subject history with onset defined as the first diagnosis by a medical professional. All subjects were free of major/unstable medical issues such as liver, kidney, or thyroid abnormality, and deficiency of vitamin B_{12}, or any cerebrovascular diseases or neurological conditions (other than PD). All subjects gave written informed consent. The study was conducted in accordance with the principles of the Declaration of Helsinki and reviewed and approved by the Penn State Hershey Institutional Review Board.

At baseline, depression was assessed using the Hamilton depression scale (Hamilton 1960), and overall cognitive ability was evaluated using the Montreal Cognitive Assessment (MoCA) (Nasreddine et al. 2005). Age, sex, smoking, MOCA, UPDRS and Hoehn-Yahr scale are provided in Table 3.

2.2 Extraction of Exosomes

ExoQuick™ Exosome Precipitation Solution kit (Systems Biosciences) was used for exosome extraction from samples. 100µl of serum was collected and centrifuged at 3000 x g for 15 minutes to remove cells and cell debris. The supernatant was then transferred to a sterile vessel and 25.2µl of ExoQuick™ Exosome Precipitation Solution was added to it. This was refrigerated at 4^0 for 30 minutes then the ExoQuick/serum mixture was centrifuged at 1500 x g

for 30 minutes. After centrifugation, the exosomes appeared as a beige or white pellet at the bottom of the vessel. The supernatant was then aspirated and the residual ExoQuick solution was then spun down by centrifugation at 1500 x g for 5 minutes. All traces of fluid were removed by aspiration. The exosome pellet was resuspended in 100µl of Phosphate Buffered Saline (PBS) with special attention not to disturb the precipitated exosomes in the pellet. Exosomes were then characterized by Transmission Electron Microscopy and NanoSight Tracking Analysis.

2.3 Exosome Identification by Transmission Electron Microscopy

Exosome identification was conducted using 10 µl of each exosome solution resuspended in 1 ml of PBS in a cuvette which was then placed in the instrument and read. Transmission electron microscopy was performed by taking 10 µl of exosome solution and placing them on parafilm. Formvar coated copper grids were then placed on top of the drops and incubated for 20 minutes. The copper grids were then incubated with a 4% solution of paraformaldehyde in 0.1M PBS for 20 minutes, washed three times with PBS for 1 minute each, incubated with 1% glutaraldehyde in 0.1M PBS for 5 minutes, washed with distilled water for 2 minutes, washed three times with PBS for 2 minutes each, contrasted with 1% Uranyl acetate for 20 seconds, and then observed by transmission electron microscopy (JEOL-1400).

2.4 Nanoparticle Tracking Analysis

Nanoparticle tracking analysis (NTA) was performed using a NanoSight NS300 (NanoSight Ltd.) equipped with a 405 nm laser. At least three 40 s videos were recorded for each sample with camera level and detection threshold set at 13. Temperature was monitored throughout the measurements. Videos recorded for each sample were analyzed with NTA 3.2 Dev Build 3.2.16 to determine the concentration and size of measured particles with the corresponding standard

error. For analysis, auto settings were used for blur, minimum track length and minimum expected particle size. Double-distilled H_2O was used to dilute the starting material.

2.5 Neuronal Enrichment of Exosomes with CD171

Anti-Human CD171 biotin-conjugated antibody (eBio5G3, Affymetrix) was used to enrich exosomes from neuronal origins together with the use of streptavidin-conjugated magnetic beads (Thermo Fisher Scientific, #10608D). IGEPAL® CA-630 (Sigma-Aldrich) was added to 1% of final concentration for lysing/extracting total RNA.

2.6 Construction of Small Library

We generated small RNA libraries using the CleanTag Small RNA Library Prep Kit (TriLink Biotechnologies). The final libraries were amplified by PCR for 27 cycles.

2.7 Agilent 2100 Bioanalyzer analysis

Library prep samples after PCR (crude or purified) were analyzed on a 2100 Bioanalyzer (Agilent Technologies). Samples were diluted 1:10 with water and then 1 μL was loaded onto a High Sensitivity DNA chip for analysis. The 2100 Expert software was used to quantitate library concentrations. All peaks within regions 140–1000 bp were included in quantification.

2.8 Pooling and Gel Size Selection

Individually barcoded libraries were mixed equimolarily into three (3) pools based on the Bioanalyzer quantification results; each of three pools included 20 from Parkinson's disease samples and 20 from control samples, by having several technical replicates of control samples in each pool.

2.9 AMPure Bead Clean Up, Gel Size Selection and Quality Control

Size selection targeting 100–200 nucleotides was performed on small RNA libraries using Agencourt AMPure XP beads (Beckman Coulter), followed by gel size selection. 1X AMPure XP magnetic beads (80 µL) were added to 80 µL of final PCR product then mixed and incubated at room temperature for 10 minutes. Tubes were then placed on a magnetic rack for 4 minutes to separate the beads. Supernatant, containing the desired product, was then transferred to a clean tube and 1.8X original PCR volume (144 µL) of beads were mixed and incubated with supernatant for 10 minutes at room temperature. Again, tubes were placed on magnetic rack for 4 minutes to separate beads and supernatant. This time, supernatant was discarded, and beads were washed twice with 500 µL of 70% ethanol on the magnetic rack. Beads were then air dried for 5 minutes on the magnetic rack before resuspending in 17 µL nuclease free water. Beads were incubated in water for 2 minutes at room temperature before placed back on magnetic rack for elution. 15 µL of eluate was collected as a purified product. These purified libraries were then size selected on a 2% Certified™ Low Range Ultra Agarose (#1613107, Bio-Rad Laboratories) gel, followed by DNA recovery using QIAquick Gel Extraction Kit (Qiagen).

Deep Sequencing Methods

2.10 MiRNA-sequencing and data processing

The size-selected libraries were subjected to deep sequencing on an Illumina HiSeq 2500. Briefly, each pool was loaded onto each lane of a flow cell for cluster formation and sequenced for 50 cycles of single end sequencing. PhiX Control library was mixed at 1% of the entire library pools and sequenced in order to quality control the sequencing run. The monitored sequencing error rate using the PhiX sequence was 0.1%. The raw .bcl files were converted into demultiplexed fastq files with Casava 1.8.2 (Illumina).

2.11 Bioinformatics cleaning and preparation of sequences for analysis

After quality filtering and adapter clipping of the reads, Chimira suite was used to align and determine differential miRNA expression. Briefly, raw sequence counts were processed in a bioinformatics pipeline including a quality control filter, trimming of adapters, and alignment of reads against reference databases using Chimira suite.

2.12 Quality control and normalization of raw sequence counts

Data analysis was done in R version 3.4.2. Exploratory analyses were performed using read count data for 333 microRNAs (miRNAs) from a total of n = 100 subjects. Both subjects and miRNAs were filtered after we observed that select subjects had low total read count and also that a number of miRNAs had zero read count across all subjects. First, we removed the subjects whose total read count fell into the lowest quartile of all total read counts. We used the first quartile (5045) as a threshold as a cut-off. This removed 25 samples, and the remaining set consists of 29 controls and 46 cases (Table 2). We then restricted to miRNAs that had at least five non-zero reads in the samples with Parkinson's disease (PD). Differential expression analyses were then performed using the edgeR R package (Robinson, McCarthy and Smyth 2010) and the resulting matrix of expression data for 66 miRNAs and n = 75 subjects was obtained.

Min.	1st Quartile	Median	Mean	3rd Quartile	Max.
351	5045	8474	13608	14840	112211

Table 2 Showing the mean and quartiles of the total read count that was obtained across samples.

2.13 Statistical Analysis

Two sample t-tests were conducted on the log transformed (base 10) read counts, and the *p*-values were controlled for multiple comparison errors using "FDR". *P*-values < 0.05 were reported as significant and fold change was used as a measure for expression changes. These tests gave 17 significant miRNAs which are differentially expressed. The significantly differentially expressed miRNAs were then subjected to further investigation. Boxplots and heatmap were constructed for those significant miRNAs using log transformed (base 10) read counts to graphically illustrate the differential expressions.

Chapter 3

RESULTS

Table 3: Demographic and clinical characteristics of study population

	PD Patients (n = 60)	**Control Patients (n = 40)**	**P-Value**
Age (years; Mean ± SD)	66.6 ± 9.9	66.3 ± 10.9	0.90
Gender (Male/Female)	(33/27)	(20/20)	0.77
Smoking (Yes/No/*)	(18/40/2)	(10/30/0)	0.75
Education (years)	16.4 ± 3.1	17.2 ± 2.9	0.16
Hoehn-Yahr Scale (I/II/II/IV/V/*)	(17/26/8/2/5/2)	N/A	N/A
PD Duration (Years) <5 y 5~ 10 y 10~ 15 y >15 y	 36 0 11 13	N/A	N/A
UPDRS Score-III	31.0 ± 23.6	5.3 ± 5.6	<0.0001
MoCA	23.0 ± 4.5	25.7 ± 2.42	0.0003
Hamilton Depression Scale	7.2 ± 5.2	2.3 ± 3.0	<0.0001

NB: * Information were not available for these subjects. All values are presented as group means and standard deviations (SD), except for the number and gender of subjects. All p-values (P(T<=t) two-tail) were obtained by excel (t-test two sample assuming unequal variances). UPDRS: Unified Parkinson's Disease Rating Scale; MoCA: Montreal Cognitive Assessment Test.

3.1. Characterization of Isolated Serum Exosomes by TEM

To ensure the efficacy and quality of the serum exosome isolation, we characterized the microvesicles by TEM. Electron microscopic analysis of the exosomes isolated from serum samples showed round structures with sizes varying between 100 and 200 nm (Figure 4(a)), consistent with previously reported characteristics of exosomes.

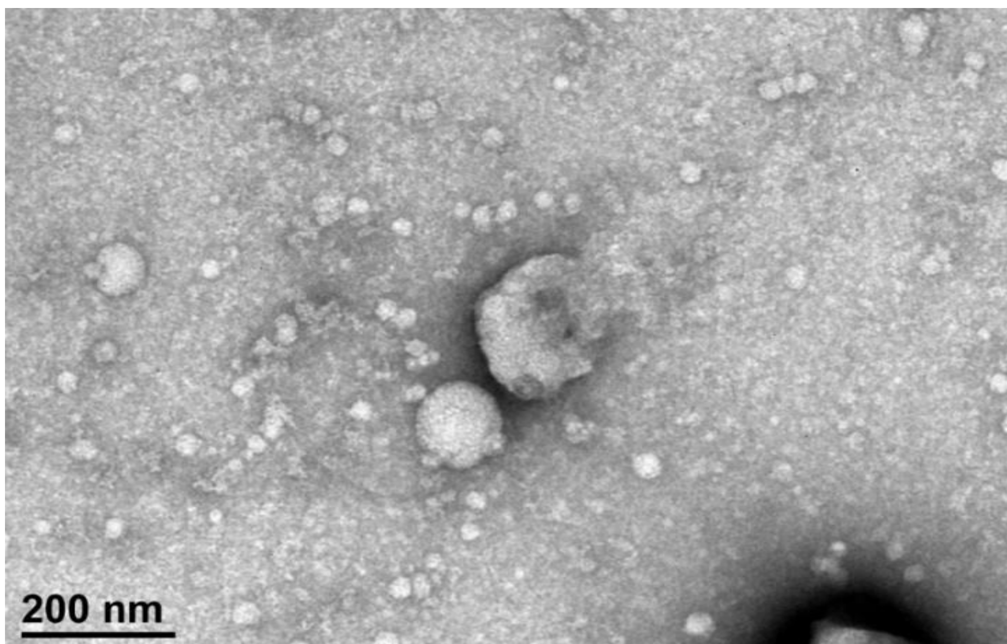


Figure 6: Validation of exosomes isolated from serum sample. Morphological characterization of exosomes isolated from serum samples by transmission electron microscopy. Bar, 200 nm. The smaller particles of <20 nm diameter observed in the surrounding field are nonspecific lipid particles.

3.2 Characterization of Exosomes by Nanoparticle Tracking Analysis (NTA)

We investigated the size distribution of extracellular vesicles from 3 PD and 3 control subjects (1:2500 dilution) using NanoSight [NTA3300] (NanoSight, Inc.). The samples were diluted using PBS, placed on ice and applied to the sample chamber of the NanoSight [NTA3300] and analyzed by NTA 3.2 software (Dev Build 3.2.16). We observed that the size of the peak diameter was comparable in the range of [185.9–235.5] ±6.9 nm as summarized in Table 4.

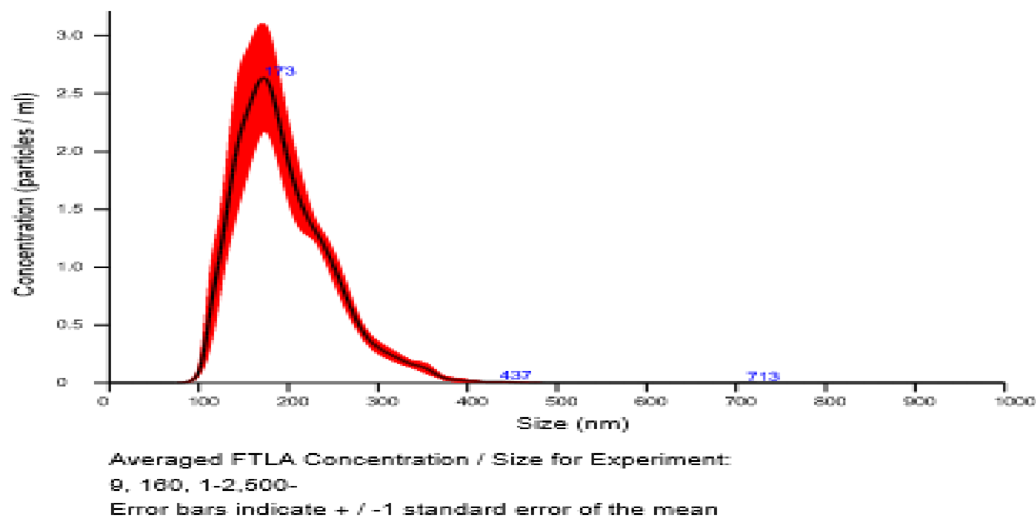


Figure 7: Graph generated by NanoSight™ showing concentration and peak size of vesicles after ExoQuick precipitation.

Table 4 Nanoparticle Tracking Analysis from NanoSight [NTA3300]		
Items (sample ID)	Peak diameter (nm)	Concentration (particles/ml)
ID182 (81 y/o, Control, Male)	208.8 +/- 6.0 nm	1.13e+009 +/- 1.60e+008 particles/ml
ID211 (81 y/o, PD, Male)	221.5 +/- 3.9 nm	3.80e+008 +/- 4.48e+007 particles/ml
ID134 (71 y/o, Control, Male)	230.8 +/- 4.6 nm	5.90e+008 +/- 9.24e+007 particles/ml
ID160 (71 y/o, PD, Male)	198.5 +/- 6.9 nm	2.95e+009 +/- 4.02e+008 particles/ml
ID7 (53 y/0, Control, Female)	185.9 +/- 2.8 Nm	5.74e+008 +/- 7.25e+007 particles/ml
ID34 (53 y/o, PD, Female)	235.5 +/- 1.2 nm	3.48e+008 +/- 2.97e+007 particles/ml
The Nanoparticle tracking analysis was used to analyze peak diameter and concentration of exosomes obtained from serum samples (1:2500 Dilution Factor). Analysis was repeated five times for each sample with similar results.		

Table 4 Nanoparticle Tracking Analysis from NanoSight [NTA3300]

3.3 Sample Bioanalyzer Results Showing miRNA

In order to confirm the presence of miRNA which was prepared using the TriLink small RNA kit, we analyzed products on DNA High Sensitivity chip in an Agilent 2100 Bioanalyzer. The electropherograms of miRNA show the size distribution in nucleotides (nt) and fluorescence intensity (FU).

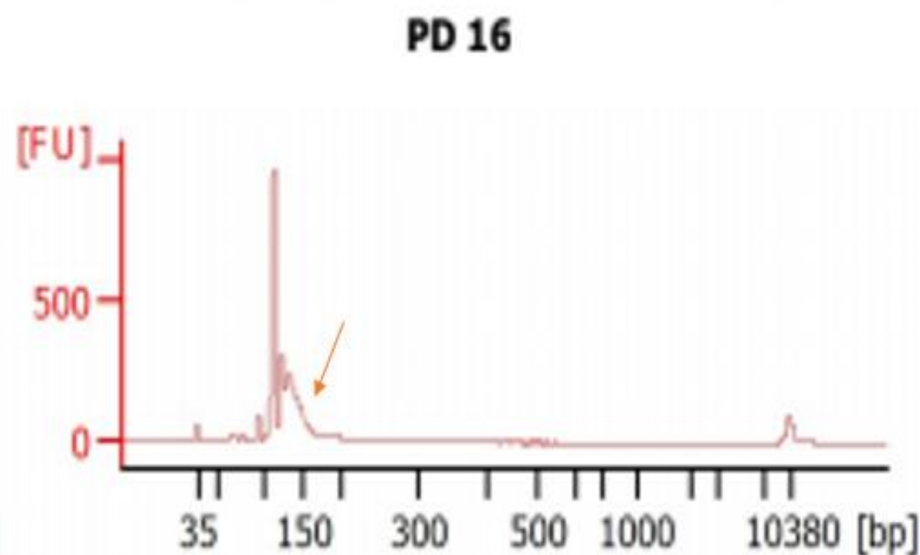
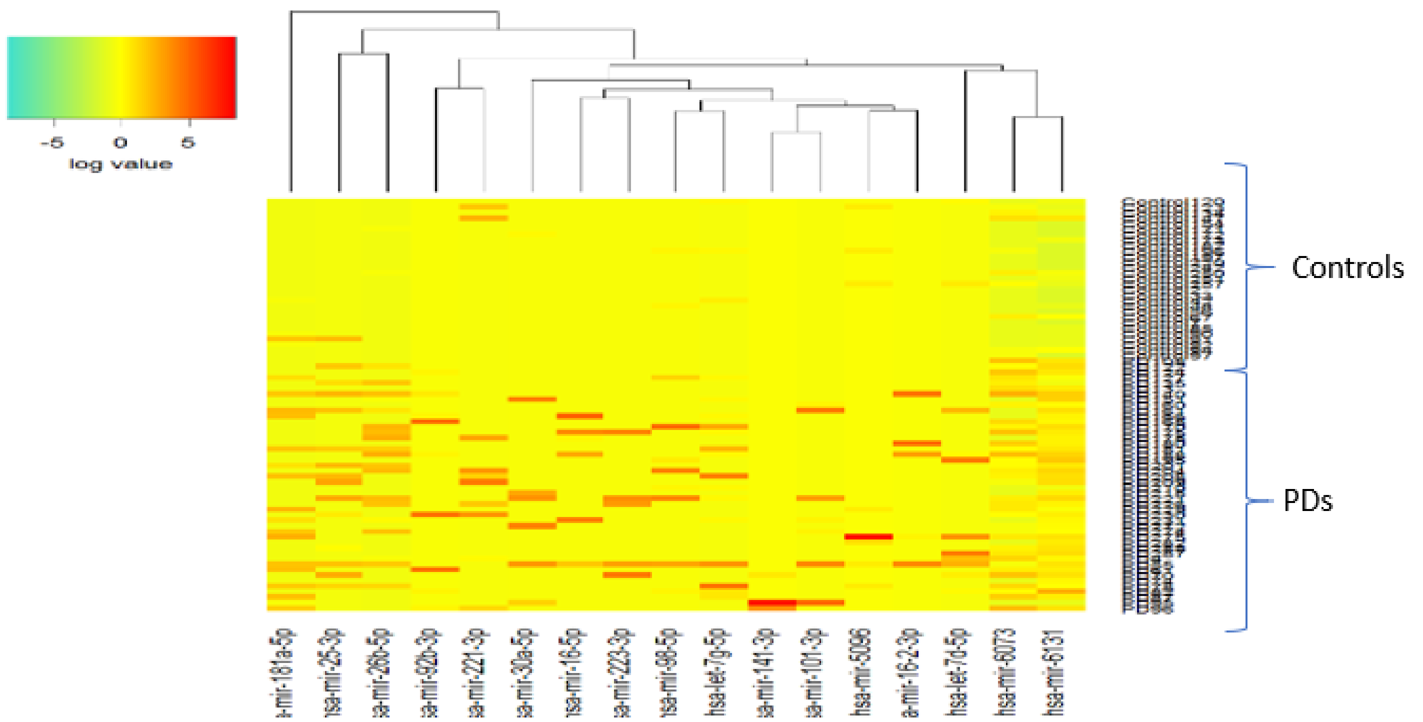


Figure 8: Analysis of purified PCR products. Bioanalyzer High-Sensitivity DNA chip showing a successful library preparation. One peak around 140 nt corresponds miRNA sequences of around 22 nucleotide length (after inclusion of adapter sequences), and the peak around 150 nt corresponds to small RNA fragments of around 30 nt length. A peak of 120 nt which represents adapter dimers was excluded by gel size selection.

3.4 Differential Expression PD versus Control

Figure 9: Hierarchical clustering of miRNAs from the healthy controls and patients with PD.



Samples are arranged in columns and miRNAs in rows. The miRNA clustering tree is presented at the bottom, and the sample clustering tree is present at the right of the heat map. Heat maps depict the relative expression intensity for each miRNA in which the base-2 logarithm of the intensity is median-centered for each row. The color coding is indicated as a horizontal bar. PD, Parkinson’s Disease; miRNA, microRNA

3.5 Boxplots showing six representative miRNAs from the Heatmap (Most consistently upregulated in PD)

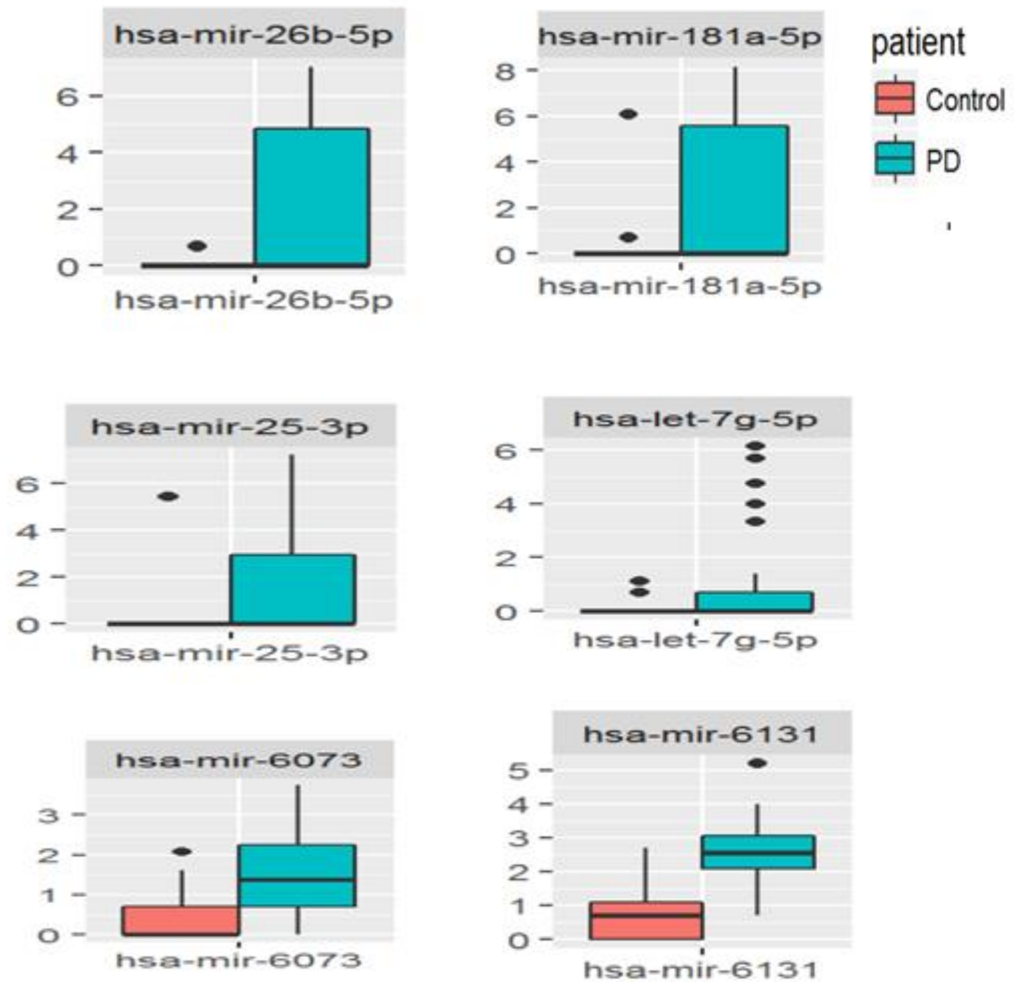


Figure 10: Boxplots showing six (6) of the seventeen (17) significantly upregulated miRNA in PD compared to controls. Red is controls and blue is Parkinson's Disease on the x-axis while the y-axis is log transformed read counts.

Chapter 4

DISCUSSION

The dysregulation of several miRNAs has been associated with PD. It has been documented that these miRNAs play important roles in the regulation of a list of PD-related pathogenic proteins namely alpha-synuclein, leucine-rich repeat kinase, Parkin, DJ-1, phosphatase and tensin homolog-induced putative kinase (Cho et al. 2013, Heman-Ackah et al. 2013). Therefore, they may play a pivotal role in further understanding the disease pathogenesis.

miRNA expression profiles in PD-diagnosed patients have been analyzed and reported (Cardo et al. 2014) (Hoss et al. 2016) and the function of miRNAs in regulating PD has been thoroughly discussed (Filatova et al. 2012, Leggio et al. 2017). Several studies have analyzed miRNA levels in body fluids (including serum, plasma, and CSF) of patients with PD compared with that in healthy controls as summarized in **Table 1**, however, the results of these previous studies were inconsistent. The discrepancy may be due to differences in miRNA stability in the studied samples, the miRNA analysis platforms and the various biofluids that have been used.

Studies have shown that miRNAs in serum are encapsulated within exosome-like microvesicles, which are secreted by pathological tissues, and are quite stable (Cheng et al. 2014). In addition, recent methods have been developed to enrich exosomes of neuronal origin in peripheral fluids such as serum using CD171, a neural cell adhesion marker (Fiandaca et al. 2015). Therefore, the aim of our study was to demonstrate the value of serum based neuronal enriched exosomal miRNAs using CD171, a neural cell adhesion marker, as a biomarker for PD. Characterization of neuron-specific exosomes in serum using the neuronal adhesion molecule CD171 showed that several exosomal miRNAs were increased in PD patients.

Our results are consistent with published studies. For example, in our study **hsa-mir-26b-5p** was significantly upregulated in PD compared to controls. The levels of hsa-miR-26b were also shown to significantly increased in PD substantia nigra pars compacta (SNpc) relative to actin

mRNA levels in another study (Alvarez-Erviti et al. 2013) and is predicted to target a chaperone mediated autophagy protein, hsc70 mRNA (Alvarez-Erviti et al. 2013). The increase in hsa-miR-26b corresponded to a significant decrease in *hsc70* (78%) mRNA levels (Alvarez-Erviti et al. 2013) and hsc70 (51%) protein levels were also previously reported in another study (Alvarez-Erviti et al. 2010). Decreased levels of the chaperone-mediated autophagy (CMA) protein hsc70 in PD brain samples suggests compromised alpha-synuclein degradation by CMA which may underpin the Lewy body pathology in PD. In addition, hsa-miR-26b-5p was patented in 2015 as one of the new diagnostic miRNA markers for PD (Keller et al. 2015).

Hsa-mir-181a-5p was also found to be significantly upregulated in our neuronal enriched exosomes in our study. This was also found to be upregulated in PD human blood (Li et al. 2013). Hsa-mir-181a-5p has been predicted to target the PD related gene, LRRK (PARK8) (Heman-Ackah et al. 2013). On the other hand, in stroke models, hsa-miR-181 regulates GRP78 and influences outcome from cerebral ischemia in vitro and in vivo (Ouyang et al. 2012).

Hsa-mir-30a-5p was also found to be significantly upregulated in neuronal enriched exosomes in PD compared to control subjects in our study. Our findings were consistent with an article where miR-30a-3p was previously found differentially expressed in PD blood specimens. Hsa-miR-30a (upregulated in peripheral blood and post-mortem serum) (Leggio et al. 2017), but this is inconsistent with a few other studies where decreased circulating miR-30a level has been demonstrated in the untreated PD-diagnosed patients compared to normal control and treated PD (Margis and Rieder 2011). Another study has shown has shown that dopamine transport regulation in PD patients is related to the has-miR-30a by its potential in targeting SLC6A3 (solute carrier family 6 – neurotransmitter transporter – dopamine) (Margis and Rieder 2011). The different expression of miR-30a was also demonstrated in other central nerve system

disorders including ischemic stroke, Huntington's disease, and schizophrenia (Darcq et al. 2015, Long et al. 2013).

Hsa-mir-16-5p was also found to be upregulated in our neuronal enriched exosomes in PD compared to control subjects was also found up-regulated in a PD brain in study performed by Hoss and colleagues (Hoss et al. 2016) and has been shown to correspond to enhanced glucocerebrosidase (GBA) protein levels (Siebert et al. 2014). It has been reported that mutations of the glucocerebrosidase gene (GBA1) are a significant risk factor for the disease. GBA deficiency has also been associated with PD (Aharon-Peretz, Rosenbaum and Gershoni-Baruch 2004). This relationship was first identified in the Ashkenazi Jewish population and began to attract attention after a number of reports (Aharon-Peretz et al. 2004, Tayebi et al. 2001).

Let 7d-5p and **let-7g-5p** were both upregulated in neuronal enriched exosomal miRNA in PD compared to control subjects. miRNA let-7 family has been shown to be highly conserved and is important and required for development, acting as regulator of oncogenes and stem cell differentiation (Bartel 2009). Upregulation of let-7 impairs glucose homeostasis and results in degeneration of neurons, while its downregulation leads to cancer. Several studies have suggested that pesticide exposure and life in rural areas are significant risks factors for Parkinsonism (Firestone et al. 2005) . The increased expression of let-7d and let-7g in hNPCs after paraquat (pesticide) exposure also provide potential targets for paraquat neurotoxicity (Huang et al. 2014).

Hsa-miR-221-3p was also found to be significantly upregulated in our neuronal enriched exosome PD compared to control subjects. This was also found to be upregulated in a study done by Nair and colleagues in 2016 where they analyzed miRNA expression changes in post-mortem PD striatum, particularly in PD putamen tissues, using a miRNA Expression Array kit with 800

probes. They analyzed 12 PD samples compared to 12 healthy controls and identified 13 dysregulated miRNAs (Nair and Ge 2016), including miR-221-3p. Computational analysis showed that this miRNA may be associated with inflammatory response and other mechanisms activated by oxidative stress in PD striatum.

Hsa-miR-223-3p was found to be upregulated in our PD patients compared to control subjects. In one study, miR-223* was upregulated in PD and MSA patients vs. healthy controls (Vallelunga et al. 2014). To date, there are no data on the physiological and pathological role of miR-223*.

Hsa-mir-25-3p was shown to be significantly upregulated in our neuronal enriched exosomes. Hsa-mir-25-3p was reported to be previously known to be associated with PD and targets syntropin beta 2 (SNTB2) mRNA. **Hsa-miR-141** was shown to be significantly upregulated in our neuronal enriched exosomes, it was also found to be associated with several cancer pathways such as pancreatic cancer (Xu et al. 2014). **Hsa-miR-92a** is shown to be specifically associated with neural progenitors (Jönsson et al. 2015) and promotes colorectal carcinoma cell proliferation, invasion, and migration by inhibiting *FBXW7* i*n vitro* and i*n vivo* (Gong et al. 2018). Hsa-miR-141 and hsa-miR-92b are not reported earlier in association with PD and hence can be considered as potential target for future study. In addition, the functions of a few of the miRNAs that were significantly upregulated in PD compared to controls is currently unknown. These include: **hsa-mir-6131**, **hsa-mir-98-5p**, **hsa-mir-6073**, **hsa-mir-16-2-3p**. **hsa-mir-101-3p** and **hsa-mir-5096** (this was only high in one sample). Future studies are needed to elucidate their function in PD.

Chapter 5

LIMITATIONS

FUTURE DIRECTIONS

CONCLUSIONS

LIMITATIONS

Studies have shown that medication affect miRNA expression in disease (Alieva et al. 2015). Patients were on medication which may be a confounding factor. It is difficult to measure each miRNA contribution to the disease. Although a single miRNA can be related with multiple pathologies, it does not mean that all pathologies will develop and the presence of miRNA in collected fluids does not directly indicate the inhibition of the translation process.

FUTURE DIRECTIONS

Experiments are further needed to validate our findings with qRT-PCR to confirm the results that were obtained. Correlation analysis are needed to assess the relationship of differentially expressed miRNA with clinical parameters such as olfaction, depression and disease duration. We will also perform western blot to validate that our CD171 antibody is capturing exosomes that are originated from the brain.

CONCLUSIONS

In conclusion, this is the first study to explore neuronal enriched exosomal miRNA as biomarker for PD. Within the last decade, detecting miRNAs in biological fluids has become a reality. A wide range of studies contributed to give robustness to the methodology, using high throughput techniques and validating the results on larger datasets. Nevertheless, there is still work needed to translate these data into clinics. Although many studies have attempted to identify miRNAs as biomarkers of PD, the results have not always been consistent with each other, especially when comparing post-mortem brain specimens with blood and CSF samples. Some consistency was found between other studies and this study when comparing PD patients

with their controls. Identification of these urgently needed non-invasive biomarkers could potentially enhance PD diagnosis and help clinicians with early treatment intervention.

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APPENDIX

LIST OF DIFFERENTIALLY EXPRESSED MIRNAS

	logFC	logCPM	F	PValue	FDR	Role	Reference
hsa-mir-26b-5p	9.648483	12.47209	36.6003	2.29E-08	7.57E-07		
hsa-mir-30a-5p	8.078975	10.71861	31.13961	1.94E-07	4.27E-06		
hsa-mir-101-3p	7.815626	10.2603	29.55223	6.28E-07	6.18E-06		
hsa-mir-92b-3p	9.443138	11.77884	29.5038	6.39E-07	6.18E-06		
hsa-mir-16-5p	9.197702	11.54388	29.41166	6.62E-07	6.18E-06		
hsa-mir-16-2-3p	7.39443	9.889321	29.15899	7.27E-07	6.18E-06		
hsa-mir-223-3p	8.379919	10.77447	29.07744	7.49E-07	6.18E-06		
hsa-mir-141-3p	8.92557	11.28529	28.47288	9.40E-07	6.89E-06		
hsa-let-7d-5p	10.58186	15.55089	25.77581	1.68E-06	1.11E-05		
hsa-mir-98-5p	6.355372	10.20629	20.05597	1.93E-05	0.000106		
hsa-let-7g-5p	5.670189	9.985224	18.22501	4.33E-05	0.00022		
hsa-mir-6131	3.444938	10.29765	16.5787	9.10E-05	0.000429		
hsa-mir-5096	6.024562	10.69434	15.63027	0.00014	0.000618		

hsa-mir-6073	2.70096	9.173071	9.166731	0.003105	0.012808		
hsa-mir-181a-5p	4.047896	13.30107	7.424932	0.007544	0.025793		
hsa-mir-221-3p	3.864349	11.33107	7.356551	0.007816	0.025793		
hsa-mir-25-3p	3.788422	12.13556	6.968416	0.009568	0.03007		

Table 5 Showing the values for the seventeen (17) significant differentially expressed miRNAs.