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IDENTIFICATION OF MICRORNA-BASED BIOMARKERS INDICATIVE OF NEUROLOGICAL EFFECTS DUE TO JET FUEL EXPOSURE IN RATS (*RATTUS NORVEGICUS*)

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14. ABSTRACT
Inhalation exposures to jet fuel can result in alterations in neurological and immunological systems, potentially disrupting neuroimmune signaling. This study utilized rat inhalation exposure models using various jet fuel to identify potential brain and blood microRNA (miRNA) biomarkers which correlate with jet fuel-mediated neurological deficit. Plasma and brain regions were examined after low, medium, and high fuel dosages to see if and which brain regions indicated significant alterations, and if those miRNA signal profiles were reflected in the blood. Study collaborators NAMRU-D also completed behavioral assays as well as electrophysiology to directly assess brain function at the neuronal levels. Specific miRNA alterations initiated by inhalation of the military fuels JP-8, Jet A, JP-5, or Fischer-Tropsch (FT) were identified in three brain regions (cerebellum, hippocampus, prefrontal cortex). As gauged by levels of miRNA alterations, it was seen that the primary brain target varied depending upon fuel type. Highest levels of differential miRNA were seen as: JP-8 (cerebellum), Jet A (cerebellum and hippocampus), JP-5 (prefrontal cortex), and FT (prefrontal cortex). While qPCR analysis of a subset of identified miRNAs failed to validate the expression changes, other miRNA discoveries (especially members of the miRNA-466 family) remain to be further examined.

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Jet fuel, neurological effects, brain, inhalation, miRNA, biomarker

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

PREFACE

This work was funded by Military Operational Medicine (MOMRP) Joint Program committee -5 (JPC-5) and conducted at the 711th Human Performance Wing, Human Effectiveness Directorate, Airman Bioengineering Division, RHDJ (now currently RHBBB) Branch at Wright-Patterson AFB, OH.

This project was funded by the MOMRP JPC-5 FY13 Injury Prevention, Physiological and Environmental Health Award (IPPEHA). Dr. Camilla Mauzy served as the Program Manager and co-Principal Investigator, along with Dr. Brian Wong, Navy Medical Research Unit Dayton (NAMRU-D) for the effort. This technical project report (Interim Work Unit report for Emerging Toxicology Work Unit H0V9) was written for this effort.

ACKNOWLEDGEMENTS

This study was conducted under an Institutional Animal Care and Use Committee (IACUC) approved protocol in a facility accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC) in accordance with the Guide for the Care and Use of Laboratory Animals (NRC, 2011). Animal studies were done according to experimental protocols approved by the AFRL Institutional Animal Care and Use Committee (Protocol Number F-WA-2014-0153A).

The authors would like to thank NAMRU-D and their staff for their collaborative efforts on this project, including Karen Mumy, Brian Wong, Joyce Rohan, Shawn McInturf, Chester Gut, CDR William Howard, and James Reboulet. The authors would also like to thank David Mattie for help on fuels acquisition and composition information.

SUMMARY

Exposures to either JP-5 or JP-8 fuels can result in significant alterations in neurological and immunological systems, potentially disrupting neuroimmune signaling which has been implicated in brain function. Changes in gene expression are also evident following jet fuel exposure. This study utilized *in vivo* inhalation exposures to various types of jet fuel to identify potential brain and blood microRNA (miRNA) biomarkers which correlate with jet fuel-mediated neurological deficit. This study measured levels of miRNA in the plasma and various brain regions after low, medium, and high fuel dosages to see if the brain miRNA signal profiles are reflected in the blood. Study collaborators NAMRU-D also completed behavioral assays as well as electrophysiology to directly assess brain function at the neuronal levels. Specific miRNA alterations initiated by inhalation of the military fuels JP-8, Jet A, JP-5, or Fischer-Tropsch (FT) were identified in three brain regions (cerebellum, hippocampus, prefrontal cortex). As gauged by levels of miRNA alterations, it was seen that the primary brain target varied depending upon fuel type. Highest levels of differential miRNA were seen as: JP-8 (cerebellum), Jet A (cerebellum and hippocampus), JP-5 (prefrontal cortex), and FT (prefrontal cortex). While analysis of a subset of miRNAs using qPCR failed to validate the expression changes, other miRNA discoveries, especially members of the miRNA-466 family, remain to be validated.

1.0 INTRODUCTION

Jet fuel exposure is a typical occupational exposure hazard among military personnel. Annual jet fuel use by the United States (US) Department of Defense (DoD) and the general economy amounts to 3 to 4 billion gallons/year and approximately 27 to 36 billion gallons/year, respectively, based on 2003 consumption.[1] The current military fuel is jet propellant-8 (JP-8) according to DoD Directive 4140.25. It is widely used by the US Air Force, Army, and Navy. The Navy also uses jet propellant-5 (JP-5) for carrier-based aircraft due to its higher flashpoint than JP-8, increasing shipboard safety. Jet A is currently the standard fuel for commercial aircraft and may replace JP-8 in military operations in the future. The first alternative jet fuel certified by the Air Force is FT, produced from coal or gas by the Fischer Tropsch (FT) process. The DoD has been interested in this environmentally cleaner fuel in an attempt to reduce the reliance on foreign crude oil and promote the use of alternative biofuel. Occupational exposure to these fuels is highly regulated so that the health of military personnel is not jeopardized. Although many precautions are in place to minimize the degree of exposure, it is unknown if neuronal degeneration occurs with unintended acute high exposures or lower level chronic exposures.

Multiple studies have documented the deleterious effects of acute and chronic exposures to jet fuels on human performance, ranging from vestibular/coordination impairments to cognitive deficits including memory defects attention decline, and information processing speed.[2,3,4] Early studies conducted in late 1970s documented cases of self-reported symptoms that include neurasthenia, dizziness, depression, headache, and memory impairment among personnel chronically exposed to the European military jet fuels MC 75 and MC 77.[5] Later studies also reported neurophysiological symptoms due to JP-5 or JP-8 exposures.[6,7] Two aviators suffered from burning eyes, poor coordination, and memory deficits due to intoxication by JP-5 fuel vapor in their cockpit.[6] Another study performed in 1997 reported that chronic low level of JP-8 exposure led to increased postural sway response suggesting effects on the vestibular and proprioception systems.[2] Members of the Air National Guard were studied at the Warfield Air National Guard Base for jet fuel effects, documenting significant impairments in attention-based tasks and information processing speed due to chronic exposure to JP-8.[4] Furthermore, an epidemiology study addressing the occupational JP-8 exposure among US Air Force personnel indicated that there were some significant differences induced by high JP-8 exposure on total recall, delayed recall, and retention tasks among the 20-29 year-old age group.[3]

A series of animal jet fuel exposure studies has also been reported. However, the jet-fuel induced neurobehavioral changes reported in rats were inconsistent, making it difficult to formulate a concrete conclusion regarding the effects of jet fuel exposure on the nervous system. A 1993 study using the Morris water maze demonstrated significant impairments of learning and memory in rats exposed to low concentrations of toluene (80 parts per million (ppm), 4 weeks, 5 days/week, 6 hours (h)/day), a component of jet fuel.[7] However, more recent studies reported no significant changes in spatial learning and memory as assessed by the Morris water maze test in rats exposed to high doses (1000-2000 milligram (mg)/ meter (m)^3) of JP-8 and JP-5.[8] In contrast, biochemical assays have been more consistent in revealing statistically significant alterations in neurotransmitter levels, particularly those of dopamine, serotonin and their metabolites, following JP-8 exposure.[8,9] Dopamine and serotonin are important neurotransmitters that play critical roles in motor coordination, mood regulation and other cognitive functions. Disruption of dopamine or serotonin regulation has been demonstrated to correlate with

various neurological disorders such as Parkinson's disease (PD) and depression/anxiety,[10] and indeed, there have been reported associations between hydrocarbon exposure and higher risk of early PD onset.[11]

Alterations in gene expression were also observed in rat exposure studies with JP-8.[12,13] Changes in genes essential for neuronal function such as acetylcholine and dopamine receptors, syntaxin, and synaptosome-associated protein 23 (SNAP23) have been reported in rat brain tissue following exposure to JP-8 vapor.[13] Although gene expression studies assessed by reverse transcriptase polymerase chain reaction (RT-PCR) and gene arrays provide critical insight into the effects of jet fuel exposure on cellular function, changes in gene expression do not always correlate with changes in protein level since transcribed genes in the form of messenger ribonucleic acid (mRNA) transcripts are often further regulated post-transcriptionally by various regulators such as miRNAs.

MicroRNAs, a class of small RNA (ribonucleic acid) molecules (approximately 22-23 nucleotides in size) that typically function as very specific regulators of mRNA degradation and/or translational repression, are highly expressed in the brain**.** A growing body of research indicates a pivotal role for miRNAs in neurological development and disease.[14,15,16] Even in cases of more molecularly subtle, non-traumatic neurological conditions such as autism, schizophrenia, and stress, distinct alterations in miRNA expression patterns have been seen.[17,18] The examination of miRNA expression for toxicological studies are fairly new, but data with hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and other drug compounds indicate that toxin/chemical exposures can and do modulate brain miRNA levels.[19,20] Studies indicate that these changes in brain miRNA expression are reflected in miRNA type and levels circulating in the blood, making them particularly appealing as non-invasive biomarkers of neurological damage to toxin exposures.[21,22] MicroRNAs could be appropriate candidate biomarkers for environmental exposure markers as: (i) miRNA expression profiles are tissue-specific; (ii) miRNAs are stable in human plasma, serum, urine, saliva and other bodily fluids; and, (iii) miRNA expression has been seen to vary in response to toxicant exposures.[23,24] In addition to ascertaining miRNA species and pattern changes in the brain and blood, the identification of specific miRNAs can be used to identify potential mRNA targets using data analyses in current databases such as TargetScan, miRanda, and TarBase. Thus, not only does miRNA discovery research identify new potential biomarkers, the data also can be used to find and examine molecular pathways altered by fuel exposures.

The authors hypothesized that alterations in the levels of blood miRNAs correlates with fuel-induced neurological deficits, and that miRNA biomarkers could be used to monitor and predict neurological impairment. Given the widespread use of jet fuel in a multitude of military settings, ongoing vigilance for potential indicators of neurological effects is vital in protecting personnel. A complete miRNA profile following jet fuel exposure could yield important insights into the effects of jet fuel on protein expression, especially those proteins relating to the nervous system. The identification of a biomarker reflecting the impact of jet fuel exposure on brain function will enable close individualized monitoring of military personnel to ensure that unforeseen and unintended high exposures to jet fuel are not inducing neurological deficits leading to cognitive impairments.

This research aimed to systematically characterize the effects of subchronic jet fuel exposure on the nervous system at the cellular and molecular levels, using electrophysiology, neurobehavioral assays, matrix-assisted laser desorption/ionization imaging (MALDI) for brain proteomic profiling, and miRNA profiling (reported here). The effort had initially proposed to first utilize MALDI as a "first pass" tool to determine which brain regions demonstrated significant proteomic alterations upon fuel inhalation exposures, and to target those regions for miRNA biomarker discovery. However, due to logistic issues

involving MALDI analysis, the miRNA profiling was limited to three brain regions (hippocampus, prefrontal cortex, and cerebellum). Finally, blood plasma was also analyzed to correlate with the expression levels of specific miRNA species upon repeated exposure to jet fuel (JP-5, Jet A, JP-8, or FT).

2.0 MATERIALS AND METHODS

2.1 Animal Exposure Model

All studies involving animals were approved by the Wright-Patterson Institutional Animal Care and Use Committee as protocol number F-WA-2014-0152, and were conducted in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International, in accordance with the *Guide for the Care and Use of Laboratory Animals*, National Research Council (1996). The study was performed in compliance with DODI 3216.1.

Male Fisher 344 rats (*Rattus norvegicus*) were exposed to Jet Propellant-8 (JP-8) Property Measurement for Fuel Research (POSF) #11747, Jet A (POSF #4658), Fischer-Tropsch (FT) POSF #5109, or Jet Propellant-5 (JP-5) POSF #12804 at various doses (0, 200, 1000, or 2000 mg/m^3) by inhalation using whole body exposure chambers (n=6 per exposure group). Jet fuel was administered as an aerosol vapor combination for 6 hours (hrs) per day, 5 days per week over a 28-day period. All rats were housed under a 12 h light/dark cycle. Food and water were provided throughout the study *ad libitum*.

All animals were evaluated for behavior alterations in spatial learning and memory using the Morris water maze test as described in Rohan et al.[25]

2.2 Study Jet Fuel Composition

Animals were exposed to one of four types of military fuels: JP-8, JP-5, Jet A, or FT. Fuels JP-8, JP-5, and Jet A are all kerosene-type, consisting of aliphatic and aromatic hydrocarbons. In addition to hydrocarbons, fuel additives are commonly found in JP-8 and JP-5 which include icing inhibitors, biocides, lubricants, and stabilizers. The fuel samples used for the *in vivo* exposures were characterized as to their hydrocarbon and additive content.

2.2.1 Jet Fuel POSF and Composition. Jet A fuel samples were analyzed at least twice. The range in percent by weight (wt%) indicates the variation in the evaluation method (**Table 1**). For the JP-8 samples, the fuel system icing inhibitor (FSII) was added at the AFRL Fuels Branch whereas the JP-5 fuel was received with FSII additive present. The exact volume (vol)% was not determined.

Table 1. POSF and composition of evaluated fuels

Fuel Name	POSF #	Additives Present	Total Aromatics (wt%, GC x GC)*	Fuel Used for GC x GC	FSII (vol% in final fuel)
Jet A	4658	None	21.25 - 22.29[a]	4658	0
JP-8	11747 (11721 + AF additives)	CI/LI, FSII, SDA	19.52	11721	0.085[b]
JP-5	12804 (Includes Navy additives)	CI/LI, FSII	18.08	12804	0.1 max[c]
FT	5109 (5108 + AF Additives)	CI/LI, FSII, SDA	0.62	5109	0.06[d]

*Gas Chromatography (GS)
[a]This fuel has been analyzed at least twice, andthe range in percent by weight gives an idea of the variation in the method.
[b]By formulation (amount added at the AFRL Fuels Branch)
[c]Fuel received with additives present; exact amount unknown
[d]By analysis (spec testing)

2.2.2 Study Jet Fuel Additives. The FSII value was formerly 0.15 vol% but has been decreased to 0.1 vol%. The amount of this additive does vary from blend to blend. Exact amounts of FSII in the test fuels JP-8, JP-5 and FT are listed in **Table 2**.

Table 2. Fuel additives found in test fuels

Additive	Additive Name/ Description	Compound or Proprietary Name	Air Force	Navy	Amount
CI/LI	Corrosion Inhibitor/Lubricity Improver	DCI-4A (proprietary)	Yes	Yes	15 mg/L
FSII	Fuel System Icing Inhibitor	diethylene glycol monomethyl ether (DIEGME)	Yes	Yes	0.1 vol% max
SDA	Static Dissipater Additive	Stadis 450 (proprietary)	Yes	No	2 mg/L

2.2.3 Comparison of Test Fuels. The sample analyses were completed using gas chromatography GC x GC analysis performed using the method described by Striebich et al. (2014).[26] All values are percent by weight (wt%, GC x GC) (**Table 3**). JP-8 fuel was analyzed without additives (POSF 11721 = 11747 without additives), as the additive GC signal would be very low in comparison to the very strong hydrocarbon signal from the fuel.

Table 3. Hydrocarbon Composition of test fuels by wt%

HYDROCARBON BINS*	Jet A		JP-8	JP-5	FT
POSF Analyzed	POSF 4658 1st Analysis	POSF 4658 2nd Analysis	POSF 11721**	POSF 12804	POSF 5109
AROMATICS					
Alkylbenzenes	13.68	13.69	10.74	11.23	0.34
Alkylnaphthalenes	1.81	1.76	0.93	1.40	0.01
Cycloaromatics	5.54	5.79	7.85	5.45	0.06
Total Aromatics	**21.04**	**21.25**	**19.52**	**18.08**	**0.41**
ALIPHATICS					
iso-Paraffins	30.16	31.34	22.52	26.86	73.87
n-Paraffins	18.26	19.00	16.14	19.54	24.71
Monocycloparaffins	23.13	22.64	30.58	26.56	0.88
Dicycloparaffins	7.33	5.73	11.18	8.90	0.14
Tricycloparaffins	0.10	0.05	0.05	0.06	<0.01
Total Aliphatics	**78.96**	**78.75**	**80.48**	**81.92**	**99.59**
TOTAL HYDROCARBONS	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**

*All values in percent by weight; **Fuel was analyzed without additives: POSF 11721 = 11747 without additives

2.2.4 Constituent Comparison of Test Fuels. Test fuels were further analyzed for specific wt% of specific hydrocarbon bins for aromatics (alkylbenzenes, diaromatics, cycloaromatics) and aliphatics (iso-parafins, n-paraffins, monocycloparaffins, dicycloparaffins, tricycloparaffins) (**Table 4**).

Table 4. Individual hydrocarbon components for test fuels

Fuel	Jet A		JP-8	JP-5	FT
POSF Analyzed	POSF 4658 1st Analysis	POSF 4658 2nd Analysis	POSF 11721**	POSF 12804	POSF 5109
HYDROCARBON BINS*					
AROMATICS					
Alkylbenzenes					
benzene (C06)	<0.01	<0.01	<0.01	<0.01	<0.01
toluene (C07)	0.15	0.16	0.10	<0.01	<0.01
C2-benzene (C08)	0.77	0.78	0.51	0.02	0.02
C3-benzene (C09)	2.20	2.24	1.59	2.01	0.07
C4-benzene (C10)	2.98	3.02	1.92	3.54	0.09
C5-benzene (C11)	2.52	2.48	1.66	2.61	0.07
C6-benzene (C12)	2.01	1.93	1.64	1.64	0.06
C7-benzene (C13)	1.22	1.19	1.19	0.92	0.04
C8-benzene (C14)	0.87	0.89	1.08	0.45	<0.01
C9-benzene (C15)	0.60	0.65	0.71	0.03	<0.01
C10+-benzene (C16+)	0.37	0.35	0.33	<0.01	<0.01
Total Alkylbenzenes	**13.68**	**13.69**	**10.74**	**11.23**	**0.34**
Diaromatics (Naphthalenes, Biphenyls, etc.)					
diaromatic-C10	0.12	0.12	0.03	0.15	<0.01
diaromatic-C11	0.42	0.42	0.12	0.52	<0.01
diaromatic-C12	0.64	0.60	0.33	0.64	<0.01
diaromatic-C13	0.42	0.40	0.26	0.09	<0.01
diaromatic-C14+	0.21	0.23	0.20	<0.01	<0.01
Total Alkylnaphthalenes	**1.81**	**1.76**	**0.93**	**1.40**	**0.01**
Cycloaromatics (Indans, Tetralins,etc.)					
cycloaromatic-C09	0.04	0.04	0.06	0.06	<0.01
cycloaromatic-C10	0.43	0.43	0.49	0.66	0.01
cycloaromatic-C11	1.09	1.13	1.53	1.45	0.03
cycloaromatic-C12	1.49	1.63	2.03	1.84	0.01
cycloaromatic-C13	1.43	1.45	1.92	1.24	<0.01
cycloaromatic-C14	0.73	0.71	1.14	0.20	<0.01
cycloaromatics-C15+	0.33	0.41	0.67	<0.01	<0.01
Total Cycloaromatics	**5.54**	**5.79**	**7.85**	**5.45**	**0.06**
Total Aromatics	**21.04**	**21.25**	**19.52**	**18.08**	**0.41**
ALIPHATICS					
iso-Paraffins					
C07 & lower -isoparaffins	0.12	0.23	0.19	<0.01	0.01
C08-isoparaffins	0.50	0.56	0.39	<0.01	1.28
C09-isoparaffins	1.33	1.08	0.71	0.04	5.71

C10-isoparaffins	3.34	3.59	2.67	3.19	8.20
C11-isoparaffins	4.86	5.12	3.20	6.36	10.01
C12-isoparaffins	4.94	5.31	3.02	5.91	11.07
C13-isoparaffins	5.19	5.25	3.29	4.78	11.49
C14-isoparaffins	4.32	4.44	3.65	4.70	11.12
C15-isoparaffins	3.12	3.10	3.06	1.77	9.81
C16-isoparaffins	1.45	1.66	1.46	0.11	4.83
C17-isoparaffins	0.64	0.69	0.59	<0.01	0.31
C18-isoparaffins	0.19	0.19	0.19	<0.01	0.02
C19-isoparaffins	0.08	0.08	0.06	<0.01	<0.01
C20-isoparaffins	0.05	0.02	0.02	<0.01	<0.01
C21-isoparaffins	<0.01	<0.01	<0.01	<0.01	<0.01
C22-isoparaffins	<0.01	<0.01	<0.01	<0.01	<0.01
C23-isoparaffins	<0.01	<0.01	<0.01	<0.01	<0.01
C24-isoparaffins	<0.01	<0.01	<0.01	<0.01	<0.01
Total iso-Paraffins	**30.16**	**31.34**	**22.52**	**26.86**	**73.87**
n-Paraffins					
n-C07 & lower	0.18	0.15	0.16	<0.01	0.04
n-C08	0.50	0.54	0.39	<0.01	1.18
n-C09	1.12	1.14	0.89	0.36	3.27
n-C10	2.45	2.55	1.92	3.34	4.49
n-C11	3.55	3.62	2.63	5.37	4.65
n-C12	3.62	3.70	2.92	4.68	4.20
n-C13	2.83	2.86	2.55	3.51	3.30
n-C14	2.03	2.17	2.27	2.04	2.35
n-C15	1.15	1.28	1.46	0.21	1.14
n-C16	0.53	0.61	0.67	<0.01	0.08
n-C17	0.21	0.27	0.22	<0.01	<0.01
n-C18	0.05	0.05	0.04	<0.01	<0.01
n-C19	0.02	0.02	0.02	<0.01	<0.01
n-C20	<0.01	<0.01	<0.01	<0.01	<0.01
n-C21	<0.01	<0.01	<0.01	<0.01	<0.01
n-C22	<0.01	<0.01	<0.01	<0.01	<0.01
n-C23	<0.01	<0.01	<0.01	<0.01	<0.01
Total n-Paraffins	**18.26**	**19.00**	**16.14**	**19.54**	**24.71**
Monocycloparaffins					
C07 & lower monocycloparaffins	0.28	0.20	0.48	<0.01	<0.01
C08-monocyclocycloparaffins	0.57	0.69	0.76	<0.01	0.12
C09-monocyclocycloparaffins	1.43	1.67	2.24	0.79	0.22
C10-monocyclocycloparaffins	3.26	3.26	3.56	4.60	0.15
C11-monocyclocycloparaffins	4.29	4.11	4.79	7.13	0.14
C12-monocyclocycloparaffins	4.08	4.07	5.77	6.06	0.12
C13-monocyclocycloparaffins	3.79	3.65	5.35	5.23	0.05

C14-monocyclocycloparaffins	2.61	2.43	3.90	2.33	0.02
C15-monocyclocycloparaffins	1.63	1.55	2.48	0.42	0.03
C16-monocyclocycloparaffins	0.74	0.64	0.90	<0.01	<0.01
C17-monocyclocycloparaffins	0.36	0.28	0.29	<0.01	<0.01
C18-monocyclocycloparaffins	0.05	0.06	0.04	<0.01	<0.01
C19+-monocyclocycloparaffins	0.02	0.03	0.02	<0.01	<0.01
Total Monocycloparaffins	**23.13**	**22.64**	**30.58**	**26.56**	**0.88**
Dicycloparaffins					
C08-dicycloparaffins	0.02	0.02	0.02	<0.01	<0.01
C09-dicycloparaffins	0.46	0.29	0.12	0.35	<0.01
C10-dicycloparaffins	0.56	0.43	0.75	0.82	0.02
C11-dicycloparaffins	1.45	1.26	1.89	2.40	0.04
C12-dicycloparaffins	1.55	1.22	2.41	2.38	0.03
C13-dicycloparaffins	1.65	1.42	3.07	2.38	0.03
C14-dicycloparaffins	0.95	0.82	2.13	0.56	0.01
C15-dicycloparaffins	0.44	0.21	0.67	0.01	<0.01
C16-dicycloparaffins	0.21	0.02	0.06	<0.01	<0.01
C17+-dicycloparaffins	0.04	0.03	0.04	<0.01	<0.01
Total Dicycloparaffins	**7.33**	**5.73**	**11.18**	**8.90**	**0.14**
Tricycloparaffins					
C10-tricycloparaffins	<0.01	<0.01	<0.01	<0.01	<0.01
C11-tricycloparaffins	0.09	0.05	0.05	0.06	<0.01
C12-tricycloparaffins	<0.01	<0.01	<0.01	<0.01	<0.01

Fuel	Jet A		JP-8	JP-5	FT
Total Tricycloparaffins	**0.10**	**0.05**	**0.05**	**0.06**	**<0.01**
Total Cycloparaffins	**30.55**	**28.42**	**41.81**	**35.52**	**1.02**
Total Aliphatics	**78.96**	**78.75**	**80.48**	**81.92**	**99.59**
Total Hydrocarbons	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**

*All values in percent by weight, **Fuel was analyzed without additives: POSF 11721 = 11747 without additives

2.3 Sample Collection

Euthanasia via rapid decapitation was conducted in accordance with the 2020 Edition of the American Veterinary Medical Association Guidelines for the Euthanasia of Animals to avoid contamination of jet fuel-induced neurologic effects by chemical anesthetics. Blood was collected via the lateral tail vein into RNAprotect® Animal Blood Tubes (Qiagen). Blood was also collected post-euthanasia by draining into PAXgene Blood RNA Tubes (BD, Cat. No. 762165). Brains were removed and the cerebellum (CRB), hippocampus (HIP), and prefrontal cortex (PFC) tissue isolated and placed on ice. Fresh tissue was immersed in RNA*later* solution and stored at 4°C overnight. Tissue was removed from RNA*later*

solution and excess solution was blotted away with a lab wipe. The RNA*later*-stabilized tissue was sectioned into smaller pieces, transferred into RNase-free microfuge tubes and stored at -80°C.

2.4 RNA Extraction

2.4.1 RNA Extraction from RNAprotect® Animal Blood Tubes. Total RNA, including miRNA, was purified from stabilized blood using RNeasy® Protect Animal Blood kits (Qiagen, Cat. No. 73224) following manufacturer's protocol "*Purification of Total RNA Including miRNA from RNAprotect Stabilized Animal Blood*." RNAprotect Animal Blood Tubes were equilibrated at room temperature (RT) for 2 h. Tubes were inverted 10 times and centrifuged for 3 min at 5,000 times gravity (x g). The supernatant was discarded, and the pellet was dissolved in 1 mL of RNase-free water. The sample was centrifuged for 3 min at 5,000 x g, and the supernatant was discarded. The pellet was dissolved in 240 µL Buffer RSB after which 200 uL Buffer RBT and 20 µL proteinase K were added. The mixture was vortexed and incubated for 10 min at 55°C and 900 rotations per minute (rpm). The sample was transferred to a QIAshredder spin column and centrifuged for 3 min at 20,000 x g. The supernatant was collected, and 690 µL of 100% ethanol was added. After vortexing, 700 µL of sample were transferred to an RNeasy MinElute spin column. The sample was centrifuged for 1 min at 8,000 x g, and the flow-through was discarded. More sample was added to the RNeasy MinElute spin column and centrifuged until the entire sample was used. The column was washed with 350 µL Buffer RWT for 15 sec at 8,000 x g. The flow-through was discarded. A mixture of 10 µL DNAse I stock solution and 70 µL Buffer RDD was added directly to the spin column membrane and incubated at RT for 15 min. The column was washed with 350 µL Buffer RWT for 15 sec at 8,000 x g, followed by 500 µL Buffer RPE. The flow-through was discarded. The column was washed with 80% ethanol for 2 min at 8,000 x g, and the flow-through was discarded. RNA was eluted by adding 30 µL Buffer REB directly to the spin column membrane and centrifuging for 1 min at 8,000 x g.

2.4.2 RNA Extraction from PAXgene Tubes. Total RNA, including miRNA, was extracted from PAXgene Blood RNA Tubes using PAXgene Blood miRNA Kits (Qiagen, Cat. No. 763134), following manufacturer's protocol. The PAXgene Blood RNA Tube was equilibrated at RT for 2 hrs, and the thawed tube was inverted 10 times. The tube was centrifuged for 10 min at 3,000 x g. The supernatant was discarded, and 4 mL of RNase-free water were added to the pellet. The tube was centrifuged a second time for 10 min at 3,000 x g, and the supernatant was discarded. 350 µL of Buffer BM1 were added, and the tube was vortexed until the pellet dissolved. 300 µL of Buffer BM2 and 40 µL of proteinase K were added. The sample was incubated for 10 min at 55°C and 600 rpm. The sample was pipetted into a PAXgene Shredder spin column and centrifuged for 3 min at 20,000 x g. The flow-through was transferred to a new 1.5 mL tube, and 700 µL of 100% isopropanol were added. The sample was vortexed, and 700 µL were pipetted into a PAXgene RNA spin column. The column was centrifuged for 1 min at 10,000 x g. The remaining sample was added to the spin column and centrifuged for 1 min at 10,000 x g. The flow-through was discarded. 350 uL Buffer BM3 were added to the spin column and centrifuged for 15 sec at 10,000 x g. The flow-through was discarded, and 80 µL of DNase I incubation mix were added directly to the PAXgene RNA spin column membrane. The sample was incubated at RT for 15 min. 350 µL of Buffer BM3 were added to the spin column, and the column was centrifuged for 15 sec at 10,000 x g. The flow-through was discarded. 500 uL Buffer BM4 were added to the column, and the column was centrifuged for 15 sec

at 10,000 x g. Another 500 uL of Buffer BM4 were added to the column, and the column was centrifuged for 2 min at 10,000 x g followed by another minute at the same speed. The column was placed into a new tube, 40 uL Buffer BR5 added, and the column centrifuged for 1 min at 10,000 x g. Another 40 uL of Buffer BR5 were added, and the column was incubated for 10 min at RT before being centrifuged for 1 min at 10,000 x g. The RNA eluate was stored at -20°C.

2.4.3 miRNA from RNAprotect Stabilized Animal Blood. RNAprotect Animal Blood Tubes were equilibrated at RT for 2 h. Tubes were inverted 10 times and centrifuged for 3 min at 5,000 x g. The supernatant was discarded, and the pellet was dissolved in 1 mL of RNase-free water. The sample was centrifuged for 3 min at 5,000 x g, and the supernatant was discarded. The pellet was dissolved in 240 µL of Buffer RSB after which 200 µL of Buffer RBT and 20 µL of proteinase K were added. The mixture was vortexed and incubated for 10 min at 55°C and 900 rpm. The sample was transferred to a QIAshredder spin column and centrifuged for 3 min at 20,000 x g. The supernatant was collected, and 690 uL of 100% ethanol were added. After vortexing, 700 µL of sample were transferred to an RNeasy MinElute spin column. The sample was centrifuged for 1 min at 8,000 x g, and the flow-through was discarded. More sample was added to the RNeasy MinElute spin column and centrifuged until the entire sample was used. The column was washed with 350 µL of Buffer RWT for 15 sec at 8,000 x g. The flow-through was discarded. A mixture of 10 uL of DNAse I stock solution and 70 uL of Buffer RDD was added directly to the spin column membrane and incubated at RT for 15 min. The column was washed with 350 µL of Buffer RWT for 15 sec at 8,000 x g, followed by 500 µL of Buffer RPE. The flow-through was discarded. The column was washed with 80% ethanol for 2 min at 8,000 x g, and the flow-through was discarded. RNA was eluted by adding 30 µL of Buffer REB directly to the spin column membrane and centrifuging for 1 min at 8,000 x g.

2.4.4 RNA Extraction from Tissue. Total RNA, including miRNA, was extracted from RNAlater stabilized lung tissue using miRNeasy Mini kits (Qiagen, Cat. No. 217004) following the manufacturer's protocol. Frozen lung tissue was pulverized using a liquid nitrogen-cooled mini mortar. Approximately 15 mg of pulverized tissue were added to a tube containing 700 µL QIAzol Lysis Reagent and 3 x 2.0 mm zirconium oxide beads (Next Advance, Cat. No. ZROB20). Tissue was disrupted for 2 min at speed 6 using a Bullet Blender® Gold (Next Advance). 140 µL of chloroform were added to the homogenate, and the mixture was shaken vigorously for 15 sec. The sample was centrifuged for 15 min at 12,000 x g at 4°C. The aqueous phase was removed and mixed with 1.5 volumes of 100% ethanol. The sample was transferred to an RNeasy spin column and centrifuged for 15 sec at 11,000 rpm. The column was washed with 350 µL Buffer RWT for 15 sec at 11,000 rpm. A total of 80 µL DNase I incubation mix (DNase in Buffer RDD) was added to the spin column and incubated at RT for 15 min. The column was washed for 15 sec at 11,000 rpm with 350 µL of Buffer RWT followed by 500 µL Buffer RPE. The column was washed a second time with 500 µL of Buffer RPE and centrifuged for 2 min at 11,000 rpm. To elute RNA, 30 µL of DNase/RNase-free water were added to the spin column membrane and incubated at RT for 2 min. The column was centrifuged for 1 min at 11,000 rpm.

2.4.5 RNA Quantification and Quality Control. RNA was quantitated using a NanoDrop-1000 spectrophotometer. An A260/280 ratio greater than 1.8 and an A260/230 ratio greater than 1.7 were considered acceptable. In addition, an aliquot of RNA was run on a 1% denaturing agarose gel to confirm ribosomal RNA integrity.

2.5 Affymetrix GeneChip miRNA 3.0 Array

2.5.1 FlashTag Biotin HSR RNA Labeling. Total RNA was labeled for microarray analysis according to kit protocol using the Affymetrix FlashTag Biotin HSR RNA Labeling kit (Affymetrix, Cat. No. 901910). A total of 2 µL of RNA Spike Control Oligos were added to 500 ng of RNA, then 5 uL of PolyA Tailing Master Mix were added to the RNA/Spike Control Oligos. The sample was incubated for 15 min at 37°C. After incubation, 4 µL of FlashTag Biotin HSR Ligation Mix and 2 µL of T4 DNA Ligase were added, and the sample was incubated at RT for 30 min. After incubation, 2.5 µL of HSR Stop Solution was added, after which 2 µL of sample were removed for Enzyme Linked Oligosorbent Assay **(**ELOSA) quality control (QC) assay with the remainder stored at -20°C.

2.5.2 ELOSA QC Assay. An ELOSA was conducted to confirm that the biotin labeling process performed appropriately. Successful biotin labeling was verified through the hybridization of Control Oligos (spiked into the sample during FlashTag labeling) to complementary Oligos immobilized onto the ELOSA microtiter plate wells. ELOSA was performed according to the FlashTag Biotin HSR RNA Labeling kit protocol. 75 µL of ELOSA Spotting Oligos were added to the wells of the plate (Flat Bottom Immobilizer Amino – 8 well strips, Fisher, Cat. No. 12-567-13) and incubated overnight at 4°C. Spotting Oligos were removed, and the plate was washed 2 x 350 uL with 1X phosphate buffered saline (PBS), 0.02% Tween-20. 150 µL of 5% bovine serum albumin (BSA) in 1X PBS were added to each well, and the plate was incubated for 1 hr at RT. BSA blocking solution was removed. A total of 50.5 µL of Hybridization Master Mix were added to 2 µL of samples and controls. All 52.5 µL of the hybridization solution/sample were added to a designated well, and the plate was incubated for 1 hr at RT. Hybridization solution/samples were removed, and the plate was washed 3 x 350 µL with 1X PBS, 0.02% Tween-20. 75 µL of 0.2 ug/mL streptavidin-horseradish peroxidase (SA-HRP) were added to each well, and the plate was incubated for 1 hr at RT. The plate was washed 3 x 350 µL with 1X PBS, 0.02% Tween-20. A total of 100 µL of 3,3',5,5'-tetramethylbenzidine (TMB) substrate were added to each well, and the plate was incubated for 15 min at RT in the dark. Lastly, 100 µL TMB Stop Solution were added to each well, and the absorbance was read immediately at 450 nm. Readings > 0.10 optical density (OD) over the negative control were considered positive.

2.5.3 Array Hybridization, Washing, Staining, and Scanning. Total miRNA expression profiling was performed on the Affymetrix GeneChip miRNA 3.0 Array (Cat. No. 902019, Affymetrix) that interrogates 486 rat pre-miRNAs and 680 rat mature miRNAs, which provides complete coverage of miRBase v17, including both mature and hairpin stem loops. RNA was hybridized to arrays according to the FlashTag Biotin HSR RNA Labeling Kit protocol. A total of 110.5 µL hybridization master mix were added to the 21.5 µL of biotin-labeled sample. The sample was heated at 99°C for 5 min and immediately cooled at 45°C for 5 min. 130 µL of sample were loaded onto the array and incubated for 16-18 hrs at 48°C and 60 rpm in an Affymetrix GeneChip Hybridization Oven 640. After hybridization, sample was removed from the array, and the array was filled completely with Array Holding Buffer. The array was washed (Wash Buffer A and B) and stained (Stain Cocktail 1 and 2) on an Affymetrix Fluidics Station 450 using protocol FS450_0002 (**Table 5**). The array was scanned using an Affymetrix GeneChip Scanner 3000 7G.

Table 5. FS450_0002 Fluidics Profile – 100 format

Wash Cycle	Buffer	Cycles	Mixes/Cycle	Temp. (°C)	Time (min)
Post Hyb #1	Wash Buffer A (non-stringent)	10	2	30	---
Post Hyb #2	Wash Buffer B (stringent)	6	15	50	---
Stain #1	SAPE Solution (Cocktail 1)	---	---	35	5
Post Stain #1	Wash Buffer A (non-stringent)	10	4	30	---
Stain #2	Antibody Solution (Cocktail 2)	---	---	35	5
Stain #3	SAPE Solution (Cocktail 1)	---	---	35	5
Post Stain #2	Wash Buffer A (non-stringent)	15	4	35	---
Hold	Array Holding Buffer	---	---	---	---

2.5.4 Microarray Data Analysis. Raw microarray signal intensity values (.CEL files) were analyzed in Affymetrix Expression Console v1.3 (EC) software. The RMA+DABG-Rat_only algorithm was used to create summarized expression values (.CHP files). Subsequently, the normalized data were analyzed using Affymetrix Transcriptome Analysis Console v2.0 software. MicroRNAs were considered differentially expressed using fold change <-2 or >2 and analysis of variance (ANOVA) *p*-value < 0.05 cut-offs (**Table 6**).

Table 6. Microarray Data Analysis Conditions

Analysis Conditions	
Analysis Type	Gene Level Differential Expression Analysis
Array Type	miRNA 3.0
Annotation File	miRNA-30-st-v1.annotations.20140513.csv
Algorithm	One-Way Between-Subject ANOVA (Unpaired)
Filter Criteria	Fold Change (linear) < -2 or Fold Change (linear) > 2
	ANOVA *p*-value (Condition pair) <0.05

2.6 Analysis using Custom TaqMan MicroRNA Cards

2.6.1 cDNA Template Preparation. cDNA templates were prepared using a TaqMan Advanced miRNA cDNA Synthesis Kit (Thermo Fisher, Cat. No. A28007) and following the manufacturer's protocol (TaqMan Advanced miRNA Assays User Guide, Publication Number MAN0016122, Revision C.0). Total RNA concentration for input into template preparation was 5 ng/μL. The poly(A) tailing reaction was performed by adding 3 μL Poly(A) Reaction mix to 2 μL total RNA. The reaction mix was incubated at 37°C for 45 min, 65°C for 10 minutes, and held at 4°C. The adaptor ligation reaction was performed by adding 10 μL Ligation Reaction Mix to the poly(A) tailing reaction. The reaction mix was incubated for 60 min at 16°C and held at 4°C. For the reverse transcription (RT) reaction, 15 μL of RT Reaction Mix were added to the adaptor ligation reaction product. The reaction mix was incubated for 15 min at 42°C, 5 min at 85°C, and held at 4°C. For the microRNA adenosine monophosphate (miR-AMP) reaction, 45 μL miR-Amp Reaction mix were added to 5 μL cDNA from the RT reaction. The reaction mix was incubated using cycling conditions comprising 10 min at 95°C followed by 14 two-step cycles of 2 min at 95°C and 2 min at 60°C, and 10 min at 99°C.

2.6.2 Real-Time Polymerase Chain Reaction. MicroRNA expression levels were examined using Custom TaqMan Array MicroRNA Cards (Thermo Fisher Cat. No. 4342249). The 384-well microfluidic cards were pre-loaded with selected PCR assays (forward and reverse primers, probe) in each reaction well (**Table 7**). Cards were composed of eight channels of 24 individual reactions. Each individual polymerase chain reaction (PCR) reaction comprised two replicates (**Figure 1**). Assays were deposited onto the cards by the manufacturer at 9×10^{-7} mole/L primer (forward and reverse) concentrations with the probe at the 2×10^{-7} mole/L**.**

Table 7. Custom TaqMan Array MicroRNA Card assays.

Well	Accession No.	miRBase ID	TaqMan Advanced MicroRNA Assay ID	Mature miRNA Sequence
1	MIMAT0000823	rno-miR-101a-3p	mmu483018_mir	UACAGUACUGUGAUAACUGAA
2	MIMAT0000843	rno-mir-137-3p	rno480924_mir	UUAUUGCUUAAGAAUACGCGUAG
3	MIMAT0000573	rno-mir-140-5p	rno480932_mir	CAGUGGUUUUACCCUAUGGUAG
4	MIMAT0000855	rno-mir-153-3p	rno481327_mir	UUGCAUAGUCACAAAAGUGAUC
5	MIMAT0000874	rno-mir-200a-3p	rno478490_mir	UAACACUGUCUGGUAACGAUGU
6	MIMAT0000790	rno-mir-21-5p	rno481342_mir	UAGCUUAUCAGACUGAUGUUGA
7	MIMAT0000552	rno-mir-301a-3p	477815_mir	CAGUGCAAUAGUAUUGUCAAAGC
8	MIMAT0000577	rno-mir-337-3p	rno481067_mir	UUCAGCUCCUAUAUGAUGCCUUU
9	MIMAT0000581	rno-mir-338-3p	rno480884_mir	UCCAGCAUCAGUGAUUUUGUUGA
10	MIMAT0000587	rno-miR-341	mmu481073_mir	UCGGUCGAUCGGUCGGUCGGU
Control	MIMAT0000069	hsa-miR-16-5p	477860_mir	UAGCAGCACGUAAAUAUUGGCG
11	MIMAT0001538	rno-miR-429	mmu481162_mir	UAAUACUGUCUGGUAAUGCCGU
12	MIMAT0005596	rno-mir-551b-3p	rno481215_mir	GGCGACCCAUACUUGGUUUCAGU
13	MIMAT0012833	rno-mir-582-5p	rno481409_mir	UACAGUUGUUCAACCAGUUACU
14	MIMAT0005335	rno-mir-758-3p	rno481136_mir	UUUGUGACCUGGUCCACUAACC
15	MIMAT0000803	rno-mir-29c-3p	rno479229_mir	UAGCACCAUUUGAAAUCGGUUA
16	MIMAT0017302	rno-mir-380-3p	rno481389_mir	UAUGUAGUAUGGUCCACAUCU
17	MIMAT0004707	rno-let-7i-3p	rno480880_mir	CUGCGCAAGCUACUGCCUUGCU
18	MIMAT0000801	rno-mir-29b-3p	rno481300_mir	UAGCACCAUUUGAAAUCAGUGUU
19	MIMAT0017106	rno-mir-34a-3p	rno481369_mir	AAUCAGCAAGUAUACUGCCCUA
20	MIMAT0000788	rno-mir-19b-3p	rno478264_mir	UGUGCAAAUCCAUGCAAAACUGA
21	MIMAT0000884	rno-miR-181a-1-3p	479405_mir	ACCAUCGACCGUUGAUUGUACC
22	MIMAT0000901	rno-miR-299a-5p	rno481489_mir	UGGUUUACCGUCCCACAUACAU
23	MIMAT0000875	rno-miR-200b-3p	rno481286_mir	UAAUACUGCCUGGUAAUGAUGAC

Position																								Replicate	Port
1	2	3	4	5	6	7	8	9	10	CTL	11	12	13	14	15	16	17	18	19	20	21	22	23	A	1
1	2	3	4	5	6	7	8	9	10	CTL	11	12	13	14	15	16	17	18	19	20	21	22	23	B	
1	2	3	4	5	6	7	8	9	10	CTL	11	12	13	14	15	16	17	18	19	20	21	22	23	C	2
1	2	3	4	5	6	7	8	9	10	CTL	11	12	13	14	15	16	17	18	19	20	21	22	23	D	
1	2	3	4	5	6	7	8	9	10	CTL	11	12	13	14	15	16	17	18	19	20	21	22	23	E	3
1	2	3	4	5	6	7	8	9	10	CTL	11	12	13	14	15	16	17	18	19	20	21	22	23	F	
1	2	3	4	5	6	7	8	9	10	CTL	11	12	13	14	15	16	17	18	19	20	21	22	23	G	4
1	2	3	4	5	6	7	8	9	10	CTL	11	12	13	14	15	16	17	18	19	20	21	22	23	H	
1	2	3	4	5	6	7	8	9	10	CTL	11	12	13	14	15	16	17	18	19	20	21	22	23	I	5
1	2	3	4	5	6	7	8	9	10	CTL	11	12	13	14	15	16	17	18	19	20	21	22	23	J	
1	2	3	4	5	6	7	8	9	10	CTL	11	12	13	14	15	16	17	18	19	20	21	22	23	K	6
1	2	3	4	5	6	7	8	9	10	CTL	11	12	13	14	15	16	17	18	19	20	21	22	23	L	
1	2	3	4	5	6	7	8	9	10	CTL	11	12	13	14	15	16	17	18	19	20	21	22	23	M	7
1	2	3	4	5	6	7	8	9	10	CTL	11	12	13	14	15	16	17	18	19	20	21	22	23	N	
1	2	3	4	5	6	7	8	9	10	CTL	11	12	13	14	15	16	17	18	19	20	21	22	23	O	8
1	2	3	4	5	6	7	8	9	10	CTL	11	12	13	14	15	16	17	18	19	20	21	22	23	P	

Figure 1. Custom TaqMan Array MicroRNA Card configuration. Card contains 23 unique assays, 1 mandatory control, 2 replicates per biological sample, and 8 unique ports/sample

Cards were loaded by mixing 50 µL cDNA with 50 µL TaqMan Fast Advanced Master Mix (Thermo Fisher, Cat. No. 4444557). The reaction mix was pipetted into the inlet port of each channel. Cards were centrifuged 2 x 1 min at 1,200 rpm, sealed, and the inlet ports trimmed following the manufacturer's instructions (TaqMan Advanced miRNA Assays User Guide, Publication Number MAN0016122, Revision C.0). Cards were run on the QuantStudio 7 Flex Real-Time PCR System (Thermo Fisher) using PCR cycling conditions comprising 10 min at 95°C followed by 40 two-step cycles of 1 sec at 95°C and 20 sec at 60°C.

2.6.3 Real-Time PCR Data Analysis. Data generated from TaqMan Array MicroRNA cards were analyzed using QuantStudio™ Real-Time PCR System Software v1.3 (Applied Biosystems by Thermo Fisher Scientific). Relative quantities were normalized using endogenous control has-miR-16-5p. miRNA expression changes were measured by calculating the delta relative threshold (ΔC_T) for all miRNAs with $C_T \leq 36$ and determining the Comparative C_T method ($\Delta\Delta C_T$) between the control and exposed groups. The software's default C_T settings were used (automatic threshold). A 95% confidence level was used to determine RQ_{min} and RQ_{max} values. If the exponential algorithm or the C_T algorithm failed, the result was rejected. The qPCR data files (EDS files) were subsequently loaded as a project into Expression Suite Software v1.1 (Applied Biosystems by Thermo Fisher Scientific). Amplification data were captured using the relative threshold algorithm with $C_T \leq 36$. Data across each array card were normalized using the global mean normalization method. *P*-values were adjusted using the Benjamin-Hochberg false discovery rate. MiRNAs were considered differentially expressed using fold change <-2 or >2 and *p*-value < 0.05 cut-offs**.**

2.7 miRNA Pathway Analysis

To identify pathways and functions previously ascribed to the differentially expressed miRNAs identified in this study, data were analyzed using Ingenuity® Pathway Analysis (IPA) software.

3.0 RESULTS AND DISCUSSION

3.1 Affymetrix GeneChip miRNA 3.0 Array Results

3.1.1 Jet Propellant-8. JP-8 inhalation exposures resulted in miRNA expression changes in the CRB, HIP, and PFC in a dose-dependent fashion. The greatest number of differentially expressed miRNAs after JP-8 exposure occurred in the cerebellum (**Figure 2**).

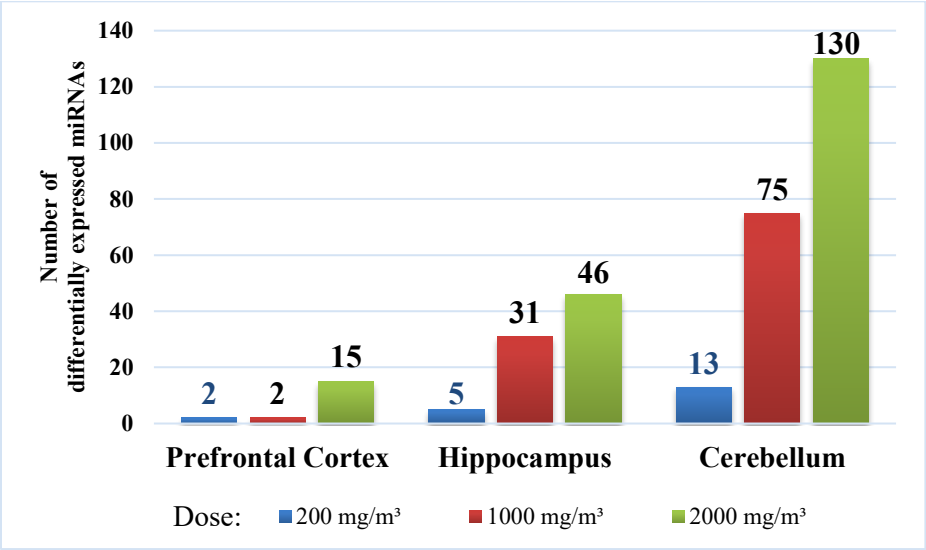


Figure 2. JP-8 exposure resulted in miRNA expression changes in all three tissues in a dose-dependent fashion. MicroRNAs were considered differentially expressed using fold change <-2 or >2 and analysis of variance (ANOVA) *p*-value < 0.05 cut-offs.

In the PFC, no miRNAs were differentially expressed across all three JP-8 concentrations. In the HIP, only rno-miR-382-star (MIMAT003202) was differentially expressed across all three JP-8 concentrations, with the highest fold change observed in the 1000 mg/m^3 dosing group (**Figure 3**). In the CRB, five miRNAs were differentially expressed across all three JP-8 concentrations (**Figure 4**). In all five instances, the largest expression change was observed in the highest dosing group (2000 mg/m^3).

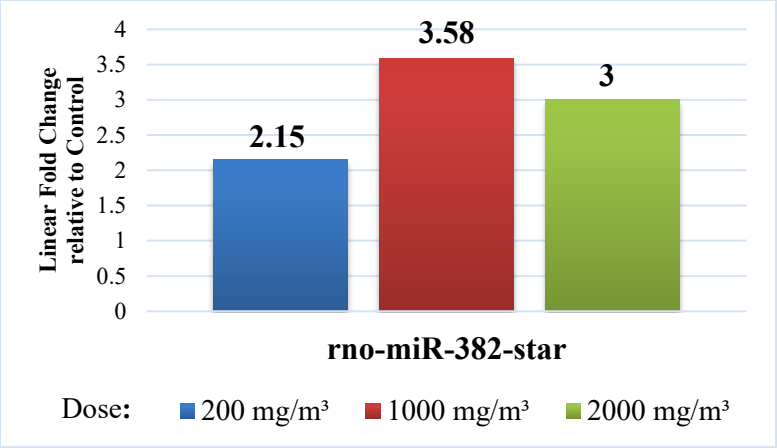


Figure 3. rno-miR-382-star was differentially expressed in the HIP post JP-8 exposure. MicroRNAs were considered differentially expressed using fold change <-2 or >2 and analysis of variance (ANOVA) *p*-value < 0.05 cut-offs

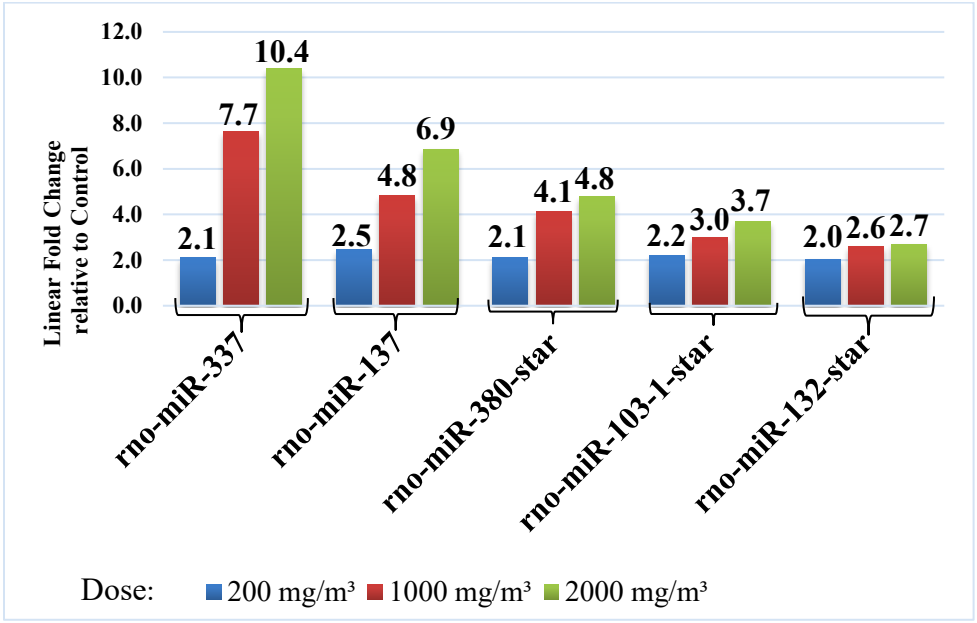


Figure 4. MicroRNAs differentially expressed in the CRB post JP-8 exposure. MicroRNAs were considered differentially expressed using fold change <-2 or >2 and analysis of variance (ANOVA) *p*-value < 0.05 cut-offs.

In addition to observing the largest number of differentially expressed miRNAs in the CRB, the largest fold changes were also observed in the CRB after JP-8 exposure (**Figure 5**). The largest expression change was observed in rno-miR-301a, a 32-fold change compared to the control group.

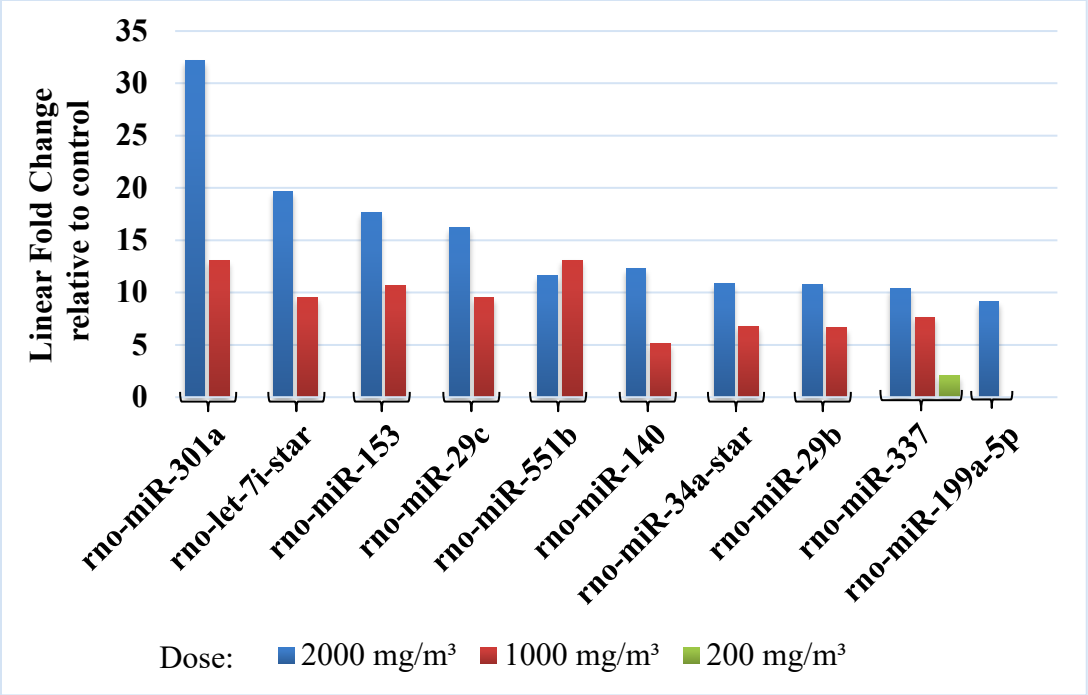


Figure 5. Top upregulated miRNAs in CRB after JP-8 exposure

3.1.2 Jet A. Jet A exposure resulted in miRNA expression changes (**Figure 6**). Jet A exposure increased the number of differentially expressed miRNAs in a dose-dependent fashion only in the cerebellum. The greatest number of differentially expressed miRNAs after Jet A exposure occurred in the cerebellum.

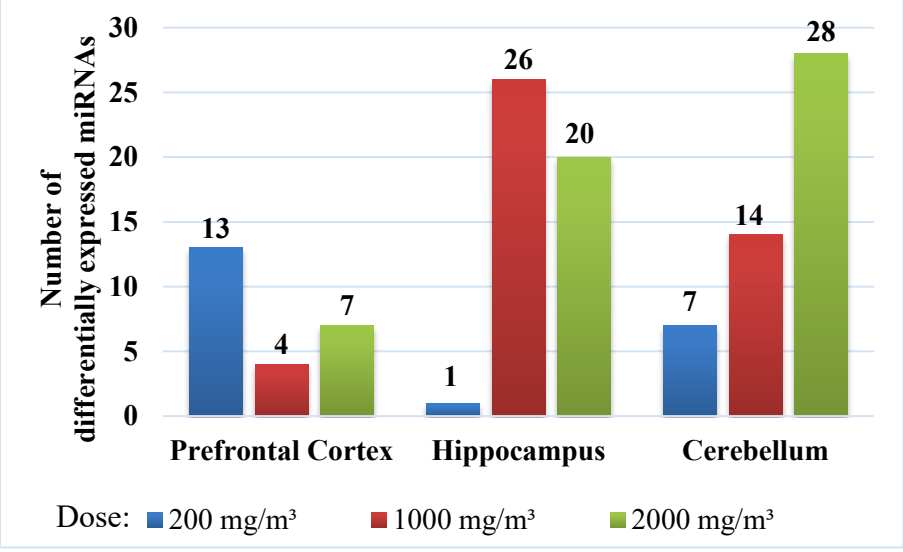


Figure 6. Jet A exposure resulted in miRNA expression changes in all three tissues

In none of the three brain regions were any miRNAs differentially expressed across all three Jet A concentrations. In the PFC, no miRNAs were differentially expressed, even across only the two highest dose groups. However, in both CRB and HIP, miRNAs were upregulated across consecutive Jet A concentrations. In the CRB, seven miRNAs were upregulated (**Figure 7**). In the hippocampus, six miRNAs were upregulated (**Figure 8**), the largest miRNA expression changes were observed in the CRB (**Figure 9**).

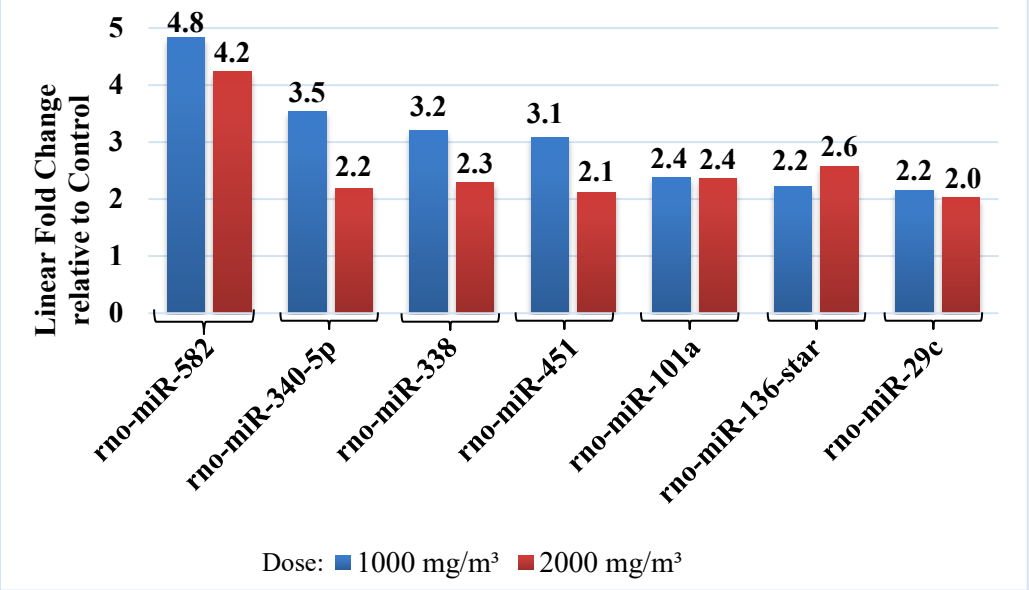


Figure 7. MicroRNAs differentially expressed across consecutive doses in the CRB after Jet A exposure

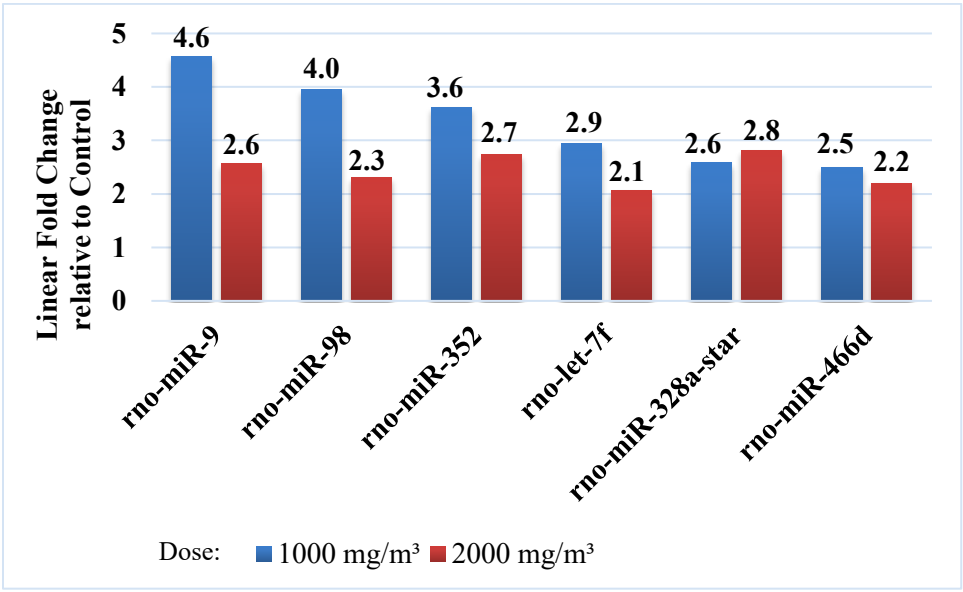


Figure 8. MicroRNAs differentially expressed across consecutive doses in the HIP after Jet A exposure

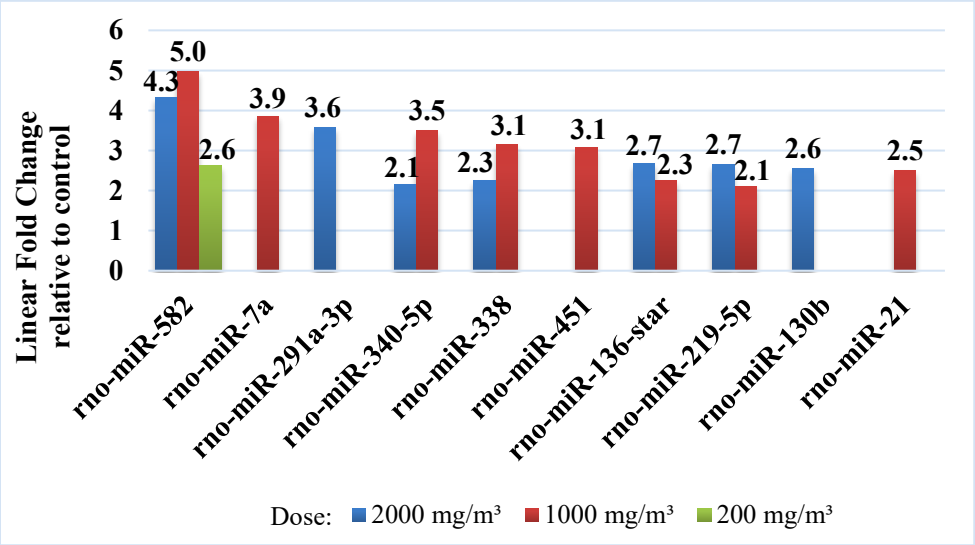


Figure 9. Top 10 upregulated miRNAs in CRB after Jet A exposure

3.1.3 Jet Propellant-5. JP-5 exposure resulted in miRNA expression changes (**Figure 10**). JP-5 exposure increased the number of differentially expressed miRNAs in a dose-dependent manner only in the PFC. The greatest number of differentially expressed miRNAs after JP-5 exposure occurred in the PFC.

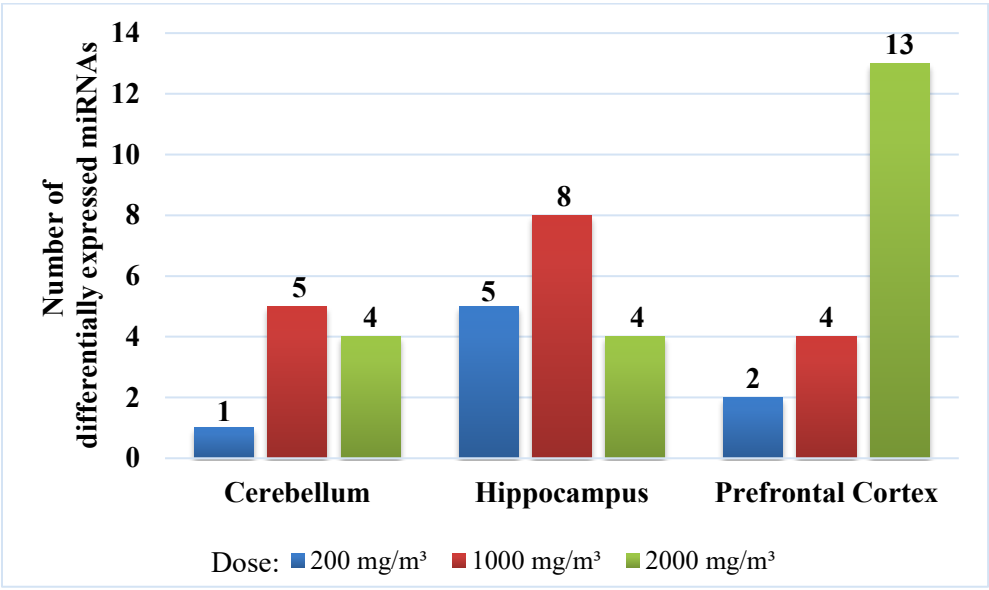


Figure 10. JP-5 exposure resulted in miRNA expression changes in all three tissues

In none of the three brain regions were any miRNAs differentially expressed across all three JP-5 concentrations. In the CRB and PFC, no miRNAs were differentially expressed even across only the two highest dose groups. However, in the HIP, miRNAs were downregulated across consecutive JP-5 concentrations. In the HIP, two miRNAs were downregulated in the two highest exposure groups (**Figure 11**). After JP-5 exposure, the largest up-regulation of miRNA was observed in the CRB (**Figure 12**). The most down-regulated miRNAs were observed in the PFC (**Figure 13**).

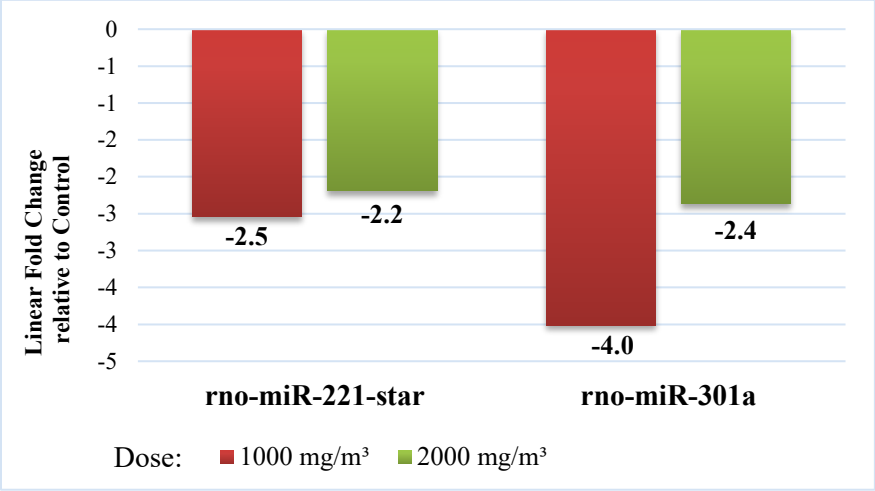


Figure 11. Differentially expressed miRNAs in HIP across JP-5 concentrations

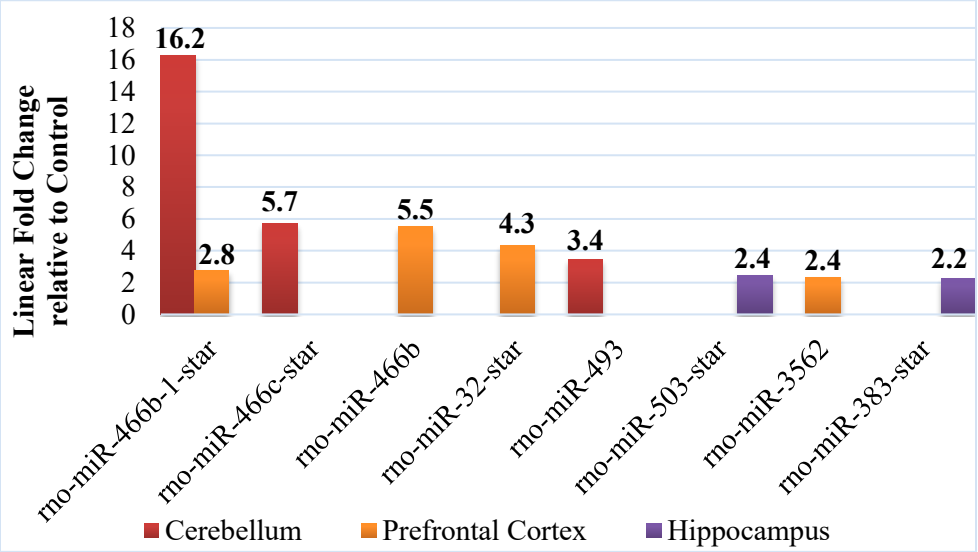


Figure 12. Highest fold upregulated miRNAs after 2000 mg/m³ JP-5 exposure

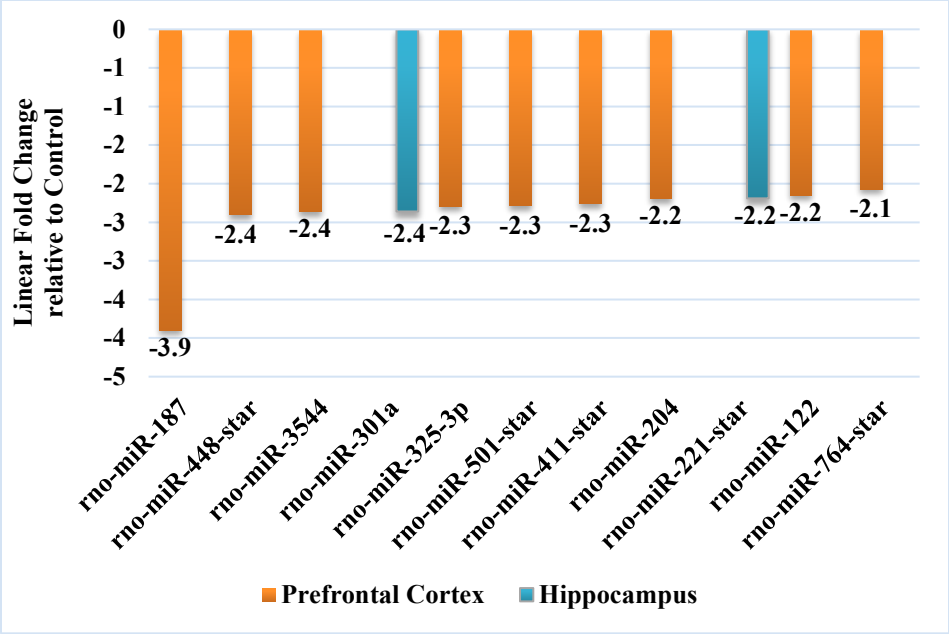


Figure 13. Highest fold down-regulated miRNAs after 2000 mg/m^3 JP-5 exposure

3.1.4 Fischer-Tropsch. FT exposure resulted in miRNA expression changes (**Figure 14**). FT exposure did not increase the number of differentially expressed miRNAs in a dose-dependent manner in any of the brain regions. The greatest number of differentially expressed miRNAs after FT exposure occurred in the prefrontal cortex.

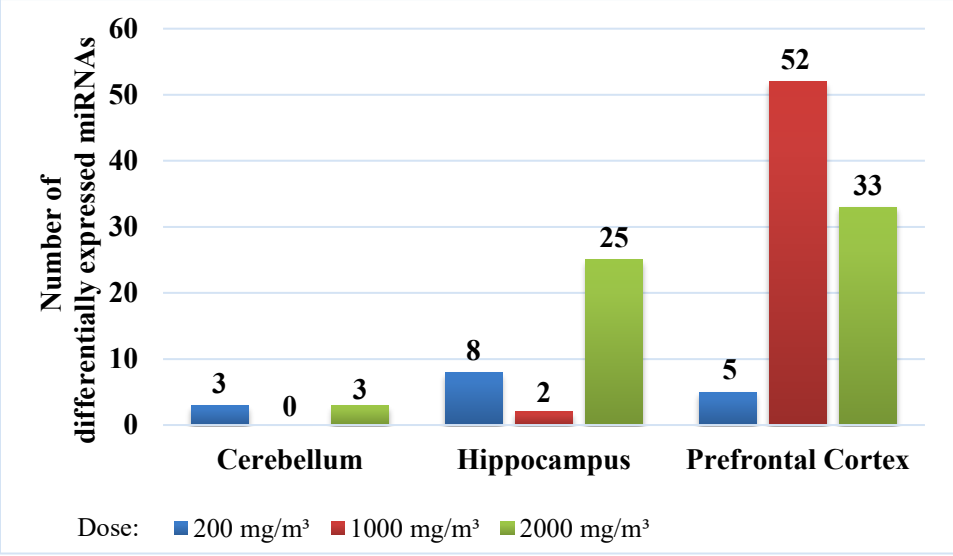


Figure 14. FT exposure resulted in miRNA expression changes in all three tissues

In none of the three brain regions were any miRNAs differentially expressed across all three FT concentrations. In the CRB and HIP, no miRNAs were differentially expressed even across only the two highest dose groups. However, in the PFC miRNAs were upregulated across consecutive FT concentrations. In the PFC, six miRNAs were upregulated and 12 miRNAs were down-regulated (**Figure 15**).

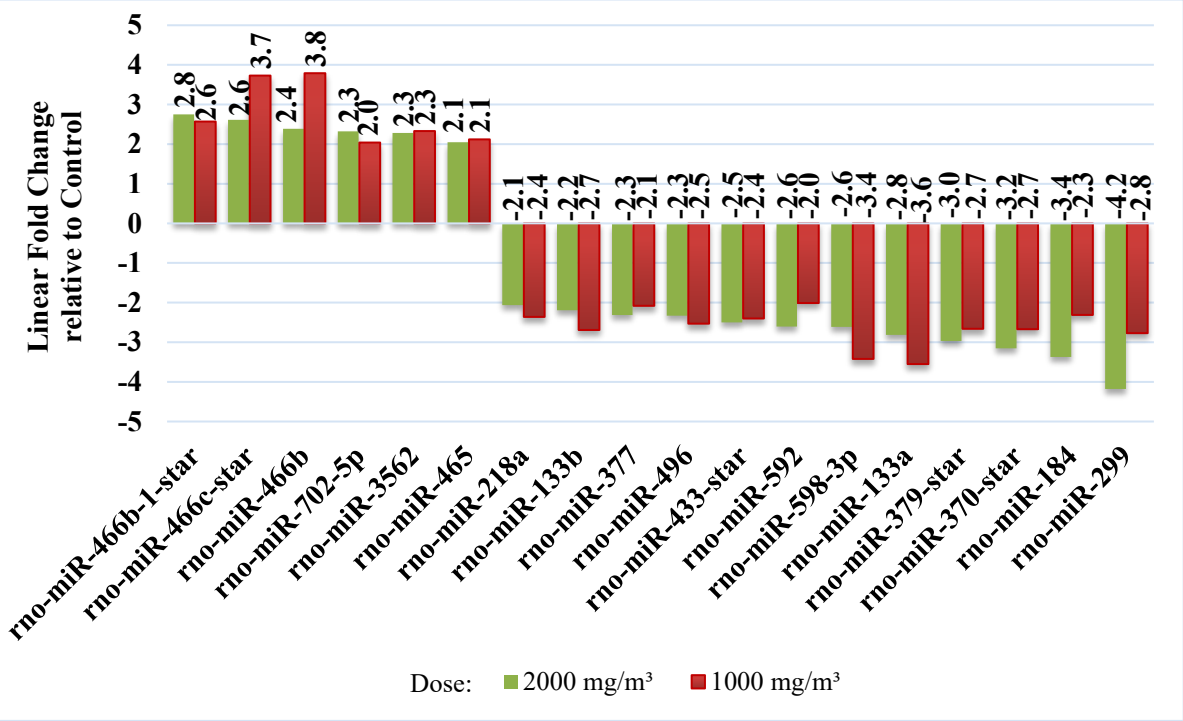


Figure 15. Differentially expressed miRNAs in PFC post FT exposure

3.2 Custom TaqMan Array MicroRNA Cards

A select group of miRNAs were examined in blood using custom TaqMan arrays (**Table 8**). A total of 23 miRNAs were selected for pre-validation due to the format options of the custom TaqMan Arrays, which permitted a run at eight samples/array for a total of 10 arrays. Differentially expressed miRNAs from all four fuels (JP-8, Jet A, FT, and JP-5) were examined. Only three of the four doses (control, 200 mg/m^3, and 2000 mg/m^3) were examined, with six animals per exposure included. The miRNAs for analysis via RT-qPCR were selected based on the following criteria:

1. Increase/decrease observed across all doses
2. Increase/decrease observed in the middle and high dose
3. Scale of increase/decrease
4. Observed in multiple fuels
5. Observed in multiple tissue types

Table 8. miRNAs selected for RT-qPCR pre-validation.

Fuel Type:	JP-8						Jet A						FT					JP-5				
Brain Region:	CRB			HIP		PFC	CRB		HIP		PFC		CRB	HIP		PFC		CRB		HIP		PFC
DOSE:	200	1000	2000	1000	2000	2000	1000	2000	1000	2000	200	2000	2000	200	2000	1000	2000	200	1000	1000	2000	1000
mo-miR-101a		5.7	7.0	3.4	3.8		2.5	2.4				2.1		-2.6	-2.4							
mo-miR-29c		9.5	16.2	2.8	6.7		2.2	2.0														
mo-miR-380-star	2.1	4.1	4.8		3.8				4.3							-2.5						
mo-miR-137	2.5	4.8	6.9		3.0																	
mo-miR-337	2.1	7.7	10.4		4.6																	
mo-miR-338			2.9	6.5	6.2		3.1	2.3	3.6							-3.1						
mo-miR-21			2.7	5.4	6.6		2.5		5.5						-2.9			-2.2		-2.2		
mo-miR-301a		13.1	32.2		7.1					-4.1		2.0	2.8		-2.0					-4.0	-2.4	
mo-let-7i-star		9.5	19.6		3.4					-3.5												
mo-miR-153		10.6	17.6		3.5								2.0									
mo-miR-29b		6.7	10.8		5.0																	
mo-miR-140		5.1	12.3		4.1																	
mo-miR-551b		13.1	11.6							-2.2												
mo-miR-341		4.2	5.5								-2.1											
mo-miR-34a-star		6.8	10.9																			
mo-miR-19b		3.7	6.0																			
mo-miR-758		3.5	5.0																			
mo-miR-181a-1-star		2.3	6.5																			
mo-miR-200a						-26.5											92.7					
mo-miR-200b						-20.7											75.3					
mo-miR-429						-47.8											80.3					
mo-miR-582							5.0	4.3														
mo-miR-299								-2.4								-2.8	-4.2					-2.6

3.2.1 Jet Propellant-8. None of the 24 miRNAs examined in blood significantly changed its levels after JP-8 exposure, either at 200 mg/m^3 or at 2000 mg/m^3. Only two miRNAs showed fold changes < -2, but these changes were not significant (**Table 9**).

Table 9. Blood miRNA expression changes post JP-8 exposure

Fuel	miRBase ID	Dose (mg/m^3)	RQ	Adjusted *p*-value
JP-8	rno-miR-153-3p	200	-2.6	0.787
		2000	-2.2	0.787
	rno-miR-200b-3p	200	-2.0	1.000
		2000	-2.4	1.000

In addition to the expression changes observed via qPCR not being significant, the (insignificant) fold changes of rno-miR-153-3p observed in blood did not trend in the same fold directions as the changes observed via Affymetrix analysis in brain tissue (**Table 10**).

Table 10. Comparison of Affymetrix analysis in brain tissue versus qPCR analysis in blood of JP-8 exposed animals

Fuel	miRBase ID	Dose (mg/m^3)	Matrix	Fold Change
JP-8	rno-miR-153-3p	200	CRB/HIP/PFC	---
			BLD	-2.6*
		1000	CRB	10.6
			HIP/PFC	---
			BLD	not tested
		2000	CRB	17.6
			HIP	3.5
			PFC	---
			BLD	-2.2*
	rno-miR-200b-3p	200	CRB/HIP/PFC	---
	rno-miR-200b-3p	200 1000	BLD	-2.0*
			CRB/HIP/PFC	---
		1000 2000	BLD	not tested
			CRB/HIP	---
		2000	PFC	-20.7
			BLD	-2.4*

*insignificant changes ($p > 0.05$)

3.2.2 Jet A. None of the 24 miRNAs examined in blood significantly changed its levels after Jet A exposure, either at 200 mg/m^3 or at 2000 mg/m^3.

3.2.3 Jet Propellant-5. None of the 24 miRNAs examined in blood significantly changed its levels after JP-5 exposure, either at 200 mg/m^3 or at 2000 mg/m^3. One miRNA, rno-miR-34a-3p, showed fold changes <-2 and >2, but these changes were not significant, and changes in this miRNA were not observed via Affymetrix analysis. In addition, at the 200 mg/m^3 dose the expression of rno-miR-34a-3p was downregulated, while at the 2000 mg/m^3 expression was upregulated (**Table 11**).

Table 11. miRNA expression changes post JP-5 exposure

Fuel	miRBase ID	Dose (mg/m^3)	RQ	Adjusted *p*-value
JP-5	rno-miR-34a-3p	200	-1.5	1.000
		2000	2.3	1.000

3.2.4 Fischer-Tropsch. None of the 24 miRNAs examined in blood significantly changed its levels after FT exposure, either at 200 mg/m^3 or at 2000 mg/m^3. Only four miRNAs showed fold changes < -2, but these changes were not significant (**Table 12**).

Table 12. miRNA expression changes post FT exposure

Fuel	miRBase ID	Dose (mg/m^3)	RQ	Adjusted *p*-value
FT	hsa-miR-181a-3p	200	-2.1	0.595
		2000	-2.2	0.595
	rno-miR-153-3p	200	-3.7	0.618
		2000	-5.3	0.595
	rno-miR-200b-3p	200	---	---
		2000	-3.3	0.595
	rno-miR-34a-3p	200	-3.3	0.664
		2000	-11.2	0.595

Has-miR-181a-3p and rno-miR-34a-3p were not identified during Affymetrix analysis. Rno-miR-153-3p was identified as significantly altered via Affymetrix Analysis (FC 2.0 in CRB). However, Rno-miR-153-3p levels using Affymetrix analysis demonstrated upregulation whereas qPCR showed insignificant downregulation. Rno-miR-200b-3p was identified as being upregulated 75 fold in the PFC of FT-exposed animals. The insignificant fold changes of rno-miR-200b-3p observed in blood did not trend in the same direction as the changes observed via Affymetrix analysis in brain tissue (**Table 13**).

Table 13. Comparison of Affymetrix analysis in brain tissue versus qPCR analysis in blood of FT exposed animals

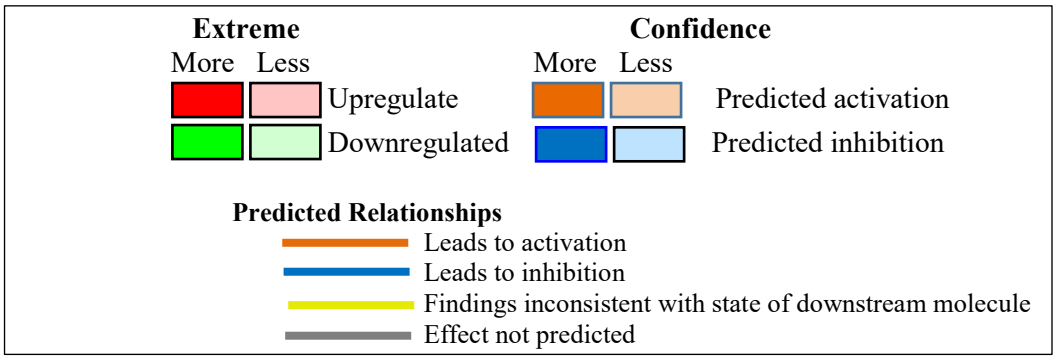
Fuel	miRBase ID	Dose (mg/m^3)	Matrix	Fold Change
FT	rno-miR-153-3p	200	CRB/HIP/PFC	NA
			BLD	-3.7*
		1000	CRB/HIP/PFC	NA
			BLD	not tested
		2000	CRB	2.0
			HIP/PFC	NA
			BLD	-5.3*
	rno-miR-200b-3p	200	CRB/HIP/PFC	NA
			BLD	NA
		1000	CRB/HIP/PFC	NA
			BLD	not tested
		2000	CRB/HIP	NA
			PFC	75.3
			BLD	-3.3*

*insignificant changes ($p > 0.05$)

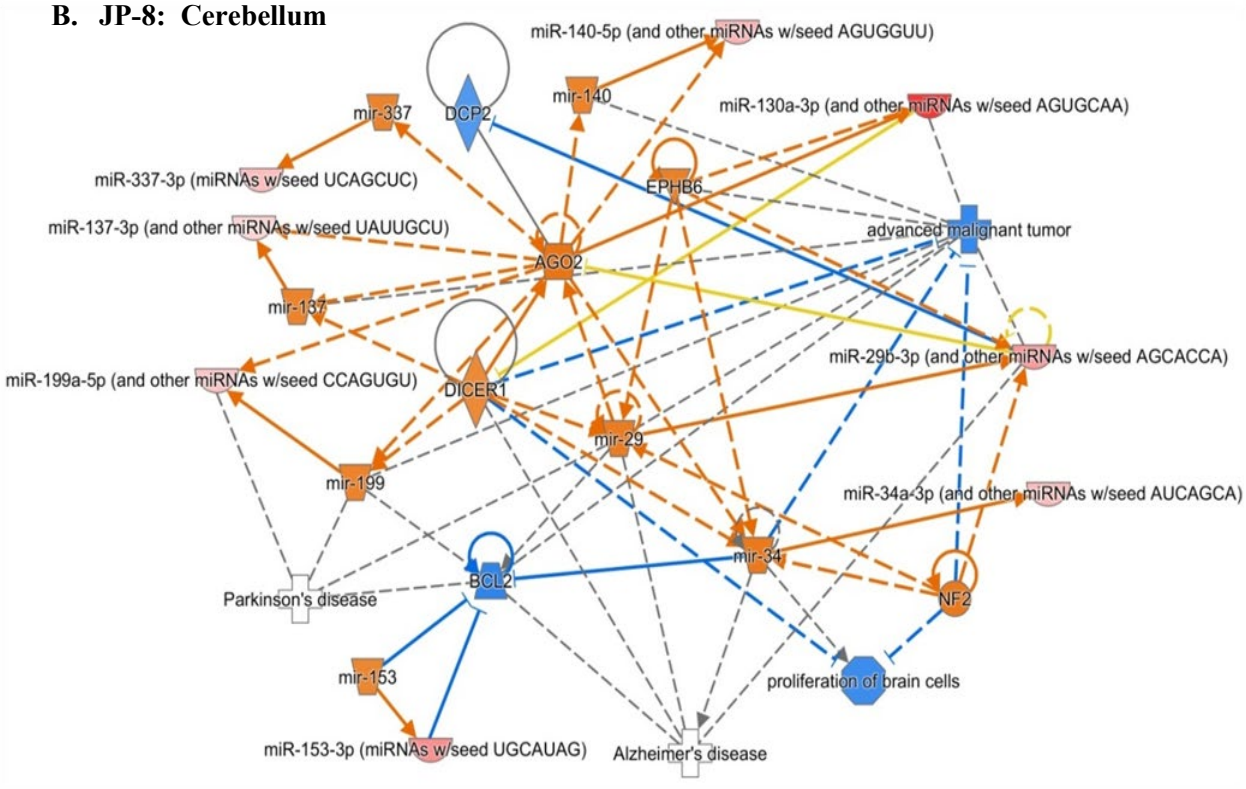
3.3 Pathway Analysis of Specific miRNA

IPA Core Analysis of significantly upregulated miRNAs in CRB shows connections between altered miRNA and biological processes. IPA Core analysis of significantly upregulated miRNAs in CRB after JP-8 and Jet A exposure shows connections to neurological deficits that mimic disease phenotypes (**Figure 16**).

A. IPA Prediction Legend



B. JP-8: Cerebellum



C. Jet A Exposure: Cerebellum

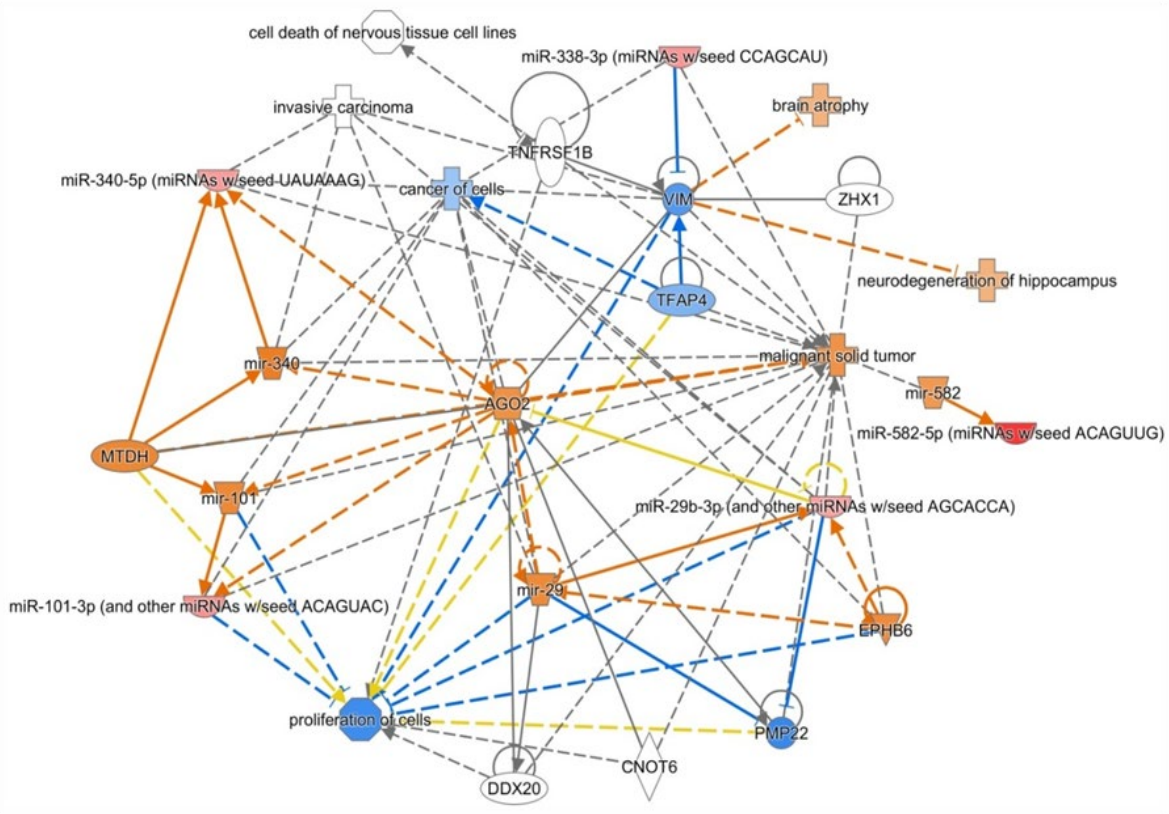


Figure 16. IPA pathway predictions for altered miRNA sets. Prediction figure legend (**A**); Pathway interactions for altered miRNA in CRB after JP-8 inhalation exposures (**B**); Pathway interactions for altered miRNA in CRB after Jet A inhalation exposures (**C**).

4.0 CONCLUSIONS

This study examined miRNA changes in specific regions in the brain initiated by inhalation of different types of common military jet fuels at low, medium, and high dosages. The dose levels used in this study were selected using literature-based *in vivo* data previously shown to initiate cognitive decrement. Unexpectedly, no significant changes in spatial learning or memory were seen at any test dose of JP-8, JP-5, or Jet A, data reported in Rohan, et al.[25] However, FT-exposed animals did demonstrate a dose response negative modulation in tested cognitive parameters of learning and memory, although full recovery was seen seven days post-training.[25] Also, unlike in previous reports which described fuel exposure-induced effects on the immune system,' this study saw no changes in the inflammatory cytokines IFN-γ, IL-1β, IL-4, IL-5, IL-6, KC/GRO, IL-10, IL-13, and TNF-α.[25,27,28] There have been indications that rats may not display immunosuppressive behavior when exposed to jet fuel kerosene.[29] Many previous studies utilized the outbred strain Sprague-Dawley rats for exposure analyses, whereas this study used the inbred strain Fischer 344, which may partly explain the fuel tolerance as strain-specific susceptibility has been previously noted.[30] With a lack of demonstrable cognitive decrement or changes in inflammatory response, a cognitive phenotype linkage to miRNA changes could not be accomplished.

In several differentially expressed miRNAs, a dose response was not seen (such as **Figure 14**, PFC). It is not uncommon in miRNA discovery studies to see lower expression at a high vs. a lower dose/condition. One potential explanation for this discrepancy in miRNA dose-response could be the initiation of feedback self-regulation of specific miRNAs, resulting in lower expression levels at higher doses. MiRNA self-regulation is not well understood, but is an activity likely to be present in some but not all miRNA species.[31]

A subset of 21 miRNA species were downselected based on dose response, expression across fuels, and/or correlative changes in blood concentrations. This subset was further examined using qPCR as a secondary method to quantitate the changes seen in the brain tissues (**Table 9**). As shown, all members of this select set failed to validate using Quant7 qPCR methodology. During the course of this study, and in conjunction with another miRNA discovery project, questions were raised on the effectiveness of hybridization methods with its reported poor sensitivity and limited multiplexing, resulting in a lack of validation of identified differentially expressed miRNAs.[32] When originally proposed, hybridization methods were considered *de rigueur* for this type of analysis and had been used for several years at this organization in numerous studies. However, with the evolution of advanced DNA sequencing methodologies and concomitant cost reductions in utilization of that method, future efforts should employ sequencing methods only. While the subset of miRNAs further examined did not validate, not all differentially expressed miRNA discoveries in this study were interrogated using qPCR due to financial constraints. Promising differentially-expressed miRNA species identified, but not further examined by qPCR, are discussed below.

JP-8 Induced Brain miRNA Alterations

While jet fuel exposure resulted in miRNA expression changes in all three brain regions examined, the CRB seems to be most sensitive to JP-8 inhalation as indicated by the total number of miRNA alterations. This outcome supports the finding that high inhalation exposure levels of jet fuel initiate the sway response in fuel-exposed personnel[33,34] as the cerebellum plays a central role in balance and movement. Interestingly, IPA core analysis of the data set for JP-8 exposure in the cerebellum indicates that similar

miRNA alterations and pathways are seen in Parkinson's disease (**Figure 16B**), a condition which also exhibits significant alterations in sway response/locomotion.[35]

A literature search for hydrocarbon or fuel-induced miRNA discoveries indicated that several miRNAs found in this study were seen in other exposure studies and identified as potential markers of exposure. However, comparisons with blood miRNA discoveries in Australian Air Force personnel with self-reported fuel exposures failed to identify any similarities.[36] Interestingly, Woeller, *et al*. examined miRNA expression in archived serum samples from the DoD serum repository.[37] In the Woeller study, five blood miRNAs were shown to be associated with polycyclic aromatic hydrocarbon (PAH) exposures, one of which (miR-140-3p) is within the same operon as miR-140 found in this study. MiRNA-140 has been shown to have tumor-suppressive effects with IGF1R as a direct functional target.[38] Increases in miR-34a (star/5p) were seen in our study as well as in a human study examining blood miRNA biomarkers and human volatile organic compound (VOCs) exposures[39] and is a potential tumor surpressor.[40]

Jet A Induced Brain miRNA Alterations

Unlike JP-8 induced miRNA brain alterations, the HIP in addition to the CRB are sensitive to Jet A inhalation as indicated by increased differential miRNA expression in both regions (**Figure 6**). As Jet A did not contain additives, the increased hippocampal miRNA activity cannot be due to the chemical components found in CI/LI, FSII, or SDA. Additionally, while the hydrocarbon components for JP-8 and Jet A are similar, JP-8 contains more cycloparaffins (41.81 wt% vs. ave 29.49 wt% for Jet A). It has been hypothesized that the inhalation of hydrocarbons >C12 is limited due low vapor pressure and blood/brain barrier effects, thus limiting neurological effects.[41,42] While one *in vivo* exposure study examining C5-C11 isoparaffinic and cycloparaffinc hydrocarbons failed to demonstrate any neurobehavioral effects,[43] other studies did exhibit subtle CNS effects.[44]

MiRNA expression in the hippocampus demonstrated a response with 1000 mg/m^3 Jet A that generated 26 significant alterations, decreasing to 20 alterations at 2000 mg/m^3. Of these, only six miRNAs were differentially expressed across mid/high doses and only one, miR-328a-star, exhibited a dose-response activity with five others decreasing at the higher dose. Interestingly, miR-328a-star (3p) is thought to be involved brain responsiveness during stroke and is upregulated during injury, a marker signature reflected in the serum.[45] MiR-328a-3p expression in the hippocampus has also been verified in human brain tissue using *in situ* hybridization.[46] While miR-328a-star was not selected for PCR validation, further evaluation is warranted based on these data.

In the cerebellum, seven miRNAs demonstrated differential expression across mid/high dose, with only miR-136-star exhibiting dose-response activity. MiR-136-star (3p) has been identified as a potential diagnostic blood biomarker for mild traumatic brain injury, linking its expression to brain injury.[47]

JP-5 Induced Brain miRNA Alterations

The miRNA profiling in this study indicated that JP-5 inhalation exposures initiate substantial dose response expression changes in the PFC (**Figure 10**), unlike JP-8 and Jet A. The PFC plays a central role in cognitive control, and impairment of this brain region results in decreases in attention, memory and impulse inhibition.[48] Interestingly, a reported case of acute JP-5 exposure in pilots showed that the victims suffered from euphoria and significant but temporary memory deficits, supporting the supposition that JP-5 inhalation targets PFC function.[6] JP-5 differed from test fuel JP-8 in lacking the SDA additive Stadis

450. JP-5 is also formulated to have a higher flash point (60° C vs 38° C for JP-8) for additional safety for Naval uses, and contains more C11-14 isoparaffins, n-paraffins, and C11-12 monocyclocyloparaffins.[49]

Of all differentially expressed miRNA discoveries with JP-5 exposure, only three were examined for qPCR validation, leaving most to be further evaluated in future studies. In the JP-5 discovery set, the highest fold change miRNAs included three in the CRB, four in the PFC, and two in the HIP (**Figure 12**). MiRNA-32 has been shown to be highly expressed in brain tissue, and may be involved in maintenance of myelin[50] through regulation of SLC45A3.[51] MiRNA-493 has been shown to suppress tumor metastasis and angiogenesis, and may regulate post-stroke angiogenesis by altering macrophage migration inhibitory factor (MIF) levels.[52] Decreases in miR-187 has been shown to promote oxidative stressed-induced apoptosis in retinal cells[53] and also to attenuate cerebral injury by regulation of the endoplasmic reticulum stress pathway.[54] Increases in miRNA-503 has been shown to promote cell growth and inhibit apoptosis by direct targeting of the PDCD4 gene.[55] Alternatively, increases in miR-383-star (3p) has been shown to promote neuronal necroptosis in intracerebral hemorrhage.[56] While JP-5 fuel initiated more overall miRNA changes in the PFC, the highest fold change miRNA, miR-466b-1-star, was found in the cerebellum. This specific miRNA was shown to increase in the rat cortex upon exposure to the anesthesia chemicals porpofol or sevoflurane.[57] Interestingly, two other components of the miR-466 operon cluster, miR-466-c-star and miR-466b were also seen to increase upon JP-5 exposure. Published research indicates that the miR-466 family are often significantly modulated in response to negative environmental stimuli[58], indicating a potential role in regulation of cellular stress responses, possibly by repression of the NeuroD1 gene.[59]

FT Induced Brain miRNA Alterations

The composition of the bio-based Fisher-Tropsch fuel is very different that JP-8, JP-5 or Jet A, with far lower aromatics (0.41 wt% vs. ave 19.96 wt%) and higher aliphatics (99.59 wt% vs. ave 80 wt%) than the other three fuels evaluated. Unlike the other test fuels, very few molecular studies have been conducted examining FT-induced neurological alterations. In light of the differences in hydrocarbon content compared to the kerosene based fuels, it was predicted that the miRNA profile signature should be unique. FT inhalation initiated the strongest miRNA response, leading to significant alterations of 52 miRNAs in the PFC at mid and high dose.

The majority of miRNA discoveries were down-regulated, expressed at 2 to 2.5 fold change. However, a set of miRNAs demonstrated higher fold changes. Among these, miR-466c-star, miR-466b-1 star, and miR-466b were shown to increase in the PFC upon exposure. Interestingly, a similar miR-466 set was also seen in JP-5 exposures. Published data indicate that miR-299 (5p) may alter neuronal survival pathways by regulating autophagy.[60] MiRNA-184 has been shown to be downregulated in adults with major depression, and *in vivo* knockout evaluation of that specific miRNA demonstrated increases in locomotor and cognitive decrements.[61] It has been reported that miR-133a is downregulated in glioma tumor tissue, with this miRNA targeting membrane-type 1 matrix metalloproteinase (MT1-MMP) mRNA to reduce levels of that protein, leading to suppression of cell proliferation.[62]

Jet Fuel Neurotoxicity and Follow-on Efforts

While this study was not able to establish a linkage to miRNA expression changes in the brain to fuel exposure-induced cognitive decrement due to a lack of exhibited phenotype, the miRNA data clearly identified different regional neurological impacts from each of the fuels evaluated. The interrogation of the target organ of interest – in this case the cerebellum, prefrontal cortex, and hippocampus – for miRNA changes versus scanning blood samples alone provides data with a stronger relationship to neurological

alterations. If evaluating blood miRNAs alone, the miRNA signature generated would reflect the inhalation exposure impact on all organs, diluting molecular discovery data. Therefore, an *in vivo* approach to determine differential miRNA signatures in the brain, followed by evaluation of specific identified miRNAs for similar expression changes in the blood, is a strong approach for neurotoxicity biomarker discovery.

While a limited set of miRNA discoveries failed to validate using qPCR, there are several miRNAs found in this study that are worthy of further evaluation. Most intriguing is the miR-466 operon family, several of which were significantly altered in JP-5 and FT fuel exposures. Examining the full JP-8 data set indicated that miR-466c-star and miR-466b-1-star increased approximately 2 fold in the cerebellum, whereas in Jet A, miR-466d (CRB, HIP), miR-466c-star (PFC), and miR-466c-star (PFC) also demonstrated ~ 2 fold changes.

The data suggest that the neurological decrements instigated by different types of fuel inhalation may be subtle and only differentiated when examined on a molecular level. It is intriguing that Jet A initiated more hippocampal-based miRNA alterations compared to JP-8, although it is not clear what chemical component contributed to the differential impact. The different miRNA profile seen with JP-5 indicates that, while similar in composition to Jet A and JP-8, the hydrocarbon composition used to alter the fuel flash point seems to shift reactivity from the hippocampus/cerebellum to the prefrontal cortex. Lastly, miRNA data generated by FT inhalation, very different than the 'traditional' kerosene-based DOD jet fuels, suggest that biofuel inhalation exposures may display entirely different adverse outcome pathways, thus different neurotoxicity risks and outcomes. As such, this study indicate that caution may be needed when attempting to apply neurobehavioral decremental phenotypes broadly across fuel types for risk assessment and modeling. We plan to conduct additional validation on these *in vivo* samples, in addition to expanding evaluation of specific miRNAs using existing human blood samples in collaboration with governmental and international partners. Both activities will aid in validation of jet-fuel induced neurotoxicological miRNA biomarkers and will provide additional data as to fuel-specific neurological pathway alterations.

5.0 REFERENCES

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LIST OF SYMBOLS, ABBREVIATIONS, AND ACRONYMS

AFB – Air Force Base
AFRL – Air Force Research Laboratory
ANOVA – analysis of variance
BLD – blood
BSA – bovine serum albumin
cDNA – complementary DNA
CRB – cerebellum
DNA – deoxyribonucleic acid
DoD – Department of Defense
DTIC – Defense Technical Information Center
ELOSA – enzyme-linked oligosorbent assay
FC – fold change
FT – Fischer Tropsch
g – gravity
h – hour
hrs - hours
HIP – hippocampus
IPA- Ingenuity Pathway Analysis
JP-5 – Jet Propellant 5
JP-8 – Jet Propellant 8
MALDI – Matrix-assisted laser desorption/ionization
m - meter
mg - milligram
min - minute
miRNA – microRNA
mRNA – message ribonucleic acid
NAMRU-D – Naval Medical Research Unit Dayton
OD – optical density
PBS- phosphate buffered saline
PCR – polymerase chain reaction
PD -Parkinson's disease
PFC – prefrontal cortex
POSF - Property Measurement for Fuel Research
RDX - hexahydro-1,3,5-trinitro-1,3,5-triazine
RNA – ribonucleic acid
RPM – rotations per minute
RT – reverse transcription
RT – room temperature
RT-PCR – reverse transcriptase polymerase chain reaction
RT-qPCR – quantitative reverse transcription polymerase chain reaction
SA-HRP – streptavidin-horseradish peroxidase
SNAP23 – synaptosome-associated protein 23
TMB – 3,3',5,5'-tetramethylbenzidine
wt% - percent by weight
% -percent
vol% - percent by volume