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Exploring Drivers of Sex-Based Disparities in Relapsing Multiple Sclerosis

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Nursing at Virginia Commonwealth University.

by

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Vita

Stephanie Buxhoeveden received her Bachelor of Science in Nursing from Villanova University in 2009, and her Master of Science in Nursing from Rutgers University in 2015. She began her nursing career as a neurosurgical intensive care nurse at Georgetown University Hospital, and later went on to be a neurological nurse practitioner, where she primarily specialized in Multiple Sclerosis (MS). She has extensive experience in advocacy and public policy through leadership positions in in organizations including the National MS Society and iConquerMS. She received her Doctor of Philosophy in Nursing from Virginia Commonwealth University in 2024, where she focused on identifying biomarkers of MS.

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Abstract

Females are three times more susceptible to relapsing multiple sclerosis (MS) and males typically have more severe disease, but the molecular underpinnings of these sex-based disease disparities are unknown and represent a critical knowledge gap. **Subject Population:** Blood samples from a demographically homogenous group of treatment naïve males and females with relapsing-MS and healthy controls. **Research Design:** Cross-sectional combinatorial omics pilot study. **Instruments:** Whole transcriptomic analysis with messenger RNA (mRNA) expression profiling using next generation sequencing (RNA-seq) and micro-RNA (miRNA) expression using NanoString technology. **Aim** 1) Identify and compare the actively expressed mRNAs in the transcriptome of males and females with relapsing-remitting MS and healthy controls using RNA-seq. 2) Analyze the miRNA profiles of males and females with relapsing MS and healthy controls and correlate miRNA and mRNA expression levels to better understand the role of miRNAs in MS etiopathogenesis. **Analysis:** Differential expression analysis of mRNA and miRNA data was performed, and Ingenuity Pathway Analysis (IPA) was used to explore the functional effects miRNA expression had on mRNA in each sample. MS females and MS males were compared to sex-matched healthy controls, than to each other to better understand the influence of sex on neuroinflammation and neurodegeneration in MS. **Results:** MS Females had unique expression of miRNA-mRNA pathways associated with neuroinflammation and may have key neuroprotective traits that contribute to their less severe disease course, while males had differential expression of pathways associated with axonal loss, cognitive decline, and increased disability. **Conclusions**: Overall, these findings revealed significant insights into sex-based differences in the pathogenesis of MS, which has helped illuminate potential biological underpinnings of the clinically observed courses of disease. These results have enhanced our understanding of MS pathophysiology and laid a crucial foundation for future research in this area.

Introduction

The title of this dissertation is Exploring Drivers of Sex-Based Disparities in Relapsing Multiple Sclerosis. A grant proposal submitted to the NINR Ruth L. Kirschstein Predoctoral Individual National Research Service Award (NRSA) will serve as the content for Chapters I-III with the approval of my advisor, Dr. Theresa Swift-Scanlan, and the VCU School of Nursing. Following the grant proposal Chapter IV will provide an overview of the findings, and Chapter V contains a discussion of the results, study strengths and weaknesses, future research directions, and end with a concluding narrative.

The aim of this study is to investigate sex-based disparities in MS to narrow the disease phenotype. MS is a chronic neurodegenerative condition that can lead to significant physical and cognitive impairments from a young age, profoundly affecting nearly all aspects of a patient's life. Although MS is about three times more prevalent in females, males tend to experience more severe forms of the disease. These significant sex-based differences in MS susceptibility and disease severity likely represent undiscovered disease phenotypes and constitute a crucial knowledge gap that this study aims to fill. This study analyzed the transcriptome of a homogeneous sample comprising males and females with MS and compared it with miRNA levels to elucidate the effects of epigenetic changes on gene expression. This approach will enhance our understanding of why females have a higher MS risk and why males tend to develop more severe forms of the disease.

Since epigenetic alterations are reversible, our findings could reveal novel therapeutic targets for personalized MS management. By advancing our understanding of the etiopathogenesis MS, this study may also provide important insights into novel mechanisms that could inform diagnosis, risk stratification, and therapeutic interventions.

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Chapters I-III: Grant Application

Specific Aims

Multiple Sclerosis (MS) affects approximately one million people in the United States and is the leading cause of disability in young adults (Lublin et al., 2014), yet little is known about its etiology and underlying pathology (Bove & Chitnis, 2013; Harbo et al., 2013). MS is currently an incurable neurodegenerative disease that is likely triggered by a combination of genetic and environmental risk factors (Bove & Chitnis, 2013). The average age-of-onset for MS is early adulthood, with most cases occurring between the ages of 20 and 40. Cognitive and physical disability can manifest early in the disease, resulting in severe and/or cumulative impacts on quality of life, and often leading to loss of employment in the prime of life. Thus, MS exacts a significant psychological, physical, and financial burden on patients and their care partners (Bove & Chitnis, 2013). Of the four subtypes of MS, relapsing-remitting MS (the terms relapsing MS and RRMS are used interchangeably in this paper) is the most common subtype, that comprises approximately 85% of the newly diagnosed population (Lublin et al., 2014). RRMS is approximately three times more common in females than males, and evidence suggests that this ratio is increasing worldwide (Bove & Chitnis, 2013; Harbo et al., 2013; Lublin et al., 2014). Although males are less susceptible to RRMS, they tend to have more severe forms of the disease, and are more likely to accumulate significant disability as a result (Bove & Chitnis, 2013; Harbo et al., 2013; Lublin et al., 2014). As the most common form of MS, it is critically important to address this knowledge gap of sex-based disparities by investigating their molecular underpinnings.

Recent studies suggest that molecular biomarkers may explain, in part, some of the differences that define MS phenotypes and different clinical courses of the disease. For example, gene expression and genome-wide association studies (RNA and DNA level studies respectively) in MS patients not segregated by sex or disease subtype have identified molecular

pathways implicated in MS pathogenesis, but these genetic variants alone only slightly increased the risk for developing MS (Bove & Chitnis, 2013; Harbo et al., 2013; Lublin et al., 2014; McCombe & Greer, 2013; Parnell & Booth, 2017). A handful of *in vivo* and *in vitro* studies suggest that messenger RNA (mRNA) and microRNA (miRNA) may be critical in understanding the observed sex-based disparities in RRMS. This study will address this knowledge gap by identifying and describing gene expression (mRNA) and microRNA profiles that may be differentially expressed between males and females with the RRMS subtype. This is the first study to narrow the phenotype of MS by using homogeneous human samples to measure both miRNA and mRNA expression in the same patient sample, which will provide detailed insights into how epigenetic changes contribute to sex-based differences. Additionally, this study will use samples from treatment naïve patients which is a key component of this study design since all MS treatments can alter miRNA and mRNA expression. Understanding these differences hold promise for advancing and optimizing personalized treatment, which could delay disease progression and limit disease-related disability.

We hypothesize that variances in gene expression influence the sex differences observed in MS. Therefore, the primary objective of this research project is to compare the human transcriptome and miRNA profiles of males and females with relapsing MS, using next generation RNA sequencing (RNA-seq) and NanoString technology, respectively. To our knowledge, this is the first study to attempt to further narrow the phenotype of RMS by exploring mRNA gene expression (transcriptomic) and miRNA profiles based on sex, in a narrowly defined demographic population. The long-term goal is to improve outcomes by identifying molecular biomarkers of sex-based differences in MS that would allow for preventative screening, and improved prognosis for individual patients. The specific, independent aims are to:

Specific Aim 1: Identify and compare the differentially expressed mRNAs in the transcriptome of males and females with relapsing MS and healthy controls. This agnostic omics study will isolate and analyze the whole transcriptome from blood samples collected from MS patients and healthy controls using RNA-seq. This broad discovery-based approach may lead to novel findings not reported by previous studies.

Specific Aim 2a: Analyze the miRNA profiles of males and females with relapsing MS, and healthy controls. miRNA profiles will be analyzed using NanoString Technology, and miRNA expression will be correlated with mRNA levels to better understand the role of miRNAs in MS etiopathogenesis.

Specific Aim 2b: Explore associations between clinical features and RNA-seq and miRNA data. Relationships between significantly differentially expressed miRNA and mRNA profiles and clinical features (MRI activity, CSF biomarkers, and disability) will be explored.

Problem and Conceptual Framework

MS is an autoimmune disease characterized by acute CD4+CD8+ T-cell driven inflammation and chronic neurodegeneration of the CNS (Harbo et al., 2013; Lublin et al., 2014). Although MS affects over two million people worldwide and is the leading cause of non-traumatic neurological disease in young adults (Bove & Chitnis, 2013; Harbo et al., 2013), little is known about its etiology and underlying pathology. Currently patients are classified as having either relapsing or progressive form MS (Harbo et al., 2013; Lublin et al., 2014). Relapsing MS is primarily characterized by acute inflammatory attacks on neurons and new neurological deficits, followed by periods of relative dormancy. Progressive MS, on the other hand, means disability steadily accumulates due to chronic neurodegeneration which is largely unresponsive to treatment. While there are over a dozen medications shown to slow the progression of relapsing MS, patients can transition to secondary-progressive MS at any time, at which point their treatment options are limited (Harbo et al., 2013; Lublin et al., 2014). The clinical

presentation, severity of symptoms, level of disability, and rate of neurodegeneration can vary widely, regardless of what type of MS the patient has (Harbo et al., 2013; Lublin et al., 2014). This variability makes MS very challenging to research and makes it extremely difficult for clinicians to predict long-term outcomes that could help guide treatment decisions (Bove & Chitnis, 2013; Harbo et al., 2013; Lublin et al., 2014). The heterogeneity of MS within these subgroups suggests that there may be several phenotypes, which could be influenced by variables such as age, ethnicity, and sex.

Sex is one of the most well-established risk factors for relapsing MS (Bove & Chitnis, 2013; Harbo et al., 2013; Lublin et al., 2014). Females are three times more susceptible to MS, and over 80% are diagnosed with the more treatable relapsing form which is predominantly characterized by acute inflammatory disease activity (Bove & Chitnis, 2013; Harbo et al., 2013). While males are less susceptible to MS, they typically have more severe forms of the disease that is characterized by progressive neurodegeneration and higher levels of physical disability, which is measured by the Expanded Disability Status Scale (EDSS) (Bove & Chitnis, 2013; Lublin et al., 2014). A recent retrospective population study of 3,028 male and 6,619 female Danish MS patients found females had a 16% higher relapse rate characterized by acute inflammatory CNS attacks, while males exhibited faster accumulation of disability (HR of 1.23 reaching EDSS or 3 or moderate disability, and 1.53 for EDSS 6, severe disability), more brain atrophy and increased mortality (Magyari & Koch-Henriksen, 2022).

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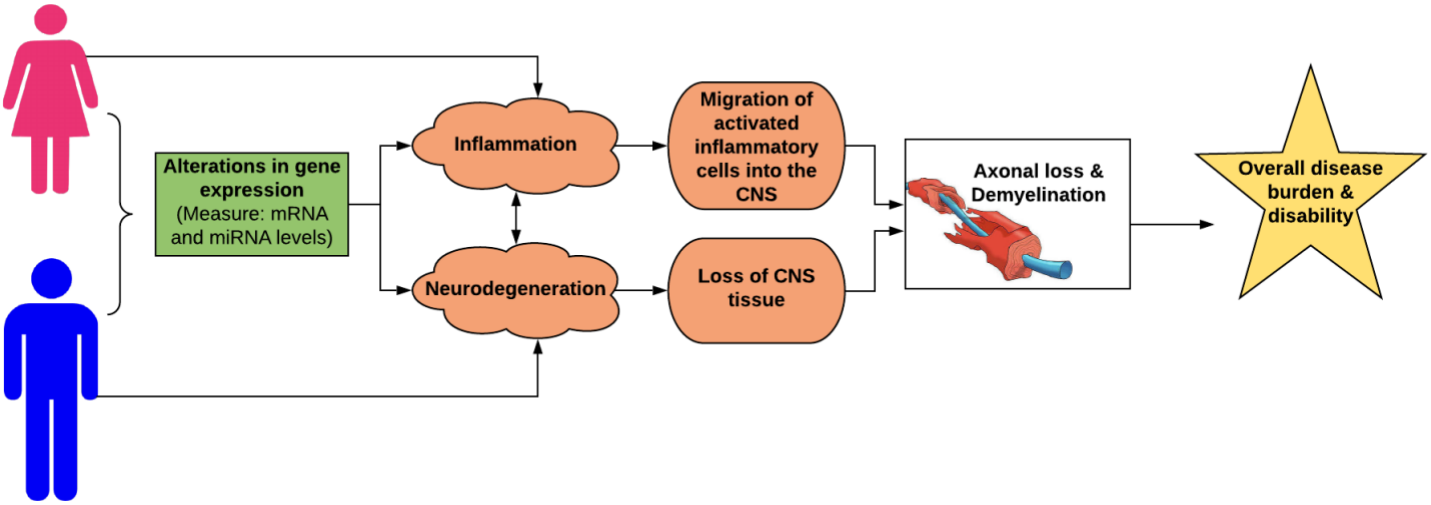

Figure 1. Conceptual framework of sex-based disparities in MS.

Scientific Premise and Background

Sexual dimorphism in the CNS and immune response play an important role in MS, but the underlying biological mechanisms are not fully understood. Prior research has provided important insights into how genetic and epigenetic mechanisms drive the increased immune activity in females and contribute to neurodegeneration in males.

Environment: Environmental risk factors may contribute to the observed sex-based disparities in MS. For example, vitamin D3 deficiencies have long been associated with a higher risk for developing MS, and for disease progression after diagnosis (Lublin et al., 2014). Interestingly, a vitamin D3 deficiency results in a seven-fold increase in female's risk for MS, but does not seem to affect males in the same way (Nashold et al., 2009). Vitamin D3 and estrogen may have a female specific synergistic relationship, which could explain this discrepancy. A study using the mouse model of MS found that female mice with vitamin D3 deficiencies were only able to increase their levels with supplements if they had sufficient levels of estrogen (Spanier et al., 2015). Other mouse studies have also shown that this relationship between vitamin D3 and estrogen plays a role in immune system homeostasis via the Foxp3 gene pathway. Insufficient

levels of Vitamin D have been shown to methylate Foxp3 leading to a more inflammatory T-cell phenotype (Spanier et al., 2015; Walecki et al., 2015), and may play a role in MS neuroinflammation. This example highlights the potential environmental impact on the epigenome and provides evidence that it could also contribute to sex-based differences in MS, which warrants further investigation in human subjects.

Sex Chromosomes: Sexual dimorphism arises from sex chromosomes and sex hormones. Females inherit two X chromosomes, one from their mother and one from their father, while males inherit one X chromosome from their mother and one Y chromosome from their father. The Y chromosome only contains about 48 genes that are primarily involved with male reproduction, while the X chromosome contains about 2000 genes. Sex-based differences in gene expression are influenced by the expression of Y chromosome genes in males, X chromosome gene dosage, and epigenetic modifications of genes in the paternal versus maternal X chromosome (Golden et al., 2019).

In females (XX) X-chromosome inactivation occurs in one of the two X chromosomes to silence duplicate genes, ensuring that males and females have the same number of gene copies. Approximately 15% of X genes in humans (and 3% in mice) escape inactivation, leading to increased expression of those genes (Golden et al., 2019; Voskuhl, 2020; Voskuhl et al., 2018). These increased dose effects that occur through failure of X chromosome silencing lead to increased gene expression in XX vs XY, and may make females more susceptible to autoimmune diseases including MS (Shansky, 2015; Sharma & Eghbali, 2014; Voskuhl, 2020; Voskuhl et al., 2018). Males with Klinefelter's syndrome (XXY) are more susceptible to autoimmune diseases with an increased female:male susceptibility including MS, lupus, and rheumatoid arthritis, illustrating the importance of X-chromosome inactivation in sex-based disease disparities (Seminog et al., 2015).

Voskuhl et al. (2018) explored the influence of X chromosomes inherited from the mother versus those inherited from the father (referred to as the parent-of-origin). Specifically, they looked at X chromosome methylation using a four core genotypes (FCG) mouse model, which allows researchers to examine the role of sex chromosomes without the confounding effects of sex hormones. They found that parent-of-origin differences in DNA methylation on the X chromosome can lead to sex differences in immune responses. Using RNA-sequencing of CD4+T lymphocytes they found increased expression of a cluster of genes in mice with a single X chromosome compared to those with two X chromosomes. This difference in expression was driven by DNA methylation, a type of epigenetic modification, that occurred more frequently on the paternal X chromosome. This methylation led to increased gene silencing in the paternal X chromosome, which is only inherited by females, and may help explain why the same sequences of genes that regulate immune responses are differentially expressed in females versus males.

Mouse models of MS have demonstrated a greater degree of neurodegeneration and disability in mice with XY chromosomes, which suggests that male chromosomes contribute, at least in part, to their more severe disease course (Du et al., 2014; Shansky, 2015; Voskuhl, 2020; Voskuhl et al., 2018). This suggests males are inherently more vulnerable to neuronal damage than females, and identifying epigenetic pathways that contribute to their more neurodegenerative disease course is an important knowledge gap.

Sex Hormones & Sex Specific Genes: There are several X chromosome genes that have been implicated in autoimmune diseases, including MS (Du et al., 2014; Voskuhl, 2020; Voskuhl et al., 2018). For instance**,** there is evidence that females have a higher rate of the MS linked HLA-DRB1*1501 allele, and that estrogen receptor polymorphisms may trigger autoimmune cascades that are regulated by the HLA genes in that region (Alcazar et al., 2010; Harbo et al., 2013; Mattila et al., 2001; Voskuhl et al., 2018).

Interestingly, there is no sex-based difference in susceptibility among pediatric MS patients, and females are only disproportionately at risk after puberty (Alcina et al., 2012; Jiang et al., 2018; Waubant, 2018). Additionally, during pregnancy disease activity in females with MS predictably slows as estrogen levels rise rapidly, followed by a significantly increased risk for relapse postpartum as estrogen drops (Avila et al., 2018; Harbo et al., 2013). This suggests that sex hormones not only influence susceptibility during childbearing years, but continue to modulate disease activity after onset (Avila et al., 2018; Jiang et al., 2018; Parnell & Booth, 2017; Spence & Voskuhl, 2012; Tomassini et al., 2005; Waubant, 2018).

In mouse studies testosterone appears to naturally regulate the Foxp3 gene, which results in a more regulatory T-cell phenotype in healthy males and may help explain why they are typically less susceptible to MS (Voskuhl et al., 2018; Walecki et al., 2015). This is supported by evidence from clinical human studies, which found that men with MS are more likely to have low testosterone levels than healthy controls (Harbo et al., 2013; Tomassini et al., 2005). Lower levels of testosterone have also been shown to positively correlate with the number of gadolinium enhancing lesions seen on MRI (Tomassini et al., 2005), which are biomarkers of active CNS demyelination. Oestradiol concentrations also seem to play a role in MS pathogenesis in men. In one study oestradiol levels positively correlated with the number of T1 and T2 lesions, or old areas of demyelinating attacks, which show the overall burden of disease (Harbo et al., 2013, 2013; Tomassini et al., 2005). Therefore, it is possible that sex hormones also drive the disease neuroinflammation and neurodegeneration in males, and may help explain why they are at a disproportionate risk for more severe MS.

Expression Quantitative Trait Loci (eQTLs) and MS Pathogenesis: Genome wide association studies (GWAS) have identified over 200 genetic risk variants, many of which are closely tied to the differentiation of B, T, natural killer, and myeloid cells, which contribute to the onset and progression of MS (Han et al., 2020; Huang et al., 2017; Lublin et al., 2014; Parnell &

Booth, 2017; Patsopoulos et al., 2019). Interactions between an individual's genome and epigenome seem to be an important component of MS pathophysiology, but are poorly understood (Gacias & Casaccia, 2014; Han et al., 2020; Huang et al., 2017; Lublin et al., 2014; Martinelli-Boneschi et al., 2012; Parnell & Booth, 2017; Patsopoulos et al., 2019). Prior RNA-seq studies evaluating the influence of MS on eQTLs by single nucleotide polymorphisms (SNP)-genes have shown that SNPs significantly regulate long noncoding RNA (lncRNAs) associated with antigen presentation and immune signaling pathways in MS (Han et al., 2020; James et al., 2018). Further research using human MS samples are needed to better understand SNP-gene relationships, and the how they influence immunological pathways in males and females.

MicroRNAs: The central dogma of genetics states that genetic information encoded in double-stranded DNA is transcribed, or "expressed", into single stranded mRNA in the nucleus. mRNA is then processed (which involves splicing to remove introns and adding a 5' cap and polyA tail) and exported from the nucleus to be translated, or further "expressed", as a polypeptide by ribosomes [Figure 2]. Pre-miRNAs are also exported from the nucleus where they become mature miRNAs capable of impacting mRNA translation. There are over 1800 human miRNAs, which are small (19-24 nucleotides) single-stranded non-coding RNAs. After transcription they can incompletely pair with a target mRNA (predominantly in the 3' untranslated region) in the cytoplasm [Figure 2] and can subsequently cause 1) mRNA degradation 2) repression or silencing of translation or 3) translation activation. miRNAs are key regulators of many biological processes, including the immune response, and modulate up to 60% of human genes. A single miRNA can target many different mRNAs, and translation of any mRNA can be blocked by many different miRNAs. Dysregulation of miRNAs have been associated with other autoimmune diseases, including rheumatoid arthritis (RA) and lupus (Shansky, 2015), and mouse models of MS and RA have shown that sex differences in miRNA expression play an important role in

immune response and levels of inflammation (Dai & Ahmed, 2014; L. Li et al., 2020; Shansky, 2015). Prior *in vivo* and *in vitro* studies have shown that the administration of estrogen and testosterone can exacerbate autoimmune responses and perpetuate miRNA dysregulation, suggesting that sex hormones and miRNA interactions can influence disease susceptibility and progression via their effects on target mRNAs, but further studies on human samples are needed to fully understand these interactions (Dai & Ahmed, 2014; Shansky, 2015).

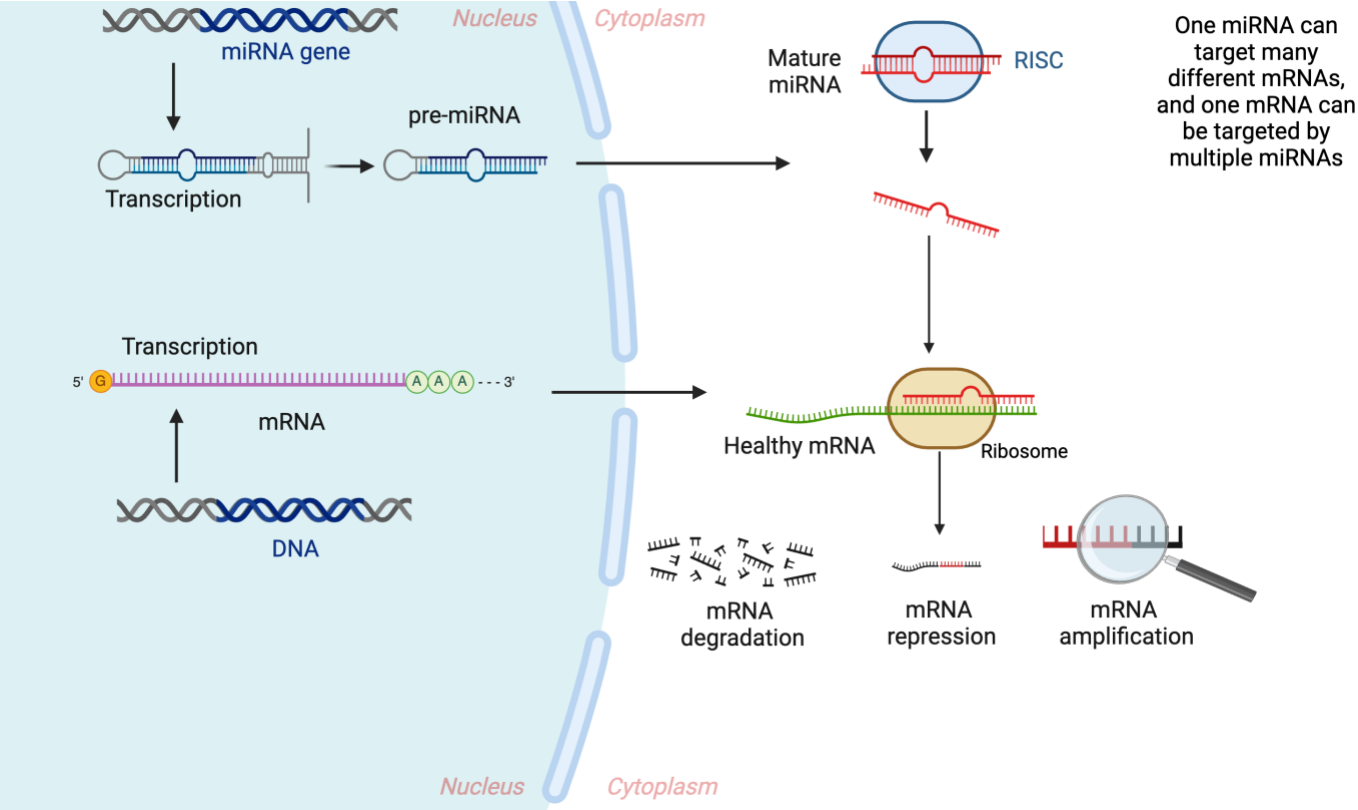

Figure 2. Epigenetic modification of mRNA by miRNA. After transcription miRNA partially binds to the target mRNA in the cytoplasm and alters translation. *Image created with Biorender.com.*

Significance

This study is significant because it will shed light on molecular underpinnings of MS and advance knowledge of MS pathophysiology. This goal is well aligned with the strategic plan and research priorities of the National Institute of Nursing Research (NINR) and American Academy of Nursing (AAN) (Eckardt et al., 2017; NINR, 2016.) Therefore, nursing research

focused on narrowing the MS phenotype to explain disease heterogeneity could elevate both nursing practice and MS care and have a positive impact on the MS population. **A novel approach** will be used to address the gap in knowledge regarding sex-based differences by exploring the effects of dysregulated miRNAs on mRNA expression within the same patient to better understand the functional impact of epigenetic pathways. By comparing gene expression pathways between males and females, we hope to gain a better understanding of the mechanisms that drive sex-based disparities in relapsing MS. Results of this study are expected to advance understanding of MS etiopathogenesis, with the potential for ultimately improving the quality of care in this population.

Innovation

This study is innovative because it is the first to use demographically homogeneous human samples and a combinatorial (RNA-seq and NanoString) omics approach to discover molecular differences based on sex in the relapsing MS population, which has well established sex-based disparities. Most research in this area has been conducted using mouse models, which often fail to translate to human studies (Lassmann & Bradl, 2017). Additionally, epigenetic studies that have utilized human samples have primarily been derived from demographically heterogeneous populations that were not powered to detect sex differences, making it difficult to narrow the phenotype of different patient groups (Gacias & Casaccia, 2014; Zheleznyakova et al., 2017). By using human samples, isolating sex as a biological variable, using treatment naïve subjects, and controlling for age and ethnicity, we will be able to shed light on the biological and molecular underpinnings and better appreciate the influence of sex. This will significantly advance knowledge of disease pathophysiology. Importantly, epigenetic changes are reversible (Zheleznyakova et al., 2017), and therefore this study can identify some of the epigenetic underpinnings of MS via miRNA biomarker profiling, which may ultimately inform individualized interventions leading to reduced disability.

The approach of this study is innovative because it will correlate mRNA and miRNA levels using advanced RNA-seq and NanoString technology to better understand the role epigenetic changes (e.g., dysregulated miRNAs) have on gene expression. Few studies have looked at whole transcriptome analysis in tandem with miRNA expression profiling within the same patient sample (see Aim 2 rationale), and to our knowledge, this is the first study to specifically focus on sex differences in relapsing MS. RNA-seq and NanoString have a higher specificity and sensitivity than microarrays (Kukurba & Montgomery, 2015), and by using two powerful omics modalities in tandem, we hope to detect novel gene expression differences between males and females not previously seen.

Grant Proposal: Research Approach and Rationale

Sex as a Biological Variable (SABV)

Considering SABV in research is crucial because males and females can exhibit differences in physiology, disease susceptibility, and response to treatments. Males are underrepresented in MS research, with about 70% of research participants being female, meaning most MS studies are not powered to detect sex differences. This has created a significant gap in knowledge of sex differences and makes it difficult to understand the mechanisms that make females more prone to inflammation, and males more vulnerable to neurodegeneration and whether they respond to treatment differently as a result. The aim of this study was to isolate the effects of sex as much as possible, and several aspects of the research design incorporated SABV. Using a narrow age range and samples from the same ethnicity increased the likelihood that differences could better be attributed to sex. An equal number of males and females were also included in this study, in contrast to many MS research studies which tend to predominantly include females. Unrelated healthy controls were also utilized which allowed males and females to be compared separately and to each other. By including controls, this study is well suited to distinguish between sex differences and disease-specific

factors. By incorporating SABV this study aims to provide more comprehensive insights into the pathophysiology of multiple sclerosis.

Sample: Biorepository Selection

Finding diverse pools of MS samples in biorepositories is a significant challenge in MS research. To isolate SABV this study was designed to control for other potential influencers of phenotype including age, ethnicity, and treatment status (only patient that are treatment naïve, or unexposed to MS medications, will be included). Several repository inventories were screened for feasibility using the described inclusion/exclusion criteria, but most did not have enough male samples for this project. The Accelerated Cure Project for MS (ACP) had 18 Caucasian male samples in the desired age range that also met the other study inclusion/exclusion criteria. Therefore, 48 blood samples (18 males, 18 females, and healthy comparison samples from 6 males and 6 females) from the ACP biorepository will be purchased and shipped to the VCU School of Nursing's Biobehavioral Research lab. The ACP's blood samples are collected in PAXgene tubes, which stabilize RNA for long-term storage at -80 ^{o}C, making it ideal for transcriptome analysis (Sawcer et al., 2011).

Sample Size Rationale

An analysis performed using the R package RNASeqSampleSize (Developer et al., 2023, version 2.12.0) showed that 15 subjects are needed to detect an estimated 3-fold change at 80% power with a False Discovery Rate (FDR) of 0.05 assuming the minimum average read count among the prognostic genes in the control group is 10, the maximum dispersion is 0.5, the ratio of the geometric mean of normalization factors is 1, the total number of genes for testing is 10000, and the top 500 genes are prognostic. While budget and repository availability were a limiting factor in the number of blood samples acquired for this pilot study, the sample size should be adequate for differential expression analysis. However, it could be boosted if the data is underpowered (due to poor sample quality or failure of an assay) by using publicly

available data from NCBI's Gene Expression Omnibus (GEO) database, such as data from GEO series GSE141804, GSE124900, GSE162051, or GSE137143.

Proposed Approach for Specific Aims

Aim 1: Identify and compare differentially expressed mRNAs in the transcriptome of males and females with relapsing MS, and healthy controls. Significance and Rationale: The underlying etiology and pathophysiology of relapsing MS is poorly understood, but it is thought to be triggered by a complex combination of genetic and environmental factors (Lublin et al., 2014). Genomic studies have identified over 200 genetic risk variants, many of which are closely tied to the differentiation of B, T, natural killer, and myeloid cells, which contribute to the onset and progression of MS (Huang et al., 2017; Lublin et al., 2014; Parnell & Booth, 2017; Patsopoulos et al., 2019). Interactions between an individual's genome and epigenome seem to be an important component of MS pathophysiology (Gacias & Casaccia, 2014; Huang et al., 2017; Lublin et al., 2014; Martinelli-Boneschi et al., 2012; Parnell & Booth, 2017; Patsopoulos et al., 2019). Therefore, gene expression (e.g., the transcriptome of MS patients) may in part explain the observed sex-based disparities (Huang et al., 2017; Lublin et al., 2014; Martinelli-Boneschi et al., 2012; Patsopoulos et al., 2019).

Characterizing different disease subtypes/phenotypes is a critical step to informing future research and clinical management. We have chosen to focus on sex differences because important genetic variants have already been identified, such as the MS-associated HLA-DRB1*1501 allele which is more common in females, and is the strongest known genetic risk factor for MS (Alcina et al., 2012). We propose that identifying differences in the actively expressed mRNAs in the transcriptomes of treatment naïve males and females will provide a better understanding of the impact of transcriptomic interactions, and advance knowledge of the molecular and biological underpinnings of MS.

Experimental Design: To characterize sex differences, we will conduct a cross-sectional omics pilot study using archived blood samples, which were collected in PAXgene blood RNA tubes by the Accelerated Cure Project, to map the mRNA of RMS patients and healthy controls. Total RNA will be purified and extracted from the PAXgene blood RNA tubes according to the Promega Maxwell® RSC blood RNA kit instructions. The quality and quantity of RNA will then be verified using a Nanodrop spectrophotometer, gel electrophoresis, and the Promega Maxwell® Quantus. Total RNA samples of sufficient quality, molecular weight, and quantity will be sent for sequencing to the VCU Sequencing Core Lab on an **Illumina next generation sequencing (NGS) platform** (targeting >20 million reads per sample). We will then look for differentially expressed genes between MS males and females, and then compare MS patient samples to healthy controls to assess whether any observed changes could be attributed to MS.

Data Analysis: Bioinformatics processing will include assessing raw data quality using FastQC (Babraham Bioinformatics, 2024) and MultiQC (Ewels et al., 2016), read trimming and filtering as needed with CutAdapt (Martin, 2011), alignment to the GRCh38 version of the human genome with STAR (Dobin et al., 2013), and transcript quantification/read counting with Salmon (summarized to the gene level) (Patro et al., 2017). Differential expression analysis will be done using the R package DESeq2 (Love et al., 2014), and genes will be considered differentially expressed if they have an adjusted p-value of <0.05 and absolute fold change of > 1.3. A pathway enrichment analysis will be performed on lists of genes that are significantly differentially expressed using Gene Set Enrichment Analysis (GSEA) (Subramanian et al., 2005), redundant pathways will be collapsed, and a heatmap of enriched pathways will be created using EnrichmentMap (Merico et al., 2010) software.

Expected Outcomes: We anticipate finding differentially expressed genes between males and females with RMS. Differentially expressed genes are important in understanding the differences between healthy people, and those with MS. This data will indicate underlying

biological differences between males and females with MS, which will help us understand sex-based disease disparities.

Potential Pitfalls and Alternatives: We recognize the sample size is modest, but if an unexpectedly large number of samples fail to produce viable RNA, or if there is not enough statistical power for data analysis, the other samples could be used to assess the suitability of other publicly available samples from GEO and boost the sample size to obtain better power. It is not uncommon to have a small percentage of the samples fail to produce complete RNA-seq data, so the ability to rerun samples was built into the study design if we run into this problem. Therefore, having a sample size of 18 in the MS male and MS female groups, and the ability to use publicly available samples from GEO if necessary, helps to ensure that enough data will be produced for differential expression analysis, even if some blood samples fail to produce high quality RNA and/or data. Importantly, the specific aims are complementary, but independent from one another, so if the RNA-seq data fails to produce data for any reason the NanoString analysis in Aim 2 could still be completed, and the study findings would still render important data.

Aim 2: Analyze the miRNA profiles of males and females with relapsing MS, and healthy controls. Significance and Rationale: Epigenetic changes, including DNA methylation, miRNA dysregulation, and altered chromatin proteins, are capable of activating autoreactive lymphocytes in the periphery, where they then migrate through the blood brain barrier (BBB) and into the CNS, causing demyelination, axonal injury, neuronal death, and even the complete loss of brain tissue (Huang et al., 2017; Lublin et al., 2014). Therefore, there is a critical need to understand the underlying epigenetic changes that drive disease activity (Gacias & Casaccia, 2014; Parnell & Booth, 2017). Due to the scope of this pilot dissertation proposal, we will focus solely on one type of epigenetic modification, differences in miRNA between males and females with MS. miRNAs can be considered an epigenetic modifier in that they can alter gene

expression without affecting the primary sequence of DNA. However, miRNA expression itself can be regulated genetically (through DNA variants) and epigenetically (via DNA methylation and histone alterations for example). The main focus of this pilot study is to examine miRNA profiles because of their direct impact differentially expressed mRNA profiles [Figure 2] (Gacias & Casaccia, 2014; Huang et al., 2017; Martinelli-Boneschi et al., 2012; Parnell & Booth, 2017; Patsopoulos et al., 2019). Several *in vitro* studies have looked at the role of miRNAs in MS, and found that they are capable of influencing cell proliferation and differentiation, as well as immune signaling (Bove & Chitnis, 2013; Gacias & Casaccia, 2014; Huang et al., 2017; Martinelli-Boneschi et al., 2012; Parnell & Booth, 2017; Patsopoulos et al., 2019). Dysregulated miRNAs may be an important component of MS pathophysiology, which suggests that mRNA-miRNA pathways are highly relevant and capable of influencing these sex-based disparities (Gacias & Casaccia, 2014; Huang et al., 2017; Lublin et al., 2014; Patsopoulos et al., 2019).

Aim 2b: Explore associations between clinical features and RNA-seq and miRNA data.

Significance and Rationale: Clinical data associated with each sample will be correlated with the RNA-seq and NanoString data to look for any associations with clinical features. Since the MS samples were taken from patients who are treatment naïve (likely at the first or second neurology encounter) most have short disease duration (0-2 years). In the early stages of MS several clinical criteria can be associated with more aggressive disease and a poorer prognosis including their disability level (measured by the EDSS score with 0-3 being mild, 3-6 being moderate, and 6-10 being severe disability), the presence and number of spinal cord lesions, a high number of enhancing T1 lesions and/or non-enhancing T2 lesions at baseline (>10 being severe), and a high number of oligoclonal bands (10 or more being severe) (Bergamaschi, 2007). The gene expression patterns we identify will be enriched by the clinical data reported for the samples [Table 1]. Although the small sample size means this study is most likely underpowered to draw formal conclusions, this could still help explore effect sizes and possible

relationships between significantly differentially expressed miRNA and mRNA profiles with these clinical features that could be used to inform a future larger study.

Experimental Design: As described in Aim 1, RNA will first be purified and extracted from PAXgene blood RNA tubes according to the Promega Maxwell® RSC blood RNA kit instructions. Using the total blood RNA described above, whole NanoString miRNA profiling will be performed to quantify miRNAs for every sample in each group. Levels of miRNA will be compared between each group and to the mRNA levels from Aim 1 to identify patterns and differences among groups.

Data Analysis: NanoString CodeSets are rigorously designed with reference miRNAs, spike-ins, and positive and negative ligation controls. Dr. Swift Scanlan and her team, in collaboration with Dr. Liu, have developed normalization pipelines for NanoString miRNA data including the use of NormFinder prior to statistical analysis. After normalization, expression levels of each miRNA will be compared in males, females, and controls within NanoString's nSolver software using a 2-tailed Student t test and analysis of variance multiple comparison tests. A cut-off adjusted p value of <0.05 and a fold change of > 1.3 will be considered significant, and positive and negative ligation controls will be used for quality assurance. These miRNA expression pathways will also be added to the heatmap from Aim 1 to visualize the different pathway components. Significantly differentially expressed miRNAs will be included in a target analysis using TargetScan (Agarwal et al., 2015), and results correlated with the set of differentially expressed gene lists from Aim 1. Whole-genome miRNA and mRNA expression profiles will be correlated for each replicate to understand the *in vivo* effects of miRNAs and to identify potential novel targets and biomarkers of RMS subtypes.

In exploratory aim 2b spearman correlation coefficients will be used to estimate the association between miRNA expression and MRI disease activity (number of T1, number of T2 lesions, number of spinal cord lesions), CSF results (number of oligoclonal bands), and

disability (EDSS score), and a 2-tailed t test to compare clinical features in MS males versus MS females.

Expected Outcomes: In Aim 1 differentially expressed genes in males and females with MS will have been identified. Aim 2 builds on this work by correlating miRNA levels with differentially expressed genes, to better understand the role of dysregulated miRNAs on gene expression. RNA-seq and NanoString data will be explored for any associations with clinical features. This will help identify the underlying variables contributing to alterations in mRNA gene expression.

Potential Pitfalls and Alternatives: The most important confounding variables including age and ethnicity will be controlled for, but other confounders including obesity, Vitamin D levels, and smoking exposure are known to affect MS susceptibility and severity and may affect miRNA expression (Bove & Chitnis, 2013). The biorepository samples do not have complete data on these potential confounding environmental variables, which is a limitation of this study. At the time of biorepository sample collection at clinical sites the principal investigators are asked to report various clinical measures and outcomes [Table 1], but if the data is missing there will not be any way of obtaining additional details which is another limitation of exploratory aim 2b. Importantly, the specific aims are complementary, but independent from one another, so if the NanoString analysis fails to produce data for any reason the RNA-seq analysis in Aim 1 could still be completed, and the study findings would still be informative.

Table 1
Description of Clinical Data Associated with Blood Samples

Demographic Information
- Age/sex/ethnicity
- Disease duration
- Height/weight/BMI

Environmental Exposures
- Tobacco use/exposure history

Clinical Assessments
- Expanded Disability Status Score (EDSS)

Neurodiagnostic Testing Results
- CSF results: oligoclonal bands, IgG index, white cell count, total protein, myelin basic protein
- MRI of the brain, cervical, and thoracic spine: number of T1 lesions, number of enhancing lesions, number of T2 lesions, location of lesions

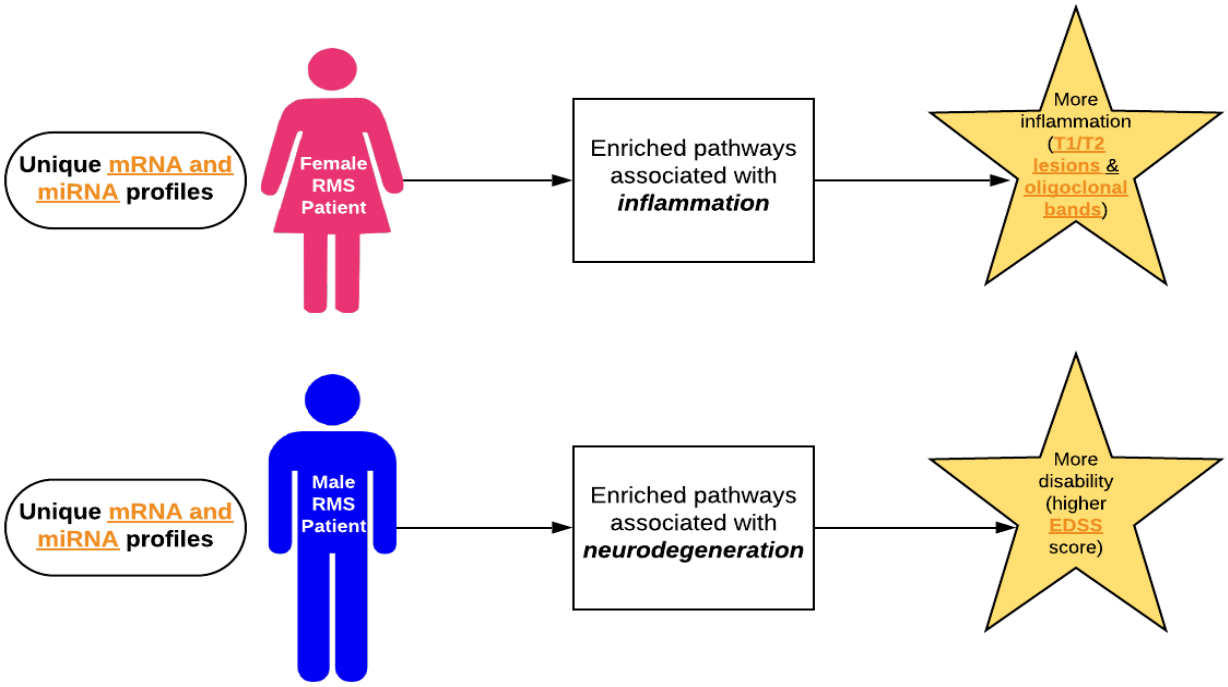

Figure 3. Specific Framework. Hypothesis: Differentially expressed miRNA-mRNA pathways drive sex-based disparities MS by impacting neuroinflammation and neurodegeneration. Variables are listed in orange.

Data Collection: Methods Used in Research

The above research proposal was submitted to the National Institute of Nursing Research (NINR). Below is a detailed description of the methods used during data collection and analysis used in this project after receiving funding, which is why the tense changes from future to past. Some bioinformatic approaches differ from the original proposal (such as the R packages used for analysis) due to updates in protocols and changes in the availability of tools/technology between the grant submission and research implementation.

Characteristics of the study sample

As described, 48 blood samples were acquired from the Accelerated Cure Project's (ACP) biorepository to use in this study (18 males, 18 females, and healthy comparison samples from 6 males and 6 females). As planned, all the participants were Caucasian, and all groups had similar ages [Table 2]. The MS male group had a slightly higher disease duration of 0.94 years, while most MS female samples were more newly diagnosed and had samples collected within a few months of their diagnosis [Table 2]. All MS samples were treatment naïve. Both the MS male and female groups had high body mass indexes (BMI) and were more likely to be smokers than the healthy control group.

Table 2
Study Sample Demographics

Group	Multiple Sclerosis		Healthy Control	
Sex	Male n=17	Female n=17	Male n=6	Female n=6
Age Mean Years(sd)	33 (6)	32.6 (5.4)	33.3 (6.7)	32 (5.1)
Disease Duration Mean Years (sd)	0.9 (3)	0.4 (1.5)	N/A	N/A
BMI Mean (sd)	49.2 (14)	38.2 (8.4)	Not reported	Not reported
No data (%)	1 (5.9%)	1 (5.9%)	6 (100%)	6 (100%)
Smoking Status				
Non-smoker (%)	6 (35.3%)	7 (41.1%)	4 (66.7%)	6 (100%)
Smoker (%)	10 (58.9%)	9 (52.9%)	2 (33.3%)	0 (0%)
No data (%)	1 (5.9%)	1 (5.9%)	0 (0%)	0 (0%)

RNA Extraction

Total RNA was extracted from PAXgene tubes using the Qiagen PAXgene blood miRNA kit as per manufacturer protocols (Qiagen, Valencia, CA). A 5µl of spike-in of osa-miRNA-414 (a non-human miRNA) was added to each sample during RNA extraction to aid with normalization. To increase RNA yield and purity an additional two-phase wash with 2-dodecanol was performed between the wash and elution steps (Jue, Witter, & Ismagilov, 2020). The quality and quantity of RNA was evaluated using a Nanodrop spectrophotometer, the Promega Maxwell® Quantus, and Bioanalyzer. To increase concentration and purity, the RNA eluate (~70µl) was washed through Amicon 3kD columns.

After the clean-up step each sample was divided into two microcentrifuge tubes, one for miRNA analysis with NanoString, and the other for RNAseq. Samples were stored at -80°C until assays were performed.

During extraction it became evident that one of the MS male PAXgene tubes had failed to preserve any viable RNA and was thus excluded from any further analysis. In addition, insufficient clinical data was available for one of the MS female samples and her MS diagnoses could not be confirmed. Therefore, this sample was also excluded, bringing the overall sample size to 46 (17 MS males, 17 MS females, 6 male controls, and 6 female controls). Even with the exclusion of these samples adequate data was collected and using the public Gene Expression Omnibus (GEO) database to boost the sample size was not required.

Once high-quality total RNA was purified in the SON BRL for each of the samples, they were aliquoted into two portions, one to be assayed for miRNA expression on the Nanostring platform in the SON BRL lab, and the other was the sent to the VCU Genomics Core for RNA-seq analysis to interrogate mRNA expression [Figure 4].

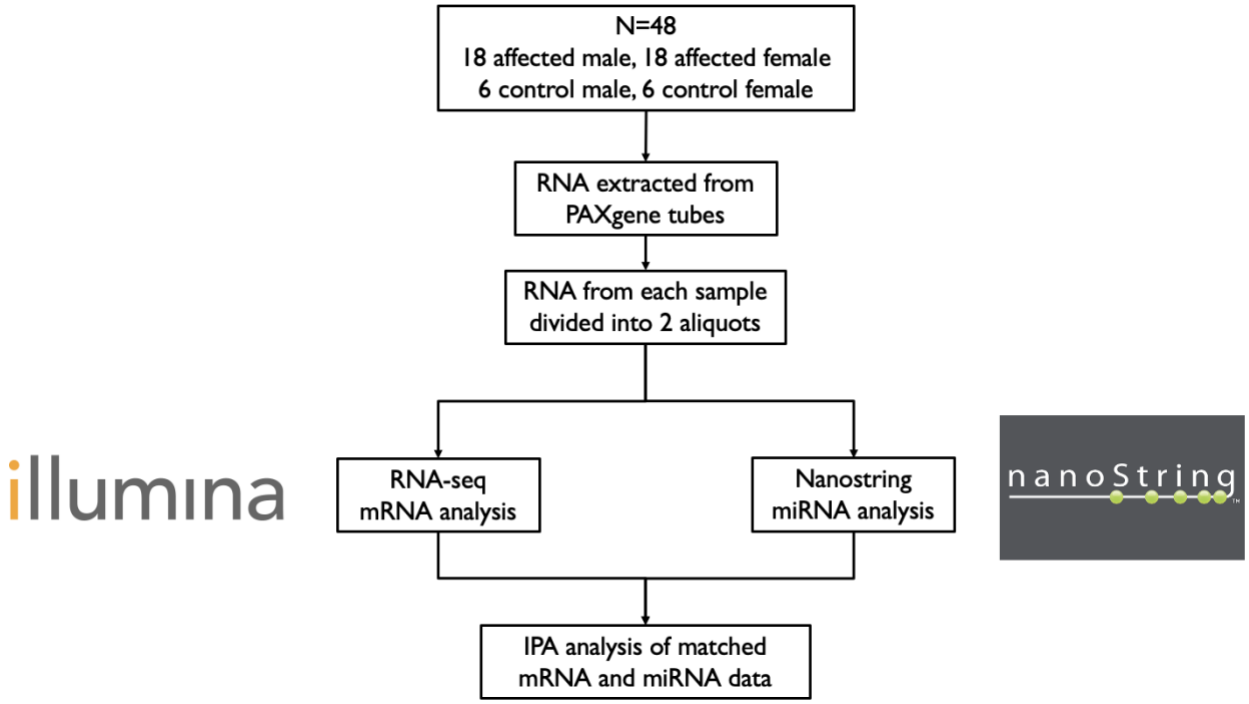

Figure 4. Flowchart of data collection procedure.

Aim 1 Procedure for Data Collection: mRNA Analysis

RNA-Seq can identify both known and novel transcripts, making it an ideal method of exploring diseases with poorly understood etiopathologies, such as multiple sclerosis. The mRNA data for this study was generated at Virginia Commonwealth University's Genomics Core facility using a polyadenylated (polyA) tail capture-based mRNA-seq, which has the advantage of not needing to fragment RNA prior to sequencing, and instead uses full length RNA providing more accurate data. Sequencing was performed on the NextSeq 2000 sequencer (Illumina Innovative Technologies) with a P3 flow cell. 200 cycles were run to achieve greater read depth and more accurately estimate fold-changes and differential expression.

Aim 2 Procedure for Data Collection: miRNA Profiling

miRNA analysis was performed using the Nanostring nCounter™ platform Human v3 miRNA Expression Assay containing probes for >800 human miRNAs as per manufacturer protocols (NanoString Technologies, Seattle, WA, USA). The NanoString assay utilizes

molecular barcodes to detect miRNAs without the use of amplification. During processing sequence-specific miRNA-tags are bound to target miRNAs using a ligase enzyme, and unbound tags are then removed through a clean-up step. Next, Reporter and Capture CodeSets are added to the samples. miR-451 is known to be overexpressed and can impair the ability to detect low-abundance targets by oversaturating the NanoString cartridge, so 5µl of DNA attenuation oligo (miR-451) were also added to each sample in improve detection of less-abundant targets. Samples were then hybridized overnight for 18h at 65°C to form target-probe complexes. Immediately after hybridization the samples are loaded into the nCounter Prep Station which washes away excess probes using a two-step magnetic bead-based purification and immobilizes target/probe complexes on the cartridge for data collection. The cartridges were then loaded into the nCounter Digital Analyzer which collects data by taking images of the immobilized fluorescent reporter probes with a CCD camera through a microscope objective lens. A high-density scan encompassing 550 fields of view is performed on each cartridge, and raw read counts are processed into RCC files, which were then downloaded from the digital analyzer and imported into the nSolver Analysis Software.

Data Management and Analysis

Management of Sex as a Biological Variable

The repository blood samples were not genotyped prior to shipment, and because sex chromosomes play an important role in MS it was crucial to confirm that all female samples were XX and the males were XY (not XXY) before proceeding with data analysis. The XIST gene (located on the X chromosome) functions to silence one of the X chromosome alleles when two X chromosomes are present. Therefore, if XIST is expressed it confirms that the sample has two X chromosomes. The presence or absence of the XIST gene expression confirmed that all the female samples were XX and none of the males had two X chromosomes. This was important because males with Klinefelter's syndrome (XXY genotype) are at an

increased risk for diseases that affect females more prominently, and could have had an impact on the data (Voskuhl, 2020).

Finally, a custom sex-informed reference genome was used to align the RNAseq data. Many genes on the X and Y chromosome can have very similar transcripts, which can result in an X chromosome gene misaligning to the Y chromosome, or vice versa. The custom genome first assessed whether the sample was female (XX) and if so, masked the Y chromosome which increased the ability to detect significantly differentiated genes on the X chromosome.

Incorporating these methods allowed for a more comprehensive analysis of sex differences in multiple sclerosis pathophysiology. Carefully considering and incorporating SABV allowed us to gain more comprehensive insights into the pathophysiology of MS and is a significant strength of this study.

Differential Expression Analysis

Differential expression (DE) in this study refers to the identification of miRNAs and mRNAs that are expressed at significantly different levels between groups. Assessing differential expression involves quantifying the expression level of each gene in the samples and applying statistical tests to determine if a gene is differentially expressed. *Fold change (FC)* is used to quantify differential expression and is calculated as the ratio of expression in one condition to another. Genes with a significant fold change are considered important, as they can provide insights into the biological processes altered in the conditions being studied. Due to the small sample size of this study a fold change of 1.3 was considered a significant enough change to warrant further functional analysis. FC data for both aims is reported in log2 which centers around 0 [Table 3]. Log2 makes FC data easier to understand and report, but requires mathematical conversion as illustrated below. In log2 a doubling of expression between groups would be -1 or 1, depending on if the gene was downregulated or upregulated. For this study a fold change of 1.3, or a log2FC of 0.33, was considered significant.

Table 3

Explanation of Log2 Fold Change

Group A Gene Expression	Group B Gene Expression	Fold Change (ratio of A/B)	Log2 Fold Change log2(A/B)
10	10	1	0
20	10	2	1
10	20	0.5	-1

Note: FC is the gene expression of group A divided by group B. It is not possible to get a negative number when dividing A/B, so all downregulation (or instances where group B is greater than group A) will fall somewhere between 0-1, making it confusing to quantify how significant a downregulated FC is. By using Log2 both downregulation and upregulation are centered around 0, making it much easier to see that both -1 and 1 equate to one group having double the gene expression as the other.

A small p-value (< 0.05) indicates that the observed fold change is unlikely to have occurred by random chance alone, leading to the conclusion that the difference in miRNA or mRNA expression is statistically significant. Statistical significance does not necessarily mean the differential expression of a gene is biologically significant, but instead serves to identify relationships that warrant further investigation.

Differentially expressed mRNAs and miRNAs from Aims 1 and 2 were assessed for potentially relevant biological significance as detailed below. The results reported in this chapter contain a focused list of the most significantly differentially expressed mRNAs and miRNA-mRNA targets. Comprehensive data from each aim can be found in Appendix 2 and Appendix 3.

Aim 1: RNA-seq Data Management

RNA-seq data was retrieved and transferred from the VCU Genomics core facility via the VCU High Performance Research Computing (HPRC) core facility utilizing Fenn, which is designed to support research using data that must comply with federal security and privacy requirements (CAT I data). It consists of 16 nodes with a total of 480 Intel cores, 4 TB of RAM, 900 TB of GPFS high performance parallel file system storage, and 54 Gb/second InfiniBand

networking. The Fenn system employs a security model that requires all access via VPN and exists on a separate virtual and physical network from other university and HPRC resources.

The pipeline for processing RNA-seq data involves several key steps, including quality control, alignment, quantification, and differential expression analysis as described below.

Quality Control (QC): The quality of the raw sequencing data was evaluated with FastQC (Babraham Bioinformatics, 2024). An appropriate read depth of around 50 million reads was achieved, with some samples in the 60-90 million read range that were more than adequate for subsequent differential expression analyses. Many samples had a high GC content and overrepresented sequences, which suggested some ribosomal RNA (rRNA) escaped the polyA tail selection during the RNA sequencing, or that there could be contamination in the samples. rRNA is the most abundant type of RNA that can overpower and complicate accurate detection of relatively smaller mRNAs and miRNAs, and therefore their removal allows for deeper sequencing of these less abundant RNAs. After removing the rRNA sequences using the software BBDuk (BBTools, 2024) that had escaped the polyA selection the percentage of overrepresented sequences and the GC content improved but was nevertheless still high [Appendix 1]. Overrepresented sequences were interrogated in the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) to determine their origin. They were confirmed to be human gene sequences (mostly hemoglobin genes) and did not seem to be caused by any contaminants. Therefore, it is possible that the issue was caused by some highly expressed human genes and they were included in the alignment step below.

Alignment: The alignment process maps the reads to the human genome/transcriptome, allowing researchers to determine the origin of each read. A custom sex chromosome informed reference genome was built with STAR (v 2.7.9a) (Dobin et al., 2013) using the methods outlined by Olney et al. (2020). First, the reference genome was built chromosome by chromosome using the ensembl GRCh38 human genome

(Homo_sapiens.GRCh38.108.chr_patch_hapl_scaff.gtf annotation file) build where the PAR regions are only loaded for chromosome X and are absent for chromosome Y, which was equivalent to the YPAR-masked reference, for genetic males (XY) as described in the aforementioned methodology. Then according to the protocol described by Olney et al. (2020) the entire Y chromosome was hard masked by converting each nucleotide to 'N' on the Y chromosome to build the Y-masked reference for genetic females (XX).

***Quantification*:** Once the reads are aligned, quantification of expression levels of genes was performed. This process involves counting the number of reads that map to each gene or transcript. Normalization and quantification were performed with FeatureCounts (v.2.0.6) (Liao et al., 2014).

***Differential Expression Analysis*:** Differential expression analysis was performed using edgeR (V4.0.16) and four comparisons 1) MS males-control males 2) MS females-control females 3) MS females-MS males and 4) control females-control males (Buxhoeveden, 2024). EdgeR is an R/Bioconductor package for analyzing RNA-seq data to identify genes that are differentially expressed between two or more conditions (Robinson et al., 2010). It starts by normalizing read counts to account for sequencing depth differences and estimates the dispersion of read counts to quantify variability in gene expression. Using a negative binomial model and empirical Bayes methods, edgeR tests for differential expression, accounting for both biological and technical variability. It returns a list of differentially expressed genes and provides their significance level (p-value), and fold changes.

Aim 2: miRNA Data Management

Raw miRNA counts were downloaded from the NanoString nCounter platform and uploaded into the nSolver Analysis Software (version 4.0) for further data processing. The NanoString miRNA CodeSet includes positive controls, "housekeeping" or reference genes used during normalization, and negative controls that quantify lane-specific variation,

differences in sample concentration, and background noise which are used to normalize the data.

Data normalization is a critical step in analysis and the parameters chosen can have a large influence on whether a miRNA's differential expression is falsely called as significant (false positive), or whether one fails to detect relevant biological changes (false negative). The miRNA data is normalized using the positive controls, negative controls, and housekeeping genes built into the NanoString assay. These controls help to ensure the accuracy, reliability, and reproducibility of gene expression data. The following parameters were utilized for the normalization of this study's miRNA data:

Positive Control Normalization*:* Nanostring includes six controls on the miRNA CodeSet - POS_A - POS_F. For this study POS_F was excluded from the analysis as the counts were close to background levels, and the data was normalized using the geometric mean of the remaining positive controls.

Rationale**:** Positive controls are synthetic RNA molecules that are added to the sample during processing. They contain a set of known RNA sequences at known concentrations. Positive controls help to assess the efficiency of the target capture, hybridization, and detection steps in the assay. They are used to monitor the overall performance of the assay and to ensure that the data obtained are reliable. According to the NanoString data analysis guidelines (2017) POS_F should be removed if it is close to background for optimal normalization, which was the case in this study.

Negative Control Normalization*:* Background threshold was set to mean of negative controls (excluding NEG_C) plus two standard deviations. NEG_C was excluded because Nanostring has reported that the counts for this control are too variable to be included in background threshold calculations.

***Rationale*:** Negative controls are also synthetic RNA molecules added to the sample during processing, but they do not have complementary sequences to any of the target genes in the assay. Negative controls help to assess background noise levels in the assay. They are used to identify and correct for any non-specific binding or background signal that may interfere with the detection of true target signals. Setting the negative threshold to the mean plus two standard deviations minimizes the number of false positives, but it is a statistically conservative approach so the presence of false negatives cannot be ruled out. To assess whether there was a high level of false negatives this stringent threshold was compared to a more liberal one and the chosen method, while more conservative, provided a list of differentially expressed miRNAs that had more significant p-values with more narrowly defined confidence intervals.

Housekeeping genes: Three reference or "housekeeping" genes, ACTB, GAPDH, RPL19, were selected for normalization because they had the lowest amount of variability (measured by %CV).

Rationale: The housekeeping genes included in the NanoString CodeSet are endogenous genes that are constitutively expressed in all cells and tissues at relatively constant levels. Housekeeping genes are important because they provide a baseline expression level that can be used to normalize the expression levels of target genes across different samples. Normalization helps to account for variations in sample input, RNA quality, and assay efficiency, ensuring that the expression levels of target genes are accurately measured and compared between samples. NanoString (2017) recommends using at least three housekeeping genes for normalization, ideally the genes with the lowest amount of variability.

***Differential Expression Analysis*:** Differential expression analysis was performed with nSolver (called "ratio data" in the program) using the same four comparisons as the mRNA data 1) MS males-control males 2) MS females-control females 3) MS females-MS males and 4) control

females-control males. Like edgeR, nSolver quantifies fold changes and provides a significance level (p-value).

Aim 2: miRNA Target Analysis: Ingenuity Pathway Analysis (IPA) Data Management

miRNA target analysis was performed with IPA using the differentially expressed mRNAs from Aim 1 and miRNAs from Aim 2. miRNAs are involved in post-transcriptional modification of gene expression [Figure 2] and miRNA target analysis involves looking for instances where an increased miRNA caused downregulation (suppression or silencing) of a target gene, or vice versa.

Qiagen's Ingenuity Pathway Analysis (IPA) uses a systematic approach to predict miRNA-mRNA interactions. After uploading mRNA (from Aim 1) and miRNA expression data (from Aim 2) IPA compares it against a vast knowledge database containing information on biological pathways, networks, and functional annotations. IPA uses machine learning to predict potential interactions between miRNAs and their mRNA targets. These algorithms consider several factors including seed sequence matching, which involves searching for complementary sequences to the seed region (positions 2-8) of the miRNA in the 3' untranslated region (UTR) of mRNA transcripts (Riolo et al., 2020). Next, target site accessibility considers the secondary structure of the mRNA 3' UTR to evaluate the accessibility of potential binding sites. IPA integrates experimentally validated data from databases such as TargetScan (Agarwal et al., 2015) to enhance the accuracy of target predictions. This comprehensive approach allows IPA to generate interaction networks and perform pathway and functional analyses, providing researchers with a deeper understanding of the biological implications of the predicted miRNA-mRNA interactions.

Once miRNA target interactions were identified in IPA further pathway analysis was performed. This involved looking for canonical pathways and functions relevant to MS. A canonical pathway is a well-defined and widely recognized biological pathway that represents a

series of molecular interactions or signaling events that lead to a specific cellular response or outcome. Canonical pathways are often characterized by their importance in normal physiological processes and their conservation across species. They are typically well-studied and characterized, making them valuable targets for research and therapeutic intervention.

miRNAs and resulting mRNA changes with a fold change of at least 1.3 (or log2FC of 0.33) and p-value of < 0.05 were considered significant, and TargetScan human (Agarwal et al., 2015) was the source of all miRNA-mRNA interactions that will be discussed in this paper. Other pathways from mouse models are included in the list in Appendix 3, but none were found to be biologically relevant after pathway analysis. The paired list of significant miRNAs that inversely correlated with significant expression of a target mRNA were examined for possible functions and pathways related to MS pathogenesis. Focused lists of the most relevant IPA miRNA target filter results are included in Chapter IV, and a comprehensive list is provided in Appendix 3.

Exploratory Aim 2b: Clinical Correlate Data Management

Clinical data from each sample reported by the collection site was compiled into an excel spreadsheet. MRI imaging is the most widely used biomarker of MS disease activity, and the current gold standard for diagnosis. Each of the MS samples had MRI outcomes that were reported by the collection site's primary investigator [Table 12]. The reported data included how many T2 lesions each patient had and their location and the number of T1 lesions, which serve as measures of MS both acute and chronic disease activity as described below:

> ***T2 lesions***: T2 MRI sequences show areas of scarring which are the hallmark of MS (multiple sclerosis literally translates "multiple scars") and are caused by demyelination of neurons. MS lesions can grow or shrink but typically do not resolve, so the T2 lesion burden serves as a blueprint of all the CNS damage accumulated by each patient. Lesions can occur anywhere in the CNS, but in general infratentorial and spinal cord

lesions tend to cause greater disability than supratentorial lesions. Table 12 shows the overall T2 lesion burden of each group, as well as their location in the CNS. The overall T2 lesion burden was considered as a surrogate marker for disease severity, and the lesion location was also explored to see if there was an association between any differentially expressed mRNA/miRNAs and lesions located in the infratentorial/spinal region, which tend to be associated with more severe disease.

T1 lesions: areas of active demyelination enhance with gadolinium contrast, which can cross the leaky blood-brain-barrier (BBB) and follow inflammatory cells to the area under attack. Lesions typically enhance for about 4 weeks while active inflammation is occurring and stops enhancing once inflammation has subsided and the BBB becomes less permeable. After this brief window of enhancement T1 lesions show up on T2 MRI sequences as “old” lesions. Thus, the presence of T1 lesions serves a surrogate marker of acute disease activity.

Spearman correlation coefficients was used to estimate the association between miRNA expression and MRI disease activity (number of T1 lesions, number of T2 lesions, location of lesions- supratentorial or infratentorial/spinal cord), CSF results (number of oligoclonal bands), and disability (EDSS score), and a 2-tailed t test was used to compare clinical features in MS males versus MS females.

Summary

MS is an incurable neurodegenerative disease, which is typically diagnosed in prime of life, and often causes significant physical and cognitive disability from a young age (Lublin et al., 2014). MS is approximately three times more common in females, but males tend to have a more severe form of the disease. These important sex-based disease disparities likely represent undiscovered disease phenotypes and represent a critical knowledge gap that is addressed by this application. Environmental exposures and sex hormones are capable of causing cellular

and molecular changes, which may drive the sex differences in MS, although the impact of these interactions is unknown (Alcina et al., 2012; Dutta & Trapp, 2012; Harbo et al., 2013; Hollenbach & Oksenberg, 2015; Kular et al., 2018; Lublin et al., 2014; Parnell & Booth, 2017; Sawcer et al., 2011; Schmidt et al., 2007). Therefore, this study will map the transcriptome of a homogeneous sample consisting of treatment naïve males and females with MS and compare it to miRNA levels to understand the impact of transcriptomic changes on gene expression. This will help us understand the sex-based disparities seen in relapsing MS. These findings have the potential to uncover novel biomarkers that may guide individualized patient management and improve screening and risk assessment for those living with MS.

Chapter IV: Findings

Aim 1: Differential Expression of mRNA Results

mRNAs were considered differentially expressed if they had a fold change of at least 1.3 (or log2FC of 0.33) and p-value of < 0.05 [Table 4]. These results were combined with the miRNA data for analysis in Ingenuity Pathway Analysis (IPA) as detailed below in Aim 2. A comprehensive list of all the differentially expressed mRNAs is in Appendix 2.

Table 4
Differentially Expressed mRNAs from Aim 1

Comparison	Number of Differentially Expressed mRNAs
MS Females vs Control Females	146
MS Males vs Control Males	157
MS Females vs MS Males	264
Control Females vs Control Males	196

Aim 2: Differential Expression of miRNA Results

The human miRNA NanoString CodeSet quantifies 800+ miRNAs. The following tables show the miRNAs that were differentially expressed with a p-value of <0.05 and fold change of at least 1.3 (or log2FC of 0.33). Positive fold changes indicate upregulation of miRNA

expression while a negative value indicates the miRNA was downregulated compared to the second group.

Table 5

Differentially Expressed miRNAs for MS Females vs MS Males

Probe Name	Mean Count MS Females	Mean Count MS Males	Log2FC	P-Value
hsa-miR-941	62.9	139.8	-2.2	0.01
hsa-miR-485-3p	139.6	80.7	1.7	<0.01
hsa-miR-223-3p	143265.1	87583.8	1.6	0.01
hsa-miR-5196-3p + hsa-miR-6732-3p	41.4	67.7	-1.6	0.01
hsa-miR-196b-5p	120.7	82.8	1.5	0.01
hsa-miR-199a-5p	227.5	158.8	1.4	0.02
hsa-miR-548n	41.4	57.0	-1.4	0.05
hsa-miR-296-5p	893.5	648.9	1.4	0.05
hsa-miR-1180-3p	472.9	348.4	1.4	0.05
hsa-miR-664b-3p	106.9	80.8	1.3	0.01
hsa-miR-1286	67.1	50.7	1.3	0.02
hsa-miR-4536-5p	41.5	54.1	-1.3	0.05

Table 6

Differentially Expressed miRNAs for MS Females vs Female Controls

Probe Name	Mean Count MS Female	Mean Count Control Female	Log2FC	P-Value
hsa-miR-941	62.9	161.6	-2.6	0.03
hsa-miR-144-3p	278.2	124.1	2.2	0.05
hsa-miR-126-3p	843.6	410.5	2.1	0.05
hsa-miR-363-3p	1410.3	736.2	1.9	0.05
hsa-miR-26b-5p	667.7	354.1	1.9	0.04
hsa-miR-454-3p	105.1	64.6	1.6	0.03
hsa-miR-183-5p	252.6	171.7	1.5	0.05
hsa-miR-28-5p	167.6	116.6	1.4	0.04
hsa-miR-1286	67.1	48.1	1.4	0.02
hsa-miR-4741	58.5	43.0	1.4	0.01

Table 7

Differentially Expressed miRNAs for MS Males vs Control Males

Probe Name	Mean Count MS Males	Mean Count Control Males	Log2FC	p-value
hsa-miR-146a-5p	126.3	76.8	1.6	0.00
hsa-miR-652-3p	998.3	626.9	1.6	0.02
hsa-miR-183-5p	192.3	122.5	1.6	0.01
hsa-miR-342-3p	1966.0	1295.8	1.5	0.01
hsa-miR-196b-5p	82.8	55.7	1.5	0.01
hsa-miR-29a-3p	108.0	79.2	1.4	0.05
hsa-miR-324-5p	70.1	52.3	1.3	0.04
hsa-miR-30d-5p	3406.3	2571.9	1.3	0.04
hsa-miR-150-5p	38184.5	28875.8	1.3	0.05
hsa-miR-99b-5p	63.3	48.5	1.3	0.02

Table 8

Differentially Expressed miRNAs for Control Females vs Control males

Probe Name	Mean Count Female Controls	Mean Count Male Controls	Log2FC	p-value
hsa-miR-342-3p	2159.8	1295.8	1.7	0.03
hsa-miR-196b-5p	90.5	55.7	1.6	0.02
hsa-miR-223-3p	105022.4	64747.2	1.6	0.05
hsa-miR-145-5p	163.0	101.8	1.6	0.02
hsa-miR-182-5p	298.2	195.0	1.5	0.04
hsa-miR-146a-5p	113.2	76.8	1.5	0.03
hsa-miR-150-5p	41955.3	28875.8	1.5	0.05
hsa-miR-183-5p	171.7	122.5	1.4	0.04
hsa-miR-942-5p	944.9	715.2	1.3	0.05

Aim 2: Ingenuity Pathway Analysis (IPA) miRNA Target Filter Results

miRNAs and resulting mRNA changes with a fold change of at least 1.3 and p-value of < 0.05 were considered significant. The paired list of significant miRNAs that inversely correlated with significant expression of a target mRNA were examined for possible functions and pathways related to MS pathogenesis. Focused lists of the most relevant IPA results are included below. A comprehensive list of all the differentially expressed miRNAs is located in Appendix 3.

IPA results

Positive fold changes indicate the miRNA expression was higher in the first comparison group, while negative indicates the miRNA expression was higher in the second group.

Table 9
IPA miRNA Target Analysis Results for MS Females vs Control Females

miRNA	miRNA log2FC	mRNA Target (log2FC, p-value)
miR-941	-2.8	TLR3 (0.9, p 0.05)
miR-26b-5p (listed as miR-26a-5p in IPA)	1.9	UQCRB (-1.4, p<0.01)
miR-183-5p	1.5	CARD16 (-0.7, p 0.05)
miR-1286	1.4	CD34 (-0.8, p 0.04)

Table 10
IPA miRNA Target Analysis Results for MS Males vs Control Males

miRNA	miRNA log2FC	mRNA Target (log2FC, p-value)
miR-29a-3p	1.4	DLGAP2 (-2.5, p <0.01)
		COL5A1 (-0.9, p 0.02)
miR- 30d-5p	1.3	DLGAP2 (-2.5, p <0.01)
		COL5A1 (-0.9, p 0.02)
		MMP21 (-0.7, p 0.04)
miR-342-3p	1.5	GSTM4 (-0.7, p 0.04)

Table 11

IPA miRNA Target Analysis Results for MS Females vs MS Males

miRNA	miRNA log2FC	mRNA Target (log2FC, p-value)
miR-1286	1.3	MMP8 (-2.0, p <0.01)
		CD34 (-0.7, p 0.02)
		DPYSL4 (-1.6, p <0.01)
		CNTNAP1 (-0.6 p 0.03)
miR-296-5p	1.4	MPO (-1.2, p <0.01)
		CD34 (-0.7, p 0.02)
miR-485-3p	1.7	TCF4 (-0.5, p 0.03)
		MELK (-1.0, 0.01)
miR-4536-5p	-1.3	JAK3 (0.3, p 0.02)
		MAPK3 (0.3, p 0.03)
miR-941	-2.2	FFAR2 (0.6, <0.01)
		NAT8L (0.7, 0.03)
miR- 223-3p	1.7	CDNF (-0.6, p 0.04)
miR-6511a-3p	1.3	MMP8 (-2.0, p <0.01
miR-664b-3p	1.3	MMP8 (-2.0, p <0.01)

Exploratory Aim 2b Results

Spearman correlation coefficients were used to test for associations between differentially expressed miRNAs/mRNAs and MRI disease activity (number of T2 lesions, number of supratentorial lesions, number of infratentorial/spinal cord lesions, and number of T1 lesions), but no statistically significant relationships were found. 2-tailed t tests were then used to compare the same MRI outcome measures in MS males versus MS females. Overall, although the male MS group appeared to have a higher T2 lesion burden, there were no statistically significant relationships between sex and MRI outcome severity.

Many of the repository samples were missing EDSS scores (a clinical measure disability severity) [Table 13], making it difficult to assess the disease severity in each group. Spinal taps for CSF analysis had been performed and reported for 70.6% of the males with MS, and 52.9% of the females with MS. While the males had more oligoclonal bands in their CSF (also a measure of disease activity and severity), the amount of missing data made it difficult to draw

any formal conclusions about whether males or females had more severe disease.

Table 12
MRI Data for Study Samples

	Male n=17	Female n=17
Overall T2 Lesion Burden		
Mild (1-6 lesions)	2 (11.76%)	8 (47.08%)
Moderate (6-10)	4 (23.53%)	1 (5.88%)
Severe (>10)	11 (64.71%)	8 (47.08%)
Number of T2 Supratentorial Lesions		
None	0	4 (23.53%)
Mild (1-6 lesions)	7 (41.18%)	6 (35.30%)
Moderate (6-10)	3 (17.65%)	1 (5.88%)
Severe (>10)	7 (41.18%)	6 (35.30%)
Number of T2 Infratentorial/Spinal Lesions		
None	5 (29.41%)	7 (41.18%)
Mild (1-6 lesions)	6 (35.29%)	9 (52.94%)
Moderate (6-10)	1 (5.88%)	0
Severe (>10)	5 (29.41%)	2 (11.76%)
Overall T1 Enhancing Lesion Burden		
None	7 (41.18%)	11 (64.71%)
Mild (1-6 lesions)	10 (58.8%)	4 (23.53%)
Moderate (6-10)	0	0
Severe (>10)	0	2 (11.76%)

Table 13
EDSS and CSF Data for Study Samples

	Male	Female
EDSS		
Mean	1.92	1.83
Range	0-5.5	1.5-2.0
Missing Data	11 (64.7%)	14 (82%)
Number of CSF Oligoclonal Bands		
Mean	6	3.67
Range	0-20	0-8
Missing Data	5 (29.4%)	8 (47.1%)

Summary

Differential expression analysis was successfully performed for the mRNA data and miRNA data from Aims 1 and 2. The RNA differential expression data was robust and did not need to be supplemented by any publicly available databases as discussed in the potential pitfalls and alternatives. Differentially expressed mRNAs and mRNAs were uploaded into IPA to explore the functional consequences of miRNAs on mRNAs within each comparison group (MS females versus control females, MS males versus control males, MS females versus MS males, and control females versus control males). Detailed pathway analysis was then performed to determine which of the miRNA-target mRNA were biologically relevant to this study. The sex-based differences found and their implications in MS are discussed in Chapter V.

Chapter V: Discussion

This study successfully identified differentially expressed mRNAs and miRNAs between MS patients and healthy controls, some of which have previously been reported in the literature, and others that are novel to MS. Additionally, the sex differences in gene expression that were found in this study among those with relapsing MS have not been described, and therefore have added to knowledge of the effect sex has on MS. Importantly, the study design helped to examine the functional impact the differentially expressed miRNAs have on the mRNAs in each patient sample, and addressed the critical gap in knowledge of sex-based drivers in MS pathophysiology.

Both neuroinflammation and neurodegeneration occur in all MS patients, resulting in demyelination and loss of CNS tissue. The conceptual framework for this study outlined the hypothesis that females tend to have more neuroinflammation than males, and that males have more neurodegeneration than females, contributing to the sex-based disparities in susceptibility and severity [Figure 1]. In general, MS medications primarily target neuroinflammation and have a minimal impact on slowing neurodegeneration which is a significant challenge in the field.

Therefore, identification of biomarkers for each pathway could identify potential therapeutic targets and tailor treatment by addressing this gap. To identify dysregulation in neuroinflammatory and degenerative pathways females and males with MS were first compared to healthy controls, then to each other. This chapter will first discuss significant findings related to neuroinflammation, followed by neurodegeneration.

Discussion Part 1: Neuroinflammation

This section will discuss several pathways by which miRNAs affect the stability of mRNAs involved in neuroinflammation. Through incomplete binding with mRNAs, miRNAs control their expression and translation of proteins post-transcriptionally [Figure 2]. These miRNA-mRNA interactions serve as both an important mechanism of maintaining homeostasis in normal conditions, and as a potential pathway for dysregulation and disease. MS females had significant dysregulation of immune related pathways, which will be discussed first, followed by a discussion of some neuroinflammatory pathways observed in the MS males.

Neuroinflammation in MS Females

The results of differential mRNA and miRNA expression comparisons between MS females and control females and MS females versus MS males shed light on several important mechanisms for the immune dysregulation seen in MS, including activation of TLR3 and the Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells (Nf-κB) signaling pathway. These mechanisms have important implications for MS pathogenesis and advance knowledge of sex-based differences in MS-related immune function.

Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells (Nf-κB) Signaling Pathways in MS Females. NF-κB is a group of transcription factors that, when activated by external stimuli, regulates the expression of genes involved in inflammation, immunity, cell survival, and other cellular processes. This pathway activation also plays a key role in driving the immune response and neuroinflammation observed in MS. The NF-κB pathway is often

activated in immune cells, such as macrophages and T cells, in response to various stimuli, including pro-inflammatory cytokines and oxidative stress. miRNAs regulate the expression of genes that, in turn, can activate or suppress the NF-κB pathway. NF-κB can also regulate miRNA, and together are powerful regulators of the immune response in MS. Once activated, NF-κB translocates to the nucleus and promotes the expression of genes encoding inflammatory mediators, such as cytokines (e.g., TNF-α, IL-1β), chemokines, and adhesion molecules. Peripheral immune cells activated by the NF-κB cascade then cross the blood-brain-barrier, where they recruit and activate immune cells within the CNS, such as microglia and astrocytes. In addition to its immune cell effects, NF-κB activation can impact oligodendrocyte survival, blood-brain barrier integrity, and under certain conditions, promote neuronal survival, highlighting its diverse effects in MS pathophysiology (Y. Zhou et al., 2020). Several medications used to treat MS either directly or indirectly regulate the NF-κB pathway, highlighting its importance as a therapeutic target (Leibowitz & Yan, 2016).

The mechanisms behind the role of miRNA and NF-κB in MS are complex. No sex-differences in the activation of NF-κB have been reported in MS, although sex hormones are thought to play a role in complex immune signaling, resulting in females being more prone to inflammatory autoimmune diseases. Interestingly, MS females in this study had multiple novel differentially expressed mRNA-miRNA pathways related to NF-κB in both the MS male and control female comparisons. The findings from the MS female to MS male comparison will be discussed first, followed by discussion of MS females versus control females.

Table 14

NF-κB Pathways in MS Females vs MS Males

miRNA Name	miRNA log2FC	mRNA Target	mRNA log2FC	P-Value
miR-4536-5p	-1.3	JAK3	0.3	0.02
miR-4536-5p	-1.3	MAPK3	0.3	0.03
miR-485-3p	1.7	TCF4	-0.5	0.03

Note: Differentially expressed miRNA and its impact on mRNA target expression. Positive fold changes indicate the RNA expression was higher in MS females, while negative indicates the mRNA expression was higher in MS males.

Downregulation of miR-4536-5p and upregulation of Janus kinase 3 (JAK3) in MS females vs MS males: IPA pathway analysis implicated miR-4536-5p in the regulation key immune-related mRNAs, including JAK3 which encodes the JAK3 protein. This protein functions as a kinase, and catalyzes phosphorylation of downstream signaling proteins, including the Signal Transducer and Activator of Transcription (STAT), post-translationally (Hu et al., 2021). In MS the JAK3-STAT signaling pathway induces pro-inflammatory cytokines and activates other pathways, such as NF-κB and MAPK, that cause damage to neurons and oligodendrocytes (Benveniste et al., 2014). While the involvement of the JAK3-STAT pathway is already known to contribute to MS pathophysiology, downregulation of miR-4536-5p is a novel activation pathway and could be a novel treatment target. The differential expression of this pathway between MS females MS males also provides important insights into sexual dimorphism of the immune response.

Downregulation of miR-4536-5p and upregulation of Mitogen-Activated Protein Kinase 3 (MAPK3) in MS females vs MS males: Downregulation of miR-4536-5p also upregulated MAPK3 expression in MS females compared to MS males. This pathway is a well-known regulator of inflammation in MS, but differential expression in MS females has not previously been reported and is an interesting mechanism that may contribute to sex-based disparities. MAPK3 is a member of the MAPK family of kinases, which are widely expressed and activated in response to various stimuli, including toll-like receptors and cytokines (Krementsov et al., 2013). MAPK3 is involved in cell proliferation, differentiation, and survival, and is required for IFN-γ production by CD4 and CD8 cells (Krementsov et al., 2013). Like JAK, MAPK3 can influence production of

cytokines post-translationally, leading to the activation of pathways that promote inflammation and cell damage in the CNS, including NF-κB (Krementsov et al., 2013). Estrogens are known to upregulate the MAPK pathway, although research on the role this plays in MS is sparse (Villa et al., 2016).

Interestingly, loss of Transcription Factor 4 (TCF4) heightens MAPK activity in EAE models (Manoharan et al., 2021), and the MS females had downregulation of TCF4 via miR-485-3p. This interaction between TCF4 and MAPK is a proposed therapeutic target for controlling neuroinflammation (Manoharan et al., 2021), and subsequently miR-4536-5p identified by this study could be of significance.

Table 15
NF-κB Pathways in MS Females vs Control Females

miRNA Name	miRNA log2FC	mRNA Target	mRNA log2FC	P-Value
miR-941	-2.6	TLR3	0.9	0.05
miR-183-5p	1.5	CARD16	-0.7	0.05
miR-1286	1.4			

Note: Differentially expressed miRNA and its impact on mRNA target expression. Positive fold changes indicate the RNA expression was higher in MS females, while negative indicates the RNA expression was higher in control females.

Downregulation of miR-941 and upregulation Toll-like receptor 3 (TLR3) in MS females vs control females: TLR3 is widely expressed on neurocytes and immune cells such as myeloid dendritic cells and macrophages (Chen et al., 2021). Upon activation, TLR3 utilizes the adaptor protein TRIF to initiate proinflammatory signaling pathways, leading to the production of IFN-β and activation of transcription factors, including NF-κB (O'Neill & Bowie, 2007). This activation, in turn, plays a role in the proliferation and recruitment of inflammatory immune cells, including TH1 cells, cytotoxic CD8+ T cells, and natural killer (NK) cells, as well as the production of proinflammatory cytokines which are all implicated in MS pathophysiology (Miranda-Hernandez & Baxter, 2013; O'Neill & Bowie, 2007). TLR3 activation in MS contributes

to immune dysregulation characteristic of the disease, highlighting its potential as a therapeutic target for modulating the immune response and reducing neuroinflammation in MS patients (Miranda-Hernandez & Baxter, 2013). No sex-differences in TLR3 expression have been previously reported in MS patients, and the regulation of TLR3 by the miR-941 is a novel pathway that may be driving the overactive immune response in females.

Upregulation of miR-183 and downregulation of Caspase Recruitment Domain Family Member 16 (CARD16) in MS females vs control females: Females exhibited upregulation of miR-183, an erythrocyte derived miRNA. Groen et al. (2020) found that increased levels of miR-183 in plasma EVs differentiated MS patients from healthy controls and positively correlated with physical disability. CARD16 is involved in IL-1 β proinflammatory cytokine signaling and its downregulation may play a regulatory role on the NF-κB pathway, but the mechanisms behind this miRNA-mRNA interaction are poorly understood and warrant further investigation. Although miR-183 has been associated with MS in other studies, no previous sex differences have been reported and the novel influence on CARD16 found in this study warrant further investigation to better understand its role in sex-based disparities and MS outcomes. This interaction is discussed in more detail in the next section of this chapter.

Upregulation of miR-1286 in MS females vs control females: In addition to being regulated by miRNAs, the NF-κB pathway can also regulate the expression of miRNAs, adding another layer of complexity to its role in immune regulation. Zhou et al. (2014) showed that the NF-κB pathway can target and regulate the expression of miR-1286 in TNF-α stimulated cell lines. The NanoString data for this study's MS females also showed upregulation of miR-1286, which can further perpetuate the IL-6, IL-1β, and

TNF-α cascade and contribute to immune dysregulation in MS (Zhou et al., 2014; Zhou et al., 2020).

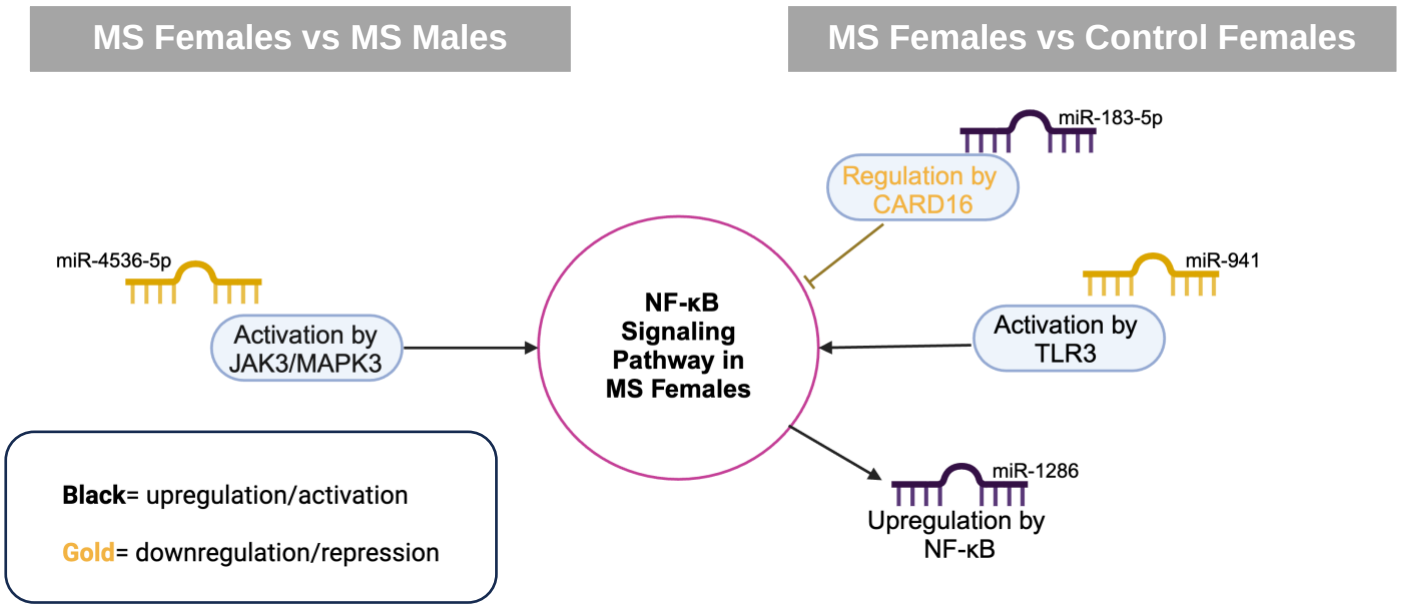

Figure 5: Statistically significant IPA findings implicated in NF-κB activation and inhibition. Pathways of MS females versus MS males are on the lefthand side, and MS females versus control females are on the right. *Image created with biorender.com.*

Epstein-Barr Virus, TLR3, and inflammatory pathways in MS Females

The Epstein-Barr Virus (EBV) is a double-stranded DNA herpesvirus known to be a key factor in the etiology of numerous cancers and autoimmune diseases (Iizasa et al., 2020). EBV infects naïve B cells, induces a viral immune response, and remains latent in memory B cells after infection (Afrasiabi et al., 2021; Skalsky, 2022). EBV is the first human virus capable of encoding miRNAs that play a role in helping the virus evade immune system detection (Afrasiabi et al., 2021; Wang et al., 2018). A study utilizing data from over 10 million military recruits (of which 955 developed MS after enlisting) revealed that EBV is a necessary component of MS development (Bjornevik et al., 2022). While the cause of MS is still likely to be multifactorial, EBV is the first common link to be found across the entire MS population, making

it the leading cause of the disease (Bjornevik et al., 2023). Bjornevik et al. (2022) also found that serum neurofilament light chain (sNfL), a marker of neuroaxonal injury associated with MS activity, was markedly increased only after EBV infection. EBV could lead to an increased risk for MS by targeting MS-associated risk genes, activating TLR and NF-κB pathways, and/or causing dysregulation of host miRNAs that lead to altering immune response and B cell function (Afrasiabi et al., 2021; Oussaief et al., 2015). Afrasiabi et al. (2021) found EBV infection dysregulates miRNAs and interacts with mRNAs *in vitro* to alter genes associated with MS pathogenesis.

The novel miRNA-mRNA interactions found in this study's MS females [Figure 5] offer interesting insights to the potential role EBV has in MS through activation of TLR3 and proinflammatory pathways including JAK, MAPK, and NF-κB (Iwakiri et al., 2009; Krementsov et al., 2013; Luo et al., 2021; Shehab et al., 2019). TLR3 is activated by foreign invaders, particularly double-stranded (i.e. viral) RNA, although direct and indirect stimulation of TLR3 by EBV (a double-stranded DNA virus) has been reported *in vitro* (Z. Li et al., 2015) and *in vivo* (mouse models) (Shehab et al., 2019). TLR3 activation by noncoding RNA released from EBV-infected cells has been observed *in vitro*, suggesting another mechanism by which it can activate proinflammatory pathways in MS (Iwakiri et al., 2009; Owens & Bennett, 2012).

The MS female samples in this study also had increased expression of miR-183, which plays a role in innate immunity and has been shown to be upregulated in latent EBV (Oussaief et al., 2015). This finding was of particular interest, because in the female samples miR-183 downregulated the CARD16 mRNA, a gene that is involved in IL-1 β proinflammatory cytokine signaling. This interaction between miR-183 and CARD16 may point to a mechanism by which EBV tries to evade the immune system. There is also a relationship between miR-183 and cancers associated with EBV, including and Burkitt's lymphoma and nasopharyngeal cancer (Oussaief et al., 2015), wherein acute downregulation of miR-183 is associated with the onset of

EBV associated cancers *in vivo* and correlate with worse outcomes, further illustrating the importance of miR-183 in EBV associated diseases, and the ability of EBV to induce both acute and chronic changes in these important pathways (Cheung et al., 2016; Oussaief et al., 2015).

Females in general have a more robust B cell response, demonstrated by higher response to vaccination and infection. Healthy human females are known to have higher titers against EBV and are more likely to be seropositive than males (Alvarez-Sanchez & Dunn, 2023), and more research on sex differences into how EBV infected B cells could activate the pathways described above could provide crucial insights into MS etiopathogenesis.

Immune Dysregulation in Males

As anticipated, distinct neuroinflammatory pathways were found in the MS male samples. Interestingly, many of the miRNAs in these pathways have previously been shown to correlate with more severe disease and/or cognitive decline but, to our knowledge, no sex differences have been reported. Therefore, these results make a significant contribution by illuminating potential pathways by which these miRNAs may cause immune dysfunction and contribute to the increased severity of MS in males.

Downregulation of miR-485-3p and Upregulation of Maternal Embryonic Leucine Zipper Kinase (MELK) in MS Males vs MS Females: miR-485-3p was differentially expressed in MS males, causing increased expression of the MELK gene, which is associated with apoptosis and cell cycle regulation. In a study of MS patients (which was not powered to detect sex differences) downregulation of miR-485-3p was associated with impaired apoptosis of autoreactive CD4+ T cells (Alizadeh-Fanalou et al., 2020). In their MS population downregulation of miR-485-3p upregulated expression of the mRNA survivin and correlated with higher disease severity (measured by the MS Severity Score) (Alizadeh-Fanalou et al., 2020).

Conversely, previous research in Alzheimer's has shown upregulation of miR-485 in activated microglia can perpetuate neuroinflammation via regulation of FBXO45, and elevated

levels of this miRNA have been detected in the serum of Parkinson's disease patients (He et al., 2023). miR-485 is known to influence neuroinflammation by targeting the expression of various mRNAs, and this novel MELK target pathway may contribute to sex differences in neuroinflammation via impaired T cell apoptosis and/or cytokine signaling. Because of its diverse effects when downregulated and upregulated, as illustrated above in the MAPK3/TCF4 pathway in MS females, miR-485-3p has important implications for both males and females.

Table 16

miR-485 and MELK mRNA Expression in MS Females vs MS Males

miRNA Name	miRNA log2FC	mRNA Target	mRNA log2FC	P-Value
miR-485-3p	1.7	MELK	-1.0	0.01

Note: Differentially expressed miRNA and its impact on mRNA target expression. Positive fold changes indicate the RNA expression was higher in MS females, while negative indicates the RNA expression was higher in MS males.

***Dysregulation of miRNAs Associated with Neuroinflammation in MS Males vs Control Males*.** miRNAs that have previously been associated with MS were found in this study and are especially relevant given the observed clinical patterns in male MS severity. These miRNAs were found to be significantly differentially expressed between MS males and control males via NanoString analysis but were not associated with biologically relevant mRNA regulation via IPA. First, miR-150 was found to be upregulated in MS males versus control males (FC 1.3, p 0.05). miR-150 is a known biomarker of MS that is involved in the production of B lymphocytes and immune signaling (Bergman et al., 2016). A study by Scaroni et al. (2022) found upregulation of miR-150 in blood myeloid extracellular vesicles to be a possible risk factor for cognitive dysfunction in MS patients. miR-146a-5p was also upregulated in MS males versus control males (FC 1.6, p <0.01), and is known to play a role in the activation of the NF-κB signaling pathway leading to altered T-cell responses, interleukin dysregulation, and apoptosis (Dominguez-Mozo et al., 2022; He et al., 2023). In a study of MS patients by Dominguez-Mozo

et al. (2022), increased expression of miR-146a-5p correlated with worse brain atrophy, higher EDSS, and lower Symbol Digit Modalities Test (SDMT) scores (a measure of cognitive function). Interestingly, miR-146a-5p is expressed on microglia in the CNS, and its overexpression was linked to phagocytosis and regulatory anti-inflammatory effects via NF-κB in brain tissue from Alzheimer's disease patients (He et al., 2023). Of note, none of the previous studies were powered to detect sex differences. As this study was designed to detect sex differences in MS the findings described herein suggest an important role for miRNAs in neuroinflammation in males and are especially relevant given their known impact on disease severity including brain atrophy, EDSS, and cognitive dysfunction which are all more prevalent in males.

Discussion Part 2: Neurodegeneration

This section will discuss several pathways by which miRNAs affect the stability of mRNAs involved in neurodegeneration. Demyelination refers to the damage or loss of the myelin sheath, which is the protective covering that surrounds nerve fibers in the CNS. Myelin is crucial for the proper functioning of nerve fibers, as it allows for efficient transmission of electrical impulses along the nerve. Oligodendrocytes are a type of glial cell in the CNS that produce myelin and provide support and nourishment to neurons, helping to maintain the health and function of the nervous system. One oligodendrocyte supports up to 50 neurons, and MS causes damage to these supportive structures as well as individual neurons. Neurodegeneration in MS leads to the permanent loss of nerve cells and their connections, contributing to the accumulation of disability over time.

Some of the following gene expression pathways occur primarily within the CNS, whereas their expression in peripheral blood is typically much lower. Extracellular vesicles (EVs) are membrane bound structures secreted by almost every cell type, including oligodendrocytes and neurons in the CNS, and play an important role in cell-to-cell communication between the

periphery to the CNS. EVs from the periphery have been shown to carry pro-inflammatory signals into the CNS (Saint-Pol et al., 2020), therefore it is possible that some of these more brain-specific mRNAs are epigenetically regulated in the blood. When the blood-brain barrier (BBB) is disrupted EVs are also more readily able to cross to and from the CNS (Cuomo-Haymour et al., 2022; Saint-Pol et al., 2020). EVs are rich in miRNAs and offer insights into the epigenetic changes seen within the CNS, and prior studies have isolated CNS derived EVs in plasma of patients with Alzheimer's and other neurogenerative diseases by selecting for those with a surface marker, such as L1CAM (Saint-Pol et al., 2020). Because MS impairs BBB integrity, it is possible that CNS-originating EVs may have been captured during RNA extraction from the samples in this study. However, there is no way to determine the origin (peripheral RNA versus CNS RNA) of the RNAs measured in this study.

This section will discuss several mechanisms that drive neurodegeneration in males including the NrF2 oxidative stress pathway, extracellular matrix dysfunction, and neuronal signaling and function. Finally, pathways associated with neuronal integrity and neuroprotection in MS females will be discussed.

Discussion of Neurodegeneration in MS Males

Nuclear Factor Erythroid 2-Related Factor 2 (Nrf2) Mediated Oxidative Stress Pathway in Males with MS. The Nrf2 mediated oxidative stress pathway plays a critical role in protecting cells from oxidative damage and is known to contribute to MS pathogenesis. Nrf2 is a transcription factor that regulates the expression of antioxidant and cytoprotective genes. Under normal conditions, Nrf2 is bound to its inhibitor protein, Kelch-like ECH-associated protein 1 (KEAP1), in the cytoplasm. In response to oxidative stress, Nrf2 is released from KEAP1 and translocates into the nucleus, where it activates the expression of genes encoding antioxidant enzymes and other protective proteins (Maldonado et al., 2022).

Oxidative stress caused by reactive oxygen species (ROS) and reactive nitrogen species (RNS) is believed to contribute to the damage by immune cells and activated glial cells, leading to demyelination and the destruction of oligodendrocytes (Maldonado et al., 2022). Activation of the Nrf2 pathway has been shown to reduce oxidative stress and protect against demyelination, and is one mechanism of action of dimethyl fumarate, a disease modifying MS treatment (Hammer et al., 2018). Although this is a known pathway, it is interesting that differential expression was only seen in the MS males, a sex difference not previously reported.

Upregulation of miR-342-3p and downregulation of Glutathione S-Transferase Mu 4 (GSTM4) in MS males vs control males: IPA target analysis implicated miR-342-3p in the canonical pathway for the Nrf2 signaling, which to our knowledge is a novel finding to MS. Mouse models of Alzheimer's disease have shown that upregulation of miR-342 in the hippocampus is associated with more oxidative stress and damage (Konovalova et al., 2019; Sun et al., 2014). Dysfunction of the Nrf2 pathway has also been shown to reduce expression of GST enzymes in mouse models of oxidative stress (Chanas et al., 2002), and the male samples in this study showed a similar downregulation of GSTM4, a member of the glutathione S-transferase (GST) family of enzymes, which play a role in detoxification and metabolism. Variations in the GSTM4 gene have been associated with susceptibility to neurodegenerative disorders, although the exact mechanisms are not fully understood (Chanas et al., 2002; Mazzetti et al., 2015). It is not clear if miR-342-3p is driving the Nrf2 dysfunction, or if Nrf2 dysfunction is altering this miRNA/mRNA relationship, but this finding warrants further investigation given the important role Nrf2 plays in MS pathophysiology and its potential as a therapeutic target.

Table 17
miR-342-3p and GSTM4 mRNA Expression in MS Males vs Control Males

miRNA Name	miRNA log2FC	mRNA Target	mRNA log2FC	P-Value
miR-342-3p	1.5	GSTM4	-0.7	0.04

Note: Differentially expressed miRNA and its impact on mRNA target expression. Positive fold changes indicate the RNA expression was higher MS Males, while negative indicates the RNA expression was higher in the control males.

Extracellular Matrix (ECM) Integrity in Males with MS. Extracellular matrices (ECM) are complex networks of proteins and other molecules that provide structural support to cells and tissues in the body. The ECM makes up 20% of the CNS and plays a crucial role in cell signaling, migration, and differentiation (Mohan et al., 2010). ECM integrity is important in MS because it is involved in the formation and maintenance of the blood-brain barrier (BBB), which is a protective barrier that separates the blood from the brain and spinal cord. In MS, the BBB becomes more permeable, allowing immune cells to enter the CNS and attack myelin (the protective covering of nerve fibers) (Latronico & Liuzzi, 2017; Samtani et al., 2023). The ECM also plays a role in the repair and remodeling of tissues damaged by the overactive immune responses observed in MS (Latronico & Liuzzi, 2017).

While dysfunction of ECM integrity has long been associated with neurodegenerative diseases, several novel pathways that may play a significant role in MS pathology are highlighted below, along with evidence that they may simultaneously contribute to sex differences in MS, that to our knowledge have not been reported in prior literature.

Gene Glycoprotein VI (GP6) Canonical Pathway**:** GP6 is a platelet-specific receptor for collagen, a key component of the ECM in tissues including the CNS. The GP6/collagen pathway may play a role in the recruitment and activation of platelets in the CNS, which interact with immune cells and contribute to inflammatory processes that cause CNS damage (Samtani et al., 2023). In mouse models of MS platelet infiltration from peripheral blood into the CNS occurs

days before T cell invasion, and depletion of platelets has been shown to prevent disease onset, highlighting their important role in immune cell trafficking (Sonia D'Souza et al., 2018). Activated platelets adhere to the endothelium resulting in BBB disruption, and release cytokines, chemokines, and reactive oxygen species that activate other immune cells (Saluk-Bijak et al., 2019). These factors can further exacerbate the inflammatory response and contribute to the progression of demyelination and neurodegeneration in MS.

Upregulation of miR-30d-5p and miR-29a-3p, downregulation of Collagen Type V Alpha 1 Chain (COL5A1) in MS males vs control males: COL5A1 was downregulated by miR-30d-5p and miR-29a-3p in MS males. IPA pathway analysis showed that decreased expression of COL5A1 is associated with the glycoprotein VI (GP6) canonical pathway. GP6 is a collagen receptor primarily expressed on platelets, where it plays a crucial role in platelet activation and aggregation. Dysfunction of GP6 is thought to be related to disease severity and may reduce the efficacy of natalizumab, a potent disease modifying therapy, although the exact mechanisms are not well understood (Al-Mojel et al., 2019). COL5A1 is a key component of ECM and produces type V collagen, which is a repair factor needed to counteract damage from CNS lesions (Mohan et al., 2010). Dysfunction of the GP6 pathway may be driving disease in these males, while also limiting response of collagen to halt or repair CNS damage.

Table 18

miR-30d-5p, miR-29a-3p and Col5A1 mRNA Expression in MS Males vs Control Males

miRNA Name	miRNA log2FC	mRNA Target	mRNA log2FC	P-Value
miR-30d-5p	1.3	COL5A1	-0.9	0.02
miR-29a-3p	1.4	COL5A1	-0.9	0.02

Note: Differentially expressed miRNA and its impact on mRNA target expression. Positive fold changes indicate the RNA expression was higher in MS males, while negative indicates the RNA expression was higher in control males.

***Matrix Metalloproteinases (MMPs*):** MMPs are a family of enzymes upregulated by inflammation that are known to degrade collagen and other components of myelin's extracellular matrices (Mohan et al., 2010). However, some MMPs also likely play a role in remyelination and repair after neuroinflammation (Latronico & Liuzzi, 2017). Interestingly, MS males had dysregulation of MMP-8 and -21 compared to MS females and control males respectively.

Downregulation of miR-1286 and miR-664b-3p, and upregulation of MMP-8: IPA target analysis showed that MMP8 was upregulated by miR-1286 and miR-664b in MS males compared to MS females. MMP-8 is thought to degrade ECM components of the BBB, and upregulation has been associated with disease severity in mouse models (Nygårdas & Hinkkanen, 2002). Interestingly, MMP-8 has also been shown to degrade collagen, so the upregulation of MMP-8 may be related to the previously discussed downregulation of COL5A1 in MS males (Mohan et al., 2010).

Table 19
miR-1286, miR-664b-3p and MMP8 mRNA Expression in MS Females vs MS Males

miRNA Name	miRNA log2FC	mRNA Target	mRNA log2FC	P-Value
miR-1286	1.3	MMP8	-2.0	<0.01
miR-664b-3p	1.3	MMP8	-2.0	<0.01

Note: Differentially expressed miRNA and its impact on mRNA target expression. Positive fold changes indicate the RNA expression was higher in MS females, while negative indicates the RNA expression was higher in MS males.

Upregulation of miR-30d-5p and downregulation of MMP-21: MMP-21 was downregulated by miR-30d-5p in MS males versus male controls. The mechanisms of MMP-21 are not well understood, but it may play a role in ECM remodeling and repair after CNS injury, and downregulation may interfere with the ability to remyelinate after MS damage occurs in males (Latronico & Liuzzi, 2017).

Table 20
miR-30d-5p and MMP-21 mRNA Expression in MS Males vs Control Males

miRNA Name	miRNA log2FC	mRNA Target	mRNA log2FC	P-Value
miR-30d-5p	1.3	MMP-21	-0.7	0.02

Note: Differentially expressed miRNA and its impact on mRNA target expression. Positive fold changes indicate the RNA expression was higher in MS males, while negative indicates the RNA expression was higher in control males.

Pathways Associated with Neuronal Function in Males with MS

IPA analysis implicated several novel pathways that affect the way neurons conduct and transmit signals, and as above males showed dysregulation when compared to both MS females and control males.

Table 21
IPA Pathways Related to Neuronal Function in MS Males vs Control Males

miRNA Name	miRNA log2FC	mRNA Target	mRNA log2FC	P-Value
miR-29a-3p	1.4	DLGAP2	-2.5	<0.01
miR-30d-5p	1.3	DLGAP2	-2.5	<0.01

Note: Differentially expressed miRNA and its impact on mRNA target expression. Positive fold changes indicate the RNA expression was higher MS males, while negative indicates the RNA expression was higher in control males.

Upregulation of miR-29a-3p and miR-30d-5p and Downregulation of Disk Large Associated Protein 2 (DLGAP2) in MS males vs control males: MS males had a marked downregulation of DLGAP2 compared to control males, which was implicated in the neurexin and neuroligin canonical pathway in IPA. Neurexins and neuroligins are cell adhesion molecules involved in mediating the communication between neurons and are critical for proper synaptic transmission and plasticity (Taylor et al., 2020). The DLGAP family (consisting of DLGAP1-4) act as scaffold proteins that regulate postsynaptic density and are closely involved with synaptic signaling via the neurotransmitter glutamate, and have thus been associated with neurological diseases

(Rasmussen et al., 2017; Taylor et al., 2020). MS is associated with synaptic abnormalities, including changes in synaptic structure and function, and neurexins and neuroligins are key molecules in maintaining synaptic integrity and function (Taylor et al., 2020).

DLGAP2 is primarily expressed in the brain as compared to blood where levels are typically very low, although a study by Li et al. (2018) did detect DNA methylation of DLGAP2 in peripheral blood of patients with schizophrenia and tardive dyskinesia, suggesting DLGAP2 expression may be epigenetically regulated in blood. In the MS males from this study DLGAP2 was regulated by both miR-29a-3p and miR-30d-5p, which can influence synaptic function. These pathway findings are novel to MS and warrant further investigation.

Table 22

IPA Pathways Related to Neuronal Function in MS Females vs MS Males

miRNA Name	miRNA log2FC	mRNA Target	mRNA log2FC	P-Value
miR-1286	1.4	CNTNAP1	-0.6	0.03
miR-1286	1.4	DPYSL4 (alias CRMP3)	-1.6	<0.01

Note: Differentially expressed miRNA and its impact on mRNA target expression. Positive fold changes indicate the RNA expression was higher MS females, while negative indicates the RNA expression was higher in MS males.

Downregulation of miR-1286 and upregulation of Contactin Associated Protein 1 (CNTNAP1) in MS males vs MS females: IPA analysis indicated miR-1286 is associated with neuronal maintenance and function. In MS males versus MS females downregulation of miR-1286 upregulated expression of the mRNA CNTNAP1, which encodes contactin-associated protein (Caspr) and is a member of the neurexin superfamily involved in nerve conduction, neurotransmitter release, and the formation of myelinated axons (Zou et al., 2017). In the CNS, CNTNAP1 (Caspr1) recruits myelin proteins, regulates the differentiation and proliferation of astrocytes, and plays a key

role in the stability of paranodal junctions of neurons (Zou et al., 2017). In early phases of MS CNTNAP1 is downregulated during acute demyelination, and upregulated during remyelination and repair efforts, making it a potential biomarker for MS staging and prognosis (Zou et al., 2017). Most of the MS samples in this study were relatively newly diagnosed, and CNTNAP1 upregulation in this study's MS males may be evidence of CNS repair mechanisms triggered by demyelination that manifest in the early stages of MS triggered by demyelination.

Downregulation of miR-1286 and upregulation of Dihydropyrimidinase Like 4 (DPYSL4) in MS males vs MS females: IPA analysis indicated miR-1286 also upregulated expression of the DPYSL4 mRNA, which is implicated in the netrin signaling canonical pathway. Similar to CNTNAP1, CRMP3 (an alias gene name for DPYSL4) is also increased in the presence of neuronal stress and cell death (Aylsworth et al., 2009), and DPYSL4 has been shown to be elevated in the damaged neurons of ALS patients (Riva et al., 2016).

While more research is needed to fully elucidate the role of netrin signaling, neurexins, and neuroligins in MS neurodegeneration, their involvement in synaptic function and immune cell interactions suggests that they may be important players in the pathogenesis of the disease and could serve as biomarkers to better assess disease severity, predict prognosis, and offer new therapeutic strategies for modulating synaptic dysfunction in MS. miR-1286 in particular was associated with upregulation of MMP-8, CNTNAP1, and DPYSL4, and could be a highly relevant biomarker of neuronal stress, disease staging, and prognosis in MS males.

Other Neurodegenerative Pathways in Males with MS

Downregulation of miR-296-5p and Upregulation of Myeloperoxidase (MPO) in MS males vs MS females: IPA target analysis showed decreased expression of miR-296-5p, leading to an

increased expression of MPO. MPO is a peroxidase enzyme encoded by the MPO gene. Invading macrophages and resident microglia that express MPO are abundant in human MS lesions in the CNS (Arnhold, 2020; Lin et al., 2024). They are thought to cause tissue necrosis, neuronal death, and blood-brain-barrier catabolism via reactive oxygen species and TNF- α, contributing to neurodegeneration and facilitating the influx of inflammatory immune cells into the CNS (Lin et al., 2024). MPO is known to be increased in humans by high levels of estrogen and low levels of testosterone, making it relevant to MS pathogenesis in both sexes (Santanam et al., 2017; Szabó et al., 2019). Targeting of MPO by miR-296-5p has not been previously reported in the literature, and although MPO is a well-established driver of MS, this sex-difference has not previously been reported. Interestingly males are known to have more lesions with increased levels of estradiol (as opposed to females who see a protective effect from high levels of estrogen during pregnancy) and low levels of testosterone, so this novel pathway has biological implications that could help explain clinical observations and warrants further exploration (Harbo et al., 2013, 2013; Tomassini et al., 2005).

Table 23

miR-296-5p and MPO mRNA Expression in MS Females vs MS Males

miRNA Name	miRNA log2FC	mRNA Target	mRNA log2FC	P-Value
miR-296-5p	1.4	MPO	-1.2	<0.01

Note: Differentially expressed miRNA and its impact on mRNA target expression. Positive fold changes indicate the RNA expression was higher in MS females, while negative indicates the RNA expression was higher in control males.

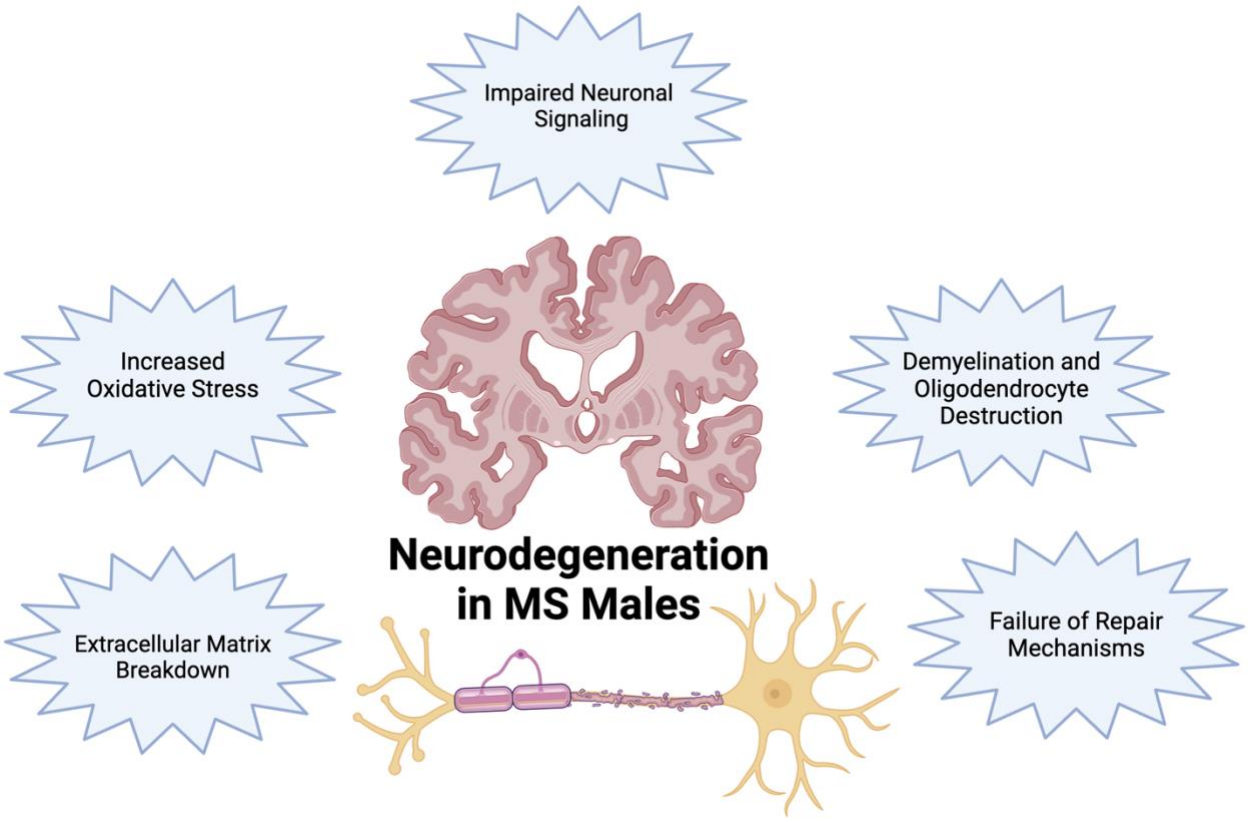

Figure 6: Summary of neurodegenerative pathways in MS males. *Image created with Biorender.com.*

Discussion of Neuronal Integrity in MS Females

Downregulation of miR-941 in MS Females vs MS Males: Interestingly, downregulation miR-941, which was associated with neuroinflammation via upregulation of TLR3 also regulated genes related to neurodegeneration and neuroprotection, indicating this novel miRNA affects both disease processes and is highly relevant in sex-based differences.

Table 24
miR-941, NAT8L and FFAR2 mRNA Expression in MS Females vs MS Males

miRNA Name	miRNA log2FC	mRNA Target	mRNA log2FC	P-Value
miR-941	-2.2	FFAR2	0.6	<0.01
miR-941	-2.2	NAT8L	0.7	0.03

Note: Differentially expressed miRNA and its impact on mRNA target expression. Positive fold changes indicate the RNA expression was higher in MS females, while negative indicates the RNA expression was higher in MS males.

Downregulation of miR-941 and upregulation of Free Fatty Acid Receptor 2 (FFAR2) in MS females vs MS males: IPA indicated this pathway is associated with the CREB cycling of neurons. FFAR2 is activated by short-chain fatty acids (SCFAs) and can influence BBB permeability, neuroinflammation, and neuronal survival (Falomir-Lockhart et al., 2019). FFAR2 can be both neurodegenerative and neuroprotective. SCFAs can have anti-inflammatory effects in the brain and support neuronal survival, and administration of SCFAs in mouse models of MS has been shown to lessen disease severity (Duarte-Silva et al., 2022). Although complex and poorly understood, acute activation of FFAR2 seems to be anti-inflammatory and protective, while chronic activation may cause neurodegeneration via the release of ROS and immune cell activation (Falomir-Lockhart et al., 2019). Interestingly, the gut microbiome plays a crucial role in regulating FFAR2 activity through the production of SCFAs. Dysbiosis, or an imbalance in the gut microbiota, can lead to alterations in SCFA production and FFAR2 signaling (Duarte-Silva et al., 2022). FFAR2 is highly expressed in adipose tissue, which is particularly relevant given the average BMI of our MS females was high. In the context of neurodegeneration and MS, the gut microbiome's influence on chronic FFAR2 activation may lead to neuronal cell death by via release of ROS and by perpetuating neuroinflammatory pathways, including MAPK (Duarte-Silva et al., 2022). This gut-brain axis is a rapidly growing area of MS research and is known to be influenced by sex. This pathway could offer important context into how the gut microbiome is epigenetically regulated in MS.

Downregulation of mir-941 and upregulation of* N-*acetyltransferase 8-Like (NAT8L): NAT8L is an enzyme that synthesizes N-acetylaspartate (NAA), a neuronal metabolite involved in several beneficial processes including supporting

oligodendrocytes, myelin synthesis, and regulation of TNF- α (Kharel et al., 2023). Mouse models looking at oxidation and knock out of NAT8L have shown that subsequent reduction in NAA is a known driver of neurodegeneration in several diseases, including multiple sclerosis (Kharel et al., 2023; Kular & Jagodic, 2020). The opposite was observed in the MS females, who had higher expression of NAT8L than MS males, indicating there were sex differences in this neuroprotective enzyme. Although NAT8L was once thought to be a brain-specific enzyme, it is also highly expressed in adipocytes (Pessentheiner et al., 2013), and it is possible that NAT8L is epigenetically regulated in blood. miR-941 is also highly expressed in the brain, and further studies on its effects on NAT8L within the CNS could better identify sex differences and the role mir-941/NAT8L interactions play in neuroprotection and neurodegeneration.

Upregulation of miR-26b-5p and downregulation of Ubiquinol-Cytochrome C Oxidoreductase Complex (UQCRB) in MS females vs control females: IPA showed miR-26b-5p is known to play a role in mitochondrial function and apoptosis, and downregulated the mRNA UQCRB, which is involved with mitochondrial function, in MS females versus control females. Downregulation of UQCRB can lead to the release of mitochondrial reactive oxygen species (ROS) which in turn induces inflammation. While novel to MS, downregulation of UQCRB mRNA has been observed in RNA-seq data from patients with other neurodegenerative diseases and is thought to be involved in both neuroinflammation and neurodegeneration (Elsadany et al., 2021).

Table 25
miR-26b-5p and UQCRB mRNA Expression in MS Females vs Control Females

miRNA Name	miRNA log2FC	mRNA Target	mRNA log2FC	P-Value
miR-26b-5p	1.9	UQCRB	-1.4	<0.01

Note: Differentially expressed miRNA and its impact on mRNA target expression. Positive fold changes indicate the RNA expression was higher in MS females, while negative indicates the RNA expression was higher in control females.

NanoString Results Relevant to Neuronal Integrity in MS Females

Two miRNAs were found to be significantly differentially expressed between MS females and control females via NanoString analysis but were not associated with mRNA regulation via IPA. However, they may play female-specific role in neuronal integrity that could help explain their better prognosis.

Table 26
miRNAs Related to Neuronal Integrity in MS Females vs Control Females

miRNA Name	Log2FC	P-Value
miR-144-3P	2.2	0.03
miR-363-3p	1.9	0.05

Note: Differentially expressed miRNA and its impact on mRNA target expression. Positive fold changes indicate the RNA expression was higher in MS females, while negative indicates the RNA expression was higher in control females.

Upregulation of miR-144-3p in MS Females: miR-144-3p has been shown to be dysregulated in the blood and CNS tissue of MS patients, and is thought to play a role in oligodendrocyte homeostasis and myelination (Roshani et al., 2021; Shin et al., 2009). Roshani et al. (2021) found that miR-144-3p was increased in their MS population (not powered to detect sex differences), which was also seen in this study's MS females. Shin et al. (2009) found that *Dicer* ablation in mouse oligodendrocytes led to downregulation of miR-144 (in addition to miR-219 and miR-32) and subsequent neurodegeneration. Therefore, it's reasonable to think miR-144-3p could have either neuroprotective or regulatory effects in MS females, although the mechanisms are not well understood.

Upregulation of miR-363-3p in MS females: Increased miR-363-3p expression in serum has been associated with female-specific neuronal survival after stroke in rat models (Selvamani & Sohrabji, 2017), and injection of miR-363-3p immediately after stroke induction in rats resulted

in less long-term cognitive decline post-infarct (Panta et al., 2020). Increased miR-363-3p expression was seen in the MS females from this study, and is novel to MS. This could shed light on why females have less neurodegeneration and cognitive decline than males, highlighting the therapeutic potential of miRNAs in MS.

Co-Expression of miR-223-3p and miR-196b-5p is Associated with MS Risk: Sex Difference or Driver of Disease?

Table 27
Expression of miR-223-3p and miR-196b-5p in MS vs Control Samples

	Control Female miRNA count	Control Male miRNA count	Log2FC	MS Female miRNA count	MS Male miRNA count	Log2FC
miR-223-3p	105,022	64,747	1.6	143,265	87,584	1.6
miR-196b-5p	91	56	1.6	121	83	1.5

The final relevant NanoString finding was the differential expression of miR-223-3p and miR-196b-5p in MS females versus MS males. These two miRNAs were also differentially expressed in control females versus control males, which would generally indicate that this is a sex difference seen regardless of disease status. However, miR-223-3p is located on the X chromosome and has been associated with increased susceptibility to MS in females, and miR-196b-5p has been previously associated with increased MS severity in females (Kiselev et al., 2015; Tufekci et al., 2010) warranting closer examination. Counts of both miRNAs were higher in MS samples than controls [Table 25], and estrogen is known to regulate both miR-196b-5p and miR-223-3p (Kiselev et al., 2015). Our findings support the potential role of these miRNAs in MS females, but provides important context given that they were also differentially expressed in healthy controls.

Strengths & Contribution to Research

Females with MS had unique expression of miRNA-mRNA pathways associated with neuroinflammation and may have key neuroprotective traits that contribute to their less severe

disease course, while males had differential expression of pathways associated with axonal loss, cognitive decline, and increased disability. Several strengths led to these important findings:

Use of Human Samples: While animal models can provide valuable insights, human samples offer a more direct translation to human disease. MS is a complex disease with diverse manifestations, and studying human samples sheds important light on the underlying mechanisms of neuroinflammation and neurodegeneration.

Isolation of Sex as a Biological Variable: MS is known to have a strong sex bias, with females being more susceptible than males. Isolating sex as a variable helps elucidate the underlying biological mechanisms driving this difference, which could lead to sex-specific treatment approaches and better outcomes for patients. By deepening our knowledge of these differences, this study has made a significant contribution to the field by addressing the critical gap in knowledge of sex-based drivers in MS pathophysiology.

Treatment Naïve Individuals: Studying treatment-naïve individuals provides insight into dysregulation inflammatory and degenerative MS pathways, potentially identifying key molecular events that drive disease progression. Treatment is often a significant confounder in biomarker studies as it can impact mRNA expression and epigenetic pathways through alterations in DNA methylation, histone modification, and miRNA expression. Therefore, the use of samples from treatment naïve individuals was a major strength of this study.

High quality RNA: High-quality RNA was extracted from PAXgene tubes, leading to excellent RNA-seq depth and NanoString data, which is crucial for accurately identifying differentially expressed genes and miRNAs.

Direct Analysis of miRNA-mRNA Interactions: Analyzing miRNA and mRNA directly in each patient sample provided a more comprehensive understanding of the gene regulatory networks involved in MS. This approach helped uncover miRNA impacts on mRNA gene expression that

are novel to MS, and that may have been missed if predicting targets (rather than mRNA expression from the same patient samples) were used.

Limitations

This study used samples from males and females with relapsing MS and healthy comparators who are of the same ethnicity (Caucasian) and age group (20-45) were selected for this study. While the sample homogeneity limits the generalizability within the total MS population, the sample is heterogeneous for sex-based differences and this pilot study is an important first step to yield important insights into the influence of sex; one of the most well-established disparities within the MS population. By studying a narrow population, we were able to better understand sub-phenotypes of MS.

During RNA extraction and data collection two samples had to be excluded due to issues with RNA and data quality, but this did not have a significant impact on the ability to perform differential expression analysis and did not have a negative impact on data quality. Additionally, this study conducted using RNA from peripheral blood, and while the peripheral immune system plays a critical role in MS pathophysiology it differs from patterns seen within the CNS. RNA expression from different tissue types will have different biological consequences, and future studies looking at other sample types (CSF or extracellular vesicles, for example) could validate and contextualize these findings.

A significant challenge faced by this study was the lack of diversity in MS biorepositories. While female MS samples are abundant, it was difficult to find a biorepository with enough male samples that fulfilled the phenotype-based criteria (age, ethnicity, and treatment naïve status), and the ACP repository had exactly 18 male samples that met all the above criteria. Given the lack of inventory, it was not feasible to also select samples with a wide range of disease severity. The research strategy for exploratory aim 2b acknowledged that the small sample size and cross-sectional design meant this study was likely not statistically

powered to draw conclusions on disease severity based on the clinical correlates. Indeed, once the samples were received and the clinical data was compiled many of the disease severity groups (mild, moderate, severe based on MRI data) had fewer than five samples [Table 12], and as a result there was not enough statistical power to assess significant differences between groups (for example females with mild disease versus females with severe disease). This exploratory pilot study provided valuable preliminary data on the effect size of sex-based differences in MS and requires confirmation in larger cohorts.

Recommendations for Future Research These results helped us better understand the underlying molecular mechanisms of this complex disease and serve as an important basis for future research that will help us further narrow the phenotype and explore biomarkers that could help identify future biomarker candidates for enhanced disease screening, risk stratification, and personalized treatment. The next phase of this research would include confirming these findings in a larger population that would be adequately powered to detect associations between miRNA-mRNA pathways and clinical outcome measures including MRI and EDSS. Since the inception of this project significant progress has also been made in MS disease biomarkers, including the use of serum neurofilament light chain (sNfL) and Glial Fibrillary Acidic Protein (GFAP) to assess disease activity and predict long term prognosis respectively. These biomarkers have recently become readily available to researchers and are an exciting opportunity to correlate epigenetic markers with disease severity. Due to the lack of diversity in MS biorepositories (underrepresentation of males and diverse ethnic groups), future studies would likely have to recruit patients instead of using repository samples. The patients used in this study have matched serum samples in the ACP biorepository that we could use to measure sNfL and GFAP, which could be combined with a larger dataset in the future.

The neurodegenerative pathways found in this study were particularly interesting and warrant further validation. In MS males versus control males upregulation of miR-30d-5p and miR-29a-3p both caused downregulation of DLGAP2 and COL5A1, and miR-30d-5p caused downregulation of MMP-21. Together this cluster of miRs and mRNA targets seem to have a significant impact on neurodegeneration in MS males and may be biomarkers of neurodegeneration, failure of repair mechanisms, and disease severity. In MS males miR-1286 was also associated with upregulation of MMP-8, CNTNAP1, and DPYSL4 which are markers of ECM impairment and neuronal stress that could help with disease staging, and prognosis. In MS females the down regulation of miR-941 upregulated the inflammatory TLR3 mRNA (MS females versus control females), FFAR2 and NAT8L (MS females versus MS males) which can have both neurodegenerative and neuroprotective effects. Next steps could include validating the co-expression of these miRNAs and target mRNAs *in vivo* using quantitative reverse transcription polymerase chain reaction (qRT-PCR), followed by validating that the miRNAs have a direct effect on the mRNAs of interest and are able to alter protein expression before evaluating their effects on biological function (Riolo et al., 2020).

Concluding Narrative

This study provided many novel insights into how miRNAs epigenetically modify expression of mRNAs and the impact of these pathways on neuroinflammation and neurodegeneration. These findings align with the clinically observed sex-based disparities in MS and help better explain why females tend to have more inflammation and better outcomes, while males have more neurodegeneration and poorer prognoses. A full list of findings that add to the current knowledge of sex-based disparities and novel results is located in Appendix 4.

MS females exhibited distinct neuroinflammatory pathways, including the novel downregulation of miR-941 which suggested previously unknown mechanisms by which TLR3

activation and the Nf-κB signaling pathway contribute to the exaggerated immune response in females with MS. Additionally, we explored the potential role of Epstein-Barr Virus (EBV) in MS pathogenesis, particularly its interaction with TLR3 and Nf-κB activation. MS males showed different miRNA expression patterns associated with immune dysfunction. Interestingly these miRNAs are associated with more severe disease and cognitive decline in MS, and this is the first study to demonstrate sex-differences in their expression.

Significant and novel neurodegenerative pathways were also uncovered. Males with MS demonstrated dysregulated Nrf2 mediated oxidative stress pathways, leaving them vulnerable to oxidative damage implicated in MS pathogenesis. Findings also highlighted importance of Extracellular Matrix (ECM) integrity in males with MS. ECM plays a crucial role neuronal and BBB maintenance and was dysregulated via the GP6 pathway by COL5A1, MMP8, and MMP21. Additionally, the study explores pathways associated with neuronal function in males with MS, such as the upregulation DLGAP2 and CNTNAP1, which are involved in synaptic function and myelinated axon formation, respectively and may help to differentiate between early and late-stage disease, as well as prognosis. On the other hand, females with MS exhibited downregulation of miR-941 and upregulation of FFAR2 and NAT8L, as well as increase expression of miR-144 and miR-363 which indicate there are female-specific neuroprotective mechanisms that may explain their less severe disease course.

These results have enhanced understanding of MS pathophysiology and laid a crucial foundation for future research in this area. This study successfully identified several potential epigenetic pathways that can be explored in a larger population. Together with future work on narrowing the phenotypes of MS, these findings will contribute to the identification of potential biomarkers for enhanced disease screening, risk stratification, and more personalized treatment strategies.

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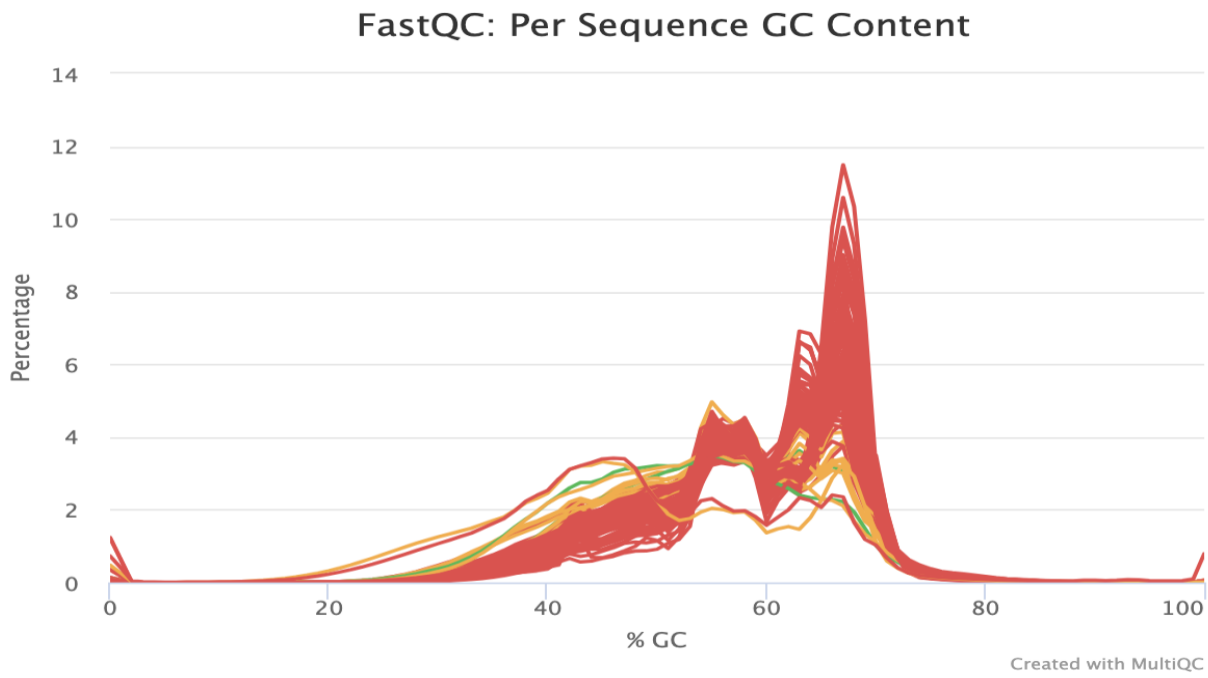
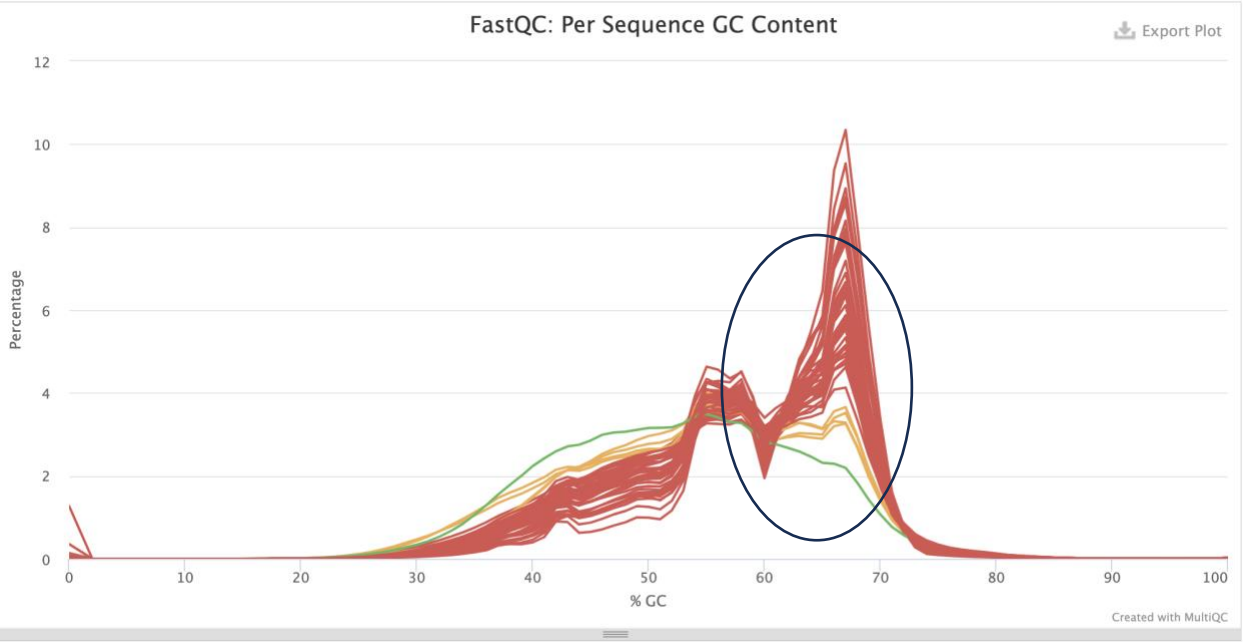
Appendix 1: GC Content before and after removal of rRNA from RNA-seq data

The GC content in RNA-seq data refers to the proportion of the nucleotides in the RNA molecule that are either guanine (G) or cytosine (C). A high GC content means that a larger proportion of the RNA molecule is made up of Gs and Cs relative to the other nucleotides (adenine (A) and uracil (U) in RNA). The typical GC content in humans ranges from 35-60%. After rRNA removal from the raw RNA-seq data the GC spike in the 60-65% range improved, indicating that at least some of the high GC content was due to rRNA that had escaped poly-A selection during RNA-seq.



Before removal of rRNA



After removal of rRNA

Appendix 2: Full results from Aim1, RNA-seq differential expression

MS Female vs MS Male		
Gene Name	**Log2 Fold Change**	**P-Value**
GTSE1	-0.79	0.01
CADM1	-0.60	0.02
MCEMP1	-0.91	0.02
UTY	-12.28	0.00
MAP7D2	3.13	0.00
SOCS3	0.74	0.01
SORCS2	-0.58	0.02
MFSD6L	0.89	0.00
NA	-0.56	0.05
TCN2	-0.59	0.05
NAT8L	0.79	0.02
FFAR3	0.84	0.04
NA	0.63	0.04
ISG15	1.06	0.03
SAXO2	1.11	0.05
RHCE	1.03	0.01
MMP23B	-0.58	0.04
PRTN3	-1.37	0.01
ASMT	0.64	0.05

AMPH	-0.75	0.02
ELANE	-1.39	0.00
ADAMTSL2	-1.22	0.00
DLGAP2	-3.22	0.00
CACNA1E	0.66	0.03
NA	0.82	0.03
EIF1AY	-10.35	0.00
DDX43	-0.99	0.01
LOC101928906	-0.95	0.01
LINC01531	0.64	0.01
LOC100128770	0.71	0.04
IGLV7-46	0.93	0.02
IGHV3-7	-0.84	0.02
IGHV3-48	-1.46	0.00
NA	0.64	0.01
RPS4XP1	1.03	0.00
RPS4XP3	0.99	0.00
EML6	-0.72	0.04
RPS3AP21	-1.07	0.05
PRSS21	0.99	0.00
LINC01857	-0.81	0.01
SLC25A5-AS1	-0.60	0.03
NA	-0.75	0.02
NA	-0.86	0.00

PRRT4	-0.80	0.00
PSPHP1	-1.89	0.02
NA	-0.90	0.00
NA	0.64	0.04
RBMY2SP	-6.52	0.00
RPS4XP2	0.69	0.04
LINC00511	-0.65	0.05
NA	-0.78	0.04
COPS8-DT	-0.68	0.01
NA	0.72	0.04
LINC01736	-1.00	0.01
NA	1.68	0.00
TTTY10	-7.05	0.00
PDC-AS1	-1.02	0.01
XIST	10.93	0.00
NA	-1.74	0.00
NA	-0.86	0.01
NA	-0.92	0.03
LINC02884	0.67	0.04
APCDD1L-DT	0.72	0.05
IGHV4-30-2	0.86	0.04
LINC00278	-9.87	0.00
NA	0.59	0.03
LOC339260	-1.27	0.00

LOC101929297	1.54	0.01
NA	0.70	0.04
BTG2-DT	0.69	0.04
NA	-0.94	0.01
NA	2.15	0.00
KIFC1	-0.63	0.01
ORC1	-0.55	0.03
DEFA3	-1.38	0.04
IGKV1-6	-3.06	0.00
CDKN2B-AS1	-0.59	0.05
NA	-0.77	0.04
NA	-7.80	0.00
ZNF702P	0.90	0.01
NA	0.86	0.00
CEACAM6	-1.81	0.00
TARM1	-1.04	0.01
TMEM158	0.92	0.00
SHANK3	-0.71	0.03
RNU6-176P	0.90	0.01
NA	1.06	0.01
NA	0.63	0.05
NA	0.80	0.01
NA	-0.67	0.03
NA	-1.45	0.03

TPX2	-0.73	0.00
NA	-0.68	0.01
NA	0.91	0.00
LINC02141	-1.30	0.02
NA	0.83	0.05
CPXM1	-0.87	0.01
RBM8A	-0.82	0.00
LOC105371745	0.64	0.05
DOC2B	-1.10	0.02
NA	0.60	0.02
BIRC5	-0.95	0.00
OTUB2	-0.87	0.03
H2BC6	0.73	0.00
DGKK	-1.17	0.00
LOC102723407	-1.47	0.01
TRBV12-5	-0.71	0.02
NA	-1.51	0.00
IGKV1D-13	-1.59	0.03
NA	0.74	0.04
NEFL	-0.69	0.00
NA	-0.90	0.01
SSTR3	0.58	0.01
NA	0.64	0.05
LINC02033	0.65	0.00

IGHV1-69-2	-1.54	0.03
WASH5P	-0.62	0.05
LINC02009	-2.17	0.00
NA	0.65	0.05
LOC389906	0.73	0.00
KIF4A	-0.78	0.03
NA	-0.59	0.05
NHIP	1.26	0.01
NA	-1.16	0.00
NRCAM	-0.80	0.02
NA	1.83	0.00
NA	0.93	0.00
NA	0.74	0.04
NA	0.65	0.02
LOC105377225	-11.16	0.00
HERC2P3	0.89	0.03
SORD2P	1.60	0.00
LOC100419170	-0.83	0.02
NA	-1.33	0.01
NA	-6.46	0.00
BCORP1	-7.55	0.00
TXLNGY	-9.18	0.00
SNHG33	-0.88	0.03
PRKY	-12.47	0.00

MMP25	0.71	0.01
AOC1	1.53	0.01
CTSG	-1.73	0.00
GINS1	-0.74	0.02
BPI	-1.45	0.00
OLFM4	-1.76	0.01
MYEF2	-0.94	0.00
ANLN	-0.83	0.03
SHD	0.87	0.01
LTF	-2.21	0.00
IFT56	0.69	0.02
KDM5D	-13.23	0.00
LSM5.00	-0.56	0.05
WNT3	-1.76	0.00
CHDH	-1.62	0.00
MS4A4A	-0.61	0.03
CDCA3	-0.58	0.04
RHAG	-0.77	0.04
KIF20A	-0.86	0.02
BTNL8	0.70	0.00
PDGFRB	-0.73	0.01
USP9Y	-10.71	0.00
GADD45A	-1.08	0.00
CDC20	-0.57	0.04

MMP8	-2.04	0.00
PMFBP1	-0.65	0.05
PPL	0.67	0.00
PGF	-0.58	0.05
KIF18A	-0.92	0.02
BHLHE41	-0.85	0.02
HJURP	-0.88	0.01
OR2B6	0.77	0.04
NRN1	1.13	0.00
DOK4	0.61	0.01
DLGAP5	-1.06	0.01
MYO16	-1.00	0.01
RNASE1	-2.22	0.00
RPS4Y1	-13.40	0.00
NECTIN2	-1.14	0.04
TOP2A	-0.85	0.01
ITGB4	0.85	0.02
HMGB1P5	-0.60	0.03
CHIT1	-0.96	0.00
EPHB2	-0.63	0.04
SLC2A11	-0.67	0.04
ANXA1	-0.65	0.04
OASL	0.88	0.02
ITGA7	-0.99	0.00

TROAP	-0.70	0.03
PRSS22	1.18	0.00
ZDHHC4	-0.61	0.02
KCTD3	-0.58	0.04
RAD51	-0.70	0.02
UACA	-0.66	0.03
CENPE	-0.94	0.02
ZCCHC2	0.58	0.02
TRAF3IP2	0.58	0.04
SLC44A3	0.78	0.01
JTB	-0.66	0.02
S100A8	-0.86	0.03
RASGRF1	-0.92	0.02
GDF7	-0.96	0.00
KDM6A	0.59	0.00
BCAT1	-0.87	0.03
LCN2	-1.82	0.00
MKI67	-0.77	0.00
ADM	0.65	0.01
MS4A3	-1.05	0.02
DPYSL4	-1.57	0.00
TMEM45B	0.68	0.01
OLAH	-2.15	0.02
PGM5	-1.37	0.00

USP43	-0.99	0.02
EPHB1	0.62	0.01
VSIG4	-1.95	0.00
UQCRB	-0.75	0.03
LRFN2	-1.03	0.02
MPO	-1.19	0.00
ERG	-0.73	0.05
COL17A1	-1.91	0.00
SPON2	-0.56	0.03
SPTBN4	-1.95	0.00
BICDL2	1.23	0.00
CIMAP2	-0.66	0.04
AKR7A3	-0.67	0.05
DDX3Y	-11.49	0.00
KIF15	-0.57	0.04
CDC25A	-0.92	0.04
CAMP	-1.31	0.00
ZFY	-6.76	0.00
HPGD	-1.56	0.00
DEFA4	-1.27	0.01
FZD6	-0.88	0.00
NLGN4Y	-5.90	0.00
MELK	-0.92	0.02
CEP57	-0.59	0.02

CDT1	-0.56	0.02
ATOH8	0.84	0.03
RNASE3	-0.92	0.04
BUB1	-0.63	0.01
HTRA3	-1.70	0.00
SHCBP1	-0.73	0.01
RRM2	-0.60	0.03
LAMB2	-0.70	0.00
AZU1	-1.21	0.00
SERHL	0.87	0.02
XKR3	1.08	0.03
EVC2	-0.66	0.04
BNC2	-0.70	0.05
TUBB8B	-1.70	0.02
OLR1	-2.33	0.00
CD34	-0.64	0.04
SH3PXD2B	-0.92	0.03
PHYHD1	-0.63	0.03
TSPEAR	0.76	0.02
TTTY14	-8.31	0.00
CAVIN1	-0.64	0.04
CD163	-1.07	0.01
CD163L1	-1.33	0.01
OPLAH	0.72	0.00

ABCA13	-1.52	0.00
RNF182	-1.46	0.04
LINC02908	0.83	0.03
SLC47A2	-0.96	0.01
SLC12A1	-1.75	0.03

MS Females vs Control Females		
Gene Name	**Log2 Fold Change**	**P-Value**
B4GALNT4	1.34	0.01
TMEM119	1.70	0.02
LINC01547	0.67	0.01
PCDH9	1.07	0.02
TMEM121	0.84	0.03
KLHL34	-1.17	0.02
NA	1.24	0.01
CYP4F12	0.76	0.05
TREML4	1.88	0.01
ZP3	0.70	0.04
TOMM7	-0.92	0.04
COL13A1	-1.20	0.02
FAM153CP	-1.13	0.00
IFITM5	2.17	0.01

ZDHHC11B	-1.65	0.00
IGLV10-54	-1.45	0.01
IGLV1-36	1.22	0.05
IGHV6-1	0.92	0.04
IGHV2-5	0.89	0.04
RPS3AP21	-1.50	0.03
LINC00612	0.92	0.02
LINC01342	0.95	0.03
IGHV4-59	1.30	0.04
LINC02470	1.92	0.03
NA	3.81	0.00
WASH8P	0.66	0.03
PSPHP1	-2.32	0.02
C14orf132	0.87	0.04
NA	-0.63	0.04
NA	2.15	0.00
PRRT3-AS1	0.82	0.05
NA	1.32	0.02
RPS23P8	-0.88	0.04
MTCO1P53	1.18	0.03
NA	1.47	0.01
NA	1.04	0.05
NA	-0.71	0.03
CDC20-DT	0.60	0.03

CD101-AS1	1.55	0.02
MTX1LP	0.63	0.01
NA	1.11	0.05
NA	0.83	0.05
PIRAT1	0.96	0.03
NA	1.38	0.05
C1orf226	-2.02	0.04
RPL13AP18	-1.13	0.04
PEG10	1.16	0.05
RPL21P11	-1.24	0.04
UPK3B	1.32	0.01
LOC157273	1.92	0.02
NA	0.76	0.03
NA	1.87	0.01
PRSS51	0.63	0.04
NA	1.13	0.04
NA	-1.84	0.02
NA	1.24	0.01
NA	1.37	0.03
NA	0.76	0.03
NA	1.39	0.02
MIR4538	0.79	0.05
NA	0.75	0.02
NA	1.04	0.02

UXT-AS1	0.86	0.05
NA	1.07	0.01
NA	1.16	0.02
OAS1	1.08	0.05
NA	0.97	0.03
NA	0.66	0.04
DGCR5	0.77	0.03
NA	1.84	0.01
NA	3.42	0.00
TBC1D3D	4.84	0.04
DGKK	-1.06	0.03
NA	-1.62	0.01
NA	3.46	0.00
NA	1.22	0.01
NA	1.10	0.02
LOC102724023	0.69	0.04
IGHV7-4-1	3.31	0.04
NA	1.63	0.01
KBTBD11-OT1	1.01	0.01
NA	-1.35	0.00
NA	0.96	0.03
NA	1.00	0.05
NA	1.22	0.02
NA	1.62	0.01

LOC101929609	0.95	0.03
NA	0.74	0.04
NA	-0.99	0.01
NA	-2.55	0.00
LOC107985892	0.90	0.04
NA	-0.94	0.04
NA	-1.40	0.04
TEKT4P2	-1.91	0.00
SGSM3	-0.88	0.03
PIN4	-0.63	0.02
CLEC11A	0.70	0.03
GSDME	0.74	0.05
MPP2	-0.89	0.01
RPL34	-1.45	0.04
SASH1	0.83	0.04
NPR3.00	-1.13	0.02
EEF1B2	-0.99	0.03
OTOF	3.21	0.02
IGFBP2	1.48	0.02
PPL	0.74	0.02
IQSEC3	2.02	0.03
DUSP4	1.31	0.00
RPL21	-0.96	0.03
IL9R	1.65	0.05

MYOM2	-2.02	0.00
GDF15	1.09	0.03
RAI2	0.82	0.05
LGALS12	0.90	0.03
UTS2	-1.81	0.01
RPS24	-1.09	0.03
FKBP10	-1.15	0.04
DMRTC2	1.30	0.03
NUP210L	-1.43	0.05
HCN3	0.63	0.04
ADAMTS16	-1.03	0.03
STYK1	0.96	0.03
LRP12	1.10	0.02
CLEC1A	-1.47	0.00
AKAP6	0.58	0.04
FAM167A	1.14	0.01
KLF10	0.60	0.03
UQCRB	-1.36	0.00
SHROOM4	1.49	0.01
TFF3	1.54	0.04
SLC45A1	0.76	0.03
IFI27	3.98	0.02
TMEM92	0.95	0.03
TBC1D16	0.68	0.05

ATOH8	1.64	0.01
GPR25	0.76	0.02
JUP	1.15	0.03
C1orf127	0.65	0.05
LPL	2.39	0.01
CASKIN2	1.27	0.01
FAM20C	0.68	0.04
RPS3AP5	-1.55	0.01
SLC47A2	-1.05	0.02
SLC12A1	-2.19	0.03
CHST13	0.94	0.02
ARHGEF10L	0.60	0.01

MS Males vs Control Males		
Gene Name	**Log2 Fold Change**	**P-Value**
SYNM	1.26	0.02
MCEMP1	1.27	0.03
TMEM119	1.48	0.04
ZNRF3	-0.87	0.04
TRAIP	-0.69	0.01
MTARC1	1.44	0.02
CENPP	-0.94	0.01

SLC35F1	-1.23	0.03
NEBL	-2.47	0.01
PARVA	-1.10	0.04
L1CAM	1.09	0.03
TMEM273	-0.87	0.00
COL11A2	-0.71	0.02
IGKJ1	0.85	0.04
IGKV6-21	1.22	0.03
IGLV3-19	1.03	0.03
IGLV2-18	1.03	0.04
IGHV3-7	1.44	0.01
IGHV4-34	0.86	0.04
NA	1.03	0.01
LINC01644	-0.95	0.05
LINC01118	-0.66	0.03
IGHV3-64	3.01	0.01
NA	-0.70	0.05
NA	0.94	0.03
NA	-0.59	0.04
NA	-1.55	0.03
NA	1.98	0.03
NA	2.61	0.02
APCDD1L-DT	-1.37	0.00
NA	-0.63	0.03

NA	-0.83	0.01
NA	-0.90	0.03
LOC339260	1.55	0.02
LINC02669	-1.26	0.04
RPL10P9	2.13	0.02
NA	-1.00	0.02
PNMA6A	-1.14	0.03
NA	2.84	0.01
IGKV1-6	3.03	0.00
C1orf226	2.55	0.05
NA	-2.64	0.02
IGKV2-24	1.36	0.01
PWP2	3.15	0.01
NA	-0.68	0.04
NA	-0.59	0.05
NA	-0.72	0.02
LOC728488	1.20	0.03
SNHG21	-0.87	0.04
ADAMTS2	4.00	0.02
NA	-0.76	0.03
TRNP1	-0.85	0.02
NA	0.80	0.02
SIGLEC12	2.24	0.01
NA	-0.98	0.03

OVCH1-AS1	-0.99	0.04
NA	-0.72	0.05
SIGLEC1	1.96	0.04
NA	-0.75	0.03
NA	-0.74	0.04
LINC02594	-1.11	0.03
NA	-0.82	0.04
NA	-0.62	0.04
ZNF595	-0.69	0.02
TBC1D3D	6.36	0.01
IGHV2-70	1.24	0.01
DGKK	-1.02	0.03
H2BC17	1.38	0.03
ARHGAP23	0.90	0.03
NA	-0.70	0.05
NA	-1.02	0.03
NA	-1.12	0.02
CFAP97D2	-0.86	0.03
ZNF511-PRAP1	-0.96	0.01
NA	1.16	0.03
LOC105375754	-0.98	0.02
NA	-1.79	0.04
NA	-0.96	0.03
LOC105376995	-1.14	0.02

NA	-1.80	0.01
NA	-1.15	0.00
GUSBP2	-0.89	0.02
NA	0.97	0.05
SLC7A8	-0.81	0.04
NA	3.17	0.02
NA	0.54	0.05
NSUN5P1	-0.74	0.02
PRSS30P	-1.04	0.02
PALM	-1.11	0.04
GGT5	-0.96	0.04
BIRC7	-1.46	0.04
EEF1A2	1.50	0.02
CDH20	-0.93	0.02
CENPI	1.39	0.01
LILRB5	-1.09	0.04
UNC5B	1.51	0.04
CCL2	2.56	0.02
FOLR3	1.54	0.01
PTK7	-0.58	0.04
MTARC2	1.39	0.02
ELOVL4	-1.00	0.04
SGIP1	2.27	0.00
LRRC7	-1.12	0.02

MMP19	1.18	0.04
MYOM2	-1.50	0.03
RAB27B	1.71	0.01
RNASE1	2.99	0.00
KIF1A	-1.22	0.00
COL5A1	-0.93	0.01
LRRC4B	-0.83	0.04
GFPT2	-1.17	0.03
RAMP1	-0.85	0.03
ITGA7	0.97	0.04
PRSS22	-1.36	0.01
TRAF3IP2	-0.80	0.03
RGS16	0.83	0.03
NUP210L	-1.42	0.04
AQP10	-0.84	0.05
SLC4A10	-1.00	0.03
ESPNL	1.29	0.03
PNLDC1	1.00	0.04
RAD9B	-0.59	0.05
OLAH	3.56	0.02
ANKRD22	1.11	0.03
PTPRK	-0.68	0.03
PTPRD	-0.72	0.05
MMP21	-0.76	0.03

EPHB1	-0.82	0.01
VSIG4	2.49	0.01
CXCL13	-0.83	0.04
TMSB15B	-1.06	0.03
GATD3	2.02	0.01
SPTBN4	2.12	0.02
MPPED2	-1.17	0.03
SLC1A7	-0.92	0.04
ZDHHC19	1.45	0.05
MATCAP2	-0.84	0.02
EML5	-0.86	0.04
RET	-0.92	0.02
IFI27	3.36	0.03
BEAN1	-1.06	0.00
RBPMS2	-0.99	0.04
EVPL	-0.83	0.04
SCT	-1.21	0.01
GSTM4	-0.77	0.03
JMJD7-PLA2G4B	1.00	0.03
GPRIN1	0.73	0.04
LDB2	-0.97	0.04
FAM153A	-0.68	0.03
RNF150	-2.44	0.01

PTCRA	-0.92	0.00
TPSAB1	-1.27	0.04
SLC2A14	1.22	0.04
CAVIN1	1.10	0.02
SAMD12	-0.65	0.05
CD163	1.28	0.03
CD163L1	2.07	0.01

Control Males vs Control Females		
Gene Name	**Log2 Fold Change**	**P-Value**
B4GALNT4	-1.67	0.01
WBP2NL	-0.58	0.04
AFMID	-0.58	0.04
GAS6	-0.90	0.02
ASCL2	-0.61	0.04
TRAIP	-0.96	0.00
UTY	-12.42	0.00
PP2D1	-0.86	0.04
TACSTD2	-1.99	0.04
MAP7D2	2.36	0.00

TMEM121	-0.96	0.04
KRT73	-1.40	0.04
HYAL3	-0.71	0.02
SHTN1	-0.91	0.03
CENPP	-0.99	0.03
ZP3	-0.81	0.04
ZFP92	-0.80	0.02
SLC35F1	-2.33	0.00
PRTN3	-2.36	0.01
ELANE	-2.29	0.00
DLGAP2	-3.66	0.00
RYR2	-1.23	0.03
EIF1AY	-10.57	0.00
L1CAM	1.14	0.04
DCLRE1A	-0.93	0.04
TMEM273	-0.91	0.02
NA	-1.39	0.01
LINC00654	-1.40	0.03
IGLV4-60	1.91	0.03
IGLV10-54	1.37	0.04
IGHG4	2.02	0.02
IGHV3-53	-1.46	0.03
RPS4XP3	1.15	0.02
MEG3	-1.98	0.02

RPS19P1	1.31	0.05
NA	-0.70	0.02
LINC00612	-1.04	0.03
CELA2B	-0.67	0.03
AP4M1	-0.61	0.01
ATXN1L	-0.96	0.02
PRRT4	-1.04	0.03
NA	-3.18	0.02
LINC00323	-2.26	0.03
MROH5	-0.98	0.02
NA	-0.92	0.04
RBMY2SP	-6.06	0.00
RPS4XP2	1.16	0.04
NA	-0.90	0.03
NA	2.09	0.02
NA	-1.73	0.02
NA	-1.28	0.05
CCDC26	-1.53	0.00
TTTY10	-7.60	0.00
XIST	10.91	0.00
NA	1.40	0.05
NA	-1.76	0.02
MTCO1P53	-1.58	0.01
NA	-0.88	0.03

LINC00278	-9.81	0.00
NA	-0.84	0.02
NA	-1.72	0.01
NA	-1.43	0.01
CDC20-DT	-0.89	0.01
NA	-0.62	0.05
SEMA3F-AS1	-0.94	0.03
NA	-1.00	0.04
PNMA6A	-1.67	0.02
NA	-0.99	0.02
NA	-0.64	0.04
LINC00106	-0.87	0.01
NA	2.98	0.02
NA	-1.28	0.05
NA	2.40	0.01
NA	-2.01	0.01
C1orf226	3.34	0.02
RPS4XP13	1.27	0.02
LINC03014	-0.78	0.03
IGKV1-16	-1.27	0.03
NA	-7.90	0.00
PWP2	3.40	0.01
IGKV2D-29	1.52	0.02
PTPRVP	-0.95	0.02

BAD	-0.68	0.02
NA	-0.84	0.03
HSD17B14	1.17	0.04
SELENOP	-1.06	0.04
SHANK3	-1.20	0.03
TRNP1	-1.12	0.02
NA	-1.76	0.02
PRSS51	-0.74	0.04
CD8B2	1.41	0.03
LINC03021	-1.68	0.03
SIGLEC12	1.97	0.04
NA	-0.88	0.01
KRT73-AS1	-1.64	0.02
LINC02356	-1.21	0.00
OVCH1-AS1	-1.41	0.02
NA	-1.16	0.01
NA	-1.02	0.03
LINC02259	-0.72	0.04
NA	-1.89	0.00
NA	-0.86	0.03
NA	-0.97	0.02
NA	1.10	0.03
NA	-1.56	0.03
NA	-1.21	0.03

SEPTIN9-DT	-1.37	0.03
NA	-0.85	0.02
NA	-0.91	0.04
NA	-1.42	0.01
TSNAX-DISC1	-1.07	0.04
MTND4P35	1.56	0.03
NA	-1.04	0.04
MILR1	-0.72	0.04
NA	-0.68	0.04
LYPD4	-1.67	0.02
BIRC5	-1.08	0.04
NA	-0.65	0.02
NA	2.07	0.01
NA	-3.10	0.02
TMEM269	-0.70	0.05
SPDYE10	-1.12	0.04
H2BC17	1.97	0.01
NA	2.16	0.01
LOC102724159	-0.98	0.05
NA	-1.34	0.05
TBC1D3F	-3.19	0.02
NA	-2.58	0.00
NA	-3.00	0.02
LOC102724023	-1.07	0.01

NA	-1.23	0.04
ZNF511-PRAP1	-0.97	0.03
NA	-1.24	0.05
NA	-1.20	0.05
NA	-2.29	0.00
NA	-0.82	0.05
PITPNM3	-1.28	0.00
NA	-1.38	0.03
LOC105377225	-11.13	0.00
NA	-1.29	0.04
TPTEP1	0.96	0.03
NA	-1.10	0.04
CEBPE	-1.22	0.03
NA	1.03	0.03
FAM238C	0.92	0.03
NA	-6.71	0.00
NA	3.50	0.02
BCORP1	-7.68	0.00
TXLNGY	-9.26	0.00
TEKT4P2	1.66	0.04
NA	-0.66	0.03
CDC45	-1.16	0.03
SCD	-0.86	0.02

PRKY	-12.62	0.00
CTSG	-2.38	0.00
SLC10A1	-0.85	0.05
NINL	-1.20	0.01
EEF1A2	1.62	0.03
BPI	-1.73	0.01
CDH20	-1.46	0.01
OLFM4	-2.67	0.02
QPRT	-0.58	0.03
ERICH1	-0.71	0.01
CLEC11A	-0.98	0.01
LILRB5	-1.57	0.02
GSDME	-1.10	0.01
KDM5D	-13.34	0.00
CPED1	-0.70	0.04
ALDH2	-0.62	0.04
ADGRD1	-0.92	0.03
USP9Y	-10.89	0.00
INO80D	-0.75	0.02
DHCR24	-0.72	0.03
DOCK7	-0.72	0.02
MTARC2	1.60	0.02
MMP8	-1.78	0.05
RARRES1	-1.09	0.03

CD274	1.27	0.03
SCPEP1	-0.68	0.00
NLN	-0.64	0.03
SLC18A1	-1.23	0.05
CD70	0.99	0.01
PZP	-0.83	0.05
RAB27B	1.51	0.05
RPS4Y1	-13.53	0.00
NECTIN2	-2.72	0.01
KIF1A	-1.14	0.01
STK33	-1.33	0.05
GDF15	-1.53	0.01
CTNNA1	-0.64	0.02
LRRC4B	-1.12	0.03
RHPN2	-1.01	0.03
SLC43A3	-0.58	0.04
CCDC146	-0.74	0.02
UTS2	2.34	0.02
LAMC3	-1.29	0.02

Appendix 3: Comprehensive IPA results by group

IPA miRNA Target Filter Analysis: MS Females vs MS Males						
miR Name	**miR name in IPA**	**Log2FC**	**Source**	**mRNA Name**	**Log2FC**	**p-value**
hsa-miR-1180-3p	miR-1180-3p (miRNAs w/seed UUCCGGC)	1.36	TargetScan Human	PCDH1	-0.6	0.02
hsa-miR-1180-3p	miR-1180-3p (miRNAs w/seed UUCCGGC)	1.36	TargetScan Human	CACNB2	-0.5	0.03
hsa-miR-1286	miR-1286 (miRNAs w/seed GCAGGAC)	1.32	TargetScan Human	UTY	-12.3	0.00
hsa-miR-1286	miR-1286 (miRNAs w/seed GCAGGAC)	1.32	TargetScan Human	MMP8	-2.0	0.00
hsa-miR-1286	miR-1286 (miRNAs w/seed GCAGGAC)	1.32	TargetScan Human	DPYSL4	-1.6	0.00
hsa-miR-1286	miR-1286 (miRNAs w/seed GCAGGAC)	1.32	TargetScan Human	MCM4	-0.4	0.01
hsa-miR-1286	miR-1286 (miRNAs w/seed GCAGGAC)	1.32	TargetScan Human	CDCA5	-0.6	0.02
hsa-miR-1286	miR-1286 (miRNAs w/seed GCAGGAC)	1.32	TargetScan Human	CD34	-0.7	0.02
hsa-miR-1286	miR-1286 (miRNAs w/seed GCAGGAC)	1.32	TargetScan Human	CNTNAP1	-0.6	0.03
hsa-miR-196b-5p	miR-196a-5p (and other miRNAs w/seed AGGUAGU)	1.46	TargetScan Human	BNC2	-0.8	0.02
hsa-miR-196b-5p	miR-196a-5p (and other miRNAs w/seed AGGUAGU)	1.46	TargetScan Human	KCNQ5	-0.5	0.02
hsa-miR-196b-5p	miR-196a-5p (and other miRNAs w/seed AGGUAGU)	1.46	TargetScan Human	CDC25A	-1.0	0.03

hsa-miR-196b-5p	miR-196a-5p (and other miRNAs w/seed AGGUAGU)	1.46	TargetScan Mouse,TargetScan Human	ERG	-0.8	0.05
hsa-miR-199a-5p	miR-199a-5p (and other miRNAs w/seed CCAGUGU)	1.43	TargetScan Human	DDX3Y	-11.5	0.00
hsa-miR-199a-5p	miR-199a-5p (and other miRNAs w/seed CCAGUGU)	1.43	TargetScan Mouse,TargetScan Human	FZD6	-0.9	0.00
hsa-miR-199a-5p	miR-199a-5p (and other miRNAs w/seed CCAGUGU)	1.43	TargetScan Mouse,TargetScan Human	MYEF2	-0.9	0.00
hsa-miR-199a-5p	miR-199a-5p (and other miRNAs w/seed CCAGUGU)	1.43	TargetScan Mouse,TargetScan Human	LARGE1	-0.6	0.01
hsa-miR-199a-5p	miR-199a-5p (and other miRNAs w/seed CCAGUGU)	1.43	TargetScan Human	FGFBP2	-0.5	0.03
hsa-miR-199a-5p	miR-199a-5p (and other miRNAs w/seed CCAGUGU)	1.43	TargetScan Mouse,TargetScan Human	CACNB2	-0.5	0.03
hsa-miR-199a-5p	miR-199a-5p (and other miRNAs w/seed CCAGUGU)	1.43	TargetScan Human	GPR89A/GPR89B	-0.4	0.04
hsa-miR-199a-5p	miR-199a-5p (and other miRNAs w/seed CCAGUGU)	1.43	TargetScan Human	C1orf21	-0.6	0.04
hsa-miR-199a-5p	miR-199a-5p (and other miRNAs w/seed CCAGUGU)	1.43	TargetScan Mouse,TargetScan Human	EIF5B	-0.3	0.04
hsa-miR-199a-5p	miR-199a-5p (and other miRNAs w/seed CCAGUGU)	1.43	TargetScan Human	ROGDI	-0.3	0.05
hsa-miR-199a-5p	miR-199a-5p (and other miRNAs w/seed CCAGUGU)	1.43	TargetScan Human	HSPA4	-0.3	0.05

hsa-miR-223-3p	miR-223-3p (miRNAs w/seed GUCAGUU)	1.64	TargetScan Human	OLR1	-2.4	0.00
hsa-miR-223-3p	miR-223-3p (miRNAs w/seed GUCAGUU)	1.64	TargetScan Human	SLC2A11	-0.7	0.02
hsa-miR-223-3p	miR-223-3p (miRNAs w/seed GUCAGUU)	1.64	TargetScan Human	CDNF	-0.6	0.04
hsa-miR-296-5p	miR-296-5p (miRNAs w/seed GGGCCCC)	1.38	TargetScan Human	MPO	-1.2	0.00
hsa-miR-296-5p	miR-296-5p (miRNAs w/seed GGGCCCC)	1.38	TargetScan Mouse,TargetScan Human	SPTBN4	-2.0	0.00
hsa-miR-296-5p	miR-296-5p (miRNAs w/seed GGGCCCC)	1.38	TargetScan Human	AZU1	-1.3	0.00
hsa-miR-296-5p	miR-296-5p (miRNAs w/seed GGGCCCC)	1.38	TargetScan Mouse,TargetScan Human	GDF7	-1.0	0.00
hsa-miR-296-5p	miR-296-5p (miRNAs w/seed GGGCCCC)	1.38	TargetScan Human	HTRA3	-1.7	0.00
hsa-miR-296-5p	miR-296-5p (miRNAs w/seed GGGCCCC)	1.38	TargetScan Human	ADAMTSL2	-1.3	0.00
hsa-miR-296-5p	miR-296-5p (miRNAs w/seed GGGCCCC)	1.38	TargetScan Human	CDT1	-0.6	0.01
hsa-miR-296-5p	miR-296-5p (miRNAs w/seed GGGCCCC)	1.38	TargetScan Human	SPON2	-0.6	0.01
hsa-miR-296-5p	miR-296-5p (miRNAs w/seed GGGCCCC)	1.38	TargetScan Mouse,TargetScan Human	LARGE1	-0.6	0.01
hsa-miR-296-5p	miR-296-5p (miRNAs w/seed GGGCCCC)	1.38	TargetScan Human	ADGRG1	-0.6	0.01
hsa-miR-296-5p	miR-296-5p (miRNAs w/seed GGGCCCC)	1.38	TargetScan Human	CHIT1	-0.8	0.01
hsa-miR-296-5p	miR-296-5p (miRNAs w/seed GGGCCCC)	1.38	TargetScan Mouse,TargetScan Human	CD34	-0.7	0.02
hsa-miR-296-5p	miR-296-5p (miRNAs w/seed GGGCCCC)	1.38	TargetScan Human	PRDX5	-0.2	0.02

hsa-miR-296-5p	miR-296-5p (miRNAs w/seed GGGCCCC)	1.38	TargetScan Mouse,TargetScan Human	PCDH1	-0.6	0.02
hsa-miR-296-5p	miR-296-5p (miRNAs w/seed GGGCCCC)	1.38	TargetScan Human	FGFBP2	-0.5	0.03
hsa-miR-296-5p	miR-296-5p (miRNAs w/seed GGGCCCC)	1.38	TargetScan Mouse,TargetScan Human	TRPM2	-0.4	0.03
hsa-miR-296-5p	miR-296-5p (miRNAs w/seed GGGCCCC)	1.38	TargetScan Human	AP1G2	-0.4	0.03
hsa-miR-296-5p	miR-296-5p (miRNAs w/seed GGGCCCC)	1.38	TargetScan Human	PGF	-0.6	0.03
hsa-miR-296-5p	miR-296-5p (miRNAs w/seed GGGCCCC)	1.38	TargetScan Human	APOL4	-0.6	0.04
hsa-miR-296-5p	miR-296-5p (miRNAs w/seed GGGCCCC)	1.38	TargetScan Human	C16orf96	-0.6	0.04
hsa-miR-4536-5p	miR-4536-5p (miRNAs w/seed GUGGUAG)	-1.3	TargetScan Human	JAK3	0.3	0.02
hsa-miR-4536-5p	miR-4536-5p (miRNAs w/seed GUGGUAG)	-1.3	TargetScan Human	MAPK3	0.3	0.03
hsa-miR-485-3p	miR-485-3p (and other miRNAs w/seed UCAUACA)	1.73	TargetScan Human	OLR1	-2.4	0.00
hsa-miR-485-3p	miR-485-3p (and other miRNAs w/seed UCAUACA)	1.73	TargetScan Human	DDX43	-1.1	0.01
hsa-miR-485-3p	miR-485-3p (and other miRNAs w/seed UCAUACA)	1.73	TargetScan Human	MELK	-1.0	0.01
hsa-miR-485-3p	miR-485-3p (and other miRNAs w/seed UCAUACA)	1.73	TargetScan Human	MS4A3	-1.1	0.01

hsa-miR-485-3p	miR-485-3p (and other miRNAs w/seed UCAUACA)	1.73	TargetScan Human	TCF4	-0.5	0.03
hsa-miR-485-3p	miR-485-3p (and other miRNAs w/seed UCAUACA)	1.73	TargetScan Human	SCP2	-0.4	0.04
hsa-miR-485-3p	miR-485-3p (and other miRNAs w/seed UCAUACA)	1.73	TargetScan Human	FBXO45	-0.5	0.04
hsa-miR-495-3p	miR-495-3p (and other miRNAs w/seed AACAAAC)	1.27	TargetScan Human	KDM5D	-13.3	0.00
hsa-miR-495-3p	miR-495-3p (and other miRNAs w/seed AACAAAC)	1.27	Ingenuity Expert Findings,TargetScan Human	BUB1	-0.7	0.01
hsa-miR-495-3p	miR-495-3p (and other miRNAs w/seed AACAAAC)	1.27	TargetScan Human	ANLN	-0.8	0.03
hsa-miR-495-3p	miR-495-3p (and other miRNAs w/seed AACAAAC)	1.27	TargetScan Human	SCP2	-0.4	0.04
hsa-miR-548n	miR-548n (miRNAs w/seed AAAAGUA)	-1.38	TargetScan Human	PROK2	0.7	0.02
hsa-miR-664b-3p	miR-579-3p (and other miRNAs w/seed UCAUUUG)	1.32	TargetScan Human	MMP8	-2.0	0.00
hsa-miR-664b-3p	miR-579-3p (and other miRNAs w/seed UCAUUUG)	1.32	TargetScan Human	CENPE	-1.0	0.01
hsa-miR-664b-3p	miR-579-3p (and other miRNAs w/seed UCAUUUG)	1.32	TargetScan Human	KIF4A	-0.9	0.02
hsa-miR-664b-3p	miR-579-3p (and other miRNAs w/seed UCAUUUG)	1.32	TargetScan Human	COBLL1	-0.6	0.02

hsa-miR-664b-3p	miR-579-3p (and other miRNAs w/seed UCAUUUG)	1.32	TargetScan Human	RSU1	-0.3	0.02
hsa-miR-664b-3p	miR-579-3p (and other miRNAs w/seed UCAUUUG)	1.32	TargetScan Human	CENPF	-0.5	0.03
hsa-miR-664b-3p	miR-579-3p (and other miRNAs w/seed UCAUUUG)	1.32	TargetScan Human	AKR7A3	-0.7	0.03
hsa-miR-941	miR-941 (miRNAs w/seed ACCCGGC)	-2.22	TargetScan Human	FFAR2	0.6	0.00
hsa-miR-941	miR-941 (miRNAs w/seed ACCCGGC)	-2.22	TargetScan Human	H2BC21	0.4	0.01
hsa-miR-941	miR-941 (miRNAs w/seed ACCCGGC)	-2.22	TargetScan Human	NAT8L	0.7	0.03

IPA miRNA Target Filter Analysis: MS Females vs Control Females						
miR Name	**miR name in IPA**	**Log2FC**	**Source**	**mRNA Name**	**Log2FC**	**p-value**
hsa-miR-1286	miR-1286 (miRNAs w/seed GCAGGAC)	1.4	TargetScan Human	CD34	-0.8	0.04
hsa-miR-1286	miR-1286 (miRNAs w/seed GCAGGAC)	1.4	TargetScan Human	ZDHHC11	-0.6	0.05
hsa-miR-183-5p	miR-183-5p (miRNAs w/seed AUGGCAC)	1.47	TargetScan Human	CARD16	-0.7	0.05
hsa-miR-26b-5p	miR-26a-5p (and other miRNAs w/seed UCAAGUA)	1.89	TargetScan Human	UQCRB	-1.4	0.00

hsa-miR-28-5p	miR-708-5p (and other miRNAs w/seed AGGAGCU)	1.44	TargetScan Human	FKBP10	-1.1	0.03
hsa-miR-28-5p	miR-708-5p (and other miRNAs w/seed AGGAGCU)	1.44	TargetScan Human	CD34	-0.8	0.04
hsa-miR-28-5p	miR-708-5p (and other miRNAs w/seed AGGAGCU)	1.44	TargetScan Mouse	NUP210L	-1.4	0.05
hsa-miR-454-3p	miR-130a-3p (and other miRNAs w/seed AGUGCAA)	1.63	TargetScan Mouse	MYOM2	-2.0	0.00
hsa-miR-454-3p	miR-130a-3p (and other miRNAs w/seed AGUGCAA)	1.63	TargetScan Mouse	NDUFA6	-0.6	0.05
hsa-miR-941	miR-941 (miRNAs w/seed ACCCGGC)	-2.57	TargetScan Human	TBC1D16	0.7	0.05
hsa-miR-941	miR-941 (miRNAs w/seed ACCCGGC)	-2.57	TargetScan Human	TLR3	0.9	0.05
hsa-miR-1226-3p	miR-1226-3p (miRNAs w/seed CACCAGC)	1.19	TargetScan Human	KLHL34	-1.2	0.02

IPA miRNA Target Filter Analysis: MS Males vs Control Males						
miR Name	**miR name in IPA**	**Log2FC**	**Source**	**mRNA Name**	**Log2FC**	**p-value**
hsa-miR-150-5p	miR-150-5p (and other miRNAs w/seed CUCCCAA)	1.32	TargetScan Human	FAM153A/FAM153B	-0.6	0.04
hsa-miR-150-5p	miR-150-5p (and other miRNAs w/seed CUCCCAA)	1.32	TargetScan Human	ACRBP	-0.4	0.04
hsa-miR-183-5p	miR-183-5p (miRNAs w/seed AUGGCAC)	1.57	TargetScan Human	FKBP1C	-0.6	0.05
hsa-miR-29a-3p	miR-29b-3p (and other miRNAs w/seed AGCACCA)	1.36	TargetScan Mouse,TargetScan Human	DLGAP2	-2.5	0.00
hsa-miR-29a-3p	miR-29b-3p (and other miRNAs w/seed AGCACCA)	1.36	TargetScan Human	BEAN1	-1.1	0.00
hsa-miR-29a-3p	miR-29b-3p (and other miRNAs w/seed AGCACCA)	1.36	TargetScan Mouse,TargetScan Human	RNF150	-2.4	0.01

hsa-miR-29a-3p	miR-29b-3p (and other miRNAs w/seed AGCACCA)	1.36	TargetScan Mouse,Ingenuity Expert Findings,TargetScan Human	COL5A1	-0.9	0.02
hsa-miR-29a-3p	miR-29b-3p (and other miRNAs w/seed AGCACCA)	1.36	TargetScan Human	PTPRK	-0.6	0.04
hsa-miR-29a-3p	miR-29b-3p (and other miRNAs w/seed AGCACCA)	1.36	TargetScan Mouse,TargetScan Human	ELOVL4	-0.9	0.04
hsa-miR-29a-3p	miR-29b-3p (and other miRNAs w/seed AGCACCA)	1.36	TargetScan Mouse,TargetScan Human	PALM	-1.0	0.05
hsa-miR-30d-5p	miR-30c-5p (and other miRNAs w/seed GUAAACA)	1.32	TargetScan Human	DLGAP2	-2.5	0.00
hsa-miR-30d-5p	miR-30c-5p (and other miRNAs w/seed GUAAACA)	1.32	Ingenuity Expert Findings	COL5A1	-0.9	0.02
hsa-miR-30d-5p	miR-30c-5p (and other miRNAs w/seed GUAAACA)	1.32	TarBase	SLC4A10	-0.9	0.02

hsa-miR-30d-5p	miR-30c-5p (and other miRNAs w/seed GUAAACA)	1.32	TargetScan Mouse,TargetScan Human	CDH20	-0.9	0.03
hsa-miR-30d-5p	miR-30c-5p (and other miRNAs w/seed GUAAACA)	1.32	TargetScan Mouse,TargetScan Human	GFPT2	-1.1	0.03
hsa-miR-30d-5p	miR-30c-5p (and other miRNAs w/seed GUAAACA)	1.32	TarBase	PTPRK	-0.6	0.04
hsa-miR-30d-5p	miR-30c-5p (and other miRNAs w/seed GUAAACA)	1.32	TargetScan Human	TRAF3IP2	-0.8	0.04
hsa-miR-30d-5p	miR-30c-5p (and other miRNAs w/seed GUAAACA)	1.32	TargetScan Mouse,TargetScan Human	SLC35F1	-1.2	0.04
hsa-miR-30d-5p	miR-30c-5p (and other miRNAs w/seed GUAAACA)	1.32	TargetScan Human	MMP21	-0.7	0.04
hsa-miR-324-5p	miR-324-5p (miRNAs w/seed GCAUCCC)	1.34	TargetScan Human	RNF150	-2.4	0.01

hsa-miR-324-5p	miR-324-5p (miRNAs w/seed GCAUCCC)	1.34	TargetScan Human	NEBL	-2.5	0.01
hsa-miR-324-5p	miR-324-5p (miRNAs w/seed GCAUCCC)	1.34	TargetScan Human	GFPT2	-1.1	0.03
hsa-miR-324-5p	miR-324-5p (miRNAs w/seed GCAUCCC)	1.34	TargetScan Human	MPPED2	-1.1	0.04
hsa-miR-342-3p	miR-342-3p (miRNAs w/seed CUCACAC)	1.52	TargetScan Human	PTCRA	-0.9	0.01
hsa-miR-342-3p	miR-342-3p (miRNAs w/seed CUCACAC)	1.52	TargetScan Human	FAM153A/FAM153B	-0.6	0.04
hsa-miR-342-3p	miR-342-3p (miRNAs w/seed CUCACAC)	1.52	TargetScan Human	GSTM4	-0.7	0.04
hsa-miR-99b-5p	miR-100-5p (and other miRNAs w/seed ACCCGUA)	1.31	TargetScan Human	NEBL	-2.5	0.01
hsa-miR-99b-5p	miR-100-5p (and other miRNAs w/seed ACCCGUA)	1.31	TargetScan Human	SLC35F1	-1.2	0.04

hsa-miR-652-3p	miR-652-3p (miRNAs w/seed AUGGCGC)	1.59	TargetScan Human	AOPEP	-0.4	0.05

IPA miRNA Target Filter Analysis: Control Females vs Control Males						
miR Name	**miR name in IPA**	**Log2FC**	**Source**	**mRNA Name**	**Log2FC**	**p-value**
hsa-miR-1226-3p	miR-1226-3p (miRNAs w/seed CACCAGC)	-1.2	TargetScan Human	GDF15	-1.5	0.01
hsa-miR-1226-3p	miR-1226-3p (miRNAs w/seed CACCAGC)	-1.2	TargetScan Human	GSDME	-1.1	0.01
hsa-miR-1226-3p	miR-1226-3p (miRNAs w/seed CACCAGC)	-1.2	TargetScan Human	OLFM4	-2.7	0.02
hsa-miR-1226-3p	miR-1226-3p (miRNAs w/seed CACCAGC)	-1.2	TargetScan Human	SCD	-0.9	0.02
hsa-miR-1226-3p	miR-1226-3p (miRNAs w/seed CACCAGC)	-1.2	TargetScan Human	TMEM51	-1.3	0.02
hsa-miR-1226-3p	miR-1226-3p (miRNAs w/seed CACCAGC)	-1.2	TargetScan Human	ARHGEF10L	-0.6	0.03
hsa-miR-1226-3p	miR-1226-3p (miRNAs w/seed CACCAGC)	-1.2	TargetScan Human	IGHMBP2	-0.5	0.04
hsa-miR-145-5p	miR-145-5p (and other miRNAs w/seed UCCAGUU)	1.6	TargetScan Human	RIN1	-0.8	0.02
hsa-miR-145-5p	miR-145-5p (and other miRNAs	1.6	TargetScan Human	NLN	-0.6	0.03

	w/seed UCCAGUU)					
hsa-miR-146a-5p	miR-146a-5p (and other miRNAs w/seed GAGAACU)	1.5	TargetScan Human	EIF1AY	-10.6	0.00
hsa-miR-146a-5p	miR-146a-5p (and other miRNAs w/seed GAGAACU)	1.5	TargetScan Mouse	OLFM4	-2.7	0.02
hsa-miR-146a-5p	miR-146a-5p (and other miRNAs w/seed GAGAACU)	1.5	TargetScan Human	CCDC146	-0.7	0.02
hsa-miR-146a-5p	miR-146a-5p (and other miRNAs w/seed GAGAACU)	1.5	TargetScan Mouse,TargetScan Human	INO80D	-0.7	0.02
hsa-miR-146a-5p	miR-146a-5p (and other miRNAs w/seed GAGAACU)	1.5	TargetScan Mouse	TACSTD2	-2.0	0.04
hsa-miR-146a-5p	miR-146a-5p (and other miRNAs w/seed GAGAACU)	1.5	TargetScan Mouse	ABCA13	-1.5	0.04
hsa-miR-146a-5p	miR-146a-5p (and other miRNAs w/seed GAGAACU)	1.5	TargetScan Mouse	BIRC5	-1.1	0.04
hsa-miR-150-5p	miR-150-5p (and other miRNAs w/seed CUCCCAA)	1.5	TargetScan Human	SPC24	-1.0	0.02

hsa-miR-182-5p	miR-182-5p (and other miRNAs w/seed UUGGCAA)	1.5	TargetScan Mouse	ADGRL1	-0.7	0.03
hsa-miR-182-5p	miR-182-5p (and other miRNAs w/seed UUGGCAA)	1.5	TargetScan Mouse	MAST4	-0.7	0.04
hsa-miR-182-5p	miR-182-5p (and other miRNAs w/seed UUGGCAA)	1.5	TargetScan Mouse	MMP8	-1.8	0.05
hsa-miR-182-5p	miR-182-5p (and other miRNAs w/seed UUGGCAA)	1.5	TargetScan Human	TCF7L2	-0.6	0.05
hsa-miR-183-5p	miR-183-5p (miRNAs w/seed AUGGCAC)	1.4	TargetScan Human	EIF1AY	-10.6	0.00
hsa-miR-183-5p	miR-183-5p (miRNAs w/seed AUGGCAC)	1.4	TargetScan Human	XK	-7.9	0.00
hsa-miR-183-5p	miR-183-5p (miRNAs w/seed AUGGCAC)	1.4	TargetScan Mouse	AKAP6	-1.0	0.00
hsa-miR-183-5p	miR-183-5p (miRNAs w/seed AUGGCAC)	1.4	TargetScan Human	MPO	-1.6	0.01
hsa-miR-183-5p	miR-183-5p (miRNAs w/seed AUGGCAC)	1.4	TargetScan Human	ERICH1	-0.7	0.01
hsa-miR-183-5p	miR-183-5p (miRNAs w/seed AUGGCAC)	1.4	TargetScan Human	RAD9B	-0.8	0.03

hsa-miR-183-5p	miR-183-5p (miRNAs w/seed AUGGCAC)	1.4	TargetScan Mouse	ADGRL1	-0.7	0.03
hsa-miR-183-5p	miR-183-5p (miRNAs w/seed AUGGCAC)	1.4	TargetScan Human	DHCR24	-0.7	0.03
hsa-miR-183-5p	miR-183-5p (miRNAs w/seed AUGGCAC)	1.4	TargetScan Human	GASK1B	-1.0	0.04
hsa-miR-183-5p	miR-183-5p (miRNAs w/seed AUGGCAC)	1.4	TargetScan Mouse	ABCA13	-1.5	0.04
hsa-miR-183-5p	miR-183-5p (miRNAs w/seed AUGGCAC)	1.4	TargetScan Mouse	FOXRED1	-0.4	0.05
hsa-miR-183-5p	miR-183-5p (miRNAs w/seed AUGGCAC)	1.4	TargetScan Mouse,TargetScan Human	TCF7L2	-0.6	0.05
hsa-miR-196b-5p	miR-196a-5p (and other miRNAs w/seed AGGUAGU)	1.6	TargetScan Mouse	SLC45A1	-1.1	0.01
hsa-miR-196b-5p	miR-196a-5p (and other miRNAs w/seed AGGUAGU)	1.6	TargetScan Mouse	HCN3	-0.9	0.02
hsa-miR-196b-5p	miR-196a-5p (and other miRNAs w/seed AGGUAGU)	1.6	TargetScan Mouse	SCD	-0.9	0.02
hsa-miR-196b-5p	miR-196a-5p (and other miRNAs w/seed AGGUAGU)	1.6	TargetScan Mouse	RAD9B	-0.8	0.03

hsa-miR-196b-5p	miR-196a-5p (and other miRNAs w/seed AGGUAGU)	1.6	TargetScan Mouse	BIRC5	-1.1	0.04
hsa-miR-196b-5p	miR-196a-5p (and other miRNAs w/seed AGGUAGU)	1.6	TargetScan Human	COL8A2	-0.7	0.05
hsa-miR-196b-5p	miR-196a-5p (and other miRNAs w/seed AGGUAGU)	1.6	TargetScan Mouse	MMP8	-1.8	0.05
hsa-miR-223-3p	miR-223-3p (miRNAs w/seed GUCAGUU)	1.6	TargetScan Mouse	BEAN1	-1.3	0.00
hsa-miR-223-3p	miR-223-3p (miRNAs w/seed GUCAGUU)	1.6	TargetScan Human	OLR1	-2.7	0.00
hsa-miR-223-3p	miR-223-3p (miRNAs w/seed GUCAGUU)	1.6	TargetScan Mouse	CRMP1	-1.0	0.03
hsa-miR-223-3p	miR-223-3p (miRNAs w/seed GUCAGUU)	1.6	TargetScan Mouse	GASK1B	-1.0	0.04
hsa-miR-223-3p	miR-223-3p (miRNAs w/seed GUCAGUU)	1.6	TargetScan Mouse	BIRC5	-1.1	0.04
hsa-miR-342-3p	miR-342-3p (miRNAs w/seed CUCACAC)	1.7	TargetScan Mouse	OLR1	-2.7	0.00
hsa-miR-342-3p	miR-342-3p (miRNAs w/seed CUCACAC)	1.7	TargetScan Mouse	DOCK7	-0.7	0.02
hsa-miR-342-3p	miR-342-3p (miRNAs w/seed CUCACAC)	1.7	TargetScan Mouse	SH3RF1	-1.0	0.04

hsa-miR-942-5p	miR-12202-3p (and other miRNAs w/seed CUUCUCU)	1.3	TargetScan Human	AKAP6	-1.0	0.00
hsa-miR-942-5p	miR-12202-3p (and other miRNAs w/seed CUUCUCU)	1.3	TargetScan Mouse	HYAL3	-0.7	0.02
hsa-miR-942-5p	miR-12202-3p (and other miRNAs w/seed CUUCUCU)	1.3	TargetScan Mouse	H6PD	-0.5	0.04
hsa-miR-942-5p	miR-12202-3p (and other miRNAs w/seed CUUCUCU)	1.3	TargetScan Mouse	SYT11	-0.5	0.05
hsa-miR-942-5p	miR-12202-3p (and other miRNAs w/seed CUUCUCU)	1.3	TargetScan Human	TCF7L2	-0.6	0.05

Appendix 4: Summary of data that added to current knowledge and novel findings

Known and Novel miRNA Data				
miRNA	**Known to MS**	**Novel to MS**	**Known Sex Difference**	**Novel Sex Difference**
mir-1286	X			X
miR-664b-3p	X			X
miR-144	X			X
miR-146a	X			X
miR-196b-5p	X		X	
miR-223-3p	X		X	
miR-26b-5p	X			X
miR-296-5p		X		X
miR-29a-3p	X			X
miR-30d-5p	X			X
miR-342-3p		X		X
miR-363-3p		X	X	
miR-150	X			X
miR-4536-5p				

miR-485-3p	X			X
miR-183-5p	X			
miR-941		X		X

Known and Novel miRNA-mRNA Interactions					
mRNA	**Novel miRNA target**	**Known to MS**	**Novel to MS**	**Known Sex Difference**	**Novel Sex Difference**
CNTNAP1	X	X			X
NAT8L	X	X			X
FFAR2	X	X			X
TLR3	X	X			X
CARD16	X		X		X
MELK	X	X			X
JAK3	X	X			X
MAPK3	X	X			X
GSMT4	X		X		X
MMP8	X	X			X
MMP21	X	X			X

DLGAP2	X	X			X
COL5A1	X	X			X
MPO	X	X			X
UQCRB	X	X			X
DPYSL4	X		X		X
TCF4	X	X			X