MicroRNA Expression in Asymptomatic Welders: Implications for Biomarker Discovery for Environmentally-Linked Neurodegenerative Disorders

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Abstract

Chronic occupational exposure to metals in welding fumes has been implicated in the etiology of neurodegenerative diseases (NDDs), including Parkinson's disease (PD) and Alzheimer's disease (AD). Changes in microRNA (miRNA) expression have been associated with various neurodegenerative conditions. Circulating miRNAs, in particular, have emerged as promising, minimally invasive biomarkers for diagnosing and monitoring disease progression. This study was designed to characterize the expression of miRNAs in neuronally-enriched serum extracellular vesicles (EVs) among welders and non-welders to explore their potential link to metal concentrations and welding exposure measures and their potential as early diagnostic biomarkers for neurodegeneration. Serum samples from 39 welders and 27 healthy individuals were collected, and EV-enclosed miRNAs were extracted and analyzed. Also, whole blood metal concentrations and welding exposure measurements were obtained. Fifty miRNAs were found to be dysregulated in welders *vs.* non-welders, of which three (miR-16-5p, miR-93-5p, miR-486-5p) showing reduced expression and two (miR-4281 and miR-4417) exhibiting positive correlations with blood metal concentrations as well as with long- and short-term welding exposure measures. The dysregulation of these miRNAs suggests that exposure to metals could disrupt important biological processes, possibly contributing to an elevated risk of NDDs. These findings highlight the need for further research to validate the causal relationship between exposure to metals in welding fumes, the dysregulation of circulating miRNAs, and their role in neurodegenerative disease development, with implications for miRNA-based biomarkers in early disease detection and prevention.

Keywords: Welder; Parkinsonism; biomarker; diagnosis; serum; extracellular vesicle; microRNA; miRNA sequencing; next-generation sequencing.

Introduction

Occupational and environmental exposure to metals has long been recognized as a significant health hazard. Welding is a predominant source of occupational metal exposure, with welders and bystanders routinely exposed to metal compounds such as manganese (Mn), iron (Fe), copper (Cu), lead, potassium, and selenium, among other constituents of the welding fumes.[1-4] Multiple studies have reported significant correlations between metal levels in welders' blood and urine and their atmospheric concentrations.[5,6] Accumulating evidence indicates that welders have a higher risk of developing neurodegenerative diseases (NDDs) due to chronic metal exposure.[1,7-12] that has been linked to an accelerated onset of neurological disorders, including Parkinson's disease (PD).[12,13]

Recent studies have highlighted the role of microRNA (miRNAs), small non-coding RNAs involved in gene regulation, in various NDDs. Changes in miRNA expression have been implicated in several pathological conditions of the central nervous system (CNS), ranging from cancer to NDDs.[14,15] Circulating miRNAs, detected in various peripheral fluids such as plasma, serum, and cerebrospinal fluid (CSF),[16] are reported to either bind to RNA-binding proteins[17] or high-density lipoproteins,[18] or to be enclosed within extracellular vesicles (EVs), shielding these miRNAs from degradation.[19] These circulating miRNAs have emerged as promising biomarkers for the early detection, diagnosis, and monitoring of disease progression due to their stability and easy accessibility.

Circulating EVs, released by most cell types, including brain cells, carry miRNA and other molecular contents that provide valuable information about cellular changes associated with health and disease,[20] making them a valuable tool for diagnosing and understanding disease pathology. These vesicles are present in multiple body fluids that can easily cross the blood-brain barrier, facilitating the transport of CNS-derived miRNAs to the periphery.[21-23] As a result, miRNAs in plasma or serum are increasingly recognized as non-invasive diagnostic biomarkers. Consequently, neuron-derived EVs in plasma show significant promise as biomarkers for diagnosing and monitoring NDDs. However, despite the growing potential of neuron-derived EV miRNAs in NDDs, the specific miRNA signatures in NDD patients remain unclear, highlighting the need for further research to fully unlock their diagnostic and prognostic potential.

This study had two objectives. The first was to identify the differential expression of miRNAs in serum neuron-enriched EVs between non-welders and welders, thus exploring correlations between miRNA expression and metal concentrations in whole blood to gain a deeper understanding of how metal exposure might influence miRNA dysregulation and its potential link to NDDs. In addition, we explored the correlations of welding exposure measures to evaluate the potential of miRNAs as early diagnostic biomarkers for neurodegeneration.

Methods

Subjects

Demographic data and miRNA measures were obtained from a subset of participants (27 non-welders and 39 welders) who were part of a larger group of participants enrolled in previous studies[24,25]. These participants were recruited from labor unions and the local community around Hershey, Pennsylvania, USA. Welders were classified as individuals who had engaged in welding

at any point in their lives, while non-welders included individuals with various occupations but no history of welding.

All participants were male, and were free of neurological disorders or significant motor dysfunction assessed by the Movement Disorders Society Unified Parkinson's Disease Rating Scale-motor score (MDS-UPDRS-III) with a threshold score <15[25]. The protocol was reviewed and approved by the Penn State Hershey Institutional Review Board. Written informed consent was obtained from all participants, and the study was conducted consistent with the Declaration of Helsinki.

Blood collection and serum preparation

For this study, we chose blood serum over plasma to reduce the presence of proteins, lipids, and sugars in the final solution. Blood samples were collected from subjects after an 8–12 h overnight fast. Within 30–60 minutes after the blood draw, samples were centrifuged at 1,500 x 1g for 15 min at 4°C. One mL aliquots of the supernatant were pipetted into cryovials on ice and then stored at −80°C. For experiments, serum samples were thawed and 100 µL was pipetted into a separate cryovial tube for the following assays.[26]

Extracellular vesicle and miRNA isolation

The extracellular vesicles (EVs), including exosomes and microvesicles, were purified from the samples to separate free circulating miRNAs from those contained within the vesicles of interest.[27] ExoQuick™ Exosome Precipitation Solution kit (Systems Biosciences) was used to extract EVs from the samples. Serum (100 µL) was centrifuged at 3,000 g for 15 min at room temperature to remove cells and cellular debris. The supernatant was transferred to a sterile vessel and 25.2 µL of ExoQuick™ Exosome Precipitation Solution was added, refrigerated at 4°C for 30 min, and centrifuged at 1,500 g for 30 min at 4°C. The supernatant was aspirated, leaving the EVs as a white pellet. Another centrifugation at 1,500 × g for 5 min was done to remove traces of ExoQuick™ by aspiration. The EV pellet was resuspended in 100 µL phosphate-buffered saline.

The resuspended pellets were analyzed to confirm that the purified EVs had the expected size and concentration measurements, and further details can be found in Morris *et al.*[26]. Briefly, the TEM measurements were used to confirm that the exosomes in the buffer had an expected size of 40–200 nm[28] and microvesicles ranging from 200–1,000 nm. EVs with neuronal origins were extracted from the solution by capturing exosomes and microvesicles expressing the neuron-specific marker, CD171 [L1 cell adhesion molecule[22]]. A biotinylated anti-human CD171 antibody (eBio5G3, Affymetrix) was used to bind vesicles expressing CD171. Then streptavidin-conjugated magnetic beads (#10608D, Thermo Fisher Scientific) were added to the solution to bind to the CD171 antibody. Neuronal exosomes and microvesicles bound to anti-CD171 and streptavidin beads were pulled down magnetically. The captured neuronally derived exosomes and microvesicles were lysed with IGEPAL® CA-630 (Sigma-Aldrich) to free the miRNAs into solution; IGEPAL® was added to 1% of the final concentration. These EVs neuronally derived miRNAs were used for sequencing library preparation.

MiRNA-Sequencing (RNA-Seq)

The miRNA sequencing libraries were generated from serum-derived EV miRNAs using the CleanTag® Small RNA Library Prep Kit for downstream miRNA expression analysis. Individually barcoded libraries were mixed equimolarly and subjected to sequencing with technical duplicates on an Illumina NovaSeq 6000. We used *Pearson's* correlation to evaluate

technical replicates to indicate that the read counts between each sequencing run on the NovaSeq 6000 run were consistent. The sequencing data and reads obtained were sent to the University of Georgia, Athens, GA, USA, for bioinformatic analysis.

Welding-related exposure assessment

Welding-related exposure was assessed by a work history (WH)[24] questionnaire that collected job information over the individual's working lifetime, emphasizing welding and other jobs associated with welding-related metal fume exposures. Responses to the WH questionnaire enabled calculation of cumulative lifetime years welding (YrsW=years spent employed as a professional welder during the participants' life). An estimate of lifetime cumulative exposure to metal fumes during welding was also calculated [ELT (units of mg*years*m^{-3})][24]. This metric integrated information about the type of welding tasks performed (*e.g.*, brazing, soldering, *etc.*), time spent performing each activity, and the use of local ventilation.[24] Information on co-exposure in the workplace, such as solvents and pesticides, was also collected.

An additional supplementary exposure questionnaire (SEQ)[24] focused on the 90-day period prior to the study visit and documented the time spent welding, types of metal welded, and type of welding performed. The exposure metrics derived from the SEQ were: hours welding [HrsW = (number of weeks worked) * (h/week) * (fraction of time worked related directly to welding, brazing, or soldering)] in the 90-day period preceding the study visit. An estimate of recent exposure to welding fumes over the 90 days (E90) prior to the study visit was also calculated. [24] Information on co-exposure over the past 90 days in the workplace, such as solvents and pesticides, was also collected.

Data processing and statistical analysis

Demographic, clinical, and welding-exposure data were compared between groups using one-way analysis of variance (ANOVA), chi-square, or Mann-Whitney U tests, as appropriate. Blood exposure data were compared between controls and welders using analysis of covariance (ANCOVA), adjusting for age and tobacco smoking history.

The deep sequencing data was subject to a series of steps from preprocessing to generating an MA plot, volcano plot, hierarchical clustering heatmap, principal coordinate analysis (PCoA) plot, and linear discriminant analysis (LDA) effect size (LEfSe) plot. The read number of each miRNA was used for deep-sequencing analysis. Any miRNAs with missing records were removed from the miRNA count matrix. Subsequently, only the miRNAs that had a read count greater than ten in more than three samples were kept. The count matrix is then converted to a DESeq object for further analysis using the DESeq2 package (version 1.40.2) in R (version 4.3.1). The DESeq2 summarizes the mean for each miRNA and calculates the log2-fold change of each miRNA between welders and non-welders based on the application of an empirical Bayes shrinkage procedure to the coefficients of a negative binomial[29]. Based on this, *p*-values and related statistics were obtained to test the significance of the differential expression of each miRNA.

For each miRNA that was significantly differentially expressed, we investigated the potential role of each in NDDs in the existing literature. Also, for each miRNA that was significantly differentially expressed, we evaluated *Pearson* partial correlations between miRNA read counts and whole blood metal concentrations (both natural log-transformed), adjusting for age and tobacco smoking history. For each miRNA that was significantly correlated with more than one blood metal concentration (after correction for multiple comparisons using the false

discovery rate, FDR), we then cross-referenced these with those identified above using existing literature to be linked with neuroinflammation, environmental, or metal exposures, Alzheimer's Disease and Related Dementias (ADRD), Parkinson's Disease (PD), or other related neurodegenerative disorders.

Additionally, we examined associations between read counts of miRNA sequences that were significantly differentially expressed and welding-related exposure measurements (HrsW, E90, YrsW, ELT) by *Pearson* partial correlation, adjusting for age and tobacco smoking history. SPSS 27.0 and R were used for correlation analyses.

Results

Demographic and clinical data

Demographic characteristics are summarized in Table 1. No significant differences were observed in age between groups [$F(1,65) = 1.70$, p-value = 0.196]. Non-welders had a significantly higher level of education compared to welders [$F(1,65) = 48.85$, p-value < 0.001]. Additionally, welders had a significantly higher Body Mass Index (BMI) than non-welders [$F(1,64) = 7.02$, p-value = 0.010] (Table 1). No significant differences were observed in UPDRS-III subscores between non-welders and welders (Table 1).

There was a significant difference in the proportion of ever-smokers, with welders exhibiting a higher frequency of smoking behavior (p-value = 0.006). However, no difference was found in mean smoking pack-years between the groups [$F(1,27) = 0.56$, p-value = 0.460]. Regarding alcohol consumption, there were no significant differences in the frequency of those consuming any alcohol *vs.* none (p-value = 0.624). Among those who consumed alcohol, there was no significant difference in average alcohol consumption (drinks per week) between non-welders and welders [$F(1,31) = 0.39$, p-value = 0.537]. On average, welders had worked approximately 25 years, and their average hours worked in the past 90 days were approximately 266 (Table 1).

Whole blood metal concentrations in welders and non-welders

The ANCOVA revealed that welders had significantly higher whole-blood Cu [$F(1,66) = 25.71$, p-value < 0.001], Fe [$F(1,66) = 20.01$, p-value < 0.001], K [$F(1,66) = 28.34$, p-value < 0.001], Mg [$F(1,65) = 8.67$, p-value = 0.005], Mn [$F(1,66) = 5.04$, p-value = 0.028], Pb [$F(1,67) = 7.08$, p-value = 0.010], Zn [$F(1,65) = 16.11$, p-value < 0.001], and Se [$F(1,67) = 19.51$, p-value <0.001], levels compared to non-welders (Table 1).

Table 1. *Demographic, clinical, welding-related exposure data, and whole blood metals concentration levels for non-welders (N=27) and welders (N=39).*

	NON-WELDERS (N=27)				WELDERS (N=39)				
	Min	***Mean (SD)***	***Median (Q1 - Q3)***	***Max***	***Min***	***Mean ± SD***	***Median (Q1 - Q3)***	***Max***	***p-value*** η_p^2
Demographic and clinical data									
Age (years)	*28*	*44 ± 10*	*42 (38 – 53)*	61	24	47 ± 10	*51 (40 – 55)*	65	0.196; 0.026
Education (years)	*12*	*16 ± 2*	*16 (16 – 18)*	22	11	13 ± 1	*12 (12 – 14)*	16	**<0.001; 0.950**
BMI (kg/m²)	*21.0*	*26.3 ± 3.4*	*26.6 (23.1 – 29.2)*	32.9	21.1	29.0 ± 4.3	*28.5 (26.1 – 30.9)*	40.7	**0.010; 0.100**
UPDRS-III (motor scores)	*0*	*1 ± 2*	*1 (0 – 2)*	8	0	2 ± 2	*1 (0 – 2)*	7	0.873; 0.000
*Smokers (n ever, % ever)**	*----*	*7, 26%*	*----*	*----*	*----*	24, 61%	*----*	*----*	**0.006**
Tobacco's smoking (pack-year)	*0.2*	*23.0 ± 29.6*	*16.5 (0.8 – 38)*	80.0	0.5	19.8 ± 21.3	*12.7 (7.4 – 28.3)*	97.5	0.460
*Any alcohol consumption n, %**	*----*	*12, 44%*	*----*	*----*	*----*	20, 51%	*----*	*----*	0.624
Alcohol consumption (drinks/week)	*2*	*6 ± 6*	*5 (2 – 6)*	20	1	5 ± 3	*5 (3 – 6)*	16	0.537
Whole blood metals concentration									
Whole blood Cu (µg/L)	*502*	*729 ± 132*	*742 (610 – 823)*	957	553	900 ± 125	*910 (827 – 996)*	1111	**<0.001; 0.293**
Whole blood Fe (mg/L)	*198*	*489 ± 79*	*504 (474 – 540)*	583	397	556 ± 55	*558 (533 – 593)*	673	**<0.001; 0.244**
Whole blood K (mg/L)	*1100*	*1736 ± 316*	*1650 (1540 – 2018)*	2300	1290	2163 ± 364	*2197 (1833 – 2460)*	2725	**<0.001; 0.314**
Whole blood Mg (mg/L)⁺	*22.3*	*35.1 ± 5.2*	*35.5 (31.6 – 38.5)*	43.9	25.6	39.2 ± 6.3	*38.4 (34.3 – 43.1)*	57.4	**0.005; 0.124**
Whole blood Mn (µg/L)	*4.9*	*8.7 ± 2.8*	*8.4 (6.8 – 9.7)*	16.5	6.2	10.6 ± 3.3	*9.9 (8.0 – 12.7)*	19.1	**0.028; 0.075**
Whole blood Na (mg/L)⁺	*998*	*2042 ± 373*	*2010 (1800 – 2283)*	2863	1510	2257 ± 384	*2286 (1984 – 2569)*	2842	0.055; 0.060
Whole blood Pb (µg/L)	*0.5*	*9.5 ± 8.8*	*8.7 (4.3 – 13.6)*	47.9	5.0	24.5 ± 19.7	*20.3 (12.5 – 28.9)*	113	**0.010; 0.103**
Whole blood Zn (µg/L)⁺	*2.8*	*5.3 ± 1.5*	*5.6 (4.1 – 6.5)*	8.1	2.9	6.6 ± 1.3	*7.0 (6.0 – 7.4)*	9.0	**<0.001; 0.209**
Whole blood Sb (µg/L)#	*2.2*	*3.3 ± 1.1*	*2.9 (2.4 – 4.1)*	6.1	2.1	3.5 ± 1.0	*2.9 (2.6 – 4.6)*	5.2	<0.404; 0.016
Whole blood Se (µg/L)⁺	*66*	*138 ± 50*	*122 (93 – 172)*	227	82	233 ± 103	*203 (155 – 296)*	527	**<0.001; 0.242**
Whole blood Sr (µg/L)⁺	*8.1*	*14.0 ± 5.2*	*11.8 (10.1 – 16.8)*	30.5	5.3	13.4 ± 6.4	*12.8 (9.6 – 14.8)*	45.7	0.227; 0.024
Welding-exposure measures									
E90 (mg-days/m³)	*0*	*0 ± 0*	*0 (0 – 0)*	*0*	0	2.3 ± 2.0	(0.6 – 2.2)	7.0	----
HrsW (hours)	*0*	*0 ± 0*	*0 (0 – 0)*	*0*	0	266 ± 203	(85 – 360)	720	----
YrsW (years)	*0*	*0 ± 0*	*0 (0 – 0)*	*0*	3	25 ± 10	(18 – 33)	40	----
ELT (mg-years/m³)	*0*	*0 ± 0*	*0 (0 – 0)*	*0*	0.17	1.14 ± 0.76	(0.31 – 1.91)	2.49	----

*Data represents the minimum (min), mean ± standard deviation (SD), median, first and third quartiles (Q1 and Q3, respectively), maximum (max) values, numbers of subjects (n), and percentages (%). One-way analysis of variance (ANOVA) Demographic and clinical data were compared using one-way analysis of variance (ANOVA), chi-square, or Mann Whitney U tests as appropriate. Analysis of covariance (ANCOVA), controlling for age and tobacco smoking history, was used to verify the effect of the group for whole blood metals concentration levels. p-values and effect size (η_p^2). Abbreviations: y = years; UPDRS-III: Unified Parkinson's Disease Rating Scale-part III (motor scores); E90 = cumulative 90-day exposure to welding fume; HrsW = hours welding; YrsW = years welding; ELT = lifetime cumulative exposure to welding fume; Cu = copper; Fe = iron; K = potassium; Mg = magnesium; Mn = manganese; Na = sodium; Pb = lead; Zn = zinc; Sb = antimony; Se = selenium; Sr = strontium. *Restricted to current or former smoking or alcohol consumption; ⁺Available data for welders = 38; #Available data for: non-welders = 21, and welders = 28.*

Differentially expressed miRNAs between welders and non-welders

To investigate the impact of chronic exposure to welding fumes and, specifically, metal exposure on miRNA expression, we conducted next-generation RNA sequencing on serum-derived EVs from welders and non-welders. Our analysis revealed the expression of 1,070 known human miRNAs and 13 PIWI-interacting RNA (piRNA)s in all samples. After filtering out miRNAs and piRNAs with fewer than ten read counts in less than three samples, applying a log-transformation, and applying a fold-change cut-off (abs. values > 0.88), we identified 173 significantly dysregulated miRNAs and 5 piRNAs in welders *vs.* non-welders. The volcano and MA plots were used to visualize the differential expression level of each miRNA between welders and non-welders (Figures 1A and 1B, respectively). The volcano plot revealed 6 miRNAs that were significantly expressed; four of them were lesser-expressed: miR-4787-5p (fold change (FC) = -24.21, *p*-value = 1.97E-32), miR-944 (FC = -23.85, *p*-value = 3.34E-27), miR-188-3p (FC = -23.03, *p*-value = 3.89E-23), and miR-7153-3p (FC = -23.77, *p*-value = 1.11E-22), and two greater expressed: miR-4707-5p (FC = 3.023, *p*-value = 1.51E-7) and miR-3125 (FC = 4. 11, *p*-value = 3.55E-7).

Then, we found 50 of these miRNAs and 1 piRNA that remained statistically significant after adjusting for multiple comparisons (adjusted *p*-value, adj. *p*-value < 0.05); with 37 greater expressed (miR-203b-5p, miR-205-5p, miR-142-5p, miR-29c-3p, miR-4775, miR-718, miR-4646-3p, miR-4632-5p, miR-4501, miR-6857-3p, miR-4281, miR-6717-5p, let-7g-5p, miR-1260b, miR-4417, miR-3182, miR-4707-5p, miR-6808-5p, miR-8056, miR-19b-3p, miR-5000-5p, miR-103b, miR-6746-5p, miR-653-5p, miR-3150a-3p, miR-639, miR-3125, miR-3616-3p, miR-597-5p, miR-22-3p, miR-378f, miR-1236-3p, miR-3658, miR-1290, miR-3646, miR-6775-3p, miR-6132) and 14 lesser expressed (miR-4787-5p, miR-188-3p, miR-944, miR-7153-3p, miR-548g-5p, miR-3977, miR-885-3p, miR-942-5p, miR-4667-5p, miR-93-5p, miR-486-5p, miR-1178-3p, miR-16-5p, and piR_000765/gb/DQ570956/Homo). Further deep-sequencing analyses were performed on these 50 significantly dysregulated miRNAs and 1 piRNA.

Hierarchical clustering heatmap analysis was performed to visualize the overall miRNA expression differences between welders and non-welders for these 50 miRNAs and 1 piRNA with an adjusted *p*-value < 0.05 (Figure 2A). This heatmap reveals that welders and non-welders cluster into separate groups except for nine non-welders and three welders. A similar pattern emerged from the PCoA analysis (Figure 2B), which illustrates a significant difference in miRNA expression between welders and non-welders. In this analysis, the PERMANOVA test was significant (*p*-value = 0.007), while the permutation test was non-significant (*p*-value = 0.284). This indicates a significant difference in the distribution between welders and non-welders and that the dispersion does not cause the difference. Lastly, the LEfSe plot, a method used for metagenomic biomarker discovery, shows the miRNAs most differently expressed between the two cohorts (Figure 2C) by coupling standard tests for statistical significance with additional tests encoding biological consistency and effect relevance.

MiRNA, exposure correlation analysis with whole blood metal measurements

Among the 50 miRNAs found to be differentially expressed, 15 miRNAs were significantly correlated with more than one whole blood metal concentration after adjusting for multiple comparisons using the FDR. Of the 9 miRNAs with read counts greater than zero in at least 10% of our participants (seven subjects), we identified five miRNAs that were previously reported in the literature to be linked to neuroinflammation, environmental or metal exposures, Alzheimer's

Disease and Related Dementias (ADRD), Parkinson's Disease (PD), or other related neurodegenerative disorders: 3 miRNAs lesser-expressed (miR-16-5p, miR-93-5p, miR-486-5p) and 2 greater-expressed (miR-4281 and miR-4417) among welders (Supplementary Material Table S2).

Correlation analysis revealed that the miRNAs, miR-16-5p, miR-93-5p, and miR-486-5p, exhibit significant negative correlations with various metals and elements, particularly Pb, Se, Cu, and K, even after FDR multiple test correction (Table 2). Conversely, the two greater-expressed miRNAs, miR-4281 and miR-4417, showed significant positive correlations with metals Cu and Pb (Table 2), even after FDR multiple test correction.

Table 2. *MiRNAs and whole blood metal pairwise Pearson partial correlation, adjusted for age and tobacco smoking history (N=66).*

MiRNA	Whole blood metal measures	R-values	Adjusted p-values
hsa-miR-16-5p vs.	**Pb**	-0.50	0.004
	Se	-0.50	0.004
	Cu	-0.48	0.011
	K	-0.40	0.043
hsa-miR-93-5p vs.	**Pb**	-0.53	0.004
	K	-0.45	0.006
	Se	-0.45	0.011
hsa-miR-486-5p vs.	**Cu**	-0.50	0.005
	Se	-0.45	0.012
	Pb	-0.45	0.015
hsa-miR-4281 vs.	**Cu**	0.41	0.045
hsa-miR-4417 vs.	**Cu**	0.49	0.006
	Pb	0.44	0.039

Abbreviations: miRNA: microRNA; R-values: R correlation coefficient value; p-value: raw statistical p-values; Cu = copper; K = potassium; Pb = lead; Se = selenium. p-values adjusted for false discovery rate.

miRNA and welding-related exposure

Our analysis revealed that YrsW and ELT (long-term welding exposure measures) were negatively correlated with the lesser-expressed miRNAs miR-16-5p, miR-93-5p, and miR-486-5p, as detailed in Table 3. Additionally, HrsW and E90 (short-term welding exposure measures) were negatively correlated with the lesser-expressed miRNA miR-93-5p (Table 3). These significant negative correlations suggest a potential less expression of these miRNAs due to increased intensity and prolonged metal exposure.

We also identified that YrsW and ELT were positively correlated with the greater-expressed miRNAs miR-4281 and miR-4417 (Table 3). Also, we found a positive correlation between HrsW and miRNAs miR-4281 (Table 3). These findings suggest that prolonged exposure in the past 90 days to welding fumes is associated with greater expression of these miRNAs.

Table 3. *MiRNAs and welding-related exposure pairwise Pearson Partial correlation, adjusted for age and tobacco smoking history (N=66).*

MiRNA	Welding exposure measures	R-values	Adjusted p-values
hsa-miR-16-5p vs.	**YrsW**	**-0.412**	**0.002**
	ELT	**-0.320**	**0.011**
	HrsW	-0.252	0.171
	E90	-0.249	0.227
hsa-miR-93-5p vs.	**YrsW**	**-0.493**	**0.002**
	ELT	**-0.432**	**0.003**
	HrsW	**-0.352**	**0.038**
	E90	**-0.334**	**0.049**
hsa-miR-486-5p vs.	**YrsW**	**-0.426**	**0.002**
	ELT	**-0.311**	**0.015**
	HrsW	-0.239	0.171
	E90	-0.226	0.228
hsa-miR-4281 vs.	**YrsW**	**0.398**	**0.003**
	ELT	**0.312**	**0.014**
	HrsW	**0.344**	**0.026**
	E90	0.269	0.129
hsa-miR-4417 vs.	**YrsW**	**0.408**	**0.007**
	ELT	**0.346**	**0.017**
	HrsW	0.294	0.104
	E90	0.221	0.252

Abbreviations: miRNA: microRNA; YrsW = years welding; ELT = lifetime cumulative exposure to welding fume; E90 = cumulative 90-day exposure to welding fume; HrsW = hours welding; R-values: R correlation coefficient value; p-values: p-values adjusted for false discovery rate.

Discussion

This study aimed to identify differential circulating miRNA biomarkers between welders and non-welders in order to gain a better understanding of the health effects linked to metal exposure. The results revealed the presence of 50 differentially expressed miRNAs in welders *vs.* non-welders. Changes in five specific miRNAs were significantly correlated with whole blood metal concentrations of lead (Pb), copper (Cu), potassium (K), and selenium (Se). These miRNAs were linked not only to metal concentrations but also to welding-related exposure measures, which have been previously reported to be involved in neurodegeneration processes. These findings, if substantiated, may further elucidate the mechanism of action of chromic metal exposure on neuropathological processes and the risk of neurodegeneration.

Our data show that welders have significantly higher whole blood concentrations of metals, including Cu, Pb, Fe, Mn, and others. This aligns with existing studies that link welding fumes to elevated metal exposure, which are known to contain a mixture of these elements[25,30-32]. Higher whole blood levels of Pb**,** Cu, and Se were negatively correlated with lower expressions of miRNAs, including miR-16-5p, miR-93-5p, and miR-486-5p. This suggests that chronic exposure to these neurotoxic metals could suppress their expression, thus impairing the miRNA's ability to regulate cellular repair and immune responses. We also observed positive correlations between

miR-4281 and miR-4417 and metals like Cu and Pb, suggesting a complex interaction between metal exposure and miRNA regulation. The greater expression of these miRNAs may represent a cellular response to the toxic effects of these metals; however, further investigation is needed to clarify their potential role in metal-induced neuroprotection.

Notably, all five miRNAs listed in Table 2 exhibited correlations with whole blood Cu, and four showed correlations with Pb, underlining the strong relationship between metal exposure and miRNA expression. Chronic exposure to metals like Pb and Cu has been strongly associated with neurotoxicity[30,33-35] and the dysregulation of miRNA expression.[36] Pb is recognized for its detrimental effects on the central nervous system, even at relatively low levels of exposure,[34] and has been linked to DNA damage, changes in miRNA expression,[37,38] and an increased risk of neurodegenerative conditions[39-42]. Although Cu is essential for many biological functions, excessive exposure can lead to neurotoxicity[43]. Previous research on miRNA expression in welders is limited, with only one previous study[44] focusing on three miRNAs linked to kidney damage, and another study examined the effect of Pb and cadmium exposure on a pre-selected panel of miRNAs in workers[38]. Our study is the first one that offered a broader exploration of miRNA expression, specifically in welders exposed to a variety of metals, and identified new miRNAs potentially involved in metal-induced toxicity. Identifying specific miRNA expression patterns in response to these metals could provide valuable insights into the health risks associated with metal exposure.

The differential expression of miRNAs in relation to both short-term and long-term welding exposure highlights the potential cumulative impact of welding fumes on miRNA profiles. Our study shows that both short-term and long-term exposure to metals significantly affects miRNA expression, primarily showing associations with long-term exposure indicators (YrsW and ELT) and the expression of miR-16-5p**,** miR-93-5p, miR-486-5p, miR-4281, and miR-4417. Long-term exposure (YrsW and ELT measures) was associated with lesser and greater expression of miRNAs that have been linked to neurodegenerative diseases, exposure to metals and other chemical compounds,[6,8,45,46] and neuroinflammatory processes.[19,47] This suggests that cumulative metal exposure can modify miRNA regulation, aligning with previous studies that propose miRNA dysregulation as a biomarker for toxicant exposure,[44,48,49] Overall, our results underscore the dynamic impact of both acute and chronic metal exposure on miRNA expression, with potential implications for early detection of toxic effects.

miRNAs are key post-transcriptional regulators of gene expression, and their altered levels can disrupt cellular pathways involved in neuroinflammation, immune response, and damage repair. Dysregulation of miRNAs has been implicated in the pathogenesis of various neurological diseases, including neurodegenerative disorders (NDDs).[45,50,51] For instance, numerous studies show that miRNA dysregulation in Parkinson's disease (PD) targets genes and pathways that contribute to disease progression,[46,52] including the regulation of α-synuclein synthesis, the hallmark protein of PD, as well as neuroinflammation, autophagy, and neural apoptosis.[53-55] This study focused on the impact of the five identified miRNAs (miR-16-5p, miR-93-5p, miR-486-5p, miR-4281, and miR-4417) on inflammation, metal exposure, and neurological disorders.

Our data demonstrated that welders exhibit significantly lesser expression of certain miRNAs, including miR-16-5p, miR-93-5p, and miR-486-5p. These miRNAs are known to play roles in cellular stress responses[1,37], neuroprotection [greater expression reduces oxidative damage and cell death[51,56]], and the regulation of neuroinflammatory processes[57]. For example, reduced expression of miR-16-5p has been linked to altered neuroinflammatory processes and infection regulation *in vitro,*[19,47] and various neurodegenerative diseases such as PD,[58] Alzheimer's Disease

(AD),[59] young-onset Alzheimer's Disease (YOAD),[2,60] severe dementia [sporadic Creutzfeldt-Jakob disease (CJD)],[18,36,59] and amyotrophic lateral sclerosis (ALS).[21,61,62] Additionally, the reduced expression of miR-16-5p in coke oven workers has been associated with increased DNA damage.[37] Furthermore, we previously demonstrated that manganese (Mn) exposure reduced exosome-mediated extracellular miR-16-5p levels in an *in vitro* PD model.[48] Lower miR-16-5p expression levels have also been observed in workers exposed to different metals[1,5,37,63] that is consistent with our findings in welders. Moreover, this miRNA has been linked to cognitive impairment[64] and depression[65] resulting from exposure to mixed heavy metals. This suggests that miR-16-5p expression may be influenced by metal-induced stress.

Similarly, reduced miR-93-5p expression has been linked to solvent exposure[8] and various NDDs, including AD,[66,67] ALS[61], CDJ[36], and glaucoma[68]. In pregnant women exposed to Pb and mercury (Hg), it has been shown that elevated patellar Pb levels are predictors of lesser expression of miR-93-5p.[50,69] Research indicates that miR-93-5p has a significant neuroprotective effect by inhibiting microglia-mediated neuroinflammation,[70,71,72,73] regulating neurogenesis,[74] neuronal growth,[74] and oxidative stress.[75,76] The dysregulation of miR-93-5p across various neurological diseases and its diverse roles in modulating inflammatory responses, protecting against cellular injury, and influencing neurogenesis and oxidative stress defense mechanisms suggest a potential therapeutic target for NDDs. Moreover, miR-486-5p has been found to be less expressed in patients with AD,[77] YOAD,[60] temporal lobe epilepsy with hippocampal sclerosis,[78] Duchenne's muscular dystrophy,[79] schizophrenia,[80] as well as in animal models of cerebral ischemia,[56] where increased levels have been shown to reduce oxidative stress and apoptosis, suggesting a neuroprotective role for miR-486-5p.[56] A study has shown that miR-486 induces reactive oxygen species-mediated neurodegeneration and recruits active inflammatory factors.[81] The role of miR-486-5p in neurological disorders and its potential implications, however, are understudied and further research is needed. In our group of welders, reduced expression of miR-16-5p, miR-486-5p, and miR-93-5p may impair the regulation of genes related to inflammation and oxidative stress, increasing their susceptibility to chronic conditions such as neuroinflammation, neurodegeneration, and other metal-related diseases.

Conversely, we observed an increase in the expression of miR-4281 and miR-4417 among welders. Although higher miR-4281 and miR-4417 expression has been reported in patients with multiple system atrophy (MSA)[82] and *in vitro* nickel exposure,[83] their role in neurodegeneration, neuroinflammation, or metal exposure is not well understood. The precise functions of these miRNAs are unclear, and further research is needed to determine whether their increased expression represents an adaptive response to metal exposure. The miRNA expression analyzed in this study was derived from neuron-enriched vesicles in serum, suggesting that the identified miRNAs may represent neuronal cell states[4]. Consequently, their presence in serum could offer valuable insights into brain conditions and neuronal function. However, additional research is needed to better understand the biological functions of these miRNAs, their response to metal exposure, and their role in increasing the risk of neurodegenerative diseases.

Our study provides important insights into the relationship between miRNA expression and metal exposure, but has several limitations. The relatively small sample size may restrict the generalizability of our findings, and our cohort was composed exclusively of male participants, necessitating the evaluation of the influence of sex as a biological factor in modulating miRNA regulation under metal exposure. Variability in exposure levels, individual work practices, and a variety of metals can interfere with miRNA expression. Furthermore, our cross-sectional study

design limits our ability to establish causality between metal exposure and changes in miRNA expression. Longitudinal studies are required to understand the potential causal links more clearly. Lastly, although miRNA expression differences were seen in welder sampl;es, the precise biological mechanisms through which these miRNAs influence neurodegenerative processes and how they are affected by metal exposure require further investigation.

Despite these limitations, the current findings have important implications. The identification of miRNAs correlated with whole blood metals may serve as potential biomarkers for monitoring toxicant exposure in occupational and environmental health risks and in the general population. Understanding the specific pathways and mechanisms through which welding fumes affect miRNA expression could provide insights into the broader health impacts of metal exposure. Future research should focus on elucidating the functional roles of the identified miRNAs in response to metal exposure and their potential contributions to disease processes, paving the way for clinical relevance.

Conclusion

This is the first study to explore the effects of exposure to multiple metals in miRNA expression derived from neuron-enriched vesicles and to offer a more comprehensive miRNA differential expression profile in asymptomatic workers exposed to welding fumes. Our findings reveal a large number of dysregulated miRNAs in welders. Of these, five miRNAs were significantly correlated with more than one blood metal concentration and have been reported to be related to NDDs, metal exposure, and inflammation. We describe how these miRNAs may regulate several important biological processes whose dysregulation may be involved in NDD processes. Further studies are essential to validate the causal link between exposure to metals in welding fumes and the dysregulation of circulating miRNAs to better understand the role of miRNAs in neurodegenerative processes.

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Figure legends

Figure 1. Deep-sequencing analysis of the serum-derived EV miRNAs from welders and healthy control.

A) Volcano plot: The *y*-axis values show the negative logarithm base 10 (log10) of the p-value; the horizontal gray dash line on the plot represents the threshold p-value set at 0.05. The *x*-axis values indicate the log2-fold change; the vertical red dash lines represent the fold change threshold set to 2. The dots on the positive side represent greater-expressed miRNA, whereas the dots within the negative area represent lesser-expressed miRNA. The significant differential expressed miRNAs are marked with blue dots, wheeas red represents the non-significantly expressed miRNAs. B) MA plot: Each dot represents one miRNA, with each triangle referring to the value beyond the *y*-axis range. The color of the dots represents significance $\alpha = 0.05$ one-tailed used for explorative research; blue means significant, while grey means not significant. The *x*-axis represents the number of counts for the miRNA, while the *y*-axis corresponds to the log2-fold change for the miRNA. The dot at the top half (positive) represents this miRNA is greater-expressed, while the dot at the bottom half (negative) represents this miRNA is lesser-expressed in the welder group compared to non-welders.

Figure 2. Deep-sequencing analysis of the 50 significantly deregulated miRNAs and 1 piRNA from welders.

A) Hierarchical clustering heatmap: Two-dimensional grid matrix derived from the functional heatmap in R displays the 50 differentially expressed miRNAs and 1 piRNA with the adj. *p*-value < 0.05 (rows) for the non-welders (blue) and welders (red) (columns). Each entry of the grid represents the mean (log2) fold change of the expression of a given miRNA on each sample. A blue grid corresponds to a low miRNA expression level (downregulation), while red represents a high miRNA expression level (upregulation), as indicated by the color key. B) PCoA plot: Investigates the resemblance of miRNA from non-welders (black) and welders (red). The lines of ellipses show 95% confidence intervals. Samples outside the ellipse line mean miRNA expression differs greatly from the rest of the samples. The *p*-value for the variance is the result of the permutation test, and the *p*-value for the mean is the result of the PERMANOVA test. C) LEfSe plot: Calculates the log LDA score for each miRNA labeled to the left. Grey bars represent miRNAs from the serum-derived EV of non-welders and the blue for welders.

Figure 1

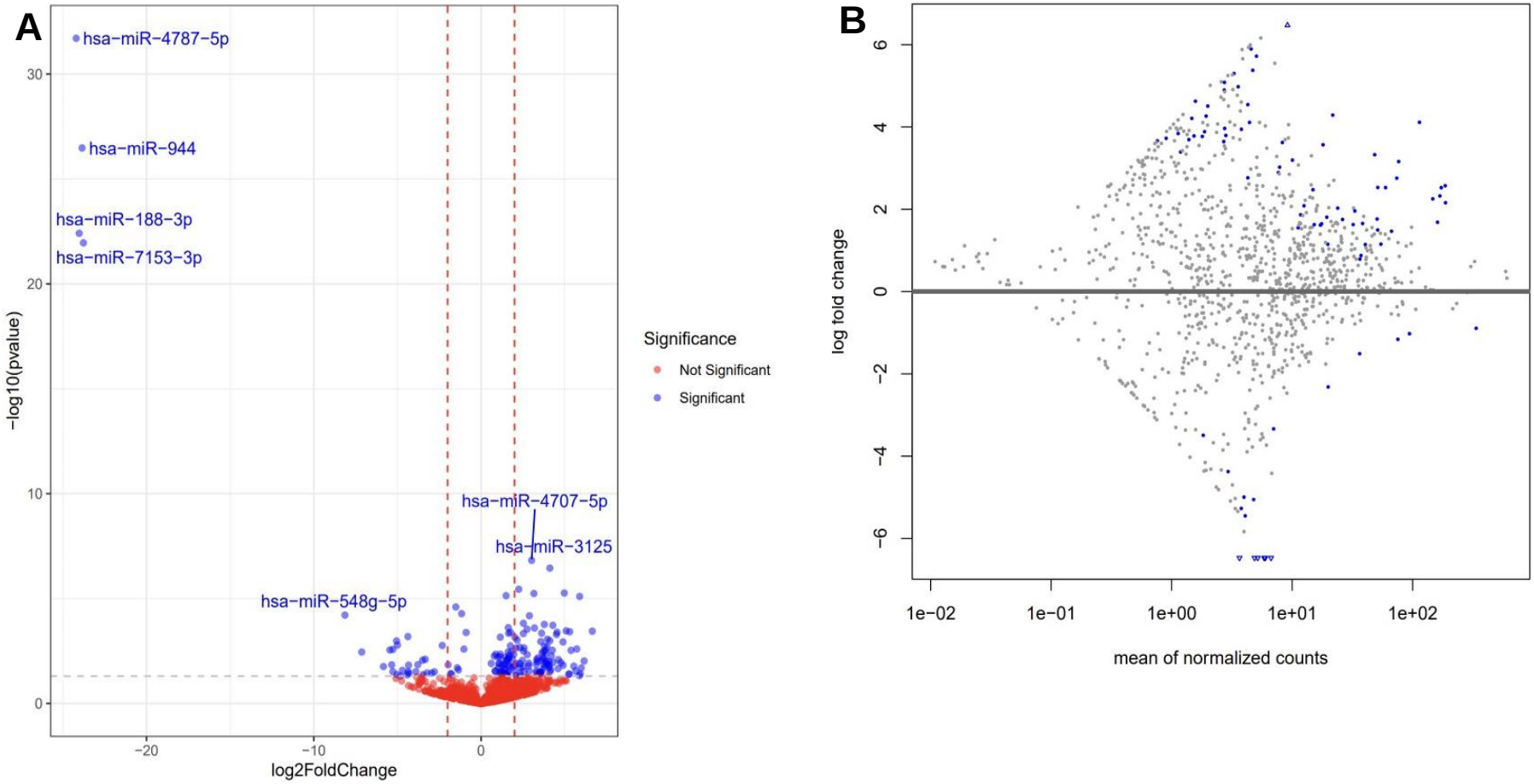
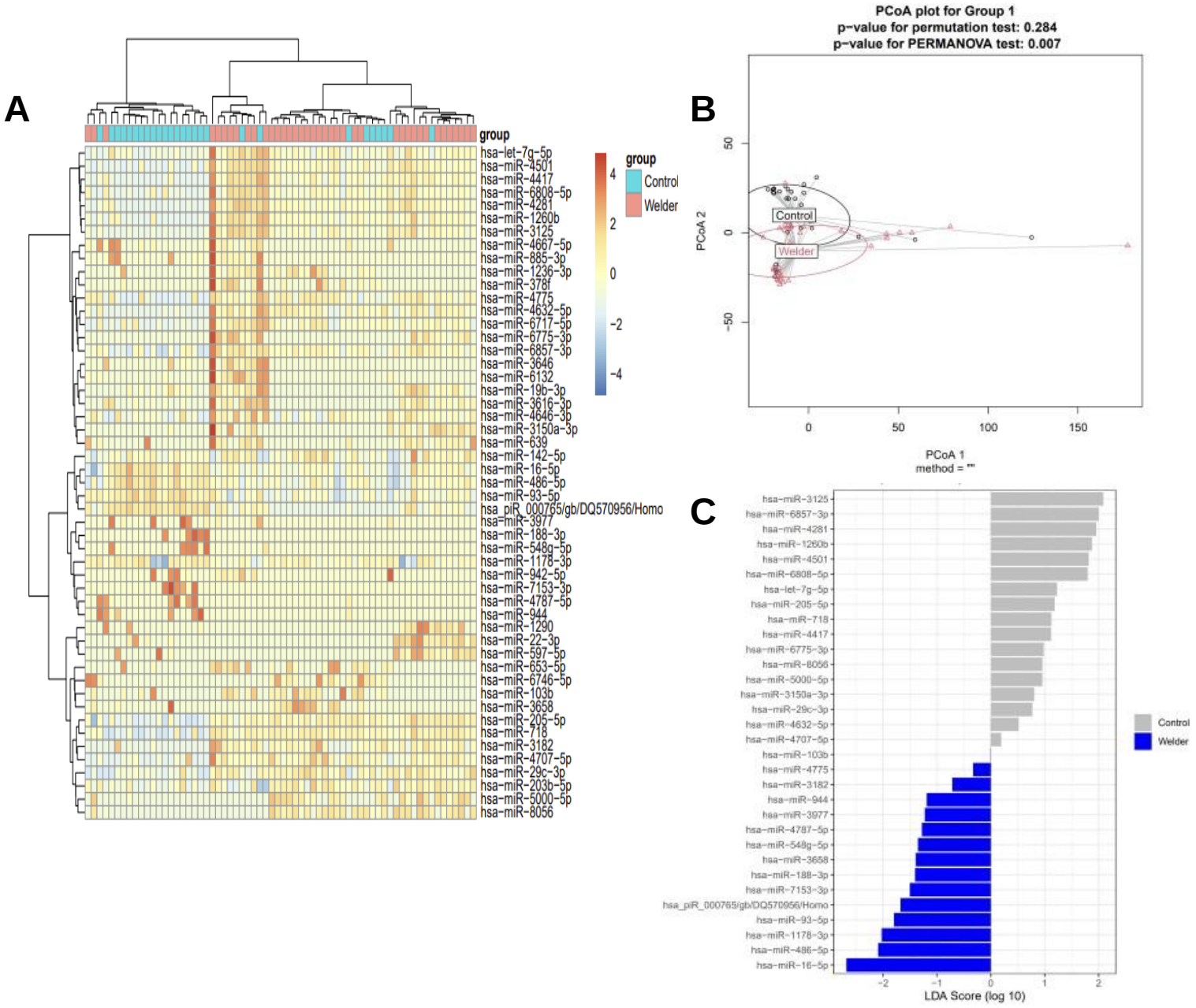

A
hsa-miR-4787-5p
hsa-miR-944
hsa-miR-188-3p
hsa-miR-7153-3p
hsa-miR-4707-5p
hsa-miR-3125
hsa-miR-548g-5p
−log10(pvalue)
log2FoldChange
Significance
Not Significant
Significant
B
log fold change
mean of normalized counts

Figure 2


A
group
hsa-let-7g-5p
hsa-miR-4501
hsa-miR-4417
hsa-miR-6808-5p
hsa-miR-4281
hsa-miR-1260b
hsa-miR-3125
hsa-miR-4667-5p
hsa-miR-885-3p
hsa-miR-1236-3p
hsa-miR-378f
hsa-miR-4775
hsa-miR-4632-5p
hsa-miR-6717-5p
hsa-miR-6775-3p
hsa-miR-6857-3p
hsa-miR-3646
hsa-miR-6132
hsa-miR-19b-3p
hsa-miR-3616-3p
hsa-miR-4646-3p
hsa-miR-3150a-3p
hsa-miR-639
hsa-miR-142-5p
hsa-miR-16-5p
hsa-miR-486-5p
hsa-miR-93-5p
hsa_piR_000765/gb/DQ570956/Homo
hsa-miR-3977
hsa-miR-188-3p
hsa-miR-548g-5p
hsa-miR-1178-3p
hsa-miR-942-5p
hsa-miR-7153-3p
hsa-miR-4787-5p
hsa-miR-944
hsa-miR-1290
hsa-miR-22-3p
hsa-miR-597-5p
hsa-miR-653-5p
hsa-miR-6746-5p
hsa-miR-103b
hsa-miR-3658
hsa-miR-205-5p
hsa-miR-718
hsa-miR-3182
hsa-miR-4707-5p
hsa-miR-29c-3p
hsa-miR-203b-5p
hsa-miR-5000-5p
hsa-miR-8056
group
Control
Welder
4
2
0
-2
-4
B
PCoA plot for Group 1
p-value for permutation test: 0.284
p-value for PERMANOVA test: 0.007
Control
Welder
PCoA 2
PCoA 1
method = ""
C
hsa-miR-3125
hsa-miR-6857-3p
hsa-miR-4281
hsa-miR-1260b
hsa-miR-4501
hsa-miR-6808-5p
hsa-let-7g-5p
hsa-miR-205-5p
hsa-miR-718
hsa-miR-4417
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hsa-miR-4707-5p
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hsa-miR-93-5p
hsa-miR-1178-3p
hsa-miR-486-5p
hsa-miR-16-5p
LDA Score (log 10)
Control
Welder