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Differential microRNA Expression Reveals Sex-Specific Neuroinflammatory and Neurodegenerative Pathways in MS

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Abstract

Relapsing–remitting multiple sclerosis (RRMS) exhibits clear sex-based disparities: females have higher incidence and inflammatory activity, whereas males have lower incidence, yet experience an accelerated disease course of neurodegeneration and disability accumulation. MicroRNAs (miRNAs). Key epigenetic regulators of immune and neuronal pathways may contribute to these differences, but sex is rarely examined as a primary biological variable in MS miRNA research. This exploratory pilot study characterized sex-specific miRNA expression in treatment-naïve individuals with RRMS using samples from 46 participants (17 males and 17 females with RRMS; 6 male and 6 female healthy controls) obtained from the Accelerated Cure Project biorepository. miRNA expression was quantified using the NanoString nCounter Human v3 panel, and differential expression ($p < 0.05$, fold-change ≥1.3) was evaluated across four comparisons: Multiple sclerosis (MS) females vs. MS males; sex-matched MS vs. control groups; and female vs. male healthy controls. Distinct sex-biased miRNA signatures emerged. Males with RRMS demonstrated dysregulation of immune-associated miRNAs, including coordinated changes in the miR-29, miR-30, and miR-199 families. Females with RRMS exhibited consistent downregulation of miR-941 and differential expression of immune-associated miRNAs including miR-223-3p, miR-485-3p, and miR-199a-5p. Several immune-regulatory miRNAs, including miR-223-3p, miR-146a-5p, miR-196b-5p, and miR-183-5p, were differentially expressed in both healthy controls and disease-related comparisons, suggesting that sexually dimorphic immune regulatory programs may precede disease onset. These findings indicate that disease-associated miRNA alterations are superimposed on pre-existing sex-specific immune networks and underscore the importance of incorporating sex as a biological variable in biomarker discovery and precision medicine for MS.

Keywords

multiple sclerosis, sex differences, neuroinflammation, neurodegeneration, epigenetics, microRNA

Introduction

Multiple Sclerosis (MS) is a chronic inflammatory and neurodegenerative disease of the central nervous system (CNS) driven by interacting genetic and environmental factors (Bove & Chitnis, 2013). Most cases begin in early adulthood, typically between ages 20 and 40, causing cognitive and physical disability that can significantly affect quality of life, employment, and financial stability from a relatively young age (Bove & Chitnis, 2013). Relapsing-remitting MS (RRMS) is the most common subtype, accounting for approximately 85% of newly diagnosed cases (Lublin et al., 2014). Although RRMS is about three times more common in females, males are more likely to experience aggressive disease with greater disability, more pronounced brain atrophy, and higher mortality (Bove & Chitnis, 2013; Harbo et al., 2013; Lublin et al., 2014; Magyari & Koch-Henriksen, 2022; Voskuhl, 2020). Clarifying the mechanisms that drive these sex-based disparities is essential to reduce outcome disparities and move toward more precise, individualized care.

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Genome-wide association studies (GWAS) have identified over 200 genetic risk variants, many within pathways related to B, T, natural killer, and myeloid cell differentiation that contribute to MS susceptibility and course (Han et al., 2020; Huang et al., 2017; Lublin et al., 2014; Parnell & Booth, 2017; Patsopoulos et al., 2019). However, these variants each confer modest risk, and together they do not fully explain the observed heterogeneity in MS presentation or the marked sex differences in disease trajectory. Recent studies, including Buxhoeveden et al. (2025), highlights that genetic risk alone cannot explain observed clinical patterns. These findings point toward epigenetic regulation as a key layer shaping sex-specific disease biology.

Epigenetic mechanisms, including microRNAs (miRNAs), offer a dynamic interface between static genomic risk and clinical phenotype (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). miRNAs are small non-coding RNAs that regulate up to 60% of human genes and coordinate numerous biological processes, including immune responses and neuronal homeostasis (Shansky, 2015). Dysregulated miRNAs are implicated in autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus, as well as in MS (Shansky, 2015).

Several miRNAs have previously been implicated in MS pathophysiology and may contribute to neuroinflammation, immune dysregulation, and neurodegeneration. For example, miR-146a-5p regulates innate immune responses and inflammatory signaling pathways, while miR-150-5p has been associated with B-cell function, disease activity, and cognitive outcomes in MS (Bergman et al., 2016; Dominguez-Mozo et al., 2022; Martinelli-Boneschi et al., 2012; Scaroni et al., 2022). Similarly, miR-223-3p has been linked to myeloid cell differentiation, microglial activation, and MS susceptibility, whereas members of the miR-29 family have been associated with immune regulation and neurodegenerative processes (Kiselev et al., 2015; Tufekci et al., 2010). Together, these findings suggest that miRNAs are involved in multiple biological pathways relevant to MS and may serve as both mechanistic contributors and potential biomarkers of disease activity.

Sex differences in miRNA expression can modulate immune function and inflammation, and sex hormones—including estrogen and testosterone—may further interact with miRNA networks to influence disease susceptibility, inflammatory activity, and neurodegeneration (Dai & Ahmed, 2014; Li et al., 2020; Shansky, 2015). Despite this biologic plausibility, relatively few studies have explicitly examined sex as a biological variable in miRNA profiling of MS, leaving a critical gap in understanding how miRNA-mediated pathways may diverge between males and females.

A more precise understanding of sex-specific pathways may help explain why some individuals, particularly males, experience accelerated neurodegeneration, while females often exhibit a stronger inflammatory profile but slower long-term decline (Buxhoeveden et al., 2025). These insights have practical implications for symptom monitoring, patient education, and personalized care, which are foundational to nursing science and clinical practice.

The purpose of this exploratory pilot study was to begin addressing the gap by characterizing sex-based disparities in MS at the miRNA level. Specifically, we aimed to identify differentially expressed miRNAs in treatment-naïve males and females with RRMS, as well as healthy male and female controls, to determine which miRNAs and pathways may underlie clinically observed differences in disease susceptibility and severity. We hypothesized that miRNAs expression patterns would diverge by sex along pathways central to neuroinflammation and neurodegeneration.

Methods

Sex as a Biological Variable (SABV)

Incorporating SABV is essential in MS research to capture differences in disease mechanisms and treatment responses between sexes. Because males are underrepresented in MS studies, gaps remain in understanding sex-specific differences. The repository blood samples used in this study were not genotyped before shipment; therefore, chromosomal sex was confirmed prior to analysis. Expression of the X-inactive specific transcript (XIST) gene — required for X-chromosome inactivation — was used to verify that samples with XIST expression were XX and that no male samples carried an additional X chromosome (XXY). This step ensured exclusion of Klinefelter syndrome (XXY), which can increase risk for conditions more common in females.

To improve read alignment on sex chromosomes, we used a custom sex-informed reference genome. Given homology between X and Y, homologous regions can misalign. For XX samples, the Y chromosome was masked, increasing sensitivity for X-linked transcripts. Together, these steps aimed to enhance the precision of differential expression estimates on sex chromosomes, an often-overlooked source of bias in MS transcriptomic studies.

Sample Size and Participants

A priori power analysis was conducted using the R package RNASeqSampleSize (version 2.12.0), which estimated that a minimum of 15 samples per group would be required to detect a 3-fold change in gene expression ($\log2FC \approx 1.6$) with 80% power at an FDR of 0.05, assuming a minimum average read count of 10, maximum dispersion of 0.5, and a testing space of 10,000 genes. While most biologically meaningful changes in complex diseases like MS are smaller (e.g., 1.3–1.5 fold), this analysis provides a benchmark for sample size adequacy under idealized assumptions. This pilot study included

48 samples (18 MS males, 18 MS females, and 12 healthy controls).

Samples

All participants provided informed consent through the Accelerated Cure Project biorepository, including permission for genomic, transcriptomic, and other molecular analyses of their samples. To best isolate the effects of sex, the study focused on a homogenous cohort that controlled for age, ethnicity, and treatment status. A total of 48 treatment-naïve Caucasian patients aged 20–45 years were selected from the Accelerated Cure Project biorepository (18 males with MS, 18 females with MS, and 12 healthy controls). These criteria minimized potential confounders: age was restricted to reduce variability in disease course, ethnicity was limited to Caucasian patients to control for genetic heterogeneity, and only treatment-naïve participants were included since disease-modifying therapies can alter miRNA expression. Two blood samples—one from the female MS group and one from the male MS group—were excluded due to poor RNA quality and incomplete clinical data, yielding a final dataset of 46 samples.

RNA Extraction/Sample Preparation

Total RNA was extracted from whole blood (PAXgene tubes) using the Qiagen PAXgene Blood miRNA Kit (Germantown, MD) per manufacturer protocol. To mitigate oversaturation of the NanoString cartridge caused by high-abundance miR-451, 5 μL of a DNA attenuation oligo targeting miR-451 was added to each sample, thereby improving detection of less abundant transcripts. RNA concentration and purity were assessed using a Nanodrop spectrophotometer (ThermoFisher, Waltham, MA) and Promega Maxwell® Quantus fluorometer (Madison, WI). RNA integrity was evaluated with the Agilent 2100 Bioanalyzer (Santa Clara, CA), and samples with RNA integrity number (RIN) ≥7 and yields above 40 ng/μL were retained for analysis. Extracted RNA was aliquoted to avoid repeated freeze-thaw cycles and stored at −80°C until use in NanoString nCounter assays.

miRNA Profiling

miRNA analysis was performed using the Nanostring nCounter™ platform Human v3 miRNA Expression Assay (NanoString Technologies, Bruker Spatial Biology Inc., Bothell, WA, USA) which includes probes for >800 human miRNAs, all of which were evaluated. Candidate miRNAs discussed in the manuscript reflect unbiased differential expression emerging from the four pre-specified statistical comparisons. The NanoString assay utilizes molecular barcodes to detect miRNAs without the use of amplification. During processing sequence-specific miRNA-tags bind to target miRNAs using a ligase enzyme, and unbound tags are then removed through a clean-up step. Reporter and Capture CodeSets are then added to each sample and the samples were hybridized overnight for 18 hours at 65°C to form target-probe complexes. Immediately after hybridization the samples were 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 was performed on each cartridge, and raw read counts were processed into RCC files, which were then downloaded from the digital analyzer and imported into the nSolver Analysis Software.

miRNA Data Processing and Differential Expression

Raw miRNA counts were processed using NanoString nSolver Analysis Software. Normalization was performed using positive and negative controls included in the code set, along with three reference ("housekeeping") genes (ACTB, GAPDH, and RPL19) selected based on their low variability (%CV). The NanoString Human v3 miRNA assay includes six synthetic positive controls (POS_A–F) spanning a range of concentrations. Consistent with NanoString guidance, POS_F was excluded from normalization because its counts are close to background levels regardless of sample or study type. Therefore, as per Nanostring guidance, assay performance was confirmed using the remaining five positive controls (POS_A–E), which demonstrated acceptable linearity across all samples. In addition, all samples met established quality control criteria for imaging performance, hybridization efficiency, and binding density, supporting the reliability of the generated expression data. Normalization was performed using the geometric mean of the five positive A-E controls. The background threshold was set to the mean of the negative controls (excluding NEG_C) plus two standard deviations. NEG_C was excluded because NanoString has reported that counts for this control are highly variable across species and should not be included in background threshold calculations.

Differential expression analysis was performed using NanoString's nSolver software package (reported as "ratio data" within the program) using four comparison groups: (1) MS males versus healthy male controls, (2) MS females versus healthy female controls, (3) MS females versus MS males, and (4) healthy females versus healthy males. Each participant sample was hybridized, scanned, and quantified individually; no biological pooling occurred. Although nSolver reports group-level "ratio data" for differential expression analyses, all results were derived from purified RNA derived from individual blood samples. Differential expression was defined as $p < 0.05$ and fold change (FC) ≥ 1.3.

Positive FC indicates upregulation relative to the comparator; negative FC indicates downregulation. All miRNAs meeting $p < 0.05$ and FC ≥1.3 for a given comparison were included in the respective tables. Non-significant miRNAs were included in the analysis but omitted from tables for clarity. The Venn diagram illustrates overlap in differential miRNA expression among the four analyses (Figure 3).

These four comparisons were selected to distinguish disease-related changes from intrinsic biological sex differences. Specifically, same-sex case-control comparisons (MS males versus healthy male controls and MS females versus healthy female controls) were used to identify disease-associated alterations. The cross-sex comparison within MS (MS females versus MS males), was used to examine sex differences in the disease state, while the cross-sex comparison in healthy controls (healthy females versus healthy males) was used to identify baseline physiological sex differences independent of MS.

Results

Demographic Characteristics

Table 1 summarizes participant characteristics. Groups were similar in age (means 32.0–33.3 years). Disease duration was modestly longer in males (mean 0.9 years) than females (mean 0.4 years); all MS participants were newly diagnosed and treatment-naïve.

MS Females vs. MS Males. This comparison was performed to identify sex differences in miRNA expression within the disease state. Fourteen miRNAs were differentially expressed between MS females and MS males (Table 2). Compared with MS males, seven miRNAs were downregulated in MS females, including miR-941, miR-5196-3p, miR-548n, miR-4536-5p, miR-627-5p, miR-1246, and miR-519b-5p. Conversely, seven miRNAs were upregulated in MS females, including miR-1286, miR-532-3p, miR-1180-3p, miR-296-5p, miR-199a-5p, miR-223-3p, and miR-485-3p. Among the differentially expressed miRNAs, miR-941 demonstrated the largest decrease in expression (log2FC = −2.4, $p < 0.01$), whereas miR-485-3p demonstrated the largest increase in expression (log2FC = 1.7, $p < 0.01$).

MS Females vs. Female Controls. This comparison was performed to identify disease-related changes specific to females. Five miRNAs were differentially expressed between MS females and healthy female controls (Table 3). Compared with healthy female controls, two miRNAs were downregulated in MS females, including miR-941 and miR-216b-5p. Conversely, three miRNAs were upregulated in MS females, including miR-1286, miR-4741, and miR-548c-5p. Among the differentially expressed miRNAs, miR-941 demonstrated the largest decrease in expression in MS females relative to healthy female controls (log2FC = −2.7, $p = 0.02$).

MS Males vs. Male Controls. This comparison was performed to identify disease-related changes specific to males. Twelve miRNAs were differentially expressed in MS males compared with healthy male controls (Table 4). Compared with healthy male controls, all twelve differentially expressed miRNAs were upregulated in MS males, including miR-146a-5p, miR-196b-5p, miR-183-5p, miR-302b-3p, miR-30e-5p, miR-29a-3p, miR-361-5p, miR-199a-3p, miR-29c-3p, miR-30d-5p, miR-324-5p, and miR-28-3p. No miRNAs were significantly downregulated in MS males relative to healthy male controls. Among the differentially expressed miRNAs, miR-146a-5p and miR-196b-5p demonstrated the largest increases in expression (log2FC = 1.7, $p < 0.01$ for both).

Female vs. Male Controls. This comparison was performed to identify baseline physiological sex differences independent of disease. Thirteen miRNAs were differentially expressed between healthy female and male controls (Table 5). Compared with healthy male controls, all thirteen differentially expressed miRNAs were upregulated in healthy female controls, including miR-342-3p, miR-196b-5p, miR-223-3p, miR-145-5p, miR-151a-3p, miR-182-5p, miR-29a-3p, miR-146a-5p, miR-150-5p, miR-361-5p, miR-625-5p, miR-183-5p, and miR-942-5p. Among the differentially expressed miRNAs, miR-342-3p, miR-196b-5p, and miR-223-3p demonstrated the largest increases in expression (log2FC = 1.8), with miR-

Table 1. Study Sample Demographics

Group	Multiple sclerosis		Healthy control	
Sex	Male (n = 17)	Female (n = 17)	Male (n = 6)	Female (n = 6)
Age Median (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
Ethnicity- Caucasian	17 (100%)	17 (100%)	6 (100%)	6 (100%)

Table 2. Differentially Expressed miRNAs for MS Female vs MS Male Cases

Probe name	Mean count MS females	Mean count MS males	Log_2FC	*p*-value
hsa-miR-941	63.7	149.6	−2.4	<0.01
hsa-miR-5196-3p*	41.3	67.3	−1.6	0.01
hsa-miR-548n	40.8	66.5	−1.6	0.05
hsa-miR-4536-5p	40.9	59.6	−1.5	0.03
hsa-miR-627-5p	46.4	66.9	−1.4	0.04
hsa-miR-1246	40.2	57.4	−1.4	0.05
hsa-miR-519b-5p*	39.9	51.9	−1.3	0.04
hsa-miR-1286	67.7	51.6	1.3	0.03
hsa-miR-532-3p	138.8	105.3	1.3	0.04
hsa-miR-1180-3p	476.9	341.5	1.4	0.04
hsa-miR-296-5p	901.1	636.3	1.4	0.05
hsa-miR-199a-5p	229.5	160.2	1.4	0.04
hsa-miR-223-3p	144,476.3	92,019.1	1.6	0.04
hsa-miR-485-3p	142.7	82.5	1.7	<0.01

Note. MS: Multiple Sclerosis cases; Probe Name: miRNA gene name; Mean Count: geometric mean miRNA counts for each group; Log2FC: Log to the base 2 fold change between MS female and male cases, respectively. Negative (−) Log2FC values signify the miRNA count was higher in MS male cases. *indicates that the miR has multiple names, for clarity we are listing one, but the others can be found in Supplemental Table 6.

196b-5p exhibiting the strongest statistical significance ($p = 0.01$).

Across all four comparisons, 36 unique miRNAs were differentially expressed. Although some transcripts were unique to individual comparisons, several—including miR-223-3p, miR-146a-5p, miR-183-5p, and miR-196b-5p—recurred across healthy and disease-related analyses, while miR-941 showed consistent female-associated downregulation. These relationships are further illustrated in the boxplot comparisons in Figure 1(A)–(D), Figure 2(A)–(D), and Figure 3, that show the intersection of differentially expressed miRNAs across all four comparisons.

Discussion

This exploratory, pilot study tested for and identified sex-biased miRNA expression patterns in RRMS that cohere with known clinical differences between males and females. Key themes include sex-specific immune regulation, distinct disease-associated molecular signatures in males and females, and substantial overlap between baseline sex differences and disease-associated miRNA expression.

MS Females vs. Female Controls

Compared with healthy female controls, five miRNAs were differentially expressed in females with MS (Table 3). The most notable finding was the downregulation of miR-941, which was also reduced in MS females compared with MS males (Table 2) and healthy controls and was one of several miRs consistently associated with the MS female phenotype. (Figure 1(A)). Although miR-941 has not previously been implicated in MS, it is a human-specific miRNA that is highly expressed in the brain and has been shown to regulate genes involved in cellular differentiation, neurotransmitter signaling, and key developmental pathways (Hu et al., 2012). More recent studies also suggest that miR-941 participates in immune regulation through effects on T-cell differentiation and neutrophil polarization and may influence oxidative stress responses through the Keap1/Nrf2 signaling pathway, although these findings have thus far been reported outside the

Table 3. Differentially Expressed miRNAs for MS Females vs Female Controls

Probe name	Mean count MS females	Mean count control females	Log2FC	*p*-value
hsa-miR-941	63.7	169.3	−2.7	0.02
hsa-miR-216b-5p	48.9	68.1	−1.4	0.04
hsa-miR-1286	67.7	48.9	1.4	0.01
hsa-miR-4741	59.0	42.0	1.4	<0.01
hsa-miR-548c-5p*	58.9	43.6	1.4	0.01

Note. MS: Multiple Sclerosis cases; Probe Name: miRNA gene name; Mean Count: geometric mean miRNA counts for each group; Log2FC: Log to the base 2 fold change between MS female cases, and female healthy controls, respectively. Negative (−) Log2FC values signify that the miRNA count was higher in female healthy controls.

Table 4. 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	127.7	74.5	1.7	<0.01
hsa-miR-196b-5p	89.3	54.3	1.7	<0.01
hsa-miR-183-5p	193.8	118.8	1.6	0.02
hsa-miR-302b-3p	135.4	84.7	1.6	0.04
hsa-miR-30e-5p	170.0	109.7	1.6	0.02
hsa-miR-29a-3p	117.2	76.8	1.5	0.01
hsa-miR-361-5p	136.3	93.4	1.5	0.04
hsa-miR-199a-3p*	80.5	56.2	1.4	0.04
hsa-miR-29c-3p	80.3	57.3	1.4	0.05
hsa-miR-30d-5p	3,424.3	2494.4	1.4	0.04
hsa-miR-324-5p	73.0	53.1	1.4	0.03
hsa-miR-28-3p	68.5	51.5	1.3	0.02

Note. MS: Multiple Sclerosis cases; Probe Name: miRNA gene name; Mean Count: geometric mean miRNA counts for each group; Log2FC: Log to the base 2 fold change between MS male cases, and male healthy controls, respectively. Positive (+) Log2FC values signify that that miRNA count was higher in MS male cases.

context of MS (Fotovat et al., 2024; Huang et al., 2026; Zhang et al., 2025). Its consistent downregulation across female-specific comparisons in our study suggests that miR-941 may represent a previously unrecognized sex-associated molecular signature worthy of further investigation.

While miR-941 was downregulated in MS females, miR-1286 expression was increased in MS Females as compared to MS males and healthy controls (Figure 1(B)). While the biological function of miR-1286 remains incompletely characterized, its appearance across multiple female-associated comparisons suggests that it may be involved in pathways relevant to sex-specific disease biology (Figure 1(B), Tables 2 and 3). Additional differentially expressed miRNAs included miR-216b-5p, miR-4741, and members of the miR-548 family. Although the role of miR-216b-5p in MS remains poorly understood, its precursor transcript has been identified among miRNAs whose expression changes in circulating CD4+ T cells following fingolimod treatment, suggesting it may participate in immune pathways responsive to disease-modifying therapy (Chen et al., 2018). The functions of miR-4741 and the miR-548 family likewise remain largely unexplored in MS.

MS Males vs. Male Controls

In contrast to females, the miRNA profile observed in males was dominated by transcripts with established or emerging roles in immune regulation and neuroinflammatory signaling, although several differentially expressed miRNAs remain poorly characterized (Table 4). Among the most notable findings was the upregulation of miR-146a-5p (Figure 1(D)),

Table 5. 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	2263.0	1,256.7	1.8	0.05
hsa-miR-196b-5p	94.8	54.3	1.8	0.01
hsa-miR-223-3p	110,038.7	62,794.3	1.8	0.04
hsa-miR-145-5p	170.7	98.7	1.7	0.03
hsa-miR-151a-3p	716.7	434.0	1.7	0.05
hsa-miR-182-5p	312.5	189.1	1.7	0.01
hsa-miR-29a-3p	123.2	76.8	1.6	0.04
hsa-miR-146a-5p	118.6	74.5	1.6	0.01
hsa-miR-150-5p	43,959.2	28,004.8	1.6	0.05
hsa-miR-361-5p	148.3	93.4	1.6	0.05
hsa-miR-625-5p	88.8	56.5	1.5	0.04
hsa-miR-183-5p	179.9	118.8	1.5	0.03
hsa-miR-942-5p	990.0	693.6	1.4	0.04

Note. Probe Name: miRNA gene name; Mean Count: geometric mean miRNA counts for each group; Log2FC: Log to the base 2 fold change between female and male healthy controls, respectively. Log2FC values signify that that miRNA count was higher in female v. male healthy controls.

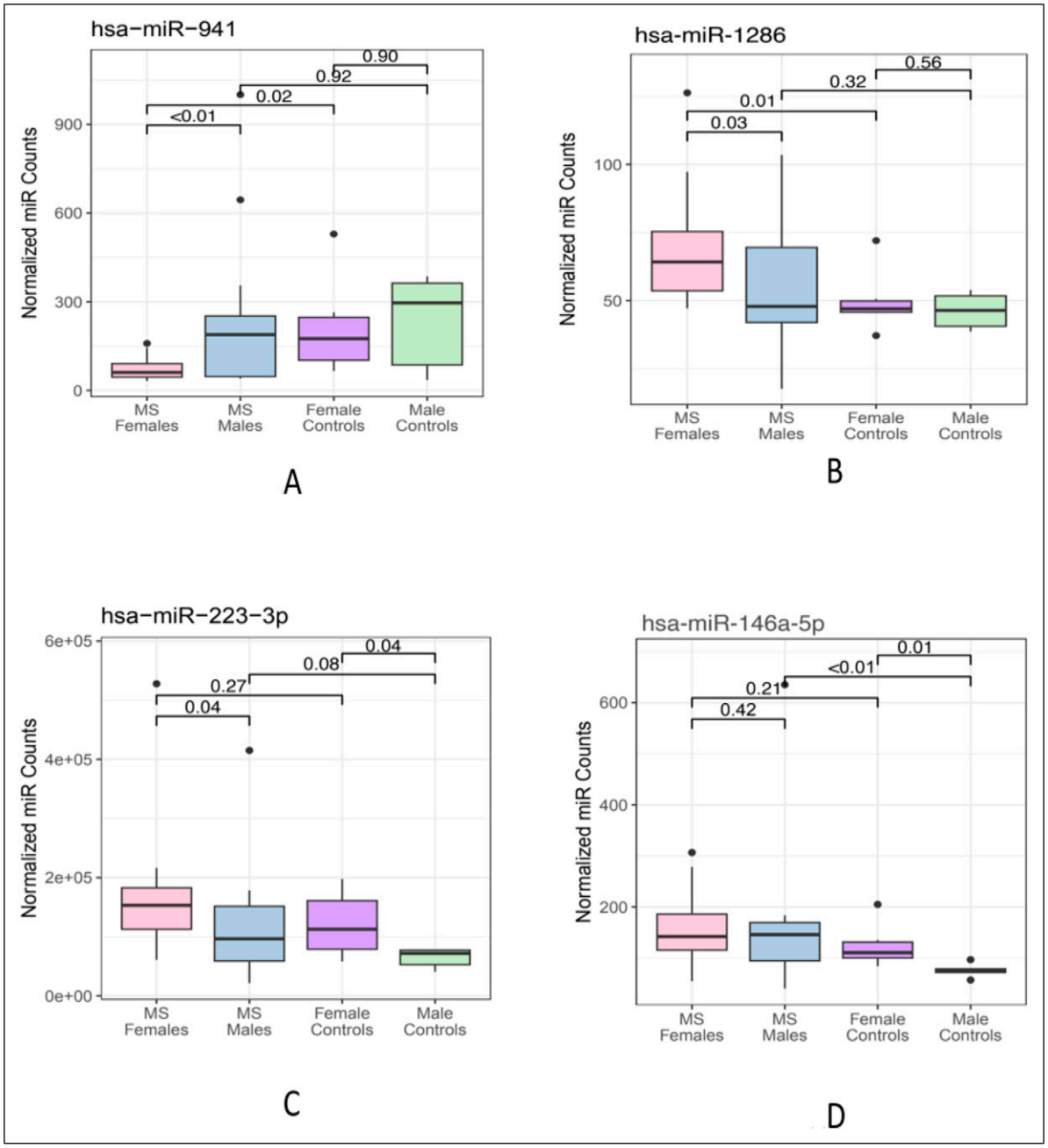

Figure 1. (A–D): Boxplots of exemplar miRNAs differentially expressed by sex between cases and controls. (A): miR-941 has lower expression in MS Females as compared to MS Males and controls. (B): miR-1289 has higher expression in MS Females as compared to MS Males and controls. (C): miR-223-3p has higher expression in MS Females as compared to MS Males and controls. (D): miR-146a-5p has higher expression in MS Males v. male controls, and in female v. male controls

a well-characterized regulator of innate immune responses that functions as a negative feedback modulator of inflammatory signaling pathways, including NF-κB (Dominguez-Mozo et al., 2022; He et al., 2023). Altered expression of miR-146a-5p has been reported in MS and other autoimmune diseases, where it is thought to influence cytokine production, immune cell activation, and neuroinflammatory processes (Dominguez-Mozo et al., 2022; He et al., 2023). As a negative regulator of NF-κB signaling, increased miR-146a expression may also reflect a compensatory response to chronic immune activation.

Additional immune-associated miRNAs included miR-196b-5p and miR-183-5p (Table 4). miR-183-5p belongs to the miR-183/96/182 cluster, which has been implicated in immune activation, pro-inflammatory cytokine pathways, and autoimmune disease (Ichiyama & Dong, 2019). Although less

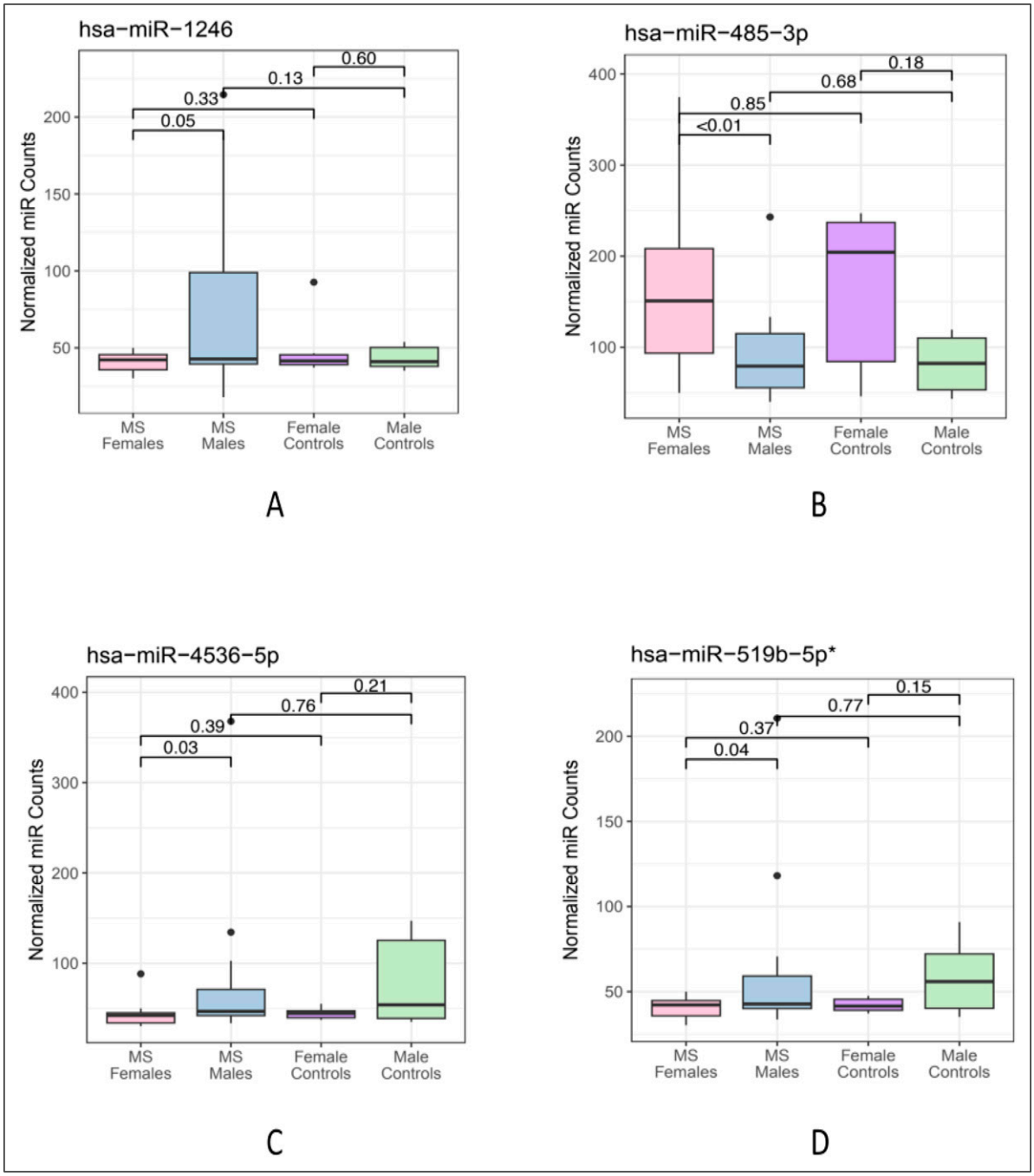

Figure 2. (A–D): Boxplots of exemplar miRNAs differentially expressed by sex between cases only. (A): miR-1246 has lower expression in MS Females as compared to MS Males. (B): miR-485-3p has higher expression in MS Females as compared to MS Males. (C): miR-4536-5p has lower expression in MS Females as compared to MS Males. (D): miR-519b-5p as lower expression in MS Females as compared to MS Males

is known about miR-196b-5p in MS, emerging evidence suggests that miR-196b participates in immune regulation, including myeloid differentiation and inflammatory signaling pathways such as TLR7/8 signaling (Hu et al., 2022; Velu et al., 2009). Notably, both miR-196b-5p and miR-183-5p were also differentially expressed between healthy female and male controls, suggesting that these transcripts may reflect an interaction between intrinsic biological sex differences and disease-associated immune responses.

Several members of the miR-29 and miR-30 families were also differentially expressed, including miR-29a-3p, miR-29c-3p, miR-30d-5p, and miR-30e-5p (Table 4). The

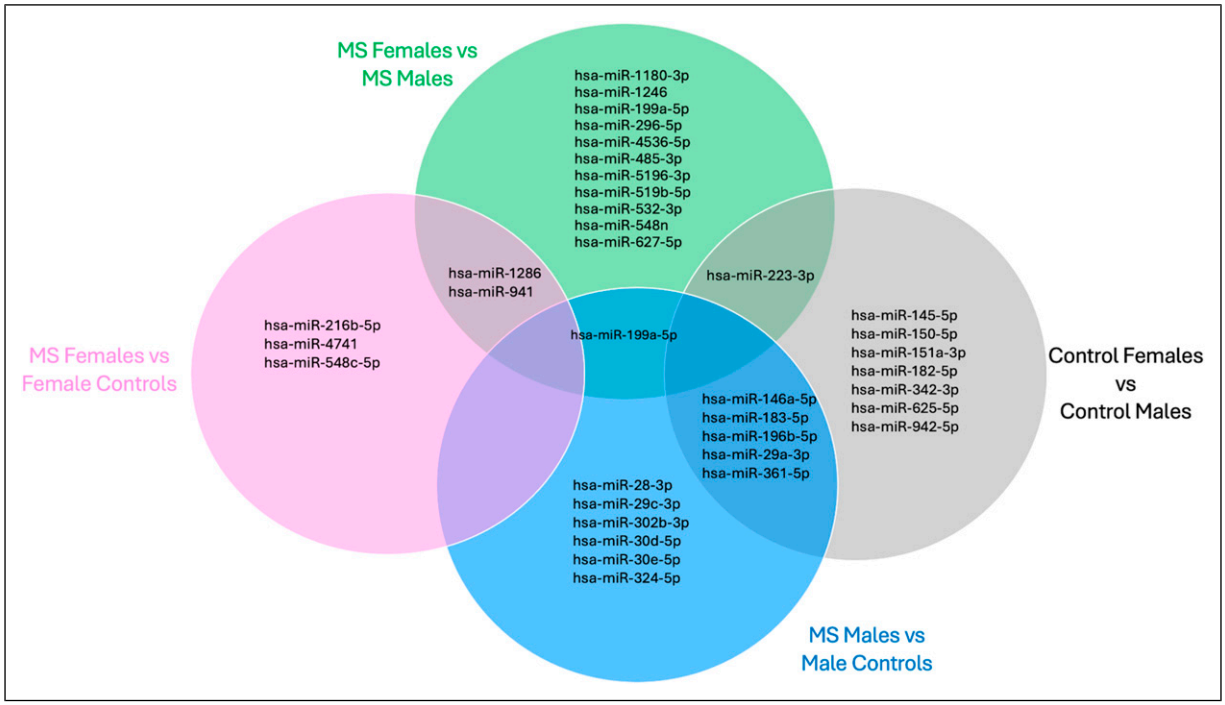

Figure 3. Venn diagram of differentially expressed miRNAs across comparison groups

differential expression of multiple members of the miR-29 and miR-30 families suggests coordinated regulation of shared biological pathways rather than isolated transcriptional events. The miR-29 family has established relevance in MS, with both miR-29a-3p and miR-29c-3p shown to be differentially expressed following interferon-β therapy, supporting roles in immune regulation, interferon signaling, and treatment-responsive pathways (Hecker et al., 2013). Evidence supporting the miR-30 family in MS is less extensive; however, miR-30e-5p has been identified as differentially expressed across independent MS cohorts (Regev et al., 2018), and the miR-30 family more broadly has been implicated in immune regulation, apoptosis, autophagy, and CNS injury models, including regulation of neuroinflammation and macrophage polarization (Mao et al., 2018; Visintin & Ray, 2022).

Additional differentially expressed transcripts included miR-199a-3p/199b-3p, miR-302b-3p, miR-324-5p, and miR-28-3p (Table 4). Of these, the miR-199 family is particularly notable because previous studies have linked circulating miR-199a-5p to clinical disability in MS. In a multicenter study of untreated individuals with MS, serum miR-199a-5p correlated with Expanded Disability Status Scale (EDSS) across independent international cohorts (Regev et al., 2018). In this study, members of the miR-199 family were differentially expressed in both the male disease comparison (miR-199a-3p/199b-3p) and the female-versus-male comparison (miR-199a-5p), suggesting that dysregulation of this family may extend beyond disease severity and potentially contribute to sex-specific molecular pathways in RRMS.

Cross-Sex Comparisons: MS Females vs. MS Males

The comparison between MS females and MS males revealed the largest number of differentially expressed miRNAs and provided the clearest evidence of sex-specific molecular differences within the disease state (Table 2, Figure 1(A)). Among these findings, miR-941 remained one of the most notable transcripts, with significantly lower expression in MS females than MS males. Its recurrence across female-associated comparisons suggests that reduced miR-941 expression may represent a stable feature of the female RRMS molecular phenotype rather than an isolated comparison-specific finding.

Additionally, miR-1246, miR-4536-5p, and miR-519b-5p expression was lower in MS Females than MS males (Figure 2(A), (C) and (D), respectively). miR-1246 has been implicated in immune dysregulation in autoimmune disease. In systemic lupus erythematosus, for instance, it is thought that repression of multiple genes by miR-1246 ultimately leads to overactive B cell function (Luo et al., 2015). Although miR-519b-5p has not previously been identified in MS studies, miR-4536-5p has been described in MS (Buxhoeveden et al., 2025) and garnered recognition as a potential biomarker found in EVs that may distinguish Myelin

Oligodendrocyte Glycoprotein Antibody-Associated Disease (MOGAD) from Multiple Sclerosis (Kim et al., 2025).

Several immune-associated miRNAs were expressed at higher levels in females than males. Among these, miR-223-3p demonstrated one of the most biologically compelling patterns (Table 2, Figure 1(C)). miR-223 is a well-established regulator of immune-cell differentiation and inflammation, with reported roles in granulocyte, dendritic cell, lymphocyte, and macrophage biology (Jiao et al., 2021). In MS specifically, miR-223 has been implicated in myeloid-derived suppressor cell function and inflammatory regulation, and MIR223 variants have also been associated with MS susceptibility in a sex-specific manner (Cantoni et al., 2023; Kiselev et al., 2015).

Additional miRNAs elevated in MS Females v. MS Males included miR-485-3p (Figure 2(B), Table 2) and miR-199a-5p (Supplemental Figure 4, Table 2). miR-485-3p demonstrated one of the largest fold changes observed in the study and has emerging relevance to MS-related immune biology. In experimental autoimmune encephalomyelitis, miR-485 was shown to regulate Th17 generation through STAT3 targeting, with miR-485 overexpression reducing Th17-associated cytokines and mitigating disease severity (Xue et al., 2023). Broader neurodegenerative disease literature also implicates miR-485 in neuroinflammation, apoptosis, and synaptic function, although its role in human MS remains incompletely defined (Ryu et al., 2023). Similarly, miR-199a-5p was elevated in females, while other members of the miR-199 family were differentially expressed in the male disease comparison, suggesting that coordinated regulation of this miRNA family may contribute to sex-specific disease biology.

MS females also demonstrated increased expression of miR-1286 (Figure 1(B), Tables 2 and 3) relative to MS males, mirroring its elevation in the comparison between MS females and healthy female controls. Because miR-1286 remains poorly characterized in MS and immune biology, this finding should be interpreted cautiously. However, its recurrence across female-associated comparisons suggests that it may represent a reproducible, hypothesis-generating feature of the female RRMS molecular phenotype.

Baseline Differences: Female Controls vs. Male Controls

Several miRNAs were differentially expressed between healthy female and male controls, indicating that substantial sex-associated molecular differences exist even in the absence of disease (Table 5, Supplemental Figure 4). Among the most notable were miR-223-3p (Figure 1(C)), miR-146a-5p (Figure 1(D)), miR-196b-5p, and miR-183-5p, all of which have established or emerging roles in immune regulation, inflammatory signaling, immune-cell differentiation, and MS pathophysiology (Cantoni et al., 2023; Dominguez-Mozo et al., 2022; Hu et al., 2022; Ichiyama & Dong, 2019; Jiao et al., 2021; Velu et al., 2009). Notably, several of these immune-associated miRNAs were also identified in one or more disease-related comparisons. In particular, miR-223-3p and miR-146a-5p were differentially expressed in both healthy controls and MS comparisons, while miR-183-5p and miR-196b-5p likewise recurred across healthy and disease comparisons. Rather than functioning solely as disease-specific biomarkers, the recurrence of these transcripts suggests that they represent components of baseline sex-associated immune regulatory networks that are subsequently modified or amplified during disease. This interpretation is consistent with broader evidence that males and females exhibit important differences in both innate and adaptive immune responses, contributing to the well-recognized sex differences in susceptibility to autoimmune diseases, including MS (Klein & Flanagan, 2016; Voskuhl, 2020).

Additional miRNAs identified in healthy controls included miR-342-3p, miR-150-5p, miR-361-5p, miR-145-5p, miR-151a-3p, miR-182-5p, miR-625-5p, and miR-942-5p (Supplemental Figure 4). Among these, miR-150-5p has previously been identified as a candidate biomarker of MS disease activity and has also been associated with cognitive impairment through its expression in circulating myeloid extracellular vesicles (Bergman et al., 2016; Scaroni et al., 2022). miR-182-5p is also notable because it belongs to the miR-183/96/182 cluster discussed above, a conserved miRNA family that regulates immune-cell activation, cytokine signaling, and autoimmune responses (Ichiyama & Dong, 2019). Emerging evidence further suggests that miR-342-3p and miR-145-5p participate in neuroinflammatory pathways. miR-342 has been identified in serum exosomal miRNA signatures associated with MS and has been shown to regulate TNF-α-induced microglial activation through NF-κB signaling, linking it to mechanisms of neuroinflammation and neurotoxicity (Brás et al., 2020; Ebrahimkhani et al., 2017). Likewise, modulation of miR-145 attenuated inflammation, immune-cell infiltration, demyelination, and axonal degeneration in experimental autoimmune encephalomyelitis, supporting a potential role in CNS autoimmunity (de Almeida et al., 2025). In contrast, the relevance of miR-361-5p, miR-151a-3p, miR-625-5p, and miR-942-5p to MS remains less well established. Nevertheless, their differential expression in healthy individuals further reinforces the concept that biological sex contributes substantially to baseline variation in circulating miRNA expression.

Integrative Interpretation

This study identified distinct sex-biased miRNA expression patterns in treatment-naïve individuals with RRMS and demonstrates that biological sex contributes substantially to molecular heterogeneity in MS. Although numerous studies

have reported dysregulated miRNAs in MS, relatively few have examined sex as a primary biological variable. By independently evaluating baseline sex-associated differences, disease-associated changes within each sex, and cross-sex differences in RRMS, our study provides a framework for distinguishing these related, but biologically distinct, sources of molecular variation. Consistent with this interpretation, the limited overlap observed among the four comparison groups (Figure 3 Venn Diagram), indicates that baseline sex-associated biology, disease-associated changes, and sex-specific disease responses represent interconnected, but distinct, molecular programs rather than a single shared transcriptional response. These findings support a broader role for biological sex in shaping immune regulation, neuroinflammatory pathways, and miRNA network activity throughout the disease process.

One of the most important observations was the overlap between baseline sex differences and disease-associated findings. Several immune-regulatory miRNAs identified in healthy female versus healthy male controls—including miR-223-3p, miR-146a-5p, miR-196b-5p, and miR-183-5p—also were differentially expressed in one or more disease-related comparisons. Rather than suggesting that MS induces entirely novel molecular programs, these findings support a model in which disease-associated miRNA alterations are superimposed upon pre-existing, sexually dimorphic immune regulatory networks. In this framework, biological sex establishes a distinct molecular landscape that may influence how immune and neuroinflammatory pathways respond once disease develops. This interpretation is consistent with well-established sex differences in innate and adaptive immunity and offers a potential mechanistic explanation for the recognized differences in MS susceptibility, inflammatory activity, disease progression, and long-term outcomes between males and females.

Beyond these baseline differences, coordinated dysregulation of multiple members of the miR-29, miR-30, and miR-199 families emerged across independent comparisons. The recurrence of multiple members within the same miRNA families suggests coordinated regulation of shared biological pathways rather than isolated transcriptional events. Similarly, recurrent identification of immune-associated transcripts—including miR-223-3p, miR-146a-5p, miR-150-5p, miR-183-5p, and miR-196b-5p—supports the concept that sex-specific molecular heterogeneity in RRMS is mediated through complex immune-regulatory and neuroimmune networks rather than individual molecular drivers. Although the functional consequences of these coordinated changes remain to be fully elucidated, they provide a biologically plausible framework through which sex may influence disease susceptibility, inflammatory responses, and neurodegeneration.

Equally noteworthy, the study also identified potentially novel miRNAs that may contribute to sex-specific disease biology. Among these, miR-941 emerged as the most reproducible finding, demonstrating consistent downregulation across multiple female-associated comparisons. Unlike many of the immune-associated miRNAs identified in this study, miR-941 has received relatively little attention in MS, yet emerging evidence suggests roles in neuronal signaling, mitochondrial function, and innate immune regulation. Together with our recurrent identification of miR-1286 and members of the miR-548 family that have been identified in other MS studies, these findings highlight the value of unbiased transcriptomic approaches for identifying previously unrecognized molecular pathways that may contribute to sex-specific disease biology. Because these miRNAs remain incompletely characterized, they should be considered hypothesis-generating targets for future mechanistic investigation.

These findings support a conceptual model in which biological sex shapes baseline immune-regulatory networks that subsequently influence the molecular response to MS. Rather than representing isolated biomarkers, many of the differentially expressed miRNAs identified in this study appear to participate in coordinated immune and neuroinflammatory pathways that differ between males and females. These observations underscore the importance of incorporating sex as a biological variable in biomarker discovery, mechanistic investigations, and precision health approaches for MS. As MS care continues to evolve toward increasingly individualized treatment strategies, understanding the molecular basis of sex-specific disease biology may ultimately improve biomarker development, patient stratification, and the design of more personalized interventions for people living with MS.

Strengths and Limitations

Several strengths and limitations of the study should be noted. A key strength was the extraction of high-quality RNA from PAXgene tubes, which provided rich data for analysis. The study was intentionally designed to detect sex differences in MS, addressing a critical gap in current research. The focus on a treatment-naïve population offered a clearer view of early disease biology, without the confounding effects of therapies. Methodological rigor was further supported by transcriptomic confirmation of biological sex through XIST expression and the use of a sex-informed reference genome, which reduced alignment errors and improved the detection of X-linked differences. Together, these features strengthened the reliability of the observed sex-biased miRNA profiles.

At the same time, several limitations should be acknowledged. Although the overall sample size was modest, the limited number of healthy controls represents a particular constraint. The small sample size also limits statistical power and increases the risk of both false-positive and false-negative findings. As a result, results should be interpreted cautiously and considered hypothesis-generating until replicated in larger, independent cohorts. The homogeneity of the cohort (Caucasian only) improves internal validity but reduces generalizability to more diverse populations, which should be

prioritized in future studies. Although statistical adjustments for multiple comparisons were not applied, this decision followed NanoString guidance for biologically focused CodeSets with correlated targets, where FDR correction may mask meaningful biological signals. Findings should therefore be interpreted in the context of pathway-level trends, and future cross-platform validation is warranted. Due to limitations in sample availability through the biorepository, we were also unable to control for potentially important confounding variables such as body mass index, smoking status, or other environmental factors. The cross-sectional design and incomplete clinical phenotyping limit the ability to directly relate miRNA differences to disease activity, progression, or clinical outcomes. Finally, because analyses were performed using peripheral blood, the observed miRNA profiles may not fully reflect molecular processes occurring within the central nervous system.

Future Directions

Future research should aim to validate these findings in larger and more diverse cohorts, ideally through longitudinal designs that can track miRNA trajectories in relation to clinical outcomes and imaging. Functional studies that connect differentially expressed miRNAs to their target mRNAs and downstream protein effects will be essential, as will integration with contemporary biomarkers such as serum neurofilament light (sNfL). Expanding these efforts across the spectrum of MS severity will provide a more comprehensive understanding of how sex-specific molecular programs influence neuroinflammation, neurodegeneration, and protective mechanisms. Ultimately, this line of inquiry has the potential to guide biomarker-driven, personalized approaches to MS care.

Conclusion

This exploratory pilot study identified sex-biased miRNA expression patterns in treatment-naïve individuals with RRMS and demonstrated that biological sex is an important source of molecular heterogeneity in MS. Several differentially expressed miRNAs were associated with immune regulation and neuroimmune signaling, while others, including miR-941, emerged as potentially novel sex-associated molecular signatures.

Notably, several miRNAs were differentially expressed in both healthy controls and disease-related comparisons, suggesting that baseline sex-associated biological differences may shape subsequent MS pathophysiology. These findings support the importance of incorporating sex as a biological variable in biomarker discovery and mechanistic studies and provide a foundation for future investigations aimed at understanding the molecular basis of sex differences in MS.

Importantly for nursing research, understanding sex-specific molecular pathways offers a foundation for developing tailored patient education, anticipatory guidance, and symptom management strategies. Nurses are central to helping patients navigate the variability of MS, and insights into sex-based differences can inform more personalized approaches to monitoring, counseling, and long-term care planning.

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Ethical Considerations

This research involved analysis of de-identified human biospecimens obtained from the Accelerated Cure Project biorepository. The study protocol, titled *"Exploring the Genetic and Epigenetic Drivers of Sex-Based Difference in Relapsing Multiple Sclerosis,"* received expedited review and approval from the Virginia Commonwealth University Institutional Review Board (VCU IRB Panel A) on March 3, 2021, under Expedited Category 5, which covers research involving previously collected data or specimens that are not collected specifically for the current research and are de-identified. IRB Study Number: HM20020589.

Consent to Participate

Because the biospecimens and associated data were fully de-identified prior to investigator access, the IRB waived the requirement for informed consent**.** No direct interaction with human participants occurred, and no identifiable information was accessed.

Author Contributions

Buxhoeveden, S contributed to conception and design contributed to acquisition, analysis, and interpretation drafted manuscript critically revised manuscript gave final approval agrees to be accountable for

all aspects of work ensuring integrity and accuracy Swift-Scanlan, T contributed to conception and design contributed to acquisition, analysis, and interpretation drafted manuscript critically revised manuscript gave final approval agrees to be accountable for all aspects of work ensuring integrity and accuracy Olex, A. contributed to conception and design contributed to acquisition, analysis, and interpretation drafted manuscript critically revised manuscript gave final approval agrees to be accountable for all aspects of work ensuring integrity and accuracy Oh, U. contributed to conception and design contributed to interpretation critically revised manuscript gave final approval agrees to be accountable for all aspects of work ensuring integrity and accuracy Carter, C. contributed to design contributed to analysis and interpretation critically revised manuscript gave final approval agrees to be accountable for all aspects of work ensuring integrity and accuracy.

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Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Data Availability Statement

This study analyzed de-identified biospecimen-derived data obtained from the Accelerated Cure Project biorepository. Due to data use agreements governing participant privacy and biospecimen governance, the dataset cannot be deposited in a public repository. Qualified researchers may request access directly from the Accelerated Cure Project, subject to approval and completion of required data use agreements.

Any other identifying information related to the authors and/or their institutions, funders, approval committees, etc, that might compromise anonymity.

The corresponding author, Stephanie Buxhoeveden, moved to a new institution since completing research and resulting manuscript. The research was conducted at Virginia Commonwealth University, and she is now affiliated with the Accelerated Cure Project, and MS research non-profit organization.

Supplemental Material

Supplemental material for this article is available online.

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