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Environmental Regulation of the Heart: The Role of Non-Coding RNA and Epigenetics in Influencing Mitochondrial and Cellular Health

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Environmental Regulation of the Heart: The Role of Non-Coding RNA and Epigenetics in Influencing Mitochondrial and Cellular Health

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Dissertation submitted to the School of Medicine at West Virginia University
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy in
Exercise Physiology

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ABSTRACT

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Quincy Alexander Hathaway

Introduction: The mitochondrion, a small but ubiquitously distributed organelle in the cell, continues to be the focus of many disease pathogeneses, tissue and organ dysfunctions, and other morbidities that occur throughout the body. By controlling major aspects of metabolism, energy homeostasis, ion concentrations, apoptosis, and inflammation, the mitochondrion serves as a multi-functional organelle that has the capacity to impact many crucial aspects of cellular homeostasis. While mitochondrial function has been broadly defined in most disease states and/or insults, a lack of understanding remains in the regulatory networks that exist between the nucleus and mitochondrion as well as the import/export dynamics of RNA and other biomolecules within the mitochondrion itself. Nuclear influence on the type, quality, and concentration of proteins shuttled to the mitochondrion (anterograde signaling) and mitochondrial regulation of metabolites produced for nuclear DNA transcription and maintenance (retrograde signaling) are poorly understood processes. Likewise, the translocation of non-coding RNAs into mitochondria and their functional relevance is lacking. The underlying regulatory network of epigenomic modifications, competitive endogenous RNAs, and other transient modifications reveal a crucial, undiscovered nature surrounding mitochondrial biology. In examining these mechanisms, the heart, with a major reliance on mitochondrial ATP production for its contractile properties, is significantly impacted by changes at the mitochondrial level. Because of the mitochondrial dense nature and high energy demands of cardiac tissue, alterations in mitochondrial homeostasis have the capacity to significantly limit cardiac function. The purpose of this work is to understand how cardiac mitochondrion are altered in disease and pathological states, specifically in their adaptation to environmentally stimulated regulatory networks, such as epigenetic modifications and promotion/inhibition of non-coding RNAs.

Methods and Results: We examined both acute stress to mitochondrial regulation (inhalation toxicology) as well as chronic (type 2 diabetes mellitus). Inhalation of particulates at the nanoscale and its impact on molecular cardiovascular biology have been poorly evaluated. Through using a FVB transgenic microRNA-378a mouse knockout model, we examined the cardiovascular impact derived from altering the innate microRNA-378a response following acute nano-TiO₂ inhalation exposure. Following inhalation exposure to nanomaterials, microRNA-378a was shown to be elevated. In complete knockout, and to a lesser extent partial knockout, of microRNA-378a, cardiac function was preserved. Through mitochondrial electron transport chain (ETC) complex activities and mitochondrial respiration, changes in substrate utilization were observed, specifically an increase in fatty acid metabolism in the knockout animals. MicroRNA-378a's regulation of mitofusin-1 (Mfn1), as well as other factors involved in mitochondrial metabolism, bioenergetics, and fission/fusion were shown to be altered in knockout and knockdown animals.

The non-coding RNA network, which may serve as the primary response during acute exposure to inhaled toxicants, is dysregulated following inhalation exposure. While inhalation exposure serves as a means for inducing localized stress and observing the systemic alterations, diabetes is a chronic, systemic disease, affecting multiple organs and organ systems. In order to better diagnosis and treat type 2 diabetes, a better understanding is required of the cellular and molecular mechanisms during the onset and progression of the disease. In atrial tissue from 50 patients (30 non-diabetic and 20 type 2 diabetic) physiological, biochemical, genomic, and epigenomic factors were assessed in an attempt to better predict the pathogenesis of the disease in the heart. Using machine learning algorithms derived from Classification and Regression Trees (CART), we were able to determine that epigenetic factors were the most critical classifiers. Importantly, total, global 5-methylcytosine content of the genome, as well as methylation at the transcription factor A, mitochondrial (TFAM) promoter region, were most highly associated with diabetic status. Through using machine learning at the DNA level, this study begins to explore the potential of precision medicine-based approaches for disease classification and novel diagnostic measures. While machine learning can aid in the prediction of novel biomarkers, there remains much to be understood regarding the mitochondrion's role in diabetes, and the mechanisms of mitochondrial RNA import/export. Next-generation sequencing was performed on human patient and FVB mouse mitochondrial and cytoplasmic non-coding RNA populations. Assessment of non-coding RNA revealed a decreased RNA biodiversity in diabetic patients. Further, using a human, mouse, and cellular model, the binding of non-coding RNA to polynucleotide phosphorylase (PNPase) was confirmed, with a specific role of the PNPase KH and S1 binding domains crucial for long non-coding RNA binding. These findings suggest a role for PNPase in the import and/or export of long non-coding RNA during normal and pathogenic conditions.

Conclusions: Ultimately, the work summarized in the preceding experiments highlights how multiple pathological insults, whether chronic or acute, can influence the underlying molecular, regulatory networks in the heart. While overt cardiovascular and mitochondrial dysfunction follow insult, an emphasis on epigenetic control and non-coding RNA regulation may prove to be primary axes for therapeutic intervention. As we continue to pursue more informed and predictive assessments of cardiovascular dysfunction, the mitochondrion remains at the heart of the issue.

LIST OF ABBREVIATIONS AND DEFINITIONS

5mC	5-methylcytosine
5hmC	5-hydroxymethylcytosine
Anterograde Signaling	Nuclear to Mitochondrial Signaling
CART	Classification and Regression Trees
ChIP	Chromatin Immunoprecipitation
CLIP	Crosslinking-Immunoprecipitation
CpG	Cytosine followed Guanine (Nucleotides)
Cyto	Cytoplasmic
DbDb	Lepr ^{db+/+} Mice
ENM	Engineered Nanomaterial
H3K4me3	Histone 3 Lysine 4 trimethylation
H3K27me3	Histone 3 Lysine 27 trimethylation
IPA	Ingenuity Pathway Analysis
KH	KH RNA Binding Domain of PNPase
LncRNA	Long Non-Coding RNA
MiRNA	MicroRNA
Mito	Mitochondrial
ML	Machine Learning
NcRNA	Non-Coding RNA
ND	Non-Diabetic
PM _{0.1} , UFP	Ultrafine Particulate Matter < 0.1 µm
PM _{2.5}	Particulate Matter < 2.5 µm
PNPase	Polynucleotide Phosphorylase
PNPT1	Polyribonucleotide Nucleotidyltransferase 1
Retrograde Signaling	Mitochondrion to Nucleus Signaling
S1	S1 RNA Binding Domain of PNPase

SHAP	SHapley Additive exPlanations
SVM	Support Vector Machines
T2DM	Type 2 Diabetes Mellitus
TiO ₂	Titanium Dioxide (Nano)
Tg	Transgenic
WT	Wild type

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Specific Aims:

Maintenance of the cardiovascular system is vital in sustaining systemic homeostasis from both disease-related and xenobiotic-induced pathology. In the heart, diabetes mellitus (5, 8, 9) and engineered nanomaterial (ENM) (15, 24, 30) exposure affect cellular adaption through alterations in mitochondrial function. Variations to substrate utilization, increased reactive oxygen species, and eventual changes in mitochondrial bioenergetic capacity contribute to cellular dysfunction and ultimately cardiovascular complications through alterations to pump function (19). What remains unclear is how these environmental changes impact the regulatory networks of the heart, primarily the involvement of non-coding RNA (ncRNA) and epigenetics in altering mitochondrial health. *The objective* of this application is to understand how environmental factors, in the context of systemic disease and xenobiotic insult, alter cardiac function through ncRNAs and epigenetics. *The long-term goal* is to detect targets in these regulatory systems and therapeutically alter their expression to positively impact mitochondrial, cellular, and heart health.

My *central hypothesis* is that changes in the epigenome through DNA methylation and histone modifications alter the ncRNA transcriptome subsequently changing the import dynamics of microRNA (miRNA), long non-coding RNA (lncRNA), and other ncRNA into the mitochondrion and affecting mitochondrial function through RNA-RNA and RNA-protein interactions; ultimately, producing a feedback loop where mitochondrial substrate availability shapes nuclear epigenetic machinery. Following both diabetes mellitus (3, 7, 13, 32) and ENM exposure (20, 21, 30) paradigms, epigenetic control of nuclear encoded-mitochondrial genes as well as transcription are altered and a shift in the ncRNA transcriptome in both the cytoplasm and mitochondrion is precipitated. *This proposal seeks to* expand the current knowledge of ncRNA and epigenetics as it relates to cardiovascular health following diabetic and ENM insult. There is a *gap in knowledge* regarding the mechanisms promoting and sustaining mitochondrial dysfunction following cardiac insult; justifying a need for research to expand our current understanding of the ncRNA/epigenetic interplay shaping environmental stimulation. This gap in knowledge will be addressed by experimentation directly assessing ncRNA altered in disease states and how these ncRNA can be targeted for therapeutic interventions, restoring mitochondrial function and ultimately cardiovascular function.

Specific Aim 1: *Determine the role of ncRNA in regulating cardiac and mitochondrial function following exposure to ENMs.* Our working hypothesis is that ENM inhalation exposure alters ncRNA transcription and subsequent downstream regulation, affecting miRNA regulation of cardiac function through inhibiting/promoting cellular pathways. MiRNA-378a knockout and wild type mice will be exposed to nano titanium dioxide (Nano-TiO₂) and cardiac and mitochondrial function determined. Regulation of the miRNA-378a host gene and miRNA-378a targeted repression will be evaluated.

- 1.1 Determine the cardiovascular function of wild type, knockdown, and knockout expression for miRNA-378a using echocardiography and speckle-tracking stress strain.
- 1.2 Examine metabolic and ultrastructure effects of inhalation nano-TiO₂ on mitochondria, and if miRNA-378a depletion can restore function.
- 1.3 Find specific targets of miRNA-378a that regulate metabolism, bioenergetics, and/or mitochondrial fission/fusion.

The ***expected outcomes*** of Specific Aim 1 are that miRNA-378a is upregulated following nano-TiO₂ exposure due to increased transcription of its host gene, repressing fatty acid metabolism, and that knockout of miRNA-378a can rescue mitochondrial function and ultrastructure through decreased regulation of constituents related to metabolism.

Specific Aim 2: *Understand the cross talk between mitochondrial and nuclear regulator networks in diabetes mellitus using machine learning predictive algorithms.* Our working hypothesis is that alterations to the genomic/epigenomic landscape in the nucleus can be correlated to mitochondrial function in patients with diabetes mellitus. Human right atrial tissue from type 2 diabetic and non-diabetic patients will be used to assess physiological, biochemical, genetic, and epigenetic markers and evaluated through predictive machine learning algorithms.

- 2.1 Tissue from 50 patients (30 non-diabetic and 20 type 2 diabetic) will be collected from the Ruby Memorial Hospital, Heart and Vascular Institute.
- 2.2 Biomarkers and risk factors measured through a variety of indices will be placed into a machine learning algorithm (SHapley Additive exPlanations) based around Classification and Regression Trees (CART).

- 2.3** The most predictive biomarkers for diabetic status will be chosen and compared with the efficiency of common diagnostics, such as glycosylated hemoglobin (HbA1c).

The *expected outcomes* for Specific Aim 2 are that changes in substrate availability in the mitochondrion, e.g. S-adenosyl-L-methionine, likely regulate DNA methylation in the nuclear genome and that predictive machine learning algorithms can be used to measure the diagnostic and therapeutic potential of genetic/epigenetic modifications in diabetic onset and progression.

Specific Aim 3: *Evaluate the mechanisms of non-coding RNA import/export involving the mitochondrion in diabetes mellitus and the role of Polynucleotide Phosphorylase (PNPase) in non-coding RNA binding.* Our working hypothesis is that mitochondrial import of ncRNAs are significantly diminished in diabetic mitochondria and that PNPase can regulate the long non-coding import/export mechanisms of the mitochondrial regulatory network through its KH and S1 RNA binding domains. Using human patient, mouse, and cell models, the mitochondrial ncRNA network will be evaluated and how proteins, such as PNPase, may contribute to changes in ncRNA flux.

- 3.1 RNA from isolated mitochondria and cytoplasmic compartments from human right atrial tissue and FVB mouse whole heart will be assessed for lncRNA presence and potential interaction capacity with miRNA, mRNA, and other RNA species.
- 3.2 Cross-linking immunoprecipitation (CLIP) of PNPase in mitochondria to determine ncRNA interaction sites and species involved.
- 3.3 Using HL-1 cells, determine how the KH and S1 RNA binding domains of PNPase alter binding capacity with ncRNA.

The *expected outcomes* of Specific Aim 3 are that diabetes induces a reduction in total non-coding RNA biodiversity, altering the ncRNA regulatory network in the mitochondrion. PNPase, through the KH and S1 RNA binding domains directly interacts with lncRNA, potentially exporting/degrading specific lncRNA involved with the mitochondrion.

The outcomes of these studies will significantly contribute toward the understanding of cardiovascular physiology, identification of therapeutic targets, and assist the development of treatment modalities for diabetic and ENM cardiovascular dysfunction.

Research Strategy

Significance: Cardiovascular diseases remain the top cause of death worldwide, with over 800,000 deaths in the United States alone, equating to 1 death every 40 seconds (2). Preservation and therapeutic treatment of the cardiovascular system is vital for mitigating co-morbidities and even death. Understanding how insults to the cardiovascular system can alter normal, homeostatic function can provide a more dynamic picture of the adaptive, molecular systems that exist; specifically, both through a systemic, chronic insult (diabetes mellitus) and through acute, direct action (engineered nanomaterial (ENM) exposure). Diabetes mellitus is a systemic pathology, resulting in cellular and tissue dysfunction across multiple organ systems (1, 4, 23, 27). Diabetic cardiomyopathy and other cardiovascular complications resulting from the disease comprise a major co-morbidity as well as the primary cause of mortality in patients with diabetes mellitus (12, 18). The cardiovascular system is also detrimentally affected through engineered nanomaterial exposure (ENM) (17, 22, 24). Inhalation exposure to ENMs produce acute cardiac abnormalities and could contribute to sustained cardiovascular and systemic dysfunction over time (10, 25). Nano-TiO₂ is one of the most prominently produced ENM (11, 16) and could pose a significant health risk occupationally, to consumers, or even environmentally.

The *scientific premise* for this dissertation work considers both cardiovascular physiology and molecular mechanisms related to mitochondrial structure and function. Currently, characterization of diabetes mellitus in the cardiovascular system has shown global epigenetic changes through DNA methylation and histone methylation (28, 31). With these changes, epigenetics have also been shown to manipulate ncRNA expression (6, 26). Though ncRNA such as miRNA and lncRNA have been shown to change following diabetic insult, their contribution to altering cardiac function has been limitedly explored, specifically in the context of mitochondrial health. Epigenetic and ncRNA alterations are less exaggerated following ENM inhalation exposure (14, 29) but the degree in alterations of the internal regulatory network provide a spectrum of ncRNA expression and epigenetic changes following cardiovascular insult. We have begun to assess the outcomes of epigenetics and ncRNA expression following both diabetic and ENM inhalation exposure. We have shown that following diabetes mellitus, the ncRNA in the mitochondrion are altered, and that specific proteins associated with the mitochondrion, Argonaut 2 (AGO2) and PNPase, are shown to be immunoprecipitated together and could provide a means

for mitochondrial ncRNA import. Additionally, the expression of miRNAs involved with metabolism are altered following inhalation exposure to nano-TiO₂, suggesting a role in regulating mitochondrial function.

Innovation: At current, no study has fully detailed the global ncRNA mitochondrial import/export pathway, the purpose of the ncRNA mitochondrial transcriptome, or how epigenetics influences the role of ncRNA and the mitochondrion. The proposed studies provide innovation conceptually, methodologically and mechanistically.

1. Conceptually Innovative: The central hypothesis proposes a regulatory network of how mitochondrial bioenergetics affect nuclear transcription, and vice versa. Through epigenetic control in the nucleus and transcription of ncRNAs which translocate to the mitochondrion and control bioenergetic regulation, the hypothesis provides a novel conceptual idea of the specific constituents involved in cellular homeostasis under pathological conditions.
2. Methodologically Innovative: While machine learning algorithms have been applied to biomarkers in predicting diabetes and other diseases, no study has used machine learning to assess patient-matched genetic/epigenetic features at base-pair resolution in diabetes. For the first time, we assess how machine learning can be used as a personalized medicine approach for identifying epigenetic changes in the diabetic heart.
3. Mechanistically Innovative: The primary ncRNA import pathway has yet to be elucidated. We propose PNPase as major constituents in the mitochondrial ncRNA import pathway. We further suggest that PNPase/lncRNA interactions through the KH and S1 RNA binding domains could explain alterations to mitochondrial ncRNA diversity.

The epigenetic/ncRNA model proposed provides not only innovative approaches, but also the propensity for therapeutic applications. AntagomiR therapy and lncRNA sponging provide simple and adaptive therapeutic intervention measures for regulating the expression of ncRNA in the mitochondrion.

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Chapter 1:

Literature Review

Inhalation Toxicology

Introduction

Irrespective of diet, physical fitness, or other controllable risk factors, the quality, and content of the air we breathe is often unavoidable. Specifically, it has been shown that combustion products, particulate matter $< 2.5 \mu\text{m}$, and emissions of engineered materials at the nanoscale, all contribute to physiological disturbances (59, 116, 117). While the pulmonary system is considered the first site of interaction with these particles, the heart and circulatory system may be subject to the most detrimental, long-term effects. Chronic and acute exposures to particulate matter $< 2.5 \mu\text{m}$ (PM_{2.5}) and ultrafine particulate matter $< 0.1 \mu\text{m}$ (PM_{0.1}, UFP) have been associated with an increased risk of cardiovascular disease, including a predisposition to cardiovascular morbidity and mortality (21, 205).

The overt, epidemiological risk of cardiovascular injury following inhalation exposure to toxicants has been well documented (21, 83) and includes increased propensity for ischemic heart disease, heart failure, arrhythmias, atherosclerosis, and other cardiovascular complications. While a large body of evidence suggests cardiovascular maladaptation following pulmonary exposure, within the last ten years studies have begun to examine the cellular and molecular mechanisms behind the induced cardiovascular decline. In this review, we will summarize those studies examining the genetic, epigenetic, proteomic, and metabolic adaptations to inhaled toxicants through both primary (the individual) and secondary (gestational, maternal and progeny) exposure. We will specifically examine the cardiovascular effects of ambient and occupational inhalation exposure from PM_{2.5}, UFP, and engineered nanomaterials (ENMs) (Table 1.1). The purpose of this review is to provide a comparative narrative to the field of molecular cardiology on the mechanisms involved in various inhalation toxicology exposure paradigms, with the hope of inciting future investigations into areas germane to understanding the effects of cardiovascular inhalation toxicology.

Table 1.1: Summarized Recent Findings

Study	Findings	Model/Exposure	Administered Concentration
PM_{2.5}			
<i>Ambient Air Pollution</i>			
(143)	Decreased methylation of Alu (carbon black) and LINE-1 (SO ₄)	Normative Aging Study, 706 individuals	PM _{2.5} data collected from Harvard School of Public Health during January 1995 to November 2007

(272)	Higher PM _{2.5} correlated with increased blood TLR2 methylation, flavonoid intake decreased TLR2 methylation	Normative Aging Study, 573 participants	PM _{2.5} data collected from Harvard School of Public Health
(47)	Fe, Ni, V were all correlated with SLFN11 cg10911913 methylation	Normative Aging Study, 646 participants	PM _{2.5} , 1 year moving average, between 1999 and 2013, Harvard University Countway Library
(18)	Fine and coarse PM _{2.5} decreased Alu and TLR4 methylation	15 local participants	PM _{2.5} collected in downtown Toronto. Administered 250 and 200 µg/m ³ per session
(119)	54 circulating miRNAs were correlated with dose of exposure and pollution type	24 non-smoking adults	PM _{2.5} collected on Oxford Street in London, 2-hour exposure
(131)	Acute exposure resulted in elevated CRP, IL-6, and TNFR2	Framingham Offspring cohort cycle 7 and 8	PM _{2.5} collected the Boston Harvard Supersite and assessed as 7-day moving average
Occupational Exposure			
(69)	Increased LINE-1 methylation with increasing dose of exposure	66 male participants	PM _{2.5} collected January 2010 to June 2012 in Boilermaker Union Local 29, Quincy, MA
(23)	Peripheral blood leukocytes mtDNA D-Loop methylation was associated with exposure	48 male participants	PM _{2.5} collected January 2007 to June 2012 in Boilermaker Union Local 29, Quincy, MA
(40)	Elemental carbon (48 genes), PM _{2.5} (49 genes) and organic carbon (260 genes) differentially effected the transcriptome	63 pick-up and delivery drivers and dock workers	PM _{2.5} collected February 2009 and October 2010 from 10 trucking terminals in the northeastern US (CT, MA, MD, NJ, NY, and PA).
(81)	ET-1 was reduced following exposure but was not modified by exercise intensity	18 male participants	PM _{2.5} collected a 5.5-kW diesel engine under a constant 2.5 kW load. Exposure of 300 µg/m ³ over 6/30-minute low and high intensity cycling periods
(155)	Nootkatone pretreatment reduced thrombosis and oxidative damage (NFE2L2) and heme turnover (HO-1)	BALB/C mice at 8 wks	Diesel exhaust particles from the National Institute of Standards and Technology, USA. 15 µg/mouse intratracheal instilled
(82)	Promoter methylation of microRNA-133a-2 in progeny and cardiovascular stress decreased microRNA 133a-2 expression	Female C57BL/6 12-14 wks – 12 wk progeny	PM _{2.5} collected a single cylinder Yanmar diesel engine, operating at 82% load ~300 µg/m ³ for 6 hrs/day for a total of 5 days during pregnancy
Animal Models			
Wild Type			
(166)	Increased ketocholesterol (7-KCh) is poorly cleared by CD36 macrophages	ApoE ^{-/-} Tg and LDLR ^{-/-} Tg mice 8 wks	PM _{2.5} collected Columbus, OH exposed for 6 months to 9.1±7.3 µg/m ³
(177)	ET-1, HSP70, CYP1A1, CYP1B1, laminin, collagen, and calcium signaling proteins increased	Male BALB/c mice 7 to 8 wks	PM _{2.5} collected winter 2008 in an urban site in Milano, Italy. Instilling intratracheally, on days 0, 3 and 6, at 0.3 mg/mouse
(111)	Increased phospho-EGFR (Tyr1068), phospho-Akt (Thr308), NLRP3, NF-κB-p52/p100, and NF-κB-p65 in heart	BALB/c mice 6 to 8 wks	PM _{2.5} collected November 2014 in a major city of central China. Intratracheal instillation of 4.0 mg/kg b.w. for 5 consecutive days.
(133)	In the heart, increased Caspase-3, Bax, and NF-κB	Male C57/BL6 mice 8 wks	PM _{2.5} was collected in, Nanjing, China. 10µg PM _{2.5} (10µl) twice a week/intranasal instillation.
(244)	Increased TNF-α and IL-1β, along with NOX4 and NOX subunits as p67 ^{phox} , p47 ^{phox} and p22 ^{phox} , in the heart	C57BL/6 mice 6 to 8 wks	PM _{2.5} collected September 2013 in Zhengzhou, Taiwan. Instilled with 1.5, 3.0, 6.0 mg/kg BW 5 days/wk for 2 wks
(163)	Increased fibrosis (COL1A1↑, COL3A1↑) oxidative stress (NOX-4↑), transcription factor binding (TGFβ1↑, SMAD3↑)	Female C57BL/6 10 month and 4 wk old mice	PM _{2.5} collected 2012 and 2013 from Taiyuan, Northern China. 3 mg/kg, b.w. PM _{2.5} oropharyngeal aspiration every other day for 4 wks
(45)	p-STAT3↑ promoted microRNA-21 expression, advocating decreased TIMP3, increased MMP9	Male Sprague Dawley rats 6 wks	PM _{2.5} collected in Beijing, China. Trachea drip, 4 mg/kg b.w. every 3 days for 36 days
(240)	Hyperlipidemic heart increased JNK and P53 phosphorylation and increased Bax and Caspase-3 expression	Male Wistar rats 8 wks on high fat diet	PM _{2.5} collected October-December 2012 in Beijing, China. Endotracheal instilling of suspension at 0, 4, 40 mg/kg
(145)	High intensity exercise stimulates reduced inflammation (eHSP70↓)	Male B6.129SF2/J mice 30 days old	PM _{2.5} collected in São Paulo, Brazil, 5 µg nasotropic instillation daily before exercise 12 wks
(266)	Endothelial dysfunction (ET-1↑, eNOS↓) and causing inflammation (COX-2↑, iNOS↑, TNF-α↑, IL-6↑)	Male C57BL/6 mice 6 to 8 wks	PM _{2.5} , collected Taiyuan, China. Intranasal instillation every other day for 6 hrs/28 days. 0.5/3.5 mg/m ³ SO ₂ , 0.2/2 mg/m ³ NO ₂ , 1/10 mg/kg PM _{2.5}
(269)	Increased DNA damage (OGG1↑, GADD153↑, MTH1↓, XRCC1↓) in the heart	Male Wistar rats	PM _{2.5} collected Taiyuan, China. Intratracheal instillation of 1.5 mg/kg b.w. PM _{2.5} , 1.6 × 10 ⁻⁴ mg/kg b.w. 1-NP, 1.2 × 10 ⁻⁴ mg/kg b.w. 9-NA
(249)	Cardiac injury through changes in serum biomarker activity (SERCA2↑, cTnT↑, ANP↑, and BNP↑)	Sprague Dawley rats 6 wks	Silica nanoparticles (SiNPs) and methylmercury (MeHg) intratracheal instillation 10 times over 30 days, 0.25 mg/kg for MeHg and 2 mg/kg SiNPs.
(73)	Heart failure markers (ANP↑, BNP↑), inflammation (CRP↑, IL-6↑, TNF-α↑) Also ANGII↑ and ET-1↑ in serum	Sprague Dawley rats 6 wks	Stöber technique was used to synthesize the SiNPs. Intratracheal instillation of 2 mg/kg body SiNPs and/ or 0.25 mg/kg of Pb
Transgenic/Supplementation			
(158)	Atherosclerosis model increased NOX-4 subunits p22 ^{phox} , p47 ^{phox} in cardiac tissue	Male ApoE ^{-/-} Tg mice 8 wks	PM _{2.5} collected June-October 2013 in Shanghai, China. 0 – 30 mg/kg b.w installation
(235)	Fibrosis↑ and collagen↑ genes, lower antioxidant genes (PRDX5↓)	Male AMPK α2 ^{-/-} Tg mice 6 to 8 wks	PM _{2.5} collected April–October 2016 in Beijing, China. 10 mg/kg intratracheal instillation/6 months
(214)	TRPA1 activation increases negative cardiovascular responses through myocardial dyssynchrony.	Trpa1 ^{mi1Kyk} /J (Trpa1 ^{-/-}) Tg female mice	PM _{2.5} collected November-December of 2014 in Triangle Park, NC. 3 hrs/day, 2 days/week, 8 total exposures through a whole-body exposure system
(268)	Diabetic model induced NF-κB, COX-2, MAPK in heart, IκB inhibitor restored function through NF-κB reduction	KKay Tg mice 7 wks	PM _{2.5} collected Polaris in Columbus, Ohio. Intratracheally administered for 6 hr/day, 5 day/week, for 8 weeks. PM _{2.5} dose of 1.6 mg/kg

(90)	Akt/eNOS phosphorylation↓ and IκBα↓, antioxidant treatment eased vascular insulin resistance/inflammation	Male ecSOD Tg mice 12 wks on high fat diet	PM _{2.5} collected June 2009 and December 2010 in Louisville, KY. 6 hr/day for 9 or 30 days, 30-120 µg/m ³ through a whole-body exposure system
(61)	Vitamin E and omega-3 polyunsaturated fatty acid administration decreased inflammation (TNF-α↓, IL-1β↓, IL-6↓)	Sprague Dawley rats 6-8 wks	PM _{2.5} collected May-September 2015 in Shanghai, China. Intratracheal instilled every other day for 6 days at 10 mg/kg b.w., 1.5 mL/kg b.w.
(74)	Mitochondrial size/cristae deformation, (FIS1↑, MFN1↑, MFN2↑, DRP1↑, OPA1↑), inflammation (TNF-α↑, IL-6↑, IL-1β↑)	Sprague Dawley rats	PM _{2.5} collected January 2013 in Taiyuan, China. Intratracheal instillation of 0.375, 1.5, 6.0, and 24.0 mg/kg b.w. performed 5 times
(261)	SeY treatment reduced inflammation markers (TNF-α, IL-1β, sICM-1)	Male Sprague Dawley rats 7 wks	PM _{2.5} collected September-November 2015 in Shanghai, China. Intratracheal instilled/40 mg/kg
(60)	N-Acetyl-L-cysteine reduced intracellular Ca ²⁺ (RyR2↓, SERCA2a↑)	Male Balb/c mice 6 to 8 wks	PM _{2.5} collected Hebei, China. Intratracheal instilled 0.5 mg at Day 0 and Day 2
(130)	SOS1, CREB, GSK3b, and GRB2 expression reduced, fucoidan treatment rescued all but CREB levels.	C57BL/6J mice 8 wks	PM _{2.5} collected November-December 2016 in Taipei, Taiwan. 100µg/m ³ , 20 days, 6 hrs/day
Gestational Exposure			
(234)	Dose-dependent increase of OPA1, MFN1, DRP1, and FIS1 in progeny.	Female Wistar rats 10 to 12 wks – 1 day progeny	PM _{2.5} collected September 2014 to March 2016 in Harbin, China. Administered dropwise, 3 doses (0.375 mg/kg, 1.5 mg/kg, and 6.0 mg/kg)
(211)	Changes in calcium dynamics (NCX↓ and Cav1.2↑)	Female FVB mice 12 wks – 14-day progeny	PM _{2.5} collected Columbus, Ohio. Exposed 6 hr/day, 5 days/week, average 91.78 µg/m ³ through a whole-body inhalation system
(212)	Acetylation (SIRT1↓, SIRT2↓), methylation (DNMT1↑, DNMT3a↑, DNMT3b↑), and Ca ²⁺ markers altered (NCX↓, p-PLN↓)	Female FVB mice 12 wks – 12 wk progeny	PM _{2.5} collected Columbus, OH. 6 hr/day, 7 days/week throughout pregnancy, average 73.61 µg/m ³ through a whole-body inhalation system
(210)	Inflammation (IL-6↑, IL-15↑, NFκB↑, CRP↑, CD26E↑, CD26P↑, VCAM-1↑, MCP-1↑), fibrosis (COL3A1↑), Ca ²⁺ regulation (SERCA2a↑, p-PLN↑), and epigenetics (DNMT1↓, SIRT1↑, SIRT2↑)	Paternal and maternal FVB mice exposure – male 12 wk old progeny	PM _{2.5} collected Columbus, OH. Average 38.58 µg/m ³ for 6 hrs/day, 5 days/week for 3 months through a whole-body inhalation system
(36)	Increased GATA4 and NKX2-5 and increased inflammation (TNF-α↑, IL-1β↑), homocysteine exacerbates effects	Female Sprague Dawley rats 10 wks – neonatal progeny	PM _{2.5} collected Fujian, China. Exposed during gestation and lactation (~42 days) to 36.5 µg/m ³ through a whole-body inhalation system.
(245)	Increased histone acetyltransferase, H3K9ac, GATA4, and MEF2c. Histone acetylation of GATA4/MEF2c promoters	Female C57BL/6 mice 12 wks – 1 day and 16 wk-old progeny	PM _{2.5} collected Chongqing, China. Gestational exposure by ultrasonic nebulization, 2 hours a day during pregnancy, at ~300 µg/m ³
UFP			
Ambient Air and Occupational Pollution			
(56)	Decreased blood plasminogen and thrombomodulin, increased CRP and SR-A. GSTM1 null participants had worse outcomes	34 participants with metabolic syndrome, ~48 years old	UFP collected US EPA facility Chapel Hill NC. Whole body exposure, average particle concentration 189,000 particles/cm ³ . 2 hour exposure
(200)	Platelet CD40L↑ and circulating CD40L↓, with increased plasma von Willebrand factor	19 type 2 diabetics, 30-60 years of age, never smoked	UFP particles were generated. Elemental carbon UFP through mouth piece for 2 hr at 50 µg/m ³
(126)	CRP↑ and TNFRII↑ significantly associated with white non-Hispanic	408 individuals aged 40-91 years	Ambient air pollution in Dorchester, South Boston
(226)	Increased oxidative stress (PRNP↑) and endoplasmic stress (GRP78↑)	30 children and young adults ~20 years of age	Ambient air pollution in Mexico City, Mexico
(123)	Increased inflammation (IL-6↑, IL-8↑, ET-1↑) in blood	Men and women (n=52) 18 to 34 years old	UFP collected Sault Ste. Marie, Ontario, Canada, in the summer of 2010. Occupational air pollution
Animal Models			
(10)	Increased AT ₁ R protein along with Acta1 and Col3a1 in the heart, with decreased HO-1 expression	Male Sprague-Dawley rats	UFP collected north of Mexico City, May to July, 2009. acute (3 days, 5 hr/day) and subchronic (8 weeks, 4 days/week, 5 hr/day) to varying concentrations.
(99)	Mitochondrial mPTP deficits, with attenuated beneficial impact of Cyclosporin A	Male Sprague-Dawley rats	UFP collected US EPA facility Chapel Hill, NC. 100 µg UFP laryngopharynx instillation
(220)	CRP and fibrinogen increased in systemic circulation	Male spontaneously hypertensive rats (SHR), 12-13 months old	UFP generation. 31 µg or 0.024 m ² UFP deposition over 24 hour time frame
(263)	Increasing UFP concentrations attenuated the beneficial effects of captopril in reducing AngII.	Spontaneously hypertensive rats (SHR)	UFP collected Beijing, China. UFP (0.15 mg/kg, 0.45 mg/kg and 1.35 mg/kg) and captopril administration intratracheal instillation.
Gestational Exposure			
(151)	Placental HSD11B2 DNA methylation, elevated IL-1β, IL-6 and MCP-1 levels	Female C57BL/6J <i>p^{um}/p^{um}</i> mice, 6 wks old. GD 17.5 progeny	UFP collected northern Mexico City collected from April to June of 2016. 12 µg or 400 µg/kg intratracheal instillation
(173)	Decreased IL-13 and IL-17 in lungs, with higher serum levels of IL-10	Female C57BL/6 and BALB/C mice. Progeny 0 to 4 wks	UFP generated. average mass concentration 101.94 µg/m ³ , 24-h daily mean dose of 25 µg/m ³
PM_{2.5} and UFP Combined Analyses			
(43)	Increased CRP and fibrinogen in blood to UFP, but not PM _{2.5}	135 patients undergoing cardiac catheterization	Ambient air pollution. New York State Department of Environmental Conservation (DEC) site in Rochester, NY
(224)	Elevated CRP and platelet count in blood for PM _{2.5} , but not UFP	3275 participants	Ambient air pollution. German Heinz Nixdorf Recall Study, three adjacent cities (Essen, Mülheim, Bochum)
(243)	Mitochondrial haplotypes differentially effected by exposure and IL-6 and TNF-α elevated. Significant to quasi-ultrafine particles <0.25 µm	36 participants, ~84 years old	Particulate matter collected from four retirement communities in the Los Angeles air basin.

(216)	Dysregulated parasympathetic response, increased IFN- γ methylation in blood to PM _{2.5} and quasi-ultrafine particles <0.50 μ m	12 healthy participants	Particulate matter collected Milan, Italy. Inhalation to mixture (PM ₁₀ , PM _{2.5} , PM _{1.0} and PM _{0.5} μ m) over 2 sessions
(68)	Oxford cohort (hsa-miR-197-3p $\uparrow$ and hsa-miR-99a-5p $\uparrow$) changes to PM _{2.5} , but not UFP	59 volunteers (20 healthy, 20 COPD, 19 ischemic heart disease)	Ambient air pollution. Oxford Street or Hyde Park, London, England. 2 hour walk
(153)	69 differentially methylated regions, 13 CpG sites, including methylation of KNDC1 and FAM50B	157 non-smoking adults	PM _{2.5} collected indoor air pollution, measured in 24-hour intervals
(137)	VEGF, UCHL1, and cortisol positively correlated with nickel, barium, and silver	53 healthy participants, 18–60 years old	Particulate matter collected in downtown Toronto, Canada. 130-min exposure
Nanotoxicology			
(105)	Blood specific activation of C3, with heart activation of the complement cascade.	Female C57BL/6 mice 8 wks	Nano-TiO ₂ , UV-Titan L181. 6.0 mg Ti/m ³ –10 mg TiO ₂ /m ³ intratracheal instillation
(100)	Inflammation (NF- κ B $\uparrow$, TNF- α $\uparrow$, IL-4 $\uparrow$, IL-6 $\uparrow$, TGF- β $\uparrow$, CK $\uparrow$, CRP $\uparrow$, IFN- γ $\uparrow$) transcriptional regulators (STAT1 $\uparrow$, STAT3 $\uparrow$, STAT6 $\uparrow$, GATA3 $\uparrow$, GATA4 $\uparrow$)	CD-1 (ICR) male mice at 4 wks	Nano-TiO ₂ prepared through hydrolysis of Ti(OBu) ₄ , nasal instillation of mice every other day for 6 months. Treatment of 1.25 – 5 mg/kg TiO ₂ NPs
(78)	Inhalation, compared to intra-arterial injection, was more detrimental to cardiac tissues. Inflammation and endothelial/epithelial pathways perturbed	Female BALB/cJ mice, 10-12 wks old	Carbon particles with primary particle diameter of 10 $\pm$ 2 nm. Intra-arterial infusion (30mm ² per animal) and inhalation (lung deposited dose 10,000mm ²). Whole body exposure for 4 or 24 hours.
(198)	mPHGPx Tg mice reduced ROS and preserved electron transport chain complex constituents in the heart	Male FVB and male mPHGPx Tg mice 10-12 wks	Nano-TiO ₂ , particle size (21 nm)/surface area (48.08 m ² /g). 11.58 $\pm$ 0.27 μ g lung deposition, 6 hr exposure through a whole-body inhalation system
(94)	microRNA-378a Tg mice preserved fusion ($\uparrow$ MFN1), metabolism, and ultrastructure in the heart	FVB and miRNA-378a knockout Tg mice aged 14-18 weeks of age	Nano-TiO ₂ , particle size (21 nm)/surface area (48.08 m ² /g). 11.09 mg/m ³ concentration for 4 hr exposure through a whole-body inhalation system
Gestational Exposure			
(95)	Increased cardiac mitochondrial proton leak, ($\uparrow$ UCP2), and fatty acid metabolism ($\uparrow$ CPT1A and $\uparrow$ PDH phosphorylation)	Female Sprague Dawley rats 10 to 12 wks – 12 wks progeny	Nano-TiO ₂ , particle size (21 nm)/surface area (48.08 m ² /g). 7.79 $\pm$ 0.26 days, 5 hr/day, 10.35 $\pm$ 0.13 mg/m ³ through a whole-body inhalation system
(199)	Decreased State 3 and State 4 mitochondrial respiration	Female Sprague Dawley rats – female 11 to 16 wks progeny	Nano-TiO ₂ , particle size (21 nm)/surface area (48.08 m ² /g). 6.8 $\pm$ 0.5 days/10.6 $\pm$ 0.3 mg/m ³ through a whole-body inhalation.
(198)	Cardiac mitochondrial epigenomic remodeling, decreased transcriptomic immune response.	Female Sprague Dawley rats 10 to 12 wks – GD 20 fetal progeny	Nano-TiO ₂ , particle size (21 nm)/surface area (48.08 m ² /g). 7.79 $\pm$ 0.26 days, 5 hr/day, 10.35 $\pm$ 0.13 mg/m ³ through a whole-body inhalation system

Table 1.1: Summarized recent findings of molecular mechanisms driving inhalation exposure in the cardiovascular system. LINE = long interspersed nuclear element, TLR = Toll-like receptor, SLFN = Schlafen Family Member, KNDC1 = Kinase Non-Catalytic C-Lobe Domain Containing, FAM50B = family with sequence similarity 50 member B, CRP = C-reactive protein, IL = interleukin, tumor necrosis factor receptor, 7-KCh = 7-ketocholesterol, CD = cluster of differentiation, ET = endothelin, HSP-70 = 70-kDa heat shock protein, CYP = cytochrome P450, EGFR = epidermal growth factor receptor, NLRP = NOD-like receptor protein, NF- κ B = nuclear factor-kappa beta, TNF = tumor necrosis factor, NOX = NADPH oxidase, COL3A1 = collagen type III alpha 1, COL1A1 = collagen type I alpha 1, TGF β = transforming growth factor beta, SMAD = SMAD transcription factor, STAT = signal transducer and activator of transcription, TIMP3 = metalloproteinases 3, MMP9 = matrix metalloproteinases 9, JNK = c-Jun NH2-terminal kinase, eNOS = endothelial nitric oxide synthase, COX = cyclooxygenase, iNOS = inducible nitric oxide synthase, OGG = 8-oxoguanine DNA glycosylase, GADD = growth arrest and DNA damage, MTH = MutT homolog, XRCC1 = X-ray repair cross- complementing group 1, cTnT = cardiac troponin T, ANP = atrial natriuretic peptide, BNP = brain natriuretic peptide, ANGII = angiotensin II, PRDX = peroxiredoxin, TRPA1 = transient receptor potential cation channel A1, MAPK = mitogen activated protein kinase, I κ B α = inhibitor of the transcription factor NF- κ B, FIS1 = mitochondrial fission 1, MFN = mitofusion, DRP 1 = dynamin-related protein, OPA1 = mitochondrial dynamin like GTPase, SeY = selenium-enriched yeast, sICAM = soluble intercellular adhesion molecule, RYR2 = ryanodine receptor 2, SERCA2a = sarcoplasmic/endoplasmic reticulum calcium ATPase 2a, SOS1 = SOS Ras/Rac Guanine Nucleotide Exchange Factor 1, CREB = cAMP response element binding protein, GSK = glycogen synthase kinase, GRB = Growth factor receptor-bound protein, NCX = Na⁺/Ca²⁺ exchanger, CaV1.2 = calcium channel, voltage-dependent, L type, alpha 1C subunit, SIRT = sirtuin, DNMT = DNA methyltransferase, p-PLN = phosphorylated phospholamban, VCAM = vascular cell adhesion protein, MCP = monocyte chemoattractant protein, GATA = GATA binding protein, NKX2-5 = homeobox protein Nkx, H3K9ac = histone 3 lysine 9 acetylation, MEF = myocyte enhancer factor, NFE2L2 = nuclear factor erythroid-derived 2-like-2, HO-1 = heme oxygenase 1, C3 = complement

factor 3, CK = creatine kinase, IFN = interferon, mPHGPx = phospholipid hydroperoxide glutathione peroxidase, UCP2 = uncoupling protein 2, CPT1A = carnitine palmitoyltransferase 1A, PDH = pyruvate dehydrogenase.

Particulate Matter 2.5 μm (PM_{2.5})

Particulate matter is the sum of all organic and inorganic compounds dispersed in the solid and liquid phases that can be carried by air (116). Decreasing the size of particulates, from 2.5-10 μm (PM₁₀) to <2.5 μm (PM_{2.5}), increases the capacity for the material to travel farther into the bronchoalveolar airways and allows for a broader interaction area (75, 247). With the heterogeneous nature of PM_{2.5}, understanding the risks posed by inhalation exposure is difficult. At a physiological level, PM_{2.5} inhalation exposure has been correlated with vascular changes (206, 207) and alteration in heart structure (7). While the cardiovascular outcomes resulting from PM_{2.5} have been thoroughly examined, the interconnectedness between PM_{2.5} exposure and the cellular and molecular mechanisms involved lacks clear integration in the literature. We provide an illustration to help summarize the DNA, protein, and cellular changes observed following toxicant inhalation exposure, including PM_{2.5} (Figure 1.1).

Figure 1.1: Cellular and Molecular Impacts on the Cardiovascular System

Figure 1.1: Summary of cardiovascular alterations observed following toxicant inhalation exposures. Changes in DNA, protein, and cellular expression levels observed in the heart, serum, and blood are displayed, followed by the number of references (if more than one) that have reported each respective change. GATA = GATA binding protein, TGF = transforming growth factor beta, DNMT = DNA methyltransferase, MEF = myocyte enhancer factor, NKX2-5 = homeobox protein Nkx, SIRT = sirtuin, SMAD = SMAD transcription factor, STAT = signal transducer and activator of transcription, CREB = cAMP response element binding protein, GRB = Growth factor receptor-bound protein, GSK = glycogen synthase kinase, SOS = SOS Ras/Rac Guanine Nucleotide Exchange Factor, TNF = tumor necrosis factor, IL = interleukin, NF- κ B = nuclear factor-kappa beta, CRP = C-reactive protein, ET = endothelin, HO = heme oxygenase, ANGII = angiotensin II, ANP = atrial natriuretic peptide, BNP = brain natriuretic peptide, CD26 = cluster of differentiation 26, COX = cyclooxygenase, CYP = cytochrome P450, HSP-70 = 70-kDa heat shock protein, IFN = interferon, iNOS = inducible nitric oxide synthase, JNK = c-Jun NH₂-terminal kinase, MCP = monocyte chemoattractant protein, NLRP = NOD-like receptor protein, p-Akt = phospho-Akt, EGFR = epidermal growth factor receptor, sICAM = soluble intercellular adhesion molecule, VCAM = vascular cell adhesion protein, eNOS = endothelial nitric oxide synthase, I κ B α = inhibitor of the transcription factor NF- κ B, FAM50B = family with sequence similarity 50 member B, KNDC = Kinase Non-Catalytic C-Lobe Domain Containing, LINE = long interspersed nuclear element, SLFN = Schlafen Family Member, TLR = Toll-like receptor, C3 = complement factor 3, cTnT = cardiac troponin T, PAI = plasminogen activator inhibitor, TNFR = tumor necrosis factor receptor, NOX = NADPH oxidase, GADD = growth arrest and DNA damage, NFE2L2 = nuclear factor erythroid-derived 2-like-2, OGG = 8-oxoguanine DNA glycosylase, mPHGPx = phospholipid hydroperoxide glutathione peroxidase, MTH = MutT homolog, PRDX = peroxiredoxin, XRCC1 = X-ray repair cross-complementing group 1, COL3A1 = collagen type III alpha 1, CaV1.2 = calcium channel, voltage-dependent, L type, alpha 1C subunit, COL1A1 = collagen type I alpha 1, RYR2 = ryanodine receptor 2, TRPA1 = transient receptor potential cation channel A1, NCX = Na⁺/Ca²⁺ exchanger, p-PLN = phosphorylated phospholamban, SERCA2a = sarcoplasmic/endoplasmic reticulum calcium ATPase 2a, DRP 1 = dynamin-related protein, FIS1 = mitochondrial fission 1, MFN = mitofusion, OPA1 = mitochondrial dynamin like GTPase, CPT1A = carnitine palmitoyltransferase 1A, p-PDH = phosphorylated pyruvate dehydrogenase, UCP2 = uncoupling protein 2, MMP9 = matrix metalloproteinases 9, TIMP3 = metalloproteinases 3.

Ambient Air Pollution

In industrial cities and large metropolitan areas, the most pervasive medium for exposing individuals to aerosolized toxicants is through the ambient air. According to the World Health Organization (WHO), over three million people die every year from outdoor air pollution and nine out of ten people are exposed to poor air quality beyond the WHO limits (157). Half of all of the deaths resulting from ambient air pollution are primarily linked to cardiovascular disease (157).

At the human exposure level, studies have begun to examine the molecular adaptations in the heart, specifically through the Normative Aging Study, established by the United States Department of Veterans Affairs. In the Normative Aging Study, hypomethylation was shown in two repetitive elements (143). Over a 90-day period, increased carbon black levels were correlated with decreased methylation of Alu, while SiO₂ was associated with decreased methylation of long interspersed nuclear element-1 (LINE-1). Toll-like receptor 2 (TLR2) methylation was

demonstrated to increase with the concentration of PM_{2.5} exposure, evidenced in a patient subset of the Normative Aging Study. Through the intake of flavonoids, TLR2 methylation was shown to decrease and ameliorate the effects on low frequency heart rate variability (272). Another study evaluating the Normative Aging Study analyzed gene pathways from blood collected from 646 participants, revealing that pathways such as cancer, signal transduction, and cell growth/death are affected by PM_{2.5} inhalation exposure (47). CpG methylation of Schlafen Family Member 11 (SLFN11), which is involved in regulating the innate response to fight cancer cells, was the most associated gene in their analysis. Other meta population studies have also investigated the cellular and molecular mechanisms of PM_{2.5} air pollution on the cardiovascular system (18, 119, 131, 153).

When measuring blood methylation levels in 15 healthy participants, the Alu repetitive transposable element was shown to be hypomethylated following concentrated ambient particles (CAPs) exposure. Decreased Alu methylation was also shown to be associated with higher diastolic blood pressure post-exposure (18). In a study examining the pollution of Oxford Street in London, England, 24 participants were recruited to give plasma samples after being exposed for 2 hours to ambient air conditions (119). The plasma evaluated contained circulating microRNAs that are involved in regulating cardiovascular function, with microRNA-133a, 145-5p, and 499a-5p differentially impacted. In a 3820 non-current smoking patient population that was part of the Framingham Heart Study, acute PM_{2.5} exposure was shown to be inversely correlated with P-selectin expression, suggesting decreased endothelial-surface cell adhesion (131). Generally, human studies evaluating exposure to PM_{2.5} have suggested global cellular alterations, including epigenome modifications, which may be indicated through transient inflammatory markers or repressive microRNA profiles. Nevertheless, the extent, and duration of epigenetic remodeling remains largely unknown in human populations.

Occupational Pollution

Most interactions with inhaled toxicants are from indirect or diluted sources, which may only interact acutely with a given individual. Alternatively, the occupational setting is one in which more concentrated, chronic exposures can result, leading to more severe cardiopulmonary outcomes. Understanding the occupational niche is important in predicating the longevity of complications arising from any given exposure.

In studies examining occupational exposure of boilermakers, decreases in heart rate variability and epigenetic alterations following exposure in 66 male participants were observed (69). Methylation of LINE-1 was significantly increased in peripheral blood leukocytes of participants when exposed to PM_{2.5}, but was not directly associated with declining heart rate variability. A following study examined blood from 48 male participants for methylation of mitochondrial DNA (23). While methylation of specific mitochondrial transcribed genes, mitochondrially encoded tRNA phenylalanine (MT-TF) and mitochondrial 12s RNA (MT-RNR1), were not associated with PM_{2.5} exposure, methylation of the mitochondrial promoter D-Loop was associated with both PM_{2.5} exposure and measures of heart rate variability (23). In blood from 63 trucking industry participants, 48 (elemental carbon), 260 (organic carbon), and 49 (PM_{2.5}) differentially regulated genes were associated with each exposure; these expression profiles indicating genes regulating apoptosis, DNA and metal binding, and chronic heart and lung disease pathways (40). Plasma examined from 18 males after cycling, revealed a significant increase in nitric oxide variants (NO_x) with a significant decrease in eET-1, though, the results suggest that acute exposure to diesel exhaust did not alter flow-mediated dilation and may not alter overall performance (81).

Diesel exhaust exposures in animals have also helped to develop a better understanding of occupational exposures through combustion particulates. Nootkatone, a sesquiterpenoid in grapefruit, was used as a pretreatment strategy for diesel exhaust exposure, reducing blood clotting (plasminogen activator inhibitor-1 (PAI-1) and fibrinogen), inflammation (IL-6), and lipid peroxidation (155). The authors suggest that the prothrombotic, oxidative environment was induced through nootkatone activation of nuclear factor erythroid-derived 2-like 2 (NFE2L2) and HO-1. Neonatal cardiomyocytes from those exposed to diesel exhaust *in utero* exhibited differential methylation of microRNA133a-2 at the promoter region. As the progeny aged, introducing stress, such as transverse aortic constriction, revealed increased microRNA-133a-2 and decreased protein tyrosine phosphatase, receptor type F (PTPRF) and peptidase domain containing associated with muscle regeneration 1 (PAMR1) expression. (82).

Animal Models

Wild Type

Animal models have been pivotal in determining mechanisms of cardiovascular dysfunction following exposure to PM_{2.5}. Examining the effects of PM_{2.5} exposure in the development of atherosclerosis, Rao, et al. discovered the increased abundance of an oxidized variant of cholesterol, 7-ketocholesterol (7-KCh). By reducing the number of CD36-positive macrophages, 7-KCh, which is inefficiently disposed of by macrophages, is more easily cleared and accumulates less within the vasculature (166). Gene expression of the 70-kDa heat shock protein (HSP-70), heme oxygenase-1 (HO-1), and myeloperoxidase (MPO) increased in the heart of mice following PM_{2.5} exposure, with a total of 181 up-regulated and 178 down-regulated genes. The heart was shown to have greater than six times more differentially affected genes compared to the lungs, with collagen, laminin, and calcium handling related genes significantly increased (177). Cardiac inflammation and fibrosis were induced in the heart of mice exposed to PM_{2.5} with elevated expression of C-X-C motif chemokine ligand 1 (CXCL1), interleukin (IL) -6, and IL-18. Nuclear factor-kappa beta (NF-κB) and epidermal growth factor receptor (EGFR)/Akt signaling may be markers involved in the initial response to inhalation exposure (111). Li et al. further highlighted this with an inhalation exposure paradigm in mice that diminished cardiac function with increased apoptosis through Caspase-3, Bax and Bcl-2 in male mice. Specifically, the connection between NF-κB activation and subsequent activation of IL-1β and IL-6 in neonatal ventricular cardiomyocytes was elucidated mechanistically (133).

The role of ROS, and particularly NADPH oxidase 4 (Nox4), in mice was examined during the development of cardiovascular disease following PM_{2.5} exposure. Measures after exposure indicated increased inflammation markers (tumor necrosis factor-α (TNF-α) and IL-1β), myocardial apoptosis, and NOX4 and NOX4 associated subunits, suggesting that the progression of cardiovascular disease, may be dictated by ROS generation and the modification of antioxidant proteins, which are initially regulated by PM_{2.5} exposure. (244). In another investigation, genes linked to fibrosis, (collagen type I alpha 1 (Col1a1) and collagen type III alpha 1 (Col3a1)) and ROS production (NOX-4 and transforming growth factor beta 1 (TGFβ1)) were shown to be increased, matching the cardiac dysfunction and prooxidative environment found in the heart (163). Vascular endothelial cell permeability pathways were assessed in rats through signal transducer and activator of transcription 3 (STAT3) phosphorylation (45). Induction of STAT3 regulated microRNA-21 expression, which in turn, caused a decrease in metalloproteinases 3 (TIMP3) and stimulated matrix metalloproteinases 9 (MMP9) expression. Exploring the effects of

hyperlipidemia during exposure, rats eating a high fat diet were predisposed to higher blood pressure and lower heart rate variability, which were accompanied by decreased expression of antioxidant proteins, including superoxide dismutase (SOD). Additionally, the authors found that apoptosis in hyperlipidemic rats was regulated through c-Jun NH2-terminal kinase (JNK) and P53 phosphorylation and downstream activation of Bax and Caspase-3 (240). The intracellular and extracellular levels of HSP-70 were used to assess inflammation through a variety of exercise modalities (145). Mice were exposed to PM_{2.5} during moderate and high intensity exercises, which revealed higher heart lipid peroxidation levels. However, only the high intensity exercise group showed an anti-inflammatory effect, reducing the extracellular to intracellular HSP70 level, or H-index.

The use of multiple models of particulate toxicity can provide a means for detecting individual toxicants responsible for the resulting physiological insult. In a co-exposure model of SO₂, NO₂, and PM_{2.5}, increased endothelin-1 (ET-1) and endothelial nitric oxide synthase (eNOS) expression suggested endothelial dysfunction, while upregulation of cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), TNF- α , and IL-6 suggested inflammatory signaling. The co-exposure contributed to decreased blood pressure and increased cardiovascular complications when compared to PM_{2.5} alone (266). In rats, PM_{2.5}, consisting of polycyclic aromatic hydrocarbons (PAHs) 1-nitropyrene (1-NP) and 9-nitroanthracene (9-NA), increased DNA damage and DNA damage response genes (8-oxoguanine DNA glycosylase (OGG1) and growth arrest and DNA damage 153 (GADD153)), as well as genes involved in ROS regulation (glutathione S-transferase (GST)), and coagulation (HO-1) (269).

Transgenic/Supplementation

Animal models of PM_{2.5} have not only allowed for the investigation of cellular adaptations in the heart following exposure, they have also provided information regarding manipulation of the biological system. In an atherosclerotic transgenic mouse model (ApoE^{-/-}), cardiac autonomic nervous system dysfunction and oxidative stress were induced following exposure to PM_{2.5}, though, the high-fat diet fed, atherosclerotic mice displayed a greater proclivity for oxidative damage (158). To examine the metabolic component of inhalation exposure, AMP-activated protein kinase $\alpha 2$ knockout (AMPK $\alpha 2$ ^{-/-}) transgenic mice, which lack this crucial gene for fatty acid oxidation, were assessed for cardiovascular function (235). AMPK $\alpha 2$ ^{-/-} mice exhibited left

ventricular dysfunction and an increased oxidative and inflammatory environment through decreased expression of peroxiredoxin 5 (PRDX5) and increased expression of NF- κ B and TNF- α . Thompson et al. showed that a transgenic mouse model for transient receptor potential cation channel A1 (TRPA1) knockout acts protectively in reducing myocardial dyssynchrony in mice when exposed to PM_{2.5} CAPs and Acrolein co-exposure (214). The authors concluded that TRAPA1 acts to increase cardiac risk, specifically in exposures with heterogeneous compositions. Supplementation of vitamins, antioxidants, and other natural and artificial compounds can provide valuable information regarding the mechanisms involved in PM_{2.5} exposure.

Through intracerebroventricular injection of an I κ B kinase-2 (IKK) analog (IMD-0354) into diabetic mice, inflammation induced by PM_{2.5} exposure through increased COX-2 and mitogen activated protein kinase (MAPK) was remediated. Increasing IKK, an inhibitor of NF- κ B, blocked the inflammatory response and reduced cardiovascular injury (268). In aortic tissue, 9 days of CAPs exposure resulted in decreased phosphorylation of Akt and eNOS, subsequently suppressing inhibitor of the transcription factor NF- κ B (I κ B α) and producing an inflammatory response. By treating with an antioxidant, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL), inflammation was alleviated, as well as insulin resistance in high fat diet fed mice (90). Supplementation of vitamin E and omega-3 polyunsaturated fatty acids prior to PM_{2.5} exposure in rats, attenuated cardiac injury and reduced inflammation through decreased expression of TNF- α , IL-1 β , and IL-6 (61). At high doses of PM_{2.5}, cardiac mitochondrial ultrastructure (size and cristae formation) changes were observed, potentially through changes in fission/fusion dynamics, with increases in mitochondrial fission 1 (FIS1), mitofusion (MFN) 1, MFN2, dynamin-related protein 1 (DRP1), and mitochondrial dynamin like GTPase (OPA1). Furthermore, these authors went on to show increased inflammation (TNF- α , IL-6, and IL-1 β) (74). Investigating the benefits of selenium yeast (SeY) supplementation, Sprague Dawley rats were pretreated with SeY for 28 days and subsequently exposed to PM_{2.5} (261). SeY pretreatment caused a significant increase in the total antioxidant capacity, total SOD expression, and a decrease in pro-inflammatory markers, TNF- α and IL-1 β as compared to the PM_{2.5} exposed group.

A prooxidative environment in mice following PM_{2.5} exposure was characterized by an increase in intracellular free calcium, and a potential decrease in cardiac contractile function due to increased ryanodine receptor 2 (RYR2) and decreased sarcoplasmic/endoplasmic reticulum

calcium ATPase 2a (SERCA2a). With inclusion of an antioxidant therapeutic approach from compound essential oils (CEOs), the heart reduced total ROS, returned the calcium levels back to control, and reduced the thrombosis factor HO-1 (60). Hypertrophy was investigated in a cohort of mice following exposure (130). Cardiac remodeling through hypertrophy and fibrosis were correlated with reduced SOS Ras/Rac Guanine Nucleotide Exchange Factor 1 (SOS1), cAMP response element binding protein (CREB), and glycogen synthase kinase 3 beta (GSK3b) expression, which was remediated through fucoidan administered therapeutically.

Gestational Exposures

Understanding the environmental impact on developing progeny is a growing field and germane to future evaluations of PM_{2.5} cardiotoxicity (Figure 1.2). One day old neonatal offspring from Wistar rats revealed higher myocardium apoptosis in a dose-dependent manner, potentially due to alterations in mitochondrial fission/fusion through OPA1, MFN1, DRP1, and FIS1 (234). In one day old neonate mice, an inflammatory and extracellular matrix remodeling phenotype (increased IL-6, IL-1 β , collagen-1, matrix metalloproteinase (MMP) 9, and MMP13 expression) was shown, revealing early fibrosis of the heart (211). Calcium regulated contraction has been shown to be affected throughout gestationally-exposed progeny development. Assessment of fourteen-day old progeny, revealed decreased cardiomyocyte contractility through calcium dynamics, with changes to proteins (SERCA-2A, phosphorylated phospholamban (p-PLN), Na⁺/Ca²⁺ exchanger (NCX), and calcium channel, voltage-dependent, L type, alpha 1C subunit (CaV1.2)) involved in calcium flux in the heart (211). At 12 weeks of age, progeny revealed cardiac dysfunction and increased collagen deposition in cardiac tissue, potentially through changes in epigenetic regulation (decreased Sirtuin (SIRT) 1 and SIRT2 and increased DNA methyltransferase (DNMT) 1, DNMT3a, and DNMT3b expression) and calcium handling proteins (decreased Ca²⁺-ATPase, p-PLN, and NCX) (212).

Figure 1.2: Gestational Inhalation Exposure Paradigm

Figure 1.2: Molecular adaptations of the heart following gestational inhalation exposure. Exposure to particulates during pregnancy can result in maladaptive alterations in progeny cardiovascular function. Epigenetic alterations in the heart have the capacity to initiate persistent functional deficits that may continue into adulthood.

In a gestational exposure setting, wherein both parents were exposed to PM_{2.5}, the resulting progeny were shown to have increased inflammation, fibrosis, and oxidative stress with an increase in calcium handling proteins (210). Interestingly, epigenetic changes suggest a repressive phenotype, with both the maintenance methyltransferase (DNMT1) and histone deacetylases (SIRT 1/2) increased in expression. Inflammation markers (TNF- α and IL-1 β) were significantly increased, along with a reduction in genes associated with cardiovascular development (GATA Binding Protein 4 (GATA4) and homeobox protein Nkx (NKX2-5)) in neonatal and weanling Sprague Dawley rats following exposure during gestation and lactation. The authors also detail the similarity in response of progeny exposed *in utero* to PM_{2.5} and those exposed through maternal intraperitoneally delivered homocysteine (36). In young adult mice following gestational exposure, cardiac hypertrophy was exhibited, with an increase in both total histone acetylation and promoter acetylation of hypertrophy genes (GATA4 and myocyte enhancer factor 2C (Mef2c)), indicating potential global epigenetic remodeling through transcriptional activation of acetylated regions (245). Interestingly, a cardiac hypertrophic/fibrotic phenotype and epigenetic remodeling have been reported in early development (210, 212, 245).

Ultrafine Particulate Matter $\leq 0.1 \mu\text{m}$ (PM_{0.1})

Similar to PM_{2.5}, ultrafine particulate matter (UFP) has the capacity to penetrate deeply into the alveoli of the tracheobronchial airways (201), but, UFP exhibit an increased capacity for interstitialization and transport systemically throughout the body due to their high surface-to-volume ratios (27, 62). While natural sources of UFP exist, UFPs can be produced in high concentrations through combustion reactions in urban settings (113) which can be especially detrimental to human health (190). Specifically, the impact on vulnerable populations (190, 228) as well as the increased susceptibility of women to inhaled UFPs (191) make the study of particulate matter at the nanoscale so important. While investigations have begun to examine the cardiovascular deficits associated with UFP inhalation toxicology (205), the molecular and cellular mechanisms driving the associated dysfunction remain unlinked between exposure paradigms. We provide an illustration to help summarize the DNA, protein, and cellular changes observed following toxicant inhalation exposure, including nanomaterial/nanoparticle (Figure 1.1).

Ambient Air and Occupational Pollution

In individuals with metabolic disorder, UFP inhalation exposure was specifically detrimental to cardiac function in those without the glutathione s-transferase mu 1 (GTSM1) protein (56). In the complete cohort exposed to UFP, C-reactive protein (CRP), serum amyloid A (SAA) were increased with a subsequent decrease in plasminogen and thrombomodulin within blood, indicating an upregulation of inflammatory pathways. In a type 2 diabetic cohort, the inflammatory response is also modified following UFP inhalation exposure, increasing CD40 ligand (CD40L) expression in platelets with a resulting decrease in soluble CD40 in blood (200). Platelet CD40L expression and blood coagulation von Willebrand factor were both elevated following acute UFP exposure, indicating the adverse predisposition of type 2 diabetics to additional insults. To understand the relationship between ambient exposure and the time/concentration of the exposure, Lane et al. examined biomarkers in patients with varying levels of microenvironment exposures (time-activity adjusted) as well as the concentration of the UFPs (annual average particle number concentration) (126). Positive associations between time/concentration of exposure and blood CRP, IL-6, and tumor necrosis factor receptor 2

(TNFRII) were found, with significant positive correlations of CRP and TNFRII with white non-Hispanic participants, but not those of East Asian descent.

While most population-derived analyses use systemic blood to assess cardiovascular impact, post-mortem ventricular autopsies were performed on 30 children and young adults to assess the impact of urban UFP pollution (226). Compared to clean air controls, proteins involved in oxidative (prion protein (PRNP)), and endoplasmic reticulum (glucose regulated protein 78 (GRP78)) stress were significantly upregulated, and even disproportionately upregulated in the left, compared to right, ventricle of the UFP exposed cohort. These data suggest a compensation by the heart to attenuate inflammatory effects of air pollution, maybe specifically for UFP (226). In an occupational study of healthy individuals near a steel mill, increases in plasma levels of IL-6, IL-8, and ET-1 were significantly associated with rises in CO, UFP and SO₂ concentrations in ambient air, with a positively correlation between ET-1 and CRP with cardiac dysfunction indexes (123). Protein ontology revealed a significant elevation of inflammation specific pathways.

Animal Models

In Sprague-Dawley rats, an acute and sub-chronic inhalation exposure to both PM_{2.5} and PM_{0.1} revealed significant upregulation of angiotensin II type 1 receptor (AT₁R) mRNA in the heart, with the sub-chronic exposure to both PMs causing an increased protein level (10). In the sub-chronic group for both PM concentrations, this also lead to decreased expression of HO-1 and increased expression of actin alpha 1 (Acta1), Col3a1, and IL-6. The authors suggest that cardiovascular complications, marked by coronary artery wall thickness, are linked to alterations within the endocrine system, specifically pathways related to angiotensin (10). UFP exposure has also been linked to mitochondrial dysfunction. Implementing a Langendorff isolated heart model in rats, ischemia/reperfusion was exacerbated in those rats exposed to UFP (99). Treatment with Cyclosporin A (CsA) in isolated hearts as well as decreased Ca²⁺ buffering in isolated mitochondria suggest that the mitochondrial permeability transition pore (mPTP) may be impacted. To understand the impacts of aging phenotypes to UFP inhalation exposure, a 12-13 month spontaneously hypertensive rats (SHRs) were exposed to ultrafine carbon particles (UFCP) (220). Increased serum CRP, plasma fibrinogen, and levels of ET-1 in the heart were all associated with UFCP inhalation exposure and increased blood pressure and heart rate. Additionally in the

SHR model, angiotensin II (AngII) was shown to be elevated in the blood following exposure to UFCP through instillation, with increasing doses of UFCP attenuating the effectiveness of captopril, an angiotensin converting enzyme inhibitor (263).

Gestational Exposures

The gestational impact of UFP on growing progeny also provides valuable mechanistic knowledge regarding the modes of action of inhaled UFP on cardiovascular function. UFP exposure to pregnant C57BL/6J dams lead to fetal resorption and increased blood pressure in adult mice progeny (151). Increases in maternal serum inflammation during gestation (i.e. elevated IL-1 β , IL-6 and MCP-1 levels) and AT₁R and angiotensin I-converting enzyme (ACE) activation in the lungs were suggested to contribute to progeny cardiovascular dysfunctions. Gestational exposure to UFP in mice was also performed to understand adaptability to immunological responses and innate immunity in progeny (173). Challenging progeny immune systems with house dust mite (HDM) allergen revealed a decreased immune response through lower IL-13 and IL-17, with higher serum levels of IL-10. This has also been demonstrated with UFP containing persistent free radicals (MCP230), which suppressed the T helper and T regulatory cells found within the lungs of offspring (239).

PM_{2.5} and UFP Combined Analyses

While many studies have examined the effects of either PM_{2.5} or UFP, some analyses allow for the direct comparison of both sizes of particulates within a single investigation. Levels of Delta-C, which is a compound known to exist in wood smoke, was found to be positively correlated with fibrinogen and myeloperoxidase (MPO), but not with PM_{2.5} or UFP (43). Additionally, the study identified PM_{2.5} or UFP as retaining a positive correlation with CRP and fibrinogen, sharing a similarity in the effects on blood coagulation with effects of Delta-C. In a meta-population study, including 3275 participants, CRP and platelet count was significantly increased in blood of participants matching long-term (2000-2008) rises in PM_{2.5}, with no direct associations found for PM_{0.1} (224). The integrity of the mitochondrial DNA defines the respiratory potential of the mitochondrion, allowing for separation of individuals into a variety of haplogroups (171). Ambient air pollution differentially effected individuals with diverse haplotype backgrounds, with

the haplogroup H (more uncoupled) compared to haplogroup U (less uncoupled) being more detrimentally effected and elevated blood IL-6 and TNF- α in both groups associated with quasi-ultrafine particles $<0.25\ \mu\text{m}$ (243).

To determine the epigenetic regulation of inflammatory markers, healthy subjects inhaled an array of particulate matter compositions (PM_{10} , $\text{PM}_{2.5}$, $\text{PM}_{1.0}$ and $\text{PM}_{0.5}$) over two sessions (216). Blood samples following the trials revealed that increased interferon γ (IFN- γ) methylation was significantly associated with $\text{PM}_{2.5}$ as well as quasi-ultrafine particles $<0.50\ \mu\text{m}$, correlating with alterations in the parasympathetic nervous system. Examining the impact of urban air pollution in healthy, COPD, and ischemic heart disease participants, microRNA profiles in blood were assessed to identify associations between isolated pollutants. Nine differentially regulated microRNAs were significantly associated with $\text{PM}_{2.5}$ in blood, while no associations were identified for UFP (68). The microRNA alterations for $\text{PM}_{2.5}$ included an upregulation of hsa-miR-197-3p and hsa-miR-99a-5p, playing potential roles in cancer (68, 256). Acute, 24 hour exposure to $\text{PM}_{2.5}$ personal air pollution resulted in significant alterations to the DNA methylation profile in 157 healthy non-smoking adults (153).

Blood collected from those subjects revealed up to 69 differentially methylated regions with altered methylation status, linking to genes such as Kinase Non-Catalytic C-Lobe Domain Containing 1 (KNDC1) and family with sequence similarity 50 member B (FAM50B), which remain relatively obscure as to their functional role in the cardiovascular system. Additionally, ambient and personal exposure to UFP resulted in 31 differentially methylated regions in DNA (153). As a means of isolating out the specific toxicants responsible for cellular and molecular dysfunction following inhalation exposure, particulate matter at the coarse (PM_{10}), fine ($\text{PM}_{2.5}$), and ultrafine ($\text{PM}_{0.1}$) range were exposed to healthy volunteers and specific metals were correlated to physiological effects (137). As a result, increased serum concentrations of VEGF, traumatic brain injury marker ubiquitin C-terminal hydrolase L1 (UCHL1), and cortisol were significantly correlated with nickel, barium, and silver metal concentrations, respectively. While reports have generally shown the increased toxicity of smaller particulate matter, Liu et al. indicated that coarse (PM_{10}) exposures to CAPs caused significant changes in blood cortisol levels, where UFPs did not reveal any changes in biomarkers surveyed (138).

Nanotoxicology

Studies investigating ultrafine particulate matter (UFP) allow for the examination of physiological perturbations in a fashion that is comparable to nanomaterials and nanoparticles. While inhalation studies examining ENMs and UFP typically differ in the number and standardization of compounds found within the mixtures, both share commonality in the nanometer scale that permits similar dispersion within the lungs during inhalation. Nanomaterials represent an ever-increasing vehicle for inhalation toxicology (121). Whether during the manufacturing of nanomaterials (121) or in their consumer use (154), production and rate of application continue to rise. Nanomaterials and nanoparticles, similar to $PM_{0.1}$, exist exclusively at a biological scale that alters the dynamics of interactions with organic matter, potentially altering toxicities compared to their micrometer-sized equivalents. We provide an illustration to help summarize the DNA, protein, and cellular changes observed following toxicant inhalation exposure, including nanomaterial/nanoparticle (Figure 1.1).

Animal Models

Savi et al. showed that a direct translocation of nanomaterials to the heart, through inhalation exposure, could be linked to subsequent cardiovascular dysfunction (179), and others have since examined the molecular consequences of this interaction (105). In the heart, following inhalation exposure, translocation of nanomaterials was suggested to initiate the complement cascade, setting off a local immune response, as well as global complement factor 3 (C3) upregulation in the blood. Chronic exposure to nano-TiO₂ in outbred mice led to a significant change in inflammation markers, including changes in transcription factors (STAT 1/3/6 and GATA 3/4) in the heart (100). Examining co-exposure models, silica nanoparticles (SiNP) and methylmercury (MeHg) induced an increase in activity of cardiac troponin T (cTnT), atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP), along with increased SERCA2 levels in rat serum (249).

In another model by the same group, Feng et al. applied a SiNPs and Pb co-exposure model and found similar effects in the heart, namely increased expression of inflammation markers (C-reactive protein (CRP), IL-6, and TNF- α) and upregulation of ANP and BNP following exposure

in the serum (73). To evaluate the mechanistic effects of inhalation compared to intra-arterial infusion on extra-pulmonary organ susceptibility, Ganguly et al. employed carbon nanoparticles in the evaluation of cardiovascular function (78). While finding that inhalation exposure induced an inflammatory response within the lungs and intra-arterial infusion produced forms of systemic inflammation, cardiovascular transcripts and proteins related to inflammation and endothelial/epithelial activation in the aorta and heart were more significantly impacted through inhalation exposure than injected carbon nanoparticles. These results provide strong evidences for indirect (neuronal or inflammatory) in contrast to direct (nanoparticle contact) mechanisms of cardiovascular dysfunctions associated with xenobiotic exposures (78).

Only recently have transgenic mouse models been applied to examining nanomaterial exposure. In a single dose inhalation exposure, a transgenic mouse model for phospholipid hydroperoxide glutathione peroxidase (mPHGPx) was used to restore mitochondrial respiratory function and ROS levels following nano-TiO₂ exposure (198). By increasing antioxidant defense, proteomics revealed that changes in mitochondrial composition, including individual electron transport chain complex constituents, were protected from the detrimental effects of exposure. In another study, a microRNA knockout transgenic mouse model (microRNA-378a) showed increased Mfn1 expression and altered mitochondrial ultrastructure through transmission electron microscopy, potentially through mitochondrial fusion dynamics (94).

Gestational Exposures

A limited number of studies have begun to examine the cardiovascular complications arising in progeny that were gestationally-exposed to nanomaterials/particles. The impact of gestational nanoparticle exposure on cardiac function and mitochondrial health, as well as transcriptomic and epigenomic landscape have been explored. Diastolic cardiac function and mitochondrial respiration were shown to be diminished in young adult progeny following nano-TiO₂ gestational exposure (95), with increases in mitochondrial proton leak, uncoupling protein 2 (UCP2), and changes in mitochondrial metabolism proteins (carnitine palmitoyltransferase 1A (CPT1A) and pyruvate dehydrogenase (PDH) phosphorylation). Previously, state 3 respiration was also shown to be decreased in the left ventricle following gestational exposure in young adult progeny (199). Ultimately, changes in global histone methylation, and the resulting epigenome, were considered for future evaluation. Using chromatin immunoprecipitation sequencing for

histone 3 lysine 4/27 trimethylation (H3K4me3 and H3K27me3) coupled with the transcriptomic profiling of fetal cardiac tissue from gestationally-exposed dams (198), major pathways including immune adaption and organismal growth, were significantly altered. In the fetal progeny, cardiovascular outcomes at the young adult stage may actually be linked to liver and fetal development, evidenced through IPA protein ontology.

Future Directions

From a morning commute, to an occupational setting, to our very homes, exposure to particulates remains a central issue for debate and therapeutic/lifestyle interventions. Industrialization and increased urbanization will continue to exacerbate issues concerning air quality and human health. Although we provide a summarization of the molecular mechanisms driving cardiovascular decline following exposure, areas of research that are crucial in driving the field of molecular inhalation cardiovascular toxicology forward, still remain.

A need exists for more encompassing “omics” based approaches. While investigators have begun to examine molecular influencers of inhalation exposure in the heart, including protein and gene markers for inflammation, ROS generation, antioxidant defense, general and specific transcription factors, non-coding RNAs, cancer predisposition, and others, few studies have examined the complete cellular protein, gene, or metabolic regulatory networks. The integration of transcriptomics with epigenomics, proteomics, etc., provides a complete snapshot of the altered molecular state. In the heart, elucidating the primary causes of insult will require multi-omics integration through the identification of pathways altered, (i.e. metal-binding, DNA damage response, epigenetic factors, mitochondrial, or other protein pathways). True mechanistic understanding requires a more complete picture to elucidate causal and compensatory actions of the cell.

A necessity also exists for cellular models of exposure interactions. While it remains outside the scope of this review, *in vitro* models can provide a very granular, and controlled approach to mechanistic evaluations. Of note, authors have recently investigated the effects of PM_{2.5} exposure (24, 46, 71, 101, 250, 251, 266), as well as the nanotoxicology (72, 103, 104, 249) in cells. Ultimately, work at the population, animal, and cellular level can all lead to better

evaluation of particulate exposure to cardiovascular outcomes. Due to limitations in the standardization of particulate matter exposures, more research establishing the relationship between individual toxicants and specific molecular outcomes is needed in order to draw conclusive statements about the exposure and/or co-exposure etiology. Moving forward, research in molecular inhalation cardiovascular toxicology needs to continue to examine what the relevant pathways are to the heart and circulatory system, including mitochondrial function, calcium regulation, and paracrine/exosome release from the heart and systemic implications. As we continue to shift the paradigm of cardiovascular research in inhalation toxicology, much of the cellular and molecular mechanisms are yet to be discerned. How these mechanisms differ for each toxicant, longevity of exposure, and concentration of dose must be considered when characterizing pathways altered in the heart (Figure 1.3).

Figure 1.3: Cellular and Molecular Impacts on the Cardiovascular System

Figure 1.3: Overview of the variables that dictate the effects induced by toxicant exposures on the downstream cellular and molecular mechanisms in the heart: source of the toxicant, particle size and dose, method of exposure, and the duration of the exposure. Changes in any one of the four levels indicated above could have serious implications on the interpretation of broad-scale cellular alterations and how we should consider the basic mechanisms involved in inhalation exposure paradigms.

Type 2 Diabetes Mellitus

Introduction

Diabetes mellitus affects 29.1 million Americans and 25.9% of Americans age 65 and older. Adults with diabetes mellitus have two to four times the rate of mortality from heart disease as compared to those without diabetes mellitus, and 68 percent of diabetic patients age 65 and older die of heart disease (1, 2). The diabetic heart is metabolically characterized by insulin resistance, reduced cellular glucose import and oxidation, and increased mitochondrial fatty acid import and oxidation (197). This chronic alteration in energy substrate utilization and loss of the dynamic ability of the myocardium to metabolically adapt to its environment, such as upregulating anaerobic glycolysis during cardiac ischemia, initiates a pathological state termed diabetic cardiomyopathy (5, 142). Increased cardiac hypertrophy and fibrosis are structural changes observed in diabetic cardiomyopathy that are accompanied molecularly by increased oxidative stress, mitochondrial dysfunction, and cardiomyocyte apoptosis (35, 64, 106, 172, 273). Diabetic heart failure is the culmination of these pathologic insults that is characterized by severe contractile dysfunction of the myocardium (57).

The proteomic alterations of the heart in the diabetic state have been well characterized. Metabolically, the decrease in glucose import and oxidation in the diabetic cardiomyocyte is associated with decreased expression of insulin-stimulated glucose transporter type 4 (GLUT4) and decreased activity of pyruvate dehydrogenase (PDH) (197). The increase in mitochondrial fatty acid import and oxidation are found in conjunction with decreased acetyl-CoA carboxylase activity, decreased malonyl-CoA concentration, increased carnitine palmitoyl transferase I activity, and increased beta-hydroxyacyl-CoA dehydrogenase activity (197). Increased reactive oxygen species (ROS) generation in diabetic cardiomyopathy results from contributors such as excess fatty acid oxidation, increased NADPH oxidase activity, and uncoupled nitric oxide synthases (NOS) (106). Increased cardiomyocyte apoptosis in the diabetic setting is contributed to by the decreased expression of the anti-apoptotic protein B-cell lymphoma 2 (Bcl-2), and increased expression of the pro-apoptotic protein p53 (168, 270). At the heart of energy substrate metabolism, ROS generation, and apoptosis lies the mitochondrion.

While transcription factor regulation of gene expression, post-translational modification, and substrate inhibition via negative feedback have shown to be foundational in regulating protein expression and activity, microRNA (miRNA) and epigenetic mechanisms of regulation have more

recently emerged (6). MiRNA are single-stranded noncoding RNA molecules, ~22 nucleotides long, that associate into a multi-protein RNA-induced silencing complex (RISC) which inhibits its target mRNA species from being translated into a functional protein (15). Differential expression of miRNAs have been characterized in a variety of cardiovascular conditions including atherosclerosis, myocardial infarction, and heart failure to modulate processes such as energy substrate metabolism, fibrosis, and cardiac remodeling (162, 192, 264). Nuclear- and, more recently, mitochondrial-genome-encoded proteins which function in the mitochondria, have been shown to be regulated by miRNAs (14, 17, 49, 109), and may alter dynamics between the mitochondrion and nucleus (Figure 1.4). Since cardiomyocyte mitochondria are central to the pathogenesis of diabetic heart disease, the first section of this review will focus on miRNA regulation of key pathologic pathways intersecting at the mitochondrion. The following section will focus on the role of regulation on the miRNA regulator, including epigenetic, exosomal, processing, and post-transcriptional control (Figure 1.5). Finally, treatment strategies for cardiomyocyte energy substrate metabolism, pharmacologic manipulation and miRNA-based therapies for the treatment of diabetic heart disease will be discussed.

Figure 1.4: Nuclear and Mitochondrial Signaling

Figure 1.4: Feedback loops existing between the mitochondrion and nucleus. By epigenetically altering the transcription efficiency of nuclear-encoded mitochondrial proteins through changes in DNA or histone acetylation (Ac), methylation (Me), phosphorylation (P), sumoylation (Su), and/or ubiquitination (Ub), the mitochondrion is affected (anterograde signaling). Alternatively, mitochondrial metabolism affects substrate availability for proteins necessary in causing epigenetic regulation (retrograde signaling).

MiRNA Regulation of the Mitochondrion in the Diabetic Heart

MiRNAs can be transcribed through two primary pathways, either a canonical or non-canonical pathway (89, 149, 242). In the canonical pathway, the pri-miRNA is transcribed from its existing exon and intron, containing one to several hairpin loop structures. After transcription, DiGeorge Syndrome critical region 8 (DGCR8) and Drosha, known as the microprocessing complex, will cut the pri-miRNA in the nucleus, resulting in a shorter (~65 nt) pre-miRNA (85, 92). The pre-miRNA is then exported through Exportin-5 and RanGTP (254).

Finally, in the cytoplasm, Dicer will edit the pre-miRNA resulting in the mature miRNA (19-22 nt) which can then associate with the RNA-induced silencing complex (RISC). In the non-canonical pathway, hairpin structures within introns can be spliced out, resulting in pre-miRNA that are referred to as mirtrons (242). After splicing, the mirtrons proceed through the same pathway as canonical miRNA in order to be exported from the nucleus.

Figure 1.5: Processes of miRNA Regulation

Figure 1.5: Regulation of miRNA regulators. The expression of miRNAs are controlled through multiple factors. Epigenetic regulation includes hypermethylation of the miRNA-184 CpG loci, pri-miRNA-208b tethering to increase EZH2 binding, and miRNA interaction with DNMT1, DNMT3a, and DNMT3b. Regulation of miRNA expression through miRNA processing is dependent on Dicer expression (miRNA-103) and protein-protein interactions with Dicer (miRNA-21). Other proteins, such as G3bp1, can affect processing (miRNA-1). LncRNA can regulate miRNA expression by acting as a “sponge,” sequestering native and exogenous miRNAs, such as miRNA-188-3p, -539, and -489. Also, exosomal release/transport of miRNA-1, -21, and -133a can alter miRNA expression. EZH2 = Enhancer

of zeste homolog 2; DNMT = DNA methyl transferase; G3bp1 = GTPase-activating protein-binding protein 1; lncRNA = long non-coding RNA.

Following export from the nucleus, miRNAs are then implicated in regulating the expression of proteins essential to mitochondrial health. Recently, miRNAs have been found to affect mitochondrial function through targeting pathways including energy substrate metabolism, ROS generation, and apoptosis. This section will focus on miRNA regulation of these processes within the cardiomyocyte in the context of the diabetic heart (Table 1.2).

Table 1.2: MiRNA Regulation of Mitochondrial Pathways in the Diabetic Heart

miRNA	Environmental Regulation	Expression in DB Heart	miRNA Target	miRNA Target Function	DB Model	Source
let-7	Pro	Upregulated	IR, IRS-2	Insulin Signaling	STZ, ischemia reperfusion	(129, 275)
miRNA-195		Upregulated	SIRT1	Insulin Signaling	STZ; db/db	(54, 271)
miRNA-216a		Upregulated	CAV2	Insulin Signaling	Human diabetic; Ischemic heart failure	(84, 125)
miRNA-199a-3p		Upregulated	CAV2	Insulin Signaling	STZ	(58, 185)
miRNA-133a	Epi, Exo	Downregulated	TR4	Fatty Acid Transport	STZ	(38, 160)
miRNA-210		Upregulated	ISCU1/2	ETC Complex I	Human diabetic; Ischemic heart failure	(30, 84)
miRNA-141		Upregulated	Slc25a3	Mitochondrial Phosphate Carrier	STZ	(17)
miRNA-378		Upregulated	ATP6	ATP Production	STZ	(109)
miRNA-29a		Downregulated	PGC-1a	Fatty Acid Oxidation	STZ	(58, 136)
miRNA-30c		Downregulated	p53	Apoptosis	Human diabetic; Rat diabetic cardiomyopathy; High glucose-treated H9C2	(168)
miRNA-181a		Downregulated	p53	Apoptosis	Human diabetic; Rat diabetic cardiomyopathy; High glucose-treated H9C2	(176)
miRNA-30d		Upregulated	FOXO3a	Survival	STZ; High glucose-treated H9C2	(132, 257)
miRNA-34a		Upregulated	BCL-2	Survival	High glucose-treated H9C2	(267)
miRNA-1	Exo, Pro	Upregulated	BCL-2	Survival	STZ; High glucose-treated H9C2	(255, 258)

Table 1.2: MiRNA regulation of mitochondrial pathways in the diabetic heart. The differential expression of each miRNA species in diabetes is indicated along with its protein target. The process or pathway the protein participates in is given. The model(s) in which the reported observations have been made are noted. Environmental regulation refers to the participation of the miRNA in either epigenetic (Epi), exosomal transport (Exo), or miRNA processing (Pro) pathways known to influence the diabetic cardiovascular system.

MiRNA Regulation of Energy Substrate Metabolism in the Diabetic Heart

MiRNA Regulation of Insulin Signaling and Glycolysis

The downregulation of insulin signaling components in the diabetic heart is influenced by miRNA regulation. Li et al. showed that let-7 miRNA was overexpressed in the myocardium of streptozotocin (STZ)-induced diabetic rats, while the protein expression of insulin-like growth factor 1 receptor (IGF-1R), insulin receptor (IR), and glucose transporter 4 (GLUT4) were significantly lower (129). Interestingly, they found that inhibition of let-7 via the administration of let-7 antimiR conferred cardioprotection against ischemia-reperfusion injury through increased expression of p-Akt and p-mTOR (129). IR and IRS2 are validated as direct targets of let-7, and the observation of a normalization of IGF-1R, IR, and GLUT4 expression upon administration of let-7 antimiR supports the assertion that let-7 targets insulin signaling and the glucose transport pathway (129, 275). Further, Greco et al. found that miRNA-216a was overexpressed in failing human hearts of patients with and without diabetes mellitus, and its expression was negatively correlated with left ventricular ejection fraction (84). MiRNA-216a targets and represses caveolin 2, a scaffolding protein and substrate of the IR that helps to recruit IRS-1 to the IR and propagate insulin signaling (84, 125). The role of miRNA-216a overexpression in regulating intermediary metabolism in HF patients with and without diabetes is unanswered and of much interest to the field. MiRNA-199a-3p has been observed to be overexpressed in STZ-induced diabetic mice, and it also has a validated target of caveolin 2 (58, 185). Zheng et al. showed that miRNA-195 is overexpressed in STZ-induced and *db/db* mouse hearts as well as in cardiomyocytes isolated from *db/db* mice, while sirtuin 1 (Sirt1) protein levels were significantly decreased (271). Inhibiting miRNA-195 increased Sirt1 expression in diabetic mice (271). Sirt1 is a downstream effector of the insulin signaling pathway indicating that miRNA not only serve to fine tune IR and IRS2, but also regulate the expression of proteins further along the insulin signaling cascade (54). Altogether, miRNA targeting of insulin signaling components of the diabetic heart may help explain the proteomic alterations of this pathway observed in the diabetic condition.

MiRNA Regulation of Fatty Acid Transport, Fatty Acid Oxidation, Citric Acid Cycle, Electron Transport Chain, and ATP Production

The upregulation of fatty acid import and oxidation in the diabetic heart is impacted by miRNA modulation. Chen et al. demonstrated that miRNA-133a was significantly decreased in the hearts of STZ-induced diabetic mice and miRNA-133a is a direct regulator of TR4 which induces the expression of lipid importer CD36 and promoter of lipid accumulation through solute

carrier family 27 member 1 (Slc27a1/FATP1) (38, 160). This could help to explain the increase in CD36 expression in cardiac tissue of STZ-induced diabetic rats that has been reported (159). Peroxisome proliferator-activated receptor alpha (PPAR- α) is the primary transcription factor responsible for the fatty acid oxidation gene expression program (181). Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α), an essential coactivator of PPAR- α and inducer of medium-chain acyl-CoA dehydrogenase, has been found to be directly regulated by the miRNA-29 family (miRNA-29a-c) (134, 136). Interestingly, miRNA-29a has been found to be decreased in STZ-induced diabetic hearts which may help explain the induction of PPAR- α and increased fatty acid oxidation (58). Greco et al. observed that miRNA-210 is upregulated in failing diabetic human hearts compared to failing non-diabetic human hearts (84). MiRNA-210 is a known negative regulator of iron-sulfur cluster assembly scaffold protein IscU 1 and 2 (ISCU1/2), which play essential roles in the function of aconitase and Complex I of the electron transport chain (30). Baseler et al. observed a significant upregulation of miRNA-141 in STZ-induced diabetic mice and found that miRNA-141 is a direct negative regulator of solute carrier family 25 member 3 (Slc25a3) which is essential for inorganic phosphate import into the mitochondrial matrix, and thus ATP synthesis (17). It has also been shown that miRNA-378 was overexpressed in interfibrillar mitochondria (IFM), mitochondria located between the myofibrils, of STZ-induced diabetic mice, and that miRNA-378 is a direct negative regulator of ATP synthase F0 component ATP6 (109). These observations provide compelling evidence that miRNAs play a critical role in the regulation of energy metabolism in the diabetic heart.

Reciprocal Regulation of miRNA and ROS in the Diabetic Heart

Oxidative stress has been shown to be a result of and cause of miRNA dysregulation. Saito et al. demonstrated that glucose fluctuations in STZ-induced diabetic rats increased cardiomyocyte miRNA-200c and miRNA-141 levels, with an accompanying increase in ROS generation, upregulation of NADPH oxidase and thioredoxin-interacting protein, while decreasing catalase and superoxide dismutase activities (175). As glycemic control has been shown to attenuate some of the peripheral diabetic symptoms, diabetic cardiomyopathy progresses even after blood glucose normalization. One possible explanation for this finding comes from a study showing that even after glycemic control in STZ-induced diabetic mice, miRNA dysregulation of many myocardial damage pathways including oxidative stress (dysregulated miRNA-221, miRNA-146a, miRNA-

34a, miRNA-210, miRNA-19b, miRNA-27a, miRNA-155) persisted (42). Due to its upregulation in cardiac fibrosis, hypertrophy, and oxidative stress in both diabetic and insulin treated groups compared to control, miRNA-125b seems to have broad-ranging effects in mediating diabetic cardiac dysfunction (42). Excess ROS production in STZ-induced diabetic cardiomyopathy has been linked to decreased expression of miRNA-499, miRNA-1, miRNA-133a, and miRNA-133b, as treatment with the antioxidant N-acetylcysteine restored the levels of these miRNA species to normal (255). The authors further demonstrated that Junctin, a key component of cardiomyocyte calcium handling, is a direct target of miRNA-1 and is consequently upregulated in the diabetic heart, which has previously been shown to impair cardiac relaxation and induce cardiac hypertrophy and arrhythmia (255). The mechanisms by which miRNAs regulate ROS production and by which oxidative stress influences differential expression of miRNAs to regulate a variety of pathophysiological pathways in the diabetic heart remains a relatively unexplored area of research.

MiRNA Regulation of Apoptosis in the Diabetic Heart

The diabetic heart, in the setting of diabetic cardiomyopathy and diabetic heart failure, experiences an increased rate of cardiomyocyte apoptosis. This increase in apoptosis is determined by a variety of factors including lipotoxicity, glucotoxicity, and increased oxidative stress. MiRNA-34b is upregulated in diabetic heart failure, and it has been shown to promote apoptosis by acting as an important downstream effector of p53 (19, 84, 97). MiRNA-30c and miRNA-181a have been shown to be downregulated in diabetic patients, a diabetic cardiomyopathy rat model, and high-glucose treated cardiomyocytes (168). P53 is a validated target of miRNA-30c and miRNA-181a, and the decreased levels of these miRNA species is correlated with increased p53-pathway activation of hypertrophy and apoptosis (168). Upregulation of miRNA-30d in high-glucose-treated cardiomyocytes and diabetic rats has also been shown to play an important role in cardiac mitochondrial-implicated pyroptosis where it has been verified to directly target and inhibit forkhead box O3 (FOXO3a), with downstream effects including decreased expression of apoptosis repressor with caspase recruitment domain (ARC) and upregulation of caspase-1 (132, 257). Zhao et al. showed that high-glucose-treated rat cardiomyocyte H9C2 cells have enhanced expression of miRNA-34a, decreased expression of the miRNA-34a target anti-apoptotic protein Bcl-2, and increased apoptosis (267). Bcl-2 has been found to be targeted and downregulated by

overexpression of miRNA-195 in a mouse model of STZ-induced type 1 diabetes, and inhibition of miRNA-195 reduced ROS production and inhibited apoptosis (271). Further, Yu et al. showed that rat cardiomyocyte H9C2 cells treated with high glucose had increased miRNA-1 expression, downregulation of the miRNA-1 target IGF-1, increased cytochrome-*c* release, and increased apoptosis (258). To add validity to the importance of miRNA-1 in high-glucose-mediated cardiomyocyte apoptosis, it has been found that high glucose *in vitro* and *in vivo* increases miRNA-1/miRNA-206 expression precipitating post-translational modification of heat shock protein-60, a protein involved in protection against diabetic myocardial injury (183). Furthermore, Katare et al. found upregulation of miRNA-1, downregulation of the miRNA-1 targets proto-oncogene serine/threonine-protein kinase (Pim-1) and Bcl2 (anti-apoptotic proteins), and increased pro-apoptotic caspase-3 activity in the hearts of STZ-induced type 1 diabetic mice (115).

MiRNA impact on energy substrate metabolism, ROS interactions within the cell, and apoptotic pathways all show the significance of the interaction between miRNAs, mitochondria, and the diabetic heart (Figure 1.6). With the significant impact to cellular and physiological health from miRNAs in the diabetic heart, a more fundamental question arises to address the driving mechanisms behind changing miRNA expression.

Figure 1.6: Signaling Pathways of the Diabetic Heart

Figure 1.6: MiRNA influence on signaling pathways in the diabetic heart. MiRNA regulation of cardiomyocyte energy metabolism (blue), survival (green), and apoptosis (red) in the diabetic heart. The up or down arrow beside each miRNA species indicates its up- or down-regulation in the diabetic heart (Table 1.2). The marked line connecting each miRNA with its target demonstrates that the miRNA inhibits its target. Subcellular components have been labeled: plasma membrane (yellow), cytoplasm (green), and mitochondrion (blue). CAV2 = caveolin 2; IR = insulin receptor; IRS2 = insulin receptor substrate 2; PI3K = phosphatidylinositol-4,5-bisphosphate 3-kinase; GLUT4 = glucose transporter type 4; FA = fatty acid; CD36 = cluster of differentiation 36; TR4 = testicular receptor 4; FAO = fatty acid oxidation; PPARα = peroxisome proliferator-activated receptor alpha; PGC1α = peroxisome proliferator-activated receptor gamma coactivator 1-alpha; Acetyl-CoA = acetyl coenzyme A; ISCU1/2 = iron-sulfur cluster assembly proteins; C1-C4 = electron transport chain complex 1-4; F0 = ATP synthase subunit F0; F1 = ATP synthase subunit F1; Slc25a3 = mitochondrial phosphate carrier protein; CytC = cytochrome C; BCL-2 = B-cell lymphoma 2; Sirt1 = sirtuin 1.

MiRNA Import into the Mitochondrion

What still remains a highly relevant, and hotly debated, topic is the process involved in miRNA import into the mitochondrion. While it has been hypothesized that some miRNAs could be transcribed through the mitochondrial genome (196), predominately, miRNA import is favored as the mechanism for miRNA accumulation in the mitochondrion. Research has demonstrated the presence of miRNA within the mitochondrion (11, 50, 120, 188, 265), and even how fluctuating concentrations within the organelle contributes toward the development of pathologies (48, 49, 63, 186, 195). Two currently proposed mechanisms of miRNA import into the mitochondria include the direct movement across the mitochondrial membrane through a chaperone, Argonaut 2 (AGO2) (49, 265), and diffusion through small RNA import machinery, Polynucleotide Phosphorylase (PNPase) (186).

Both Das et al. and Zhang et al. have shown that AGO2 is present in the mitochondria, and that crosslinking immunoprecipitation reveals associations with miRNAs. (49, 265). While it has been suggested that AGO2 could have a localization sequence on its N-terminus for mitochondrial targeting (12), no definitive research has validated the precise mechanisms involved in AGO2/miRNA mitochondrial import. A new mechanism proposed for mitochondrial miRNA import includes the mitochondrial inner membrane protein PNPase, and potential associations with AGO2. Wang et al., has demonstrated the importance of PNPase in facilitating the import of RNase P, 5S rRNA, and MRP RNAs (232, 233), suggested through stem-loop recognition. Recently, our group has shown that the regulation of PNPase can alter miRNA-378 levels within the mitochondrion (186), indicating that the function of PNPase may extend to other small RNA species. The expression of PNPase is also decreased during diabetes mellitus. Though this novel mechanism of miRNA import has not been fully characterized mechanistically, it provides a basis for future research.

Regulation of miRNAs Affecting the Diabetic and Non-Diabetic Cardiovascular System

As described above, many cardiovascular diseases including diabetic cardiomyopathy and diabetic heart failure have unique miRNA expression signatures. These regulatory molecules have an impact in modulating cellular pathways in health and disease. Although miRNAs are highly active in post-transcriptional repression and degradation with complex downstream effects, the miRNA expression landscape is just as complexly regulated by environmental stimuli; including

mechanisms of epigenetic modulation, exosomal transport between cells and tissues, processing of pri- and pre-miRNA pathways, long non-coding RNA (lncRNA) “sponging,” and many others (Table 1.3).

Table 1.3: Regulation of miRNA in the Heart and Circulatory System

miRNA	Type of Regulation	Pathway	Expression in Heart or Circulatory System	Disease Model	Source
miRNA-133a	Epigenetic	Class I and IIb HDACs decrease the expression of miRNA-133a	Downregulated	Mouse heart – transverse aortic constriction model	(169)
miRNA-184	Epigenetic	DNA hypermethylation through DNMT1	Downregulated	HL-1 cardiomyocytes – Arrhythmogenic cardiomyopathy model	(88)
pri-miR-208b	Epigenetic	Pri-miRNA tether to EZH2	Upregulated	Mouse heart – transverse aortic constriction model	(150)
*miRNA-375	Epigenetic	CpG Island methylation	Upregulated	Human plasma	(31, 203)
*miRNA-145	Epigenetic	Promoter hypomethylation	Upregulated	Human saphenous vein smooth muscle	(170)
*miRNA-125b	Epigenetic	Suv39h1 3' UTR binding, decreasing H3K9me3 expression	Upregulated	Mouse microvascular smooth muscle cells – db/db model	(227)
*miRNA-101	Epigenetic	Binding to EZH2, decreasing H3K27me3 expression	Upregulated	Human fetal embryonic stem cells of the umbilical cord vein	(74)
*miRNA-133a	Epigenetic	Binding to DNMT1, DNMT3A, and DNMT3B	Downregulated	Ins2(+/-) Akita diabetic mice and HL-1 cardiomyocytes	(34)
miRNA-1 and miRNA-133a	Exosomal Transport	Potential biomarker for cardiomyocyte death, released from injured myocardium	Upregulated release into serum	Human serum from acute myocardial infarction patients	(124)
miRNA-142-3p	Exosomal Transport	Endothelial barrier integrity breakdown through RAB11/FIP2 interaction	Upregulated release into serum	Human serum from heart transplant surgery patients	(202)
miRNA-21	Exosomal Transport	Binds PDCD4, offering cardioprotection	Upregulated release into media	Mouse cardiac progenitor cells under oxidative stress	(246)
miRNA-30a	Exosomal Transport	HIF-1 α upregulates miRNA-30a, potentially offering cardioprotection from autophagy	Upregulated release into serum	Human serum from acute myocardial infarction patients	(252)
miRNA-143	Exosomal Transport	Upregulation of miRNA-143-3p, compared to miRNA-143-5p.	Upregulated release into media	Human pulmonary artery smooth muscle cells from pulmonary arterial hypertension patients	(55)
miRNA-194	Exosomal Transport	Contribution to the p53-response and potential biomarker for ischemic heart failure	Upregulated release into serum	Human serum from acute myocardial infarction patients	(148)
*miRNA-1 and miRNA-133a	Exosomal Transport	Levels positively correlated with predicting myocardial steatosis in type 2 diabetic patients	Upregulated release into media	HL-1 cardiomyocyte – lipid-loaded preconditioning	(53)
*miRNA-7	Exosomal Transport	Changes in serum levels were not significantly contributed by exosomes	Upregulated in serum, not changing in exosomes	Human serum from patients with microvascular complications	(230)
*miRNA-455, miRNA-29b, miRNA-323-5p and miRNA466	Exosomal Transport	Downregulation of MMP9	Upregulated release into serum	Mouse – db/db model with exercise	(32)
*miRNA-320	Exosomal Transport	Targets IGF-1, Hsp20 and Ets2, producing an anti-angiogenic effect in cardiac endothelial cells	Upregulated release from cardiomyocytes	Rat cardiac endothelial cell and cardiomyocyte co-culture – Goto-Kakizaki (GK) model	(241)

miRNA-21	MiRNA Processing	DICER1 and phospho-SMAD-2 interactions increase mature miRNA-21 expression	Upregulated	Mouse – transverse aortic constriction model	(79)
miRNA-103	MiRNA Processing	Downregulation of Dicer leads to decreased NF-κB activation through decreased miRNA-103/KLF4 binding	Downregulated	Mouse – high fat diet apolipoprotein E knockout model	(93)
miRNA-1	MiRNA Processing	G3bp1 binds the stem loop of miRNA-1, decreasing its expression	Downregulated	Rat cardiomyocyte – transverse aortic constriction model	(98)
*let-7a, miRNA-130, miRNA-142-3p, miRNA-148, miRNA-338, miRNA-345-3p, miRNA-384-3p, miRNA-433, miRNA-450, miRNA-451, miRNA-455, miRNA-494, miRNA-499, miRNA-500, miRNA-542-3p, miRNA-744, and miRNA-872	MiRNA Processing	Dicer was shown to be downregulated, though some miRNAs showed increased expression	Upregulated	Ins2(+/-) Akita diabetic mice	(33)
miRNA-188-3p	LncRNA Sponging	Increased expression of APF lncRNA decreases miRNA-188-3p levels while increasing ATG7	Downregulated	Mouse cardiomyocytes – anoxia/re-oxygenation model	(236)
miRNA-539	LncRNA Sponging	Increased expression of CARL lncRNA decreases miRNA-539 levels while increasing PHB2	Downregulated	Mouse cardiomyocytes – anoxia/re-oxygenation model	(238)
miRNA-489	LncRNA Sponging	Increased expression of CHRFL lncRNA decreases miRNA-489 levels while increasing Myd88	Downregulated	Mouse – angiotension II treatment	(237)

Table 1.3: Regulation of miRNA in the heart and circulatory system. Each miRNA which is regulated, or involved in mechanisms altering miRNA expression, is listed for the heart and/or circulatory system. The type of regulation, the specific pathway involved, and the expression change of the miRNA are included. The disease model is stated, with an asterisk (*) next to the miRNA name, denoting the use of a diabetic model.

Regulation of miRNAs Altering the Epigenome of the Cardiovascular System

What are Epigenetics?

The most described forms of epigenetic regulation consist primarily of two transient alterations: DNA methylation and histone modification (28). DNA methylation proceeds through the addition of a 5' methyl group which occurs on cytosine nucleotides, where it is added by DNA methyltransferases (DNMT1, DNMT3a, and DNMT3b) (20). Most often, this methyl group addition will occur at CpG islands (paired cytosine and guanine nucleotides within a DNA sequence). The addition of methyl groups to a gene sequence attenuates transcription by decreasing

the propensity of transcription factors and other transcription initiation proteins to bind to the sequence (112).

Histone modifications, such as acetylation, methylation, phosphorylation, etc., change the conformation of chromatin by either relaxing or condensing the DNA/protein structure (16). The processes involved in histone modifications include histone methyltransferases (HMTs), which generally condense chromatin, and histone acetyl transferases (HATs), that primarily relax chromatin (274). The interplay of DNA methylation and histone modifications can significantly alter gene expression, specifically in diseases such as type 2 diabetes mellitus, such that the epigenome can be completely reshaped (229).

The Role of Epigenetics in Altering miRNA Expression

Of the variety of mechanisms known to regulate miRNA expression, the beginning of the regulatory chain starts at transcription. MiRNAs can either be intergenic, found within the introns of genes, or intragenic, found within the coding region of a gene, and can assume multi-directional or overlapping features with the gene (41, 146). At each gene locus containing a miRNA, the expression of the miRNA can be governed both by the DNA methylation of a promoter/intergenic/intragenic region or through histone conformations around the reading frame (178). Researchers have begun to investigate how miRNAs can be controlled epigenetically in a variety of pathological conditions. Conversely, miRNAs can be implemented as a direct mechanism for altering cellular epigenetics. The epigenetic machinery of the cell contains multiple forms of epigenetic regulation: polycomb repressor complex 1 (PRC1), PRC2, histone deacetylases (HDACs), HATs, HMTs, and DNMTs (107, 178).

MiRNAs have been found to directly affect major constituents of the epigenetic machinery and many miRNAs have been identified with changed expression levels through epigenetic regulation. Ingenuity Pathway Analysis (IPA) can be implemented as a protein/miRNA ontology system through the use of known molecular association data, combined with predictive software analyzing sequence homology. Figure 1.7 depicts how mature miRNAs are known to interact with the DNMT machinery, and also reveals miRNAs associated with the mitochondrion of the diabetic heart that theoretically could interact with DNMTs through their seed sequence regions.

Figure 1.7: DNA Methylation and MiRNA Involvement

Figure 1.7: Predicted interactions of miRNA with DNMT1. Ingenuity Pathway Analysis (IPA) provides potential areas for future epigenetic/miRNA analyses. The predicted connections include mature miRNAs and miRNA seed sequences, which are suggested to interact with DNMT1, DNMT3a, or DNMT3b. MiRNAs with listed seed sequences can account for one or many miRNAs which contain similar regions suggested to promote protein/miRNA or mRNA/miRNA interactions with the DNMTs. DNMT1 = DNA methyltransferase 1, DNMT3a = DNA methyltransferase 3a, DNMT3b = DNA methyltransferase 3b.

In human femoral artery atherosclerotic plaques, Aavik et al. measured global DNA methylation levels and found hypomethylation of the genome and two-thirds of the genes exhibiting a significant, differential methylation pattern, compared to normal mammary artery (3). One of the major findings was the 14q32 locus being significantly hypomethylated, which contains miRNAs -127, -136, -410, -431, -432, and -433 (3). The authors suggest that this epigenetic paradigm in atherosclerotic plaques is a potential mechanism for initiating the progression of the lesions. In the heart, during overload-induced fibrosis through transverse aortic constriction, class I and IIb HDACs were shown to negatively impact the heart through the regulation of miRNA-133a (169). HDACs are known to increase in a pressure overload-induced model and by introducing HDAC inhibitors to overload-induced mice, miRNA-133a expression was restored and functional deficits were ameliorated.

Arrhythmogenic cardiomyopathy has been linked to mutations in specific genes, including plakophilin 2 (PKP2) (8, 223). A PKP2 knockdown model in HL-1 cardiomyocyte cells was used to evaluate miRNA expression, revealing 59 differentially regulated miRNAs (88). Of these differentially regulated miRNAs, miRNA-184 showed the most significant downregulation. The authors suggest that miRNA-184 expression is controlled through DNA hypermethylation of its

gene loci through DNMT1. A newly proposed mechanism of miRNA/epigenetic interaction suggests that pri-miRNA can be used as tethers to recruit histone modification proteins to specific DNA sites (147). Enhancer of zester homolog-2 (EZH2) was shown to bind to the bi-directional promoter at the myosin heavy (MHC) chain genes in the heart and promote a switch from α -MHC to the antisense β -MHC. Through RNA-chromatin immunoprecipitation experiments, the authors demonstrated the capacity for pri-miR-208b to tether specific genomic sites and recruit chromatin modification machinery.

The Impact of Epigenetics and miRNA During Diabetes Mellitus

With the progression toward type 2 diabetes mellitus (i.e. insulin resistance, increased adiposity, increased body-mass-index (BMI)), changes in the epigenome can be observed. A patient cohort (n = 10,261) revealed that genome-wide changes in DNA methylation in blood were linked to an increased BMI (229). The resulting differential methylation was found to encompass genes involved in lipid metabolism, inflammatory signaling, and substrate transport. The group also found that changes in genome-wide DNA methylation can be used as a predictive measure for the development of type 2 diabetes mellitus. Methylation of genes involved in the progression of type 2 diabetes mellitus also varies between ethnicities (76, 122, 152), with Indian Asians showing increased levels of DNA methylation at ABCG1, PHOSPHO1, SOCS3, SREBF1, and TXNIP. The increased methylation of these genetic loci in the Indian Asian population correlates to the increased risk of developing type 2 diabetes mellitus (29).

Pheiffer et al., found that miRNA expression in type 2 diabetes mellitus can be controlled through DNA methylation (161). Specifically, this group established the importance of intergenic regions in controlling miRNA expression and showed that most differentially regulated peaks between diabetic and non-diabetic groups were found in the intergenic regions, as opposed to the promoter or intragenic regions. These findings raise a number of new questions concerning miRNAs and epigenetics, such as how does epigenetic regulation occur in a specific pathology, such as diabetes mellitus, or tissue, such as the heart, and how can therapeutics take advantage of these processes? In plasma from type 2 diabetic patients, miRNA-375 was increased (203). Further, miRNA-375 has 17 different CpG islands within or surrounding the DNA loci, in which some of these regions were differentially regulated between diabetic and control populations. Other studies focusing on two different populations in China, Han and Kazak, revealed differences

in the methylation pattern of miRNA-375 (31). The authors postulate that this may be a potential mechanism for the increased prevalence of type 2 diabetes mellitus in the Han population.

The role that miRNAs and epigenetics play in defining pathological states, such as diabetes mellitus, and how this interaction occurs in a variety of tissue types has been well-studied. What remains to be fully understood is the impact of the epigenetic/miRNA interplay within the diabetic heart and cardiovascular system. In the saphenous vein of human diabetic and non-diabetic patients, smooth muscle cells were cultured and miRNA-145 promoter methylation was measured (170). Expression of miRNA-145 was increased in the diabetic phenotype, but highly variable CpG island methylation between groups did not allow for any overall significant changes between groups. Though, specific CpG loci were shown to exhibit hypomethylation, irrespective of group. Microvascular smooth muscle cells (MVSMCs) from *db/db* and control mice were used to assess the effects of miRNA-125b, which is overexpressed in the diabetic phenotype (227). MiRNA-125b was validated to bind to the suppressor of variegation 3-9 homolog 1 (Suv39h1) 3' UTR controlling the expression of histone 3 lysine 9 tri-methylation (H3K9me3). Increased miRNA-125b expression resulted in less restrictive H3K9me3 marks, ultimately increasing the expression of inflammatory genes.

Gestational diabetes mellitus can affect the health and future function of the growing progeny. In human fetal embryonic stem cells of the umbilical cord vein (HUVECs), EZH2 was decreased in mothers with gestational diabetes mellitus while miRNA-101 was increased (74). EZH2 is responsible for the tri-methylation of histone 3 lysine 27 (H3K27me3). EZH2 is also directly targeted by miRNA-101, resulting in decreased H3K27me3 in HUVECs from gestational diabetic mothers and subsequent altered gene transcriptional patterns. Through the use of Ins2(+/-) Akita diabetic mice and HL-1 cardiomyocytes, Chavali et al. defined the role of miRNA-133a in regulating DNMTs (34). The group found that DNMT1, DNMT3a, and DNMT3b were inversely correlated to miRNA-133a (i.e. silencing of miRNA-133a induced overexpression of the DNMTs while expression of miRNA-133a inhibited DNMT expression). In the Akita model, miRNA-133a is decreased, with DNMT1 and DNMT3b being induced. Through involvement in both the processes of DNA methylation and histone modifications, the unique miRNA/epigenetic signature involved in the progression of a pathology, such as diabetes mellitus, could be an invaluable predictor of cardiac function and cardiovascular health.

Exosomal Release, Transport, and Uptake of miRNA in the Cardiovascular System

Exosomes and Exosomal Cargo

Exosomes are cell derived vesicular carriers utilized for cell signaling and communication and have been implicated in numerous disease states, including diabetic cardiomyopathy (174) and various cancers (9). Generally, exosomes range in size from 50 to 100 nm (167, 193, 213), but small discrepancies in size are common in the literature. The central role of exosomes is the delivery of various cargo types including lipids, nucleic acids, proteins, RNAs, and miRNAs to different cells (22, 213, 218, 221). Exosomes transport cargo, transmitting a unique biochemical signal from the host cell to a specific target cell or tissue or, more systemically, by the blood stream. Exosomal cargo is of importance due to the specificity of the cargo loaded into each vesicle by the host cell. This cargo is then delivered to targeted acceptor cells and the exosomes release their protein and lipid cargo to activate downstream signaling events, or simply deliver genetic material including DNA and various RNAs (194). Secretion of exosomes occurs via multiple mechanisms, such as release upon exocytic fusion of multivesicular bodies with the plasma membrane and miRNA release through a ceramide-dependent mechanism (218). The different pathways available for exosome secretion depend on the cell types (213) and, currently, a lack of classification of the mechanisms of exosome biogenesis exists.

Exosomal Transport of miRNA

The transport of miRNAs via exosomes can directly impact cellular dynamics. Exosomal transport in the immune system through macrophages has also proven to be an important mechanism for changing cellular dynamics (4, 51, 108, 209), specifically through the regulation of inflammation during cardiac injury (231). This section will focus explicitly on the regulation of exosomal transport of miRNA within the heart and circulatory system.

MiRNA-1 and miRNA-133a are increased in the serum of patients who have suffered from an acute myocardial infarction (124). In a mouse model of myocardial infarction, the infarcted and peri-infarcted tissue display a significant reduction of miRNA-1 and miRNA-133a. Through measuring the exosomal release of miRNA-133a in dead, H9c2 cardiomyoblasts, the authors suggest that injured myocardium releases miRNA-133a and that this marker may be used in the serum to account for cardiomyocyte death. Sukma Dewi et al., measured the miRNAs of exosomes

taken from the serum of patients who underwent heart transplant surgery, both with and without experiencing acute cardiac allograft rejection (202). These authors found miRNA-142-3p to be significantly increased in the exosomes. When the exosomes from the serum were used to treat endothelial cells, miRNA-142-3p also increased in the endothelial cells. After acute cardiac allograft rejection, the authors concluded that exosomes in the serum can affect endothelial barrier integrity through the interaction of miRNA-142-3p with RAB11 family interacting protein 2 (RAB11FIP2). The capacity for exosomes released from the heart to affect the systemic circulation and peripheral tissues has been explored (66). Serum was taken from patients before and after undergoing coronary artery bypass grafting, the authors found an increase in the total plasma concentration of exosomes, as well as increased miRNA cargo. They observed that high sensitivity cardiac troponin I was positively correlated with the plasma expression of total exosomes.

Cardiac progenitor cells (CPCs) were used to understand how exosomal release from cells can contribute toward alleviation of oxidative stress (246). During oxidative stress, it has been shown that CPCs released more miRNA-21, which targets the protein programmed cell death 4 (PDCD4), ultimately reducing the cleaved version of caspase-3. These authors suggest that CPCs may offer cardioprotection during oxidative stress through release of exosomal miRNA-21 to surrounding tissue. After acute myocardial infarction, exosomes isolated from the serum of patients contained increased levels of miRNA-30a (252). MiRNA-30a is also released in exosomes from cardiomyocytes after hypoxic conditioning. Hypoxia inducible factor (HIF)-1 α regulates the expression of miRNA-30a in exosomes released by the cell, which directly target autophagy machinery. In this way, cardiomyocytes may protect surrounding tissue in a hypoxic environment by releasing miRNA-30a and decreasing autophagy. During pulmonary arterial hypertension, the selective upregulation of miRNA-143-3p compared to miRNA-143-5p was shown in pulmonary artery smooth muscle cells (PASMCs) (55). PASMCs also excreted more miRNA-143-3p through exosomes to influence vascular endothelial cells. Blocking the actions of miRNA-143-3p resulted in decreased pulmonary hypertension in mouse models.

Following acute myocardial infarction, Matsumoto et al., examined the role of circulating miRNAs as a contributing factor to the incidence of heart failure (148). Eighteen days post-infarction, serum was collected and miRNA-192 was shown to be significantly increased, which plays a role in the p53 response. In the p53 pathway, miRNA-194 was shown to be significantly

elevated in exosomes and the authors correlated serum levels of miRNAs-192, -194, and -34a in patients who experience acute myocardial infarction with the development of heart failure.

Exosomal Transport of miRNA in Diabetes Mellitus

Through the progression of diabetes mellitus, it is well documented that the miRNA profile changes throughout the body (13, 67, 87, 118, 182, 260). What is not entirely known is how the miRNA profile changes within exosomes. Questions concerning the transport of miRNAs in diabetes mellitus, the protective or detrimental effects of exosomes during the pathology from a temporal standpoint, and how exosomal transport of miRNAs may differ mechanistically between tissues, have begun to be explored.

In uncomplicated type 2 diabetes mellitus, the role of serum miRNAs in predicting myocardial steatosis was evaluated (53). These authors found that miRNA-1 and miRNA-133a were positively correlated with myocardial steatosis, and that miRNA-133a was elevated in type 2 diabetic patients. Using HL-1 cardiomyocytes, miRNA-1 and miRNA-133a were found to be higher in exosomes released from lipid-loaded preconditioning. Patients with type 2 diabetes mellitus may exhibit microvascular complications (230). MiRNA-7 was shown to be overexpressed in the serum of patients with type 2 diabetes mellitus or type 2 diabetes mellitus with microvascular complications compared to non-diabetics. These authors found that miRNA-7 was not primarily packaged in exosomes, but free floating in the serum. The authors concluded that miRNA-7 serum levels may have the potential to predict microvascular complications in type 2 diabetes mellitus. Examining the generational effects of diabetes mellitus and exosome delivery, Shi et al., found that mice made type 1 diabetic through STZ injections before mating had serum containing increased levels of exosomes with miRNAs involved in cardiac developmental regulation (187). They also showed that these exosomes can be directly transferred to the fetal pups, which may account for the observed increased in congenital heart defects. By injecting the exosomes of the diabetic mothers into the control pups, they also observed an increase in congenital heart defects in the F₁ generation.

The effects of exercise on the diabetic condition and how complications of the disease can be mitigated were explored through matrix metalloprotease 9 (MMP9) regulation (32). MMP9 was shown to be significantly decreased in a *db/db* model after exercise. The authors demonstrated that exosomal transport of miRNAs decreasing the expression of MMP9 (i.e. miRNA-455, miRNA-

29b, miRNA-323-5p and miRNA466) were increased after exercise. In the Goto-Kakizaki (GK) rat, a model of type 2 diabetes mellitus, cardiac endothelial cells (MCECs) co-cultured with cardiomyocytes from diabetic rats resulted in a decrease in proliferation and migration as compared to control, Wistar rats (241). Inhibition of exosome formation from diabetic mice restored function of MCECs, which was correlated with a significant decrease in miRNA-320 within MCECs. The importance of miRNA-320 is that it binds to targets which decrease angiogenesis. The authors concluded that in diabetes mellitus, cardiomyocytes may be involved in producing an anti-angiogenic effect on the surrounding environment.

MiRNA Processing, LncRNA, and Other Regulators of miRNA in the Cardiovascular System

Mechanisms of miRNA Processing and Post-transcriptional Control

Indirect mechanisms of miRNA regulation, such as epigenomic changes and alterations in exosomal transport, are not the only ways that miRNA levels can be affected in the diabetic heart. More direct means of miRNA alteration within the cell can include miRNA processing as well as regulation by small biomolecules or other RNA species. The milieu of the pathology, such as changes in glucose and other metabolite concentrations within the cell, can directly alter the efficiency of miRNA processing and modify the dynamics of cellular processes.

After transcription of miRNA, post-transcriptional regulation through long-noncoding RNAs (lncRNAs) can occur. It has been shown that lncRNAs have binding sites for miRNAs (110). One mechanism, referred to as “sponging,” involves the expression of lncRNA in order to sequester miRNAs (96). This lncRNA/miRNA is considered part of a larger network of competing endogenous RNA (ceRNA), in which the cell uses both coding and non-coding RNA to expand the functional genetic information that a cell can produce (176). At current, this field is underexplored, with few lncRNAs and even fewer lncRNA/miRNA interactions characterized. Ultimately, the potential for information regarding the control, sequestering, and further post-transcriptional regulation of miRNAs is immense.

MiRNA Regulation

MiRNA Processing

The most well-known and studied role of Drosha and Dicer are to regulate the transcription of miRNAs in developed organisms. Studies have shown that a complete depletion of Dicer from an embryonic time point can significantly alter the development of cardiac tissue (180, 189), likely through a depletion of most, if not all, miRNAs. Additionally, miRNA processing through Dicer has been shown to have epigenetic control (222). During hypoxic conditions, the demethylases KDM6A/B have decreased expression, resulting in increased H3K27me3. Other epigenetic mechanisms, such as DNA methylation or further histone modifications, could also likely control the expression and processing of Dicer or Drosha.

García et al. demonstrated that myocardial remodeling by miRNA-21 may be mechanistically controlled by the presence of SMAD2/3 and DICER1 (79). In mice undergoing pressure overload, SMAD2/3 and DICER1 expression was increased in the left ventricle, which was also upregulated in patients with aortic stenosis. MiRNA-21 was isolated bound to DICER1, while DICER1 and phosphorylated SMAD2/3 were shown to have protein-protein interactions. The authors concluded that this TGF β -dependent mechanism of phosphorylated SMAD2/3 and DICER1 regulating the increased, mature miRNA-21 processing could be important in myocardial remodeling. In some tissues, the downregulation of Dicer may be beneficial. It was shown that in endothelial cells where Dicer is downregulated, it decreased lesional macrophage content and monocyte adhesion in high fat diet apolipoprotein E knockout mice (93). Mechanistically, downregulation of Dicer also decreases miRNAs, such as miRNA-103, which binds Krüppel-like factor 4 (KLF4). KLF4 through NF- κ B leads to an exacerbated atherosclerotic pathology, suggesting that increased Dicer expression is a maladaptation in endothelial cells.

Other than the canonical Drosha and Dicer miRNA editing pathway, other mechanisms have been proposed for editing miRNA. GTPase-activating protein (SH3 domain) binding protein 1 (G3bp1) is upregulated in cardiac hypertrophy and binds to the stem loop of miRNA-1 (98). MiRNA-1 is known to regulate important transcription and translational machinery in the cell. Overexpression of G3bp1 can lead to decreased miRNA-1 levels and therefore increased transcriptional and translational capacity of the cell. Though G3bp1 does not directly induce cardiac hypertrophy, this novel post-transcriptional regulation of miRNA by an endoribonuclease may be mimicked by other proteins as well.

MiRNA Post-Transcriptional Regulation

Gene regulation through lncRNA is becoming recognized as a mechanism for the control of pathological development. lncRNA named autophagy promoting factor (APF lncRNA) promotes autophagy in the heart through targeting miRNA-188-3p (236). MiRNA-188-3p targets autophagy-related protein 7 (ATG7) suppressing autophagy. Thus, regulation of APF lncRNA in the heart could be beneficial in reducing autophagy-related heart conditions, such as myocardial infarction and heart failure. Wang et al. also investigated the role of lncRNA named cardiac apoptosis-related lncRNA (CARL lncRNA) in regulating mitochondrial fission and apoptosis (238). CARL lncRNA targets miRNA-539, acting as a sponge, to increase the expression of the miRNA-539 target prohibitin-2 (PHB2). PHB2 is suggested to play a role in suppressing apoptosis and increasing mitochondrial fission. In mice, after angiotension II treatment, miRNA-489 expression was found to be reduced (237). By overexpressing miRNA-489 in mouse cardiomyocytes, the hypertrophic response was decreased and miRNA-489 was shown to target myeloid differentiation primary response gene 88 (Myd88) as the mechanism. MiRNA-489 expression was further found to be regulated by a lncRNA named cardiac hypertrophy related factor (CHRF lncRNA) which can act as an endogenous sponge and increase the hypertrophic response.

Acute myocardial infarction was experimentally induced in swine (114). Using global run-on sequencing, RNA was taken from both the infarcted area and the peripheral, healthy cardiac tissue. In the infarcted tissue, 450 lncRNAs were differentially regulated. Some of the differentially regulated lncRNAs were novel, conserved sequences found within myocardial transcription factors in an antisense orientation. The sequencing experiment demonstrates the breadth of lncRNAs that could potentially impact miRNA and mitochondrial function. The regulation of miRNAs and the dynamic of cellular homeostasis in the heart and circulatory system surpass the scope of this review paper, with research teams reporting miRNA regulation through transcription factors (26, 102, 164), gene expression (37, 88), other non-coding RNAs (80, 253), and even the microbiome (225).

MiRNA Processing and lncRNA Regulation in the Diabetic Heart

Limited research has been conducting in exploring the diabetic heart and the mechanisms of miRNA processing and post-transcriptional regulation. During gestational diabetes mellitus and pregnancy, it was demonstrated that the content of DGCR8, Drosha, and Dicer were increased

compared to non-pregnant controls (165). Further, DGCR8, Drosha, and Dicer were more highly expressed in gestational diabetes mellitus than in those with normal pregnancies, suggesting a role for increased miRNA processing in diabetes mellitus. An increase in Dicer expression is also observed in Insulin2 mutant (Ins2^{+/-}) Akita mice (33). In Akita hearts, even though Dicer expression is increased, let-7a, miRNA-130, miRNA-142-3p, miRNA-148, miRNA-338, miRNA-345-3p, miRNA-384-3p, miRNA-433, miRNA-450, miRNA-451, miRNA-455, miRNA-494, miRNA-499, miRNA-500, miRNA-542-3p, miRNA-744, and miRNA-872 expression are decreased.

LncRNAs have been connected to the diabetic heart, being independent predictors of grade I diastolic dysfunction and left ventricular mass to left ventricular end-diastolic volume ratio in human blood (52). Also, blocking the function of lncRNA NONRATT021972 could serve as a therapeutic for diabetic cardiac autonomic neuropathy, decreasing tumor necrosis factor alpha (TNF- α) and increasing insulin receptor substrate 1 (IRS1) expression (248). Though no studies at current have implicated lncRNAs in controlling miRNA expression in the diabetic heart, the impact of lncRNAs on the pathogenesis of the diabetic heart is noted, and miRNA targets are likely present.

Therapeutic Applications of miRNA in Diabetes Mellitus

Due to the significant cardiovascular complications that arise from diabetes mellitus, investigating the therapeutic applications that are available, or could be feasible options in the future, are important. The purpose of this section is to evaluate both current and potential future therapeutic applications in the diabetic heart. We will evaluate how direct targeting of miRNAs, exosomal transport of miRNAs, epigenetic manipulation of/by miRNAs, and other therapeutics involving miRNAs could impact patient health.

Targeting dysregulated energy substrate metabolism in the viable, but diseased myocardium, has been extensively investigated as a potential therapy for heart disease patients. Diabetic cardiomyopathy and diabetic heart failure are characterized by decreased glycolysis, decreased glucose oxidation, and increased fatty acid metabolism. Previous strategies to address these metabolic alterations have been to pharmacologically improve myocardial glucose import, increase pyruvate dehydrogenase activity to allow for greater glucose oxidation, and decrease fatty acid oxidation (208). These drugs include glucose transport activators (GLP-1 mimetics/agonists),

pyruvate dehydrogenase activators (dichloroacetate, L-carnitine), carnitine palmitoyltransferase I inhibitors (perhexiline, etomoxir), and fatty acid oxidation inhibitors (ranolazine, trimetazine) (91, 140, 141, 144). Due to extensive regulation of fatty acid metabolism by the peroxisome proliferator-activated receptor (PPAR) family of transcription factors, PPAR agonists are being considered for improving mitochondrial function in diabetic cardiomyopathy (127). Interestingly, metoprolol, a beta-blocker, has been shown to inhibit fatty acid oxidation and improve cardiac function in STZ-induced diabetic rats (184). Recent studies of antianginal drugs have shown effects in improving myocardial oxygen utilization and efficiency of energy substrate metabolism (39).

MiRNA-Directed Therapies

As differential miRNA expression in cardiovascular disease have proven to modulate the expression of components of energy substrate metabolism, one may ask if normalizing the expression of miRNAs that target the most important metabolic players through the administration of antimiRs/antagomiRs or miRNA mimics would recalibrate energy substrate metabolism back to healthy flux. This strategy of pharmacologically regulating the regulators (miRNAs) has been successfully deployed preclinically to improve pathological cardiac abnormalities found in diabetic cardiomyopathy or diabetic heart failure. As an example, Thum et al. showed that administering an antagomiR specific for miRNA-21 reduced cardiac fibrosis in a mouse pressure-overload-induced model (215). Others have reported that antagomiR-mediated inhibition of miRNA-199b inhibited and reversed cardiac hypertrophy and fibrosis in mouse models of heart failure (44). Therapeutic inhibition of miRNA-208a via antimiR action has shown much promise in preventing pathological cardiac remodeling in hypertension-induced heart failure in Dahl hypertensive rats, and in improving systemic insulin sensitivity and glucose tolerance in a mouse model of high fat diet-induced obesity (86, 150). To our knowledge, there have been no preclinical miRNA-based studies to investigate the potential of normalizing the differential expression of miRNAs targeting components of energy substrate metabolism in the diseased diabetic heart. Because metabolic dysregulation is foundational to the etiology of diabetic heart disease and miRNA-based therapeutics may offer precise regulatory potential, we believe the field of miRNA-based therapies targeting energy substrate metabolism in diabetic cardiomyopathy and diabetic heart failure has a very bright future.

Epigenetics in miRNA Regulation during Therapeutic Intervention

In *ob/ob* mice, unacylated ghrelin was used as a therapeutic to increase resistance against oxidative stress (217). Unacylated ghrelin increased the expression of miRNA-126, leading to indirect increases in SIRT1 and decreased histone 3 lysate 56 deacetylation. The results of the study showed that unacylated ghrelin can reduce cell senescence by protecting endothelial cells from ROS damage. Curcumin (diferuloylmethane) has been implicated as a potential drug for modifying both epigenetic and miRNA expression. Curcumin supplementation has been suggested to inhibit DNA methylation through inhibition of the catalytic thiolate of DNMT1 (139). Restriction of DNA methylation may be a contributing factor toward modified miRNA expression after curcumin supplementation (204, 262). EGCG, which is an ester of epigallocatechin and gallic acid, has shown very similar results as curcumin. EGCG is a direct inhibitor of DNMT1 and also has been shown to alter as many as 61 miRNAs and their expression (70, 128, 219).

Exosomes in miRNA Regulation during Therapeutic Intervention

Exosome based therapeutics currently revolve around the idea of collecting exosomes from healthy cell populations to directly transfer to those in a pathological setting. Gallet et al. showed that in pigs, cardiosphere-derived cell (CDC) exosomes, which were delivered through open-chest intramyocardial injection, improved the condition of acute and convalescent myocardial infarction subjects (77). Alleviation of conditions included decreased scarring and improved left ventricle ejection fraction. The content of CDCs has also been profiled, revealing that, aside from traditional miRNAs, exosomes derived from CDCs also contain non-coding RNA species, such as Y RNA, which are understudied (25). Future applications for the heart could even include the use of other modified exosomes. One such study examined the transport of a cargo protein through injected macrophage exosomes across the blood brain barrier (259). With the capacity to alter exosomal cargo, such as introducing specific miRNA or miRNA sponges, the potential therapeutic applications in the heart are numerous.

MiRNA Processing and Other Mechanism of Regulation during Therapeutic Intervention

In both diabetic human patients and mice, DICER1 protein levels were found to be increased in peripheral blood mononuclear cells (PBMCs) after treatment with Metformin (156).

Metformin was shown to post-transcriptionally regulate the expression of RNA binding protein AU-binding factor 1 (AUF1), disrupting binding with DICER1 mRNA and leading to increased expression of DICER1 protein. Metformin also resulted in an increase in miRNA-20a, miRNA-34a, miRNA-130a, miRNA-106b, miRNA-125, and let-7c, as well as a decrease in genes associated with senescence. Elgheznawy et al. demonstrated that platelets from diabetic human patients and mice had a lower expression of miRNA-142, miRNA-143, miRNA-155, and miRNA-223 (65). In diabetic platelets, DICER1 expression was decreased, which the authors hypothesized was due to calpain cleavage of the enzyme. When diabetic mice were treated with a calpain inhibitor, miRNA-223 expression was restored.

Though lncRNAs have not yet been implemented in diabetic cardiovascular studies, their application could be effective. For cancer therapy, an interfering lncRNA (lncRNAi) was designed to contain specific complementary miRNA sequences which impacted the disease progression (135). The results included decreased abilities for proliferation, migration, and invasion of hepatocellular carcinoma cells.

Conclusions

MiRNAs are key players in the regulation, maintenance, and function of the cardiovascular system. The impact of miRNA on mitochondrial function in the diabetic heart is evidence that managing the expression of these small endogenous RNAs is important, and that the onset of pathologies may largely be controlled, and sustained, by miRNA. This review has examined how the miRNA profile is altered in the diabetic heart, and how the changes in miRNA expression specifically target mitochondrial function, through energy substrate utilization, apoptosis, and ROS production. Further, we examined how miRNA may be controlled in the cardiovascular system and diabetes mellitus through epigenetics, exosomes, processing, and post-transcriptional sequestration. While research concerning miRNA expression is maturing, studies focusing on the regulation of the regulating miRNAs is in its infancy. Much information can be gained mechanistically of disease and pathologies, specifically in the heart and diabetes mellitus, through additional research in this area. Therapeutically, regulating the regulators provides a method with the potential for improved treatment strategies and health effects with greater longevity.

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Chapter 2: Specific Aim 1

MiRNA-378a as a Key Regulator of Cardiovascular Health Following Engineered Nanomaterial Inhalation Exposure

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Running Title: MiRNA-378a and ENM exposure

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Abstract

Nano-titanium dioxide (nano-TiO₂), though one of the most utilized and produced engineered nanomaterials (ENMs), diminishes cardiovascular function through dysregulation of metabolism and mitochondrial bioenergetics following inhalation exposure. The molecular mechanisms governing this cardiac dysfunction remain largely unknown. The purpose of this study was to elucidate molecular mediators that connect nano-TiO₂ exposure with impaired cardiac function. Specifically, we were interested in the role of microRNA (miRNA) expression in the resulting dysfunction. Not only are miRNA global regulators of gene expression, but miRNA-based therapeutics provide a realistic treatment modality. Wild type and MiRNA-378a knockout mice were exposed to nano-TiO₂ with an aerodynamic diameter of 182 ± 1.70 nm and a mass concentration of 11.09 mg/m³ for four hours. Cardiac function, utilizing the Vevo 2100 Imaging System, electron transport chain complex activities, and mitochondrial respiration assessed cardiac and mitochondrial function. Immunoblotting and qPCR examined molecular targets of miRNA-378a. MiRNA-378a-3p expression was increased 48 hours post inhalation exposure to nano-TiO₂. Knockout of miRNA-378a preserved cardiac function following exposure as revealed by preserved E/A ratio and E/SR ratio. In knockout animals, complex I, III, and IV activities (~2-6 fold) and fatty acid respiration (~5 fold) were significantly increased. MiRNA-378a regulated proteins involved in mitochondrial fusion, transcription, and fatty acid metabolism. MiRNA-378a-3p acts as a negative regulator of mitochondrial metabolic and biogenesis pathways. MiRNA-378a knockout animals provide a protective effect against nano-TiO₂ inhalation exposure by altering mitochondrial structure and function. This is the first study to manipulate a miRNA to attenuate the effects of ENM exposure.

Introduction

The advent and advancement of clinical applications continues to drive utilization of engineered nanomaterials (ENMs) in consumer goods (5, 19). As such, titanium dioxide (TiO₂) has become one of the most prolifically implemented ENMs (25, 32). While the beneficial manufacturing properties of ENMs are clear, the rate of incorporation in new products and devices outpaces our understanding of the toxicology of these materials. Though progress has been made outlining the consequences on organ systems and the physiological impact of ENM exposure, the molecular mechanisms governing their toxicity are poorly defined. Understanding how to manage the untoward consequences of ENM inhalation exposure therapeutically involves the exploitation of molecular pathways central to the etiology.

Systemic health effects following ENM exposure are linked to mitochondrial dysfunction, not only in the lungs (58) but also in the cardiovascular system (29, 52, 69). The heart has been established as a significant contributor to the overall pathophysiological outcomes elicited by ENM exposure (33, 38, 45, 68). Cardiac mitochondrial function is crucial in maintaining/restoring cardiac homeostasis after ENM insult, as it is a central determinant of metabolic and bioenergetic capacity. Recently, particulate matter (PM_{2.5}) air pollution has been shown to alter microRNA (miRNA) expression in the serum of a human cohort in Shanghai, China (13). The correlation between serum miRNA expression and inflammatory cytokines involved in cardiovascular health reveals the clinical relevance of miRNAs in shaping the effects of inhalation exposure. Because miRNAs can influence numerous biological target axes, therapeutic interventions targeting their regulation offer distinct advantages to approaches designed to restore a single molecular target as they provide the potential for a coordinated response to pathological insults at numerous loci.

MiRNAs are transcribed as pri-miRNA species within the nucleus (processed through Drosha) and travel to the cytoplasm as pre-miRNA (processed through Dicer) where they eventually assume their mature miRNA conformation (64). The mature, miRNA species (~22 nt) that originate in both intragenic and intergenic regions of the genome are known to individually function in regulating multiple genetic and epigenetic pathways (60). The manipulation of a miRNA that controls metabolic and mitochondrial function provides a potentially novel therapeutic strategy for controlling cellular insults precipitated by ENM exposure. In the heart, and other mitochondrially dense tissue, peroxisome proliferator-activated receptor gamma coactivator

1-beta (Ppargc1b or PGC-1 β) is highly expressed (66), and is central in the control of metabolism (11). MiRNA-378a, which encodes both the 3p and 5p mature variants, resides within the first intron of the PGC-1 β gene.

MiRNA-378a is involved in regulating apoptosis (39), insulin sensitivity (40), angiogenesis (34), metabolism (11), and other cellular processes (23, 73) implicating it in the coordination of cellular responses. Though multiple miRNAs regulate heart function (18, 22), the involvement of miRNA-378a with shifting substrate metabolism and regulation of mitochondrial bioenergetics (35) provides an intimate, and novel, connection between its expression and mitochondrial health in the heart. Carrer et al. reported on the propensity of miRNA-378a knockout mice to protect against metabolic insult (11). These researchers found that a high-fat diet induced obesity in wild type mice, but not miRNA-378a knockout mice, suggesting miRNA-378a may repress metabolic genes in response to environmental stimuli. The role of miRNA-378a in regulating metabolism and its high abundance in cardiac tissue (42) indicates that this small RNA may represent a therapeutic target for attenuation of metabolic disturbances and mitochondrial dysfunction resulting from pathological insult.

The objective of the current study was to determine whether manipulation of miRNA-378a expression could alter adverse cardiovascular and metabolic outcomes associated with ENM inhalation exposure. Our findings indicate that knockout, and knockdown to a lesser extent, of miRNA-378a improves cardiac and mitochondrial function by altering global bioenergetic and respiration capacity following nano-TiO₂ inhalation exposure. This is the first study to determine the impact of altered miRNA expression on the response to ENM exposure *in vivo*.

Materials and Methods

MiRNA-378a Knockout Mouse Model

The West Virginia University Animal Care and Use Committee approved all animal studies, which conformed to the most current National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals manual. Specifics about the animal model have been previously published (11). Briefly, miRNA-378a is located within the first intron of the PGC-1 β gene (Figure S2.1). Removal of the miRNA-378a loci was achieved by replacing the region with a neomycin cassette and LoxP sites. The recombination removed the miRNA-378a transcribed region but retained PGC-1 β gene function. MiRNA-378a animals have been shown to be more metabolically active, specifically through mitochondrial fatty acid oxidation (11). Sperm, which was cryopreserved from the miRNA-378a knockout 129SvEv/C57BL/6 mouse line, was used for *in vitro* fertilization of FVB mice. The progeny heterozygous miRNA-378a FVB mice were bred for greater than 10 generations, establishing the congenic line.

Experimental Model

Mice were housed in the West Virginia University Health Sciences Center Animal Facility and given access to a rodent diet and water *ad libitum*. To verify presence of the knockout allele, traditional PCR was performed amplifying the first intronic region of the PGC-1 β gene (Table S2.1). The WT allele amplifies a 400 bp product while the transgenic allele amplifies a 470 bp product. Between 14-18 weeks of age, mice were subjected to a single exposure of either control, filtered air (Sham exposure) or nano-TiO₂ aerosol (Ex exposure). Animals used in the analyses, unless stated, included Con Sham (n = 6), Con Ex (n = 6), Het Sham (n = 7), Het Ex (n = 8), KO Sham (n = 4), and KO Ex (n = 4). Both male and female mice were used in the analyses.

Engineered Nanomaterial

Nano-TiO₂ P25 powder, purchased from Evonik (Aeroxide TiO₂, Parsippany, NJ), was prepared through drying, sieving, and storing, as previously described (41, 54), and was composed of anatase (80%) and rutile (20%) TiO₂ with a primary particle size of 21 nm. This particle has

been previously characterized: Zeta potential = -56.6 mV (52) and specific surface area = 48.08 m²/g (54, 59) determined.

Inhalation Exposure System

Nano-TiO₂ aerosols were created with a customized high-pressure acoustical generator (HPAG). TiO₂ bulk nano-powders were aerosolized with the HPAG and fed into the whole-body exposure chamber. Figure 2.1A shows a representative transmission electron micrograph of nano-TiO₂ aerosols sampled from the chamber, imaged with the JEOL 1400 transmission electron microscope (Tokyo, Japan). A personal DataRAM (pDR-1500; Thermo Environmental Instruments Inc., Franklin, MA) sampled the exposure chamber air and utilized light scattering techniques to estimate the mass concentration in real-time. Feedback loops within the software automatically adjusted the acoustic energy to maintain a stable mass concentration during the exposure. A target DataRAM concentration of 10 mg/m³ for a period of 240 minutes was chosen in order to achieve a similar lung burden (7 - 10 µg/exposure) as that in previous studies (52, 67). The DataRAM signal during the exposure is shown in Figure 2.1B. A concurrent gravimetric measurement indicated an average mass concentration of 11.09 mg/m³ during the 240 minute period. Aerosol size distributions were examined from the exposure chamber with: 1) a high-resolution electrical low-pressure impactor (ELPI+; Dekati, Tampere, Finland), (2) a scanning particle mobility sizer (SMPS 3938; TSI Inc., St. Paul, MN), and (3) an aerodynamic particle sizer (APS 3321; TSI Inc., St. Paul, MN). The ELPI+ data (Figure 2.1C) and the combined SMPS-APS data (Figure 2.1D) both indicated log-normal aerosol size distributions with Count Median Diameters of 182 nm and 190 nm, respectively. Bedding material soaked with water was used in the exposure chamber to maintain a comfortable humidity during the exposure. Control animals were exposed to HEPA filtered air with similar chamber conditions in terms of temperature and humidity.

Echocardiography—M-mode, B-Mode and PW Doppler Imaging

Both one week prior to exposure and within 24 hours following exposure to control filtered air or nano-TiO₂, ultrasound imaging was performed. Animals were initially anesthetized using

2.5% isoflurane, with a sustained rate of 1% isoflurane during imaging, in order to more appropriately replicate physiological conditions (36). To monitor the condition of the mice, a rectal temperature probe was used. Motion-mode (M-mode), Brightness-mode (B-mode), and Pulse Wave (PW) Doppler imaging acquisition and analysis have been previously described by our laboratory (29, 52, 53, 63, 70). Briefly, images were procured with a linear array transducer at 32–40 MHz, with a frame rate of 233–401 frames/second. Ultrasound images were taken using the Vevo 2100 Imaging System (Visual Sonics, Toronto, Canada). Briefly, imaging of the left ventricle in long-axis B-mode was achieved through placing the transducer left of the sternum. Rotation 90°, perpendicular to the animal, allowed for short-axis B-Mode images. Gating midway between the short-axis B-Mode images allowed for acquisition of M-mode parameters. Pulse Wave Doppler echocardiography measured mitral valve function, determining early (E-wave) and late (A-wave) diastolic filling of the left ventricle. Ejection Fraction is considered the percent of blood leaving the heart following each contraction and fractional shortening the change in left ventricular diameter. Total cardiac output was measured as the heart rate times the stroke volume per beat (volume of blood leaving the heart). All analyses were performed by one analyst. Three replicate analyses were performed for each echocardiographic measure per animal.

Stress Strain-Speckle Tracking

In both the short and long axis, left ventricular visualization B-Mode images were used for speckle-tracking-based strain analyses. The procedure for speckle-tracking-based strain analysis have been previously described by our laboratory (29, 52, 63). As a summary, strain was measured as a function of total deformation length divided by the original length of a segment, computed for each cardiac cycle (7, 57). Peak strain and strain rates were measured across radial, circumferential, and longitudinal dimensions. Displacement length, velocity, strain, and strain rate were measured in the endocardium in the short (radial and circumferential dimensions) and long (radial and longitudinal) axes over a minimum of three cycles. Short-axis images of the left ventricle were split into anterior free, lateral, posterior, inferior free, posterior septum, and anterior septum while long-axis images were split into anterior base, anterior mid, anterior apex, posterior apex, posterior mid, and posterior base. The average of segmental values was used for analyses in this study. Assessment of Global Longitudinal Strain (GLS) in the left ventricle provides a speckle tracking-

based valuation of myocardium contraction which correlates with left ventricular function (6, 21), and can be combined with PW Doppler E-wave (E/SR) to make clinical prognostics of left ventricular health (72).

Mitochondrial Isolations

48 hours post-exposure to control filtered air or nano-TiO₂, mice were euthanized, the heart, lungs, and liver were excised, and cardiac and lung mitochondrial subpopulations were isolated for analyses as previously described (56), with modifications by our laboratory (3, 4, 16). Samples were prepared through differential centrifugation, allowing for the compartmentalization of nuclear, cytoplasmic, and mitochondrial fractions. Mitochondrial subpopulations, subsarcolemmal (SSM) and interfibrillar (IFM) were combined to form a total mitochondrial population.

Mitochondrial Respiration

State 3 and state 4 respiration rates were analyzed in isolated mitochondria as previously described (15, 70) with adaptations. The Bradford method (9), with standardization to bovine serum albumin, provided normalization of protein concentrations for cardiac mitochondria loading. To measure oxygen consumption of mitochondria, a multi-unit (8 channel) Oxytherm Peltier Electrode apparatus (Hansatech Instruments, Norfolk, England) was utilized. Calibration of the machine was performed through maximal oxygen concentration in respiration buffer (80mM KCl, 50mM MOPS, 1 mmol/l EGTA, 5 mmol/l KH₂PO₄ and 1 mg/ml BSA) and zero oxygen concentration through NaHSO₃. Oxygen concentration measurements were assessed using the S1 Clark type polarographic oxygen electrode disc, with a central platinum cathode and a concentric silver anode. Mitochondria were loaded into 500 mL of respiration buffer in the Peltier Chamber. Changes in oxygen levels were measured in real-time, providing both nmol O₂/mL consumption and rates per 10s, 30s, and 1min. Maximal complex I-mediated respiration was initiated by the addition of glutamate (5 mM) and malate (5 mM) for glucose mediated pathways or palmitoyl-carnitine (50 µM) and malate (5mM) for fatty-acid mediated pathways. Data for state 3 (250mM

ADP) and state 4 (ADP-limited) respiration were expressed as nmol of oxygen consumed/mL/min, standardized to protein concentration.

Electron Transport Chain Complex Activities

Maximal activities of electron transport chain (ETC) complexes I, III, IV and V were measured as previously described (29, 62). The ETC is comprised of five protein complexes which transport electrons, in a redox fashion, to create a proton (H^+) gradient which contributes toward the production of ATP. Complexes I (ubiquinone oxidoreductase), III (succinate dehydrogenase), and IV (cytochrome c reductase) can complex together to form a respirasome. Complex V (ATP Synthase) produces ATP from ADP using the proton gradient. The Bradford method (9), with standardization to bovine serum albumin, provided normalization of protein concentrations for cardiac and lung mitochondria loading. Activity was measured in complex I (reduction of decylubiquinone), complex III (reduction of cytochrome c), complex IV (oxidation of reduced cytochrome c), and complex V (oligomycin-sensitive ATPase activity through pyruvate kinase and phosphoenolpyruvate.).

Quantitative PCR

RNA was isolated from 20 mg of cardiac, lung, and liver tissue or from isolated cardiac mitochondria, using the miRNeasy Mini Kit (product no.: 217004, Qiagen, Hilden, Germany) per manufacturer's instructions, with minor modifications. For tissue, 700 μ L QIAzol was used for homogenization with a Polytron PowerGen 500 S1 tissue homogenizer (Fisher Scientific, Hampton, NH). Total RNA was isolated from both the whole tissue and mitochondrial samples and used in subsequent analyses. RNA was converted to cDNA with the First-strand cDNA Synthesis kit for miRNA (product no.: HP100042, Origene, Rockville, MD), per manufacturer's instructions. Differential expression of miRNA-378a-3p, miRNA-378a-5p, PGC-1 β , mitofusin 1 (Mfn1), and mediator complex subunit 19 (Med19) were measured. Primer design for qPCR is provided (Table S2.1). Briefly, primers for mRNA transcripts were derived using Primer-BLAST through NCBI, while miRNA forward primers were designed to span the 5' region of the sequence with a standard reverse to flank the poly-A tail following cDNA synthesis. Expression was

normalized to GAPDH in whole tissue and U6 in isolated mitochondria. Experiments were performed on the Applied Biosystems 7900HT Fast Real-Time PCR system (Applied Biosystems, Foster City, CA), using 2X SYBR Green Master Mix. Quantification was achieved using the $2^{-\Delta\Delta C_T}$ method (46).

Western Blot Analyses

Immunoblotting was performed on 4 –12% gradient gels through MES SDS-PAGE, as previously described (3, 4, 16, 53, 70). The Bradford method (9), with standardization to bovine serum albumin, provided normalization of protein concentrations for the cytoplasmic portion of cardiac tissue for loading. Primary antibodies used in the study included: anti-GAPDH (product no.: ab8245, Abcam, Cambridge, MA), anti-optic atrophy 1 (OPA1, product no.: ab42364, Abcam, Cambridge, MA), and anti-peroxisome proliferator-activated receptor alpha (PPAR α , product no.: ab2779, Abcam, Cambridge, MA). All primary antibodies were used at a 1:1000 concentration. Secondary antibodies used included the following: goat anti-mouse IgG (H&L) horseradish peroxidase (HRP) conjugate (product no. 31430, Thermo Fisher, Waltham, MA) and goat anti-rabbit IgG (H&L) HRP conjugate (product no. ab6721, Abcam, Cambridge, MA). Standardization was determined through GAPDH expression. Pierce® ECL Western Blotting Substrate (product no.: 32106, Thermo Fisher) was used per manufacturer's instructions to detect tagged proteins through a G:Box Bioimaging system (Syngene, Frederick, MD). Data were captured using GeneSnap/GeneTools software (Syngene). Densitometry was analyzed using Image J Software (NIH, Bethesda, MD). All values were expressed as optical density with arbitrary units.

Mitochondrial Size, Internal Complexity, and Membrane Potential

Flow cytometry was used to assess structural and functional parameters of mitochondria as previously described (15-17, 75), with modifications. Briefly, the LSRFortessa (BD Biosciences, Franklin Lakes, NJ) was used to perform flow cytometric analyses. To stain viable mitochondria, MitoTracker™ Deep Red FM/633 (Thermo Fisher) was used to produce a threshold for gating. Measurements of size were assessed through Sphero AccuCount Blank Particles, 2.0 μ m (SpheroTech Inc., Lake Forest, IL) sizing beads. Forward Scatter (FSC PMT-H) measured the

absolute size of the mitochondria while the Side Scatter (SSC-H) measured the granularity of the mitochondria. The ratiometric mitochondrial dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol carbocyanine iodide, Molecular Probes, Carlsbad, CA) was used to measure mitochondrial membrane potential. Changes in membrane potential were assessed as the ratio of orange to green fluorescence of JC-1. All flow cytometric measures were performed at the West Virginia University Flow Cytometry & Single Cell Core Facility. FSC files were processed using the FCS Express Flow Research Edition (De Novo Software, Glendale, CA).

Transmission Electron Microscopy of Mitochondria

After excision of cardiac tissue, ~1-2 mm³ pieces were cut and sent to the WVU Electron Microscopy Shared Facilities where they were processed and further imaged at the WVU Electron Microscopy Histopathology and Tissue Bank. Briefly, cardiac tissue was fixed in 2.5% glutaraldehyde in 0.1 M PBS (pH 7.4) for 60 minutes. After washing in PBS, samples were incubated in 1% osmium tetroxide containing 1% potassium ferricyanide for 60 minutes. Following additional PBS washes, tissue was dehydrated using a graded series of alcohol dilutions and propylene oxide. Samples were then incubated overnight in a 1:1 mix of propylene oxide and EPON. Samples were incubated in pure EPON for 4 washes, each for 60 minutes. Samples were cured in pure EPON inside the BEEM® Embedding Capsules Size 3 (Electron Microscopy Sciences, Hatfield, PA) starting at 37°C for 24 hours and then 60°C for 48 hours. Tissue was sectioned using an Ultramicrotome Leica EM UC7 (Leica Microsystems, Wetzlar, Germany) and stained with uranyl acetate and lead citrate. Sections were imaged using the JEOL JEM-2100 Transmission Electron Microscope (JOEL, Akishima, Tokyo, Japan). Semi-quantitative analyses of mitochondrial size was processed through Fiji (NIH, Bethesda, MD) and the Trainable Weka Segmentation (TWS) plugin by providing a probability map by which particle size could be determined. Selection of 5 mitochondria per group were used to perform analyses.

In Situ Hybridization (ISH) for miRNA-378a

After excision of cardiac tissue, the apex of the heart was removed (~5 mm³) and prepared per manufacturer's instructions (Qiagen) (31, 61), with slight modifications. Tissue was treated

with freshly prepared 4% paraformaldehyde in PBS and was incubated at 4°C overnight. Tissue was then directly transferred to 0.5M Sucrose/PBS and was incubated at 4°C overnight. Following incubation, tissue was flash frozen and samples taken to the WVU Pathology Laboratory of Translational Medicine to embed and section tissue. Tissue was cut in 12-14 μ M sections and mounted on Superfrost/Plus slides. Sections were further fixed in 4% paraformaldehyde in PBS and was incubated at 4°C for 10 minutes and then washed with PBS 3 times for 5 minutes each. For epitope retrieval, slides were immersed in a citric acid bath (10 mM citric acid, pH 6.0) at 95°C for 15 minutes and then washed in PBS for 5 minutes at room temperature. Sections were acetylated (2.33 mL of triethanolamine and 500 μ L acetic anhydride in 197 mL water) for 10 minutes. Slides were washed 3 times with PBS for 5 minutes each. Slides were hybridized using a hybridization buffer (50% deionized formamide, 0.3M NaCl, 20 mM Tris HCl, pH 8.0, 5 mM EDTA, 10 mM Na₃PO₄, pH 8.0, 10% Dextran Sulfate, 1X Denhardt's solution, and 0.5 mg/mL of yeast RNA) (74) and 40 nM of miRNA-378a-3p 3'-DIG labeled probe (Qiagen).

Total hybridization mixture was heated to 65°C to linearize the probe and 100 μ L of solution was added to slides, cover slipped, and hybridized at 56°C for 18 hours in a HybEZ™ Hybridization System (Advanced Cell Diagnostics, Newark, CA). Coverslips were removed in 5X SSC buffer at room temperature for 20 minutes. Slides were then washed twice in 50% formamide, 0.1% Tween-20, and 1X SSC at 56°C. Following two washes in PBS for 15 minutes each, blocking solution (3% Donkey serum, 0.1% Titron-100X, in PBS) was applied for 1 hour at room temperature. AP conjugated anti-DIG Fab fragments (1:1000) (Sigma-Aldrich, St. Louis, MO) were then applied to the blocking solution for 2 hours at room temperature. Following two washes in 0.1% Tween/PBS for 30 minutes each and 1X PBS for 10 minutes each, BM Purple AP Substrate (Sigma-Aldrich) and 2mM Levamisole were applied to slides for 6 days. Staining was quantified through light microscopy using the EVOS™ FL Auto Imaging System (Thermo Fisher). Briefly, using images derived with the same light intensity, Fiji (NIH, Bethesda, MD) was applied to measure intensity of BM Purple staining by using the log(max intensity/mean intensity) of Purple (R: 0.4643, G: 0.8303, B: 0.3083).

Citrate Synthase Activity

Citrate synthase is key to metabolism through the production of citrate from acetyl-CoA and oxaloacetate, initiating the tricarboxylic acid (TCA) cycle. To determine the number of mitochondria in heart tissue, a colorimetric Citrate Synthase Assay (Sciencell, San Diego, CA) was employed per manufacturer's instructions (35). The Bradford method (9), with standardization to bovine serum albumin, provided normalization of protein concentrations for cardiac mitochondria. Briefly, 1 μ g of mitochondria was used to measure the colorimetric change produced from 5', 5'-Dithiobis 2-nitrobenzoic acid (DTNB) and CoA-SH forming of TNB at an absorbance maximum of 412 nm.

Hydrogen Peroxide (H₂O₂) Assay

To measure the concentration of H₂O₂ in the heart we used an Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Thermo Fisher, Waltham, MA) per manufacturer's instructions and as previously described (52), with modifications. Heart tissue was homogenized in NP-40 buffer (150 mM NaCl, 50 mM Tris-HCl, 1% Triton-100X, and pH adjusted to 7.4) and 50 μ g of protein, determined through the Bradford method (9), was used in the assay. The reaction of the Amplex® Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) with H₂O₂ occurs at a stoichiometric ratio of 1:1. Sample H₂O₂ concentrations were determined through running a standard curve (0 to 20 μ M H₂O₂), with absorbance measured at 560 nm. Data was normalized to the number of mitochondria for each sample, using citrate synthase activity (described above).

TargetScan

The miRNA-mRNA binding prediction software TargetScan 7.1 Server was implemented in order to determine seed-sequence binding affinities (1, 44). Briefly, binding affinities were examined in the 3' untranslated region (UTR) of Mfn1 and Med19, where context++ scores (CSs) predicted the propensity for binding. CSs are derived from a variety of variables including 3' UTR length, supplementary pairing, local AU content, and conservation of target sequence. Seed-sequence regions begin with the second base pair on the miRNA, starting from the 5' end, and can identically bind to eight (8mer), seven (7mer), or six (6mer) consecutive base pairs on the mRNA strand. http://www.targetscan.org/vert_71/

IntaRNA

RNA-RNA interaction software IntaRNA 2.0 was used to assess miRNA binding in the 3' UTR of Mfn1 (mouse), MFN1 (human), Med19 (mouse), and MED19 (human) (10, 47, 76). Unlike TargetScan 7.1, IntaRNA considers free energy binding as well as intramolecular forces between base pairs. Ensembl was used to obtain the 3' UTR sequence for Mfn1 and Med19. The 3' UTR sequences were then run against the miRNA-378a-3p sequence (5' – ACUGGACUUGGAGUCAGAAGG – 3') to determine complementarity. The interaction energy is a measure of binding potential.

Mfold

The UNAFold Web Server was used to evaluate miRNA folding, specifically through using the mfold software (78). The pre-miRNA-378a (5' – AGGGCUCCUGACUCCAGGUCCUGUGUGUUACCUCGAAAUAGCACUGGACUUGGAGUCAGAAGGCCU – 3'), mature miRNA-378a-3p (5' – ACUGGACUUGGAGUCAGAAGG – 3'), and mature miRNA-378a-5p (5' – CUCCUGACUCCAGGUCCUGUGU – 3') sequences from miRBase release 21 were used.

Ingenuity Pathway Analysis (IPA)

QIAGEN's IPA Server (December 2017 update) provides protein ontology and the capacity to correlate gene expression data through molecular pathways experimentally verified. The "PPAR α /RXR α Activation" pathway and graphical illustrations were used in the analysis www.qiagen.com/ingenuity.

Statistics

Significance was determined using either a two-tailed Student's t-test or one-way analysis of variance (ANOVA), where appropriate. Tukey's multiple comparisons test was implemented

following the ANOVA to derive significance between multiple groups. Differences between groups were considered statistically different ($P \leq 0.05$, denoted by *). All data are presented as the mean $\pm$ standard error of the mean (SEM).

Results

MiRNA-378a Expression following Nano-TiO₂ Exposure

Because the pre-miRNA-378a gene actually contains both a 3' (miRNA-378a-3p) and a 5' (miRNA-378a-5p) arm mature miRNA (42), expression of both miRNAs needed to be assessed in total heart homogenates and within isolated mitochondria. This tissue and organellar-specific approach allowed for the determination of miRNA-378a's involvement in regulating a variety of pathways (whole tissue) or those specifically related to mitochondrial bioenergetics and mitochondrial transcription (mitochondria). In whole heart tissue (Figure 2.2A), the expression of miRNA-378a-3p was significantly increased following exposure, while the 5p strand was unchanged. Using *in situ* hybridization, miRNA-378a-3p was also shown to be significantly elevated in cardiac tissue, displaying universal, not localized, expression of the miRNA throughout the cell (Figure 2.2B). The gene that encodes these two mature miRNAs, PGC-1 β , was also shown to have increased expression following exposure (Figure S2.2A). The heart, in comparison to the lung and liver, was the only tissue where miRNA-378a-3p was increased, with a trend toward decreased expression in both the lung and liver following exposure (Figure S2.2B).

In mitochondria (Figure 2.2C), the 3p strand remained unchanged, while miRNA-378a-5p was increased after nano-TiO₂ exposure. Though alterations in mitochondrial miRNA-378a-5p could potentially change dynamics surrounding mitochondrial metabolism (35), the relative abundance of the 5p strand to the 3p strand was significantly lower (Figure S2.2C) in both the whole heart tissue and the mitochondrion, likely contributing little to systemic cellular mechanisms. Genotyping was performed using PCR and gel agarose electrophoresis for the genic region surrounding miRNA-378a for wild type (WT), heterozygous knockout (Het) and total knockout (KO) animals; the size and characteristic folding of the pre-miRNA and mature strands are also represented (Figure 2.2D). The expression of miRNA-378a-3p and 5p were then confirmed both in whole heart tissue and mitochondria through qPCR in transgenic animals (Figure S2.2D). The increased expression of miRNA-378a-3p in the whole heart homogenates following exposure provided a pathway for examining how miRNAs can alter cardiac function following exposure to nano-TiO₂ through a transgenic model.

A list of all M-mode parameters are provided in Table 2.1. Of note, a decrease in the ejection fraction (percentage of total blood leaving the heart) and fractional shortening (change in left ventricular diameter) between the WT Sham and WT Ex group was observed, though preserved in the Het and KO groups. The WT groups also had larger systolic volumes than both the Het and KO groups. M-mode characteristics are also provided for Het and KO mice pre-exposure, validating the sham exposure model (Table S2.2). PW Doppler was implemented to measure mitral valve function and filling parameters (Figure 2.3A). The E wave (early diastolic filling of the left ventricle) was shown to not be significantly altered between groups, with the Het group exhibiting a higher A wave (late diastolic filling of the left ventricle) following exposure (Figure 2.3B). As seen in other studies following nano-TiO₂ exposure (29, 52), the WT group had an E/A ratio of ~2. When the E/A ratio increases, it indicates either more passive (early) filling or less active (late) filling of the left ventricle; this signifies a pathological shift in blood flow in the left ventricle and indicates diastolic dysfunction and increased left atrial pressure.

While the KO group preserved E/A ratio, the Het group had a significant decrease in E/A ratio. Short axis radial strain and strain rate were significantly decreased following exposure to nano-TiO₂ in the WT and Het groups, respectively, while no changes were shown in the KO (Figure 2.3C). Also, long axis diastolic radial strain was increased in the WT group after exposure. Generally, decreases in systolic measures of strain infer impaired contractile properties of the left ventricle, while increases in diastolic measures of strain could suggest aberrant relaxation of the left ventricle. Strain measures represent the contraction and relaxation of the myocardium and subsequent cardiomyocyte contractility. The segmental changes in short axis systolic radial strain are illustrated as an example of the segmental analysis of speckle tracking stress-strain (Figure 2.3D).

In the long axis, global longitudinal strain (GLS) during end-diastole provides significant prognostic value in assessing cardiac health (21) (Figure 2.3E). By combining both the E wave and end-diastolic GLS (E/SR ratio), it provides both cardiac strain and mitral valve function in a prognostic assessment (72). An increased E/SR ratio in the WT and Het indicates increased susceptibility to adverse cardiac events, while the KO group remained unchanged (Figure 2.3E). Cardiac function following exposure is further captured in the left ventricle myocardial

performance index $\left(\frac{\text{isovolumic relaxation time (IVRT)} + \text{isovolumic contraction time (IVCT)}}{\text{left ventricle ejection time (LVET)}}\right)$, revealing increased function in the KO group compared to the WT (Figure 2.3E). All other measures of diastolic (Table S2.3 and S2.4) and systolic (Table S2.5 and S2.6) stress-strain are included. Decreases in miRNA-378a expression attenuates the effect of nano-TiO₂ exposure to the heart, preserving cardiac function following acute nano-TiO₂ exposure.

Mitochondrial Bioenergetics and miRNA-378a

In the WT, changes to ETC activities and mitochondrial respiratory capacity were observed following exposure. Complex III activity and glucose-mediated state 3 respiration were decreased following nano-TiO₂ exposure (Figure S2.3A and S2.3B). When examining bioenergetic capacity of all the groups, a distinctive metabolic profile was observed. Irrespective of exposure, complex I, III, and IV activities were shown to be significantly increased in the Het and KO groups (Figure 2.4A), potentially contributing to the cardioprotection observed after exposure. Unlike in the WT mice, glucose-mediated metabolism was not the most significantly impacted metabolic pathway in the Het or KO animals (Figure 2.4B); the main pathway affected was fatty-acid metabolism (Figure 2.4C). Both the Het and KO groups displayed a more robust fatty acid metabolic profile, which is illustrated in Figure 2.4D.

While exposure primarily affected glucose substrate utilization pathways in WT animals (Figure S2.3B), the Het and KO animals displayed decrements in fatty acid substrate utilization in state 3 and state 4 respiration, respectively. The increased utilization of fatty acid metabolites has been previously shown in the liver of miRNA-378a knockout animals (11), supporting the parallels described in the heart and suggesting a metabolic contribution toward attenuating the pathology. Lung tissue of the Het and KO animals showed conserved ETC complex activity following exposure (Figure S2.4A and S2.4B), indicating preservation of cardiac function following inhalation exposure may be a function of protection to multiple organ systems in the miRNA-378a knockout/knockdown animals. The increase in complex activities and fatty acid-driven state 3 and state 4 respiration in Het and KO animals revealed a bioenergetic role for cardioprotection following nano-TiO₂ exposure.

Mitochondrial Ultrastructure

Mitochondrial size, through flow cytometry (Figure S2.5A), was shown to increase following exposure in the WT group (Figure 2.5A), as well as in both the Het and KO groups, irrespective of exposure. The shape and size of mitochondria were also examined through transmission electron microscopy (TEM), confirming the increased size of mitochondria following ENM inhalation exposure in the WT group using a semi-quantitative approach (Figure 2.5B). Citrate synthase activity, used as a common marker of mitochondrial number/content and shown to be highly correlative (43), revealed decreased mitochondrial number in the WT group after exposure, while the Het and KO were protected from the effect (Figure S2.5B). In the WT group following exposure, though the size of the mitochondria increased, decreased mitochondrial number suggests a role in contributing to the dysfunctional respiratory capacity of the mitochondria (37). Alternatively, in the Het and KO groups, mitochondrial size was shown increased while mitochondrial size and number were unaffected by the exposure paradigm. The increased size and unchanging number of mitochondria in the Het and KO groups could relate to the increased bioenergetic capacity and potential protective mechanism observed following nano-TiO₂ inhalation exposure. Other measures of mitochondrial ultrastructure, such as internal complexity (Figure S2.5C) and membrane potential (Figure S2.5D) were measured and found to be unchanged. Measurements of H₂O₂ were performed to further understand mitochondrial stress/dysfunction. In the WT group following exposure, the concentration of H₂O₂ in cardiac tissue was significantly increased (Figure S2.5E). Following inhalation exposure to nano-TiO₂, the density, size, and impact of ROS is altered, but knockout, or even knockdown, of miRNA-378a produces a protective phenotype.

Targets of miRNA-378a and Pathways Affected

Gene transcripts, which were related to mitochondrial function and revealed highest 3' UTR binding scores by miRNA-378a, were examined for further *in-silico* binding and expression. Mfn1 and Med19 were identified to be directly targeted by miRNA-378a-3p using bioinformatics software IntaRNA and TargetScan. MiRNA-378a-3p binding to the 3' untranslated region (UTR) was evaluated through both free energy binding affinity (IntaRNA) (Figure 2.5D) and seed sequence complementarity (TargetScan) (Figure S2.6A). The mRNA of Mfn1 and Med19 were

found to be significantly increased following exposure in the Het and KO groups (Figure 2.5D), suggesting that the negative regulation of miRNA-378a-3p was degrading transcripts and limiting translation of these proteins. While Med19 is involved in general transcriptional activation of RNA Polymerase, Mfn1 is involved prolifically in mitochondrial fusion dynamics and the health of mitochondria.

Opal, a direct mediator with Mfn1 of inducing mitochondrial fusion, was shown to be significantly increased in the Het and KO groups following inhalation exposure; likewise, expression of the fatty acid metabolism protein Ppar α was also increased (Figure S2.6B). The knockdown/knockout of miRNA-378a-5p, which acts as a negative regulator of estrogen-related receptor gamma (EER γ) and GA-binding protein alpha chain (GABPA), could promote the expression of PGC-1 β and subsequently interact with Ppar α (20). In the KO animal, PGC-1 β is found to be significantly increased compared to the WT group, following inhalation exposure (Figure S2.6C). Following maternal nano-TiO₂ exposure, PGC-1 β displayed increased histone 3 lysine 4 tri-methylation (H3K4me3) association at its promoter region, suggesting an increased expression of both the gene and miRNA-378a through epigenetic mechanisms (Figure S2.6D) (67). Suppressing miRNA-378a during inhalation exposure could diminish cardiac dysfunction by offering cardioprotection through pathways including transcription, bioenergetics, fatty acid metabolism, and mitochondrial fusion (Figure 2.6A and 2.6B).

Discussion

The scope and impact of research examining the potential human consequences of ENM exposure continues to grow, with considerations into the dose, physiochemical characteristics, route, and time course of exposure and the impact on human health and safety adding multiple layers of complexity. Nano-TiO₂ remains a significant contributor to total ENM production, affecting both those involved in manufacturing and utilization of the material. The life cycle of ENMs, including byproducts of manufacturing, disposal through waste and recycling, use by consumers, and other mediums, eventually carry ENMs into the environment (26). The environmental sequestration of ENMs and their byproducts could then have secondary or tertiary impacts on human health (8). We provide evidence to support the therapeutic benefit of miRNA-378a downregulation as a means of mediating the ENM toxicological response through metabolism, mitochondrial function, and other cellular processes during ENM inhalation exposure. For the first time, this study illustrates the impact of the manipulation of a miRNA *in vivo* on controlling toxicity to ENMs.

Under non-pathological states, altered miRNA content may have no overt consequences to basal physiological processes. As a stressor is introduced, such as disease, xenobiotic exposure, or caloric changes, specific miRNAs are needed to mediate the response and attempt to return the cell to homeostasis (11, 49, 55). In the transgenic miRNA-378a knockout and knockdown animals, this has been highlighted in both caloric changes (11) and currently with ENM inhalation exposure. Adaptation to a stress has significantly diverse outcomes in transgenic versus wild type animals, though little or no genetic or phenotypic changes exist at a basal condition. Because miRNAs exist in a complicated web of regulatory non-coding RNA, compensatory mechanisms are present which can attenuate loss of function from a gene or genes (27, 71). In the transgenic animals used in this study, it is likely that the loss of miRNA-378a is compensated through increased expression of endogenous miRNAs that regulate similar functional pathways, such as the insulin response and fatty acid metabolism pathways. Additionally, epigenetic remodeling could occur, repressing the expression of previously targeted miRNA-378a transcripts.

Cellular and systemic dysfunction has been attributed to ENM-induced changes in miRNA expression (2, 14, 28, 50, 51, 65). While the contribution of miRNA expression in advancing ENM related insult is beginning to be understood, what lacks clarity are the explicit mechanisms

governing these changes in expression. The interplay between the epigenome and miRNA expression provides a new area of research and hierarchy of control, which could potentially explain the regulation of multiple pathways. MiRNAs are known to directly regulate, and to be regulated by, epigenetic function (30, 60).

In one of the first studies to examine the effects of inhalation of ENM and miRNA expression, Halappanavar et al. showed that following inhalation exposure, miRNA-1 and -449 were elevated (28). MiRNA-1 is known to target histone deacetylase 4 (HDAC4) while miRNA-449 has been shown to target HDAC1, resulting in global changes to the epigenetic histone profile in the cell (12). Down regulation of HDACs following nano-TiO₂ exposure would suggest the activation of genes through open histone conformations, which we have reported in progeny following maternal exposure (67). Alternatively, the miRNA expression can be altered by epigenetic machinery. The expression of miRNA-378a is regulated through methylation in TGF- β 1-treated LX-2 cells (77). In the current study, both the expression of the host gene PGC-1 β and miRNA-378a-3p were shown to be increased following inhalation exposure to nano-TiO₂, with epigenetic mechanisms known to alter the expression of the PGC-1 β gene. The epigenetic landscape of miRNA-378a, and other altered miRNAs, could provide valuable information regarding transient and, potentially, sustained effects of ENM exposure on the heart and other organ systems.

The animal models employed in the study examine miRNA-378a across a stratum of normal (WT), knockdown (Het), and knockout (KO) expression levels. Intuitively, one could hypothesize that the health outcomes going from poorest to best prognosis would trend in a linear fashion from WT to Het to KO. While we expect the KO model to have increased mitochondrial fatty acid metabolism (24) and overall better health (11) following ENM exposure, the knockdown expression of miRNA-378a may still be prevalent enough to regulate maladaptive metabolic pathways, meaning that the linearity of functional measures in the three models may not exist in all cases. MiRNA's are multifaceted transcriptional and translational repressors that target multiple genic groups, making it difficult to speculate the intermediary effects that knockdown expression may have on any one cardiac, mitochondrial, or molecular measure (48). That being said, in general, we expected to see overall better function in the Het model compared to the WT control.

The current study proposes the idea that targeting miRNAs can alter cardiac and mitochondrial function and adaptation to ENM inhalation exposure. A noted limitation to the study is the examination of systemic miRNA-378a knockout and knockdown when examining cardiovascular health following ENM inhalation exposure. The current work focuses on cardiac adaptation to ENM inhalation exposure, but does so through systemic knockdown of miRNA-378a; a cardiac-specific knockout model could provide further indication into the absence of localized miRNA-378a expression following insult. We point to miRNA-378a as a target for attenuation of nano-TiO₂ inhalation exposure, though it is likely that multiple miRNAs, and other coding and non-coding RNAs, are involved in the response to the insult. Our current work may be limited through the exploration of a single miRNA, but the outlined methodology provides an archetype for transgenic animal use in examining ENM toxicology and signifies the importance of non-coding RNA in regulating the cardiovascular response.

The goal of ENM inhalation toxicology is to decrease the severity of biologic effects and/or prevention of exposure, ultimately increasing the positive impact of ENM utilization in human endeavors. The ability to therapeutically target a miRNA, or even group of miRNAs, could beneficially protect those exposed to ENMs. We show through the heterozygous knockout of miRNA-378a that even partial downregulation of a miRNA can beneficially alter the response to ENM inhalation exposure, producing a therapeutic effect. Ultimately, further investigation into the role of miRNAs following ENM exposure is needed as well as understanding their interconnection with the epigenome. Future studies could examine the epigenetic regulation of genes encoding miRNAs, such as the promotor region of PGC-1 β , and determining if ENM exposure provides transient or sustained epigenetic control of crucial genes and non-coding RNA.

Understanding the role of miRNAs in regulating the response to ENM inhalation exposure is important in defining the molecular consequences involved in the response. In this study, we discovered that miRNA-378a, a small non-coding RNA, was directly involved in regulating cardiac function following nano-TiO₂ inhalation exposure. The knockout, and to a lesser extent knockdown, of miRNA-378a expression was associated with preserved cardiac function following exposure and increased bioenergetic capacity when compared to wild type animals. The mechanisms governing this functional difference are likely through bolstered mitochondrial health, including increased mitochondrial fusion and fatty acid metabolism, as well as changes to

cellular transcription. This study investigates for the first time, the *in vivo* role of a miRNA in attenuating cardiovascular consequences following ENM inhalation exposure, setting miRNA-378a as a potential target for future investigations. Manipulation of miRNA-378a, or other miRNAs found in the heart or systemically, have the potential to improve health outcomes following ENM inhalation exposure.

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Declaration of Interest

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Disclaimer

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention.

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Table 2.1: M-Mode Cardiac Function

Calculation	Units	WT Sham ¹	WT Ex ²	Het Sham ³	Het Ex ⁴	KO Sham ⁵	KO Ex ⁶
CO	mL/min	13.04 ± 1.15	11.00 ± 1.41	12.11 ± 2.22	12.79 ± 1.97	12.34 ± 0.82	14.35 ± 0.62
Diameter;d	mm	2.33 ± 0.06	2.15 ± 0.13	2.52 ± 0.15	2.58 ± 0.14	2.62 ± 0.07	2.82 ± 0.29
Diameter;s	mm	1.01 ± 0.03	1.03 ± 0.08	0.83 ± 0.09	0.85 ± 0.08	0.79 ± 0.08	0.99 ± 0.21
EF	%	87.39 ± 1.16	84.17 ± 1.75 6	89.07 ± 1.54	87.43 ± 1.89	90.05 ± 0.79	91.60 ± 0.45
FS	%	56.44 ± 1.62	52.14 ± 2.11 6	57.88 ± 2.06	55.86 ± 2.50	59.86 ± 0.80	62.96 ± 2.74
LV Mass	mg	108.88 ± 10.24	111.87 ± 10.05	100.21 ± 6.94	132.61 ± 8.47	122.29 ± 10.26	108.32 ± 9.97
LV Mass Corr	mg	87.10 ± 8.20	89.50 ± 8.04	80.17 ± 5.55	106.08 ± 6.78	97.84 ± 8.21	86.66 ± 7.97
SV	uL	27.16 ± 1.38	22.06 ± 2.62	22.24 ± 3.19	23.62 ± 3.26	24.22 ± 1.46	31.49 ± 4.82
V;d	uL	30.86 ± 1.44	29.65 ± 1.92	25.13 ± 3.13	25.18 ± 2.13	25.41 ± 1.66	29.66 ± 2.82
V;s	uL	2.63 ± 0.36 3,4,5,6	2.93 ± 0.29 3,4,5,6	1.45 ± 0.40	1.55 ± 0.28	1.19 ± 0.31	1.68 ± 0.14

Table 2.1: Ultrasound imaging following nano-TiO₂ inhalation exposure. Measurements were taken for at least three consecutive systolic and diastolic peaks and troughs, respectively, for each animal. Significance ($P \leq 0.05$) is denoted for a specific category through bold text and reference to the sample number that it is significantly different from. Sample numbers are listed in the column heading. WT = wild type, Het = heterozygous for the miRNA-378a allele, KO = knockout for the miRNA-378a allele, Sham = control filtered air exposed, Ex = nano-TiO₂ exposed, CO = cardiac output, Diameter;d = diastolic diameter, Diameter;s = systolic diameter, EF = ejection fraction, FS = fractional shortening, LV Mass = left ventricular mass, LV Mass Corr = left ventricular mass corrected, SV = stroke volume, V;d = volume during diastole, V;s = volume during systole.

Figure 2.1

Figure 2.1: Nano-TiO₂ aerosol characterizations. **(A)** Representative transmission electron micrograph of aerosolized agglomerates collected on a filter. Bar is 50 nm. **(B)** Real-time estimation of the aerosol mass concentration measured with a light scattering device. The red line represents the target concentration of 10 mg/m³. The black line is the measured concentration during the exposure period. Note that computerized feedback system (see Methods) maintains a stable concentration. Mean DataRAM aerosol concentration during this exposure was 9.88 ± 0.03 mg/m³. Data was sampled every 2 seconds. **(C)** High resolution ELPI aerodynamic diameter measurements. The red line designates the log normal distribution obtained with the log probability plot method. This produced a count median diameter of 182 nm with a geometric standard deviation of 1.70. **(D)** SMPS (light gray bars) and APS (dark gray bars) mobility diameter measurements. The red line designates the log normal distribution obtained with the log probability plot method. This produced a count median diameter of 190 nm with a geometric standard deviation of 1.88. Note: APS bar values are nominal and difficult to visualize.

Figure 2.2

Figure 2.2

Figure 2.2: Expression of miRNA-378a following nano-TiO₂ inhalation and miRNA-378a animal models. Following inhalation exposure to control filtered air or nano-TiO₂ qPCR was implemented to assess miRNA-378a-3p and miRNA-378a-5p expression in **(A)** whole heart tissue. **(B)** Representative images of *in situ* hybridization of miRNA-378a-3p in cardiac tissue performed under light microscopy (n = 4 for both groups). **(C)** qPCR was also used to measure miRNA-378a-3p and miRNA-378a-5p expression in isolated mitochondria **(D)** Genotyping through PCR amplification and agarose gel electrophoresis for the miRNA-378a loci in WT, Het, and KO animals. Additionally, RNA folding software Mfold evaluated the likely folding characteristics of the miRNA-378a species. Groups are considered significantly different if $P \leq 0.05 = *$. All data are presented as the mean $\pm$ standard error of the mean (SEM). WT = wild type, Sham = control filtered air exposed, Ex = nano-TiO₂ exposed.

Figure 2.3

Figure 2.3

Figure 2.3: Cardiac function following nano-TiO₂ inhalation exposure. **(A)** PW Doppler images illustrate changes in E and A wave velocities following exposure. **(B)** E and A wave velocities, as well as E/A wave ratios, are represented from PW Doppler imaging. Speckle-tracking analyses from B-Mode short and long axis images for **(C)** systolic and diastolic strain and strain rate. **(D)** An illustration of dyssynchrony of the endocardium during short axis systolic radial strain (indicated by color) in the WT nano-TiO₂ exposed animals compared to conserved strain parameters in the KO exposed animals. **(E)** The diastolic longitudinal strain rate coupled with the E wave velocities provided a prognostic cardiac health index for the left ventricle (LV), while the LV myocardial performance index (MPI) further provided a prognostic index of the left ventricular ejection parameters. Groups are considered significantly different if $P \leq 0.05 = *$. All data are presented as the mean $\pm$ standard error of the mean (SEM). WT = wild type, Het = heterozygous for the miRNA-378a allele, KO = knockout for the miRNA-378a allele, Sham = control filtered air exposed, Ex = nano-TiO₂ exposed, SA = left ventricular short axis, LA = left ventricular long axis, Long = longitudinal, Rad = radial.

Figure 2.4

Figure 2.4

Figure 2.4

Figure 2.4: Bioenergetic function of cardiac mitochondria following nano-TiO₂ inhalation exposure. **(A)** Electron transport chain complex activities were measured for complexes I, III, IV, and V. Mitochondrial State 3 and State 4 respiration were measured through **(B)** glucose mediated pathways and **(C)** fatty acid mediated pathways. **(D)** State 3 and State 4 respiration changes between a representative WT and KO animal illustrates changes in oxygen consumption when using p-carnitine for fatty acid mediated respiration. Groups are considered significantly different if $P \leq 0.05 = *$. All data are presented as the mean $\pm$ standard error of the mean (SEM). WT = wild type, Het = heterozygous for the miRNA-378a allele, KO = knockout for the miRNA-378a allele, Sham = control filtered air exposed, Ex = nano-TiO₂ exposed, P-Carnitine = palmitoyl carnitine.

Figure 2.5

Figure 2.5

Figure 2.5

Figure 2.5: Mechanisms governing mitochondrial function and molecular targets of miRNA-378a following inhalation exposure. **(A)** Mitochondrial size was determined through forward scatter, gating mitochondria positively stained with MitoTracker™ Deep Red FM/633; representative images of the WT sham and nano-TiO₂ exposure are illustrated. **(B)** Representative Transmission Electron Microscopy (TEM) images depicting changes to mitochondrial size (n = 1 for each group). **(C)** Binding interactions and qPCR for both human (MFN1 and MED19) and mouse (Mfn1 and Med19) using IntaRNA 2.0. Interaction energies are calculated as binding propensity through free energy binding near the seed sequence region. Groups are considered significantly different if $P \leq 0.05 = *$. All data are presented as the mean $\pm$ standard error of the mean (SEM). WT = wild type, Het = heterozygous for the miRNA-378a allele, KO = knockout for the miRNA-378a allele, Sham = control filtered air exposed, Ex = nano-TiO₂ exposed, FSC PMT-H = forward scatter, Mfn1 = mitofusin 1, Med19 = mediator complex subunit 19, 3' UTR = 3' untranslated region.

Figure 2.6

Figure 2.6: General schema of miRNA-378a interactions following inhalation exposure. **(A)** Transgenic mouse model, illustrating miRNA-378a wild type and knockout alleles. Mice were exposed to a total nano-TiO₂ deposition of 9.88 ± 0.03 mg/m³. Cardiac issue was excised for subsequent experimental procedures. **(B)** Molecular changes in the KO nano-TiO₂ exposed heart compared to WT exposed. Med19 was shown to be significantly elevated, an important cofactor in regulating cellular transcription. Metabolically, fatty acid metabolism and electron transport chain complex activities were elevated, likely through mediators such as Ppar α and Ppargc1b and enhanced mitochondrial ultrastructure. Increased mitochondrial fusion (Mfn1 and Opa1) contributes to the integrity of mitochondria following exposure. These molecular pathways altered with the repression of miRNA-378a contribute to elevated cardiac function in KO compared to the WT mice. WT = wild type, Het = heterozygous for the miRNA-378a allele, KO = knockout for the miRNA-378a allele, Mfn1 = mitofusin 1, Med19 = mediator complex subunit 19, 3' UTR = 3' untranslated region, OPA1 = optic atrophy 1, PPAR α = peroxisome proliferator-activated receptor alpha, Ppargc1b or PGC-1 β = peroxisome proliferator-activated receptor gamma coactivator 1-beta.

Table S2.1

Gene Name	Sequence	Orientation
Ppargc1b	CAGCCTCAGTTCCAGAAGTCAG	Forward
Ppargc1b	CACCGAAGTGAGGTGCTTATGC	Reverse
Mfn1	CCAGGTACAGATGTCACCACAG	Forward
Mfn1	TTGGAGAGCCGCTCATTCACCT	Reverse
Med19	ATGCTGTCTGGTTTCCGCCTCC	Forward
Med19	GCGGCTCTGTTTGTGCTTATGC	Reverse
Gapdh	CATCACTGCCACCCAGAAGACTG	Forward
Gapdh	ATGCCAGTGAGCTTCCCGTTCAG	Reverse
miRNA-378a-3p	GCAGACTGGACTTGGAGT	Forward
miRNA-378a-5p	TCCTGACTCCAGGTCCT	Forward
Standard Reverse	GAACATGTCTGCGTATCTC	Reverse
miRNA-378a		
Genotype	GGCCAACTTGGGAAATGTA	Forward
miRNA-378a		
Genotype	AGCTCACATGCAAACACAGG	Reverse

Table S2.1: Primer design for qPCR and genotyping. Primers were designed using Primer-BLAST through NCBI and Primer3. Sequence information was compiled from the University of Santa Cruz (UCSC) genome browser from the mouse GRCm38/mm10 assembly. MiRNA sequences were analyzed based on the miRBase 21 release.

Table S2.2

Calculation	Units	Het Sham Pre	Het Sham Post	Het Ex Pre	Het Ex Post	KO Sham Pre	KO Sham Post	KO Ex Pre	KO Ex Post
CO (LV Trace)	mL/min	13.12 ± 1.43	12.11 ± 2.22	13.66 ± 1.32	12.79 ± 1.97	10.96 ± 2.03	12.34 ± 0.82	12.48 ± 2.62	14.35 ± 0.62
Diameter;d (LV Trace)	mm	2.54 ± 0.16	2.52 ± 0.15	2.84 ± 0.18	2.58 ± 0.14	2.54 ± 0.21	2.62 ± 0.07	2.52 ± 0.20	2.82 ± 0.29
Diameter;s (LV Trace)	mm	0.68 ± 0.06	0.83 ± 0.09	1.12 ± 0.19	0.85 ± 0.08	0.81 ± 0.13	0.79 ± 0.08	0.71 ± 0.11	0.99 ± 0.21
EF	%	87.17 ± 0.51	89.07 ± 1.54	86.62 ± 1.44	87.43 ± 1.89	89.47 ± 0.69	90.05 ± 0.79	88.63 ± 0.90	91.60 ± 0.45
FS	%	55.33 ± 0.88	57.88 ± 2.06	54.30 ± 1.54	55.86 ± 2.50	56.80 ± 0.98	59.86 ± 0.80	57.22 ± 1.35	62.96 ± 2.74
LV Mass (LV Trace)	mg	101.51 ± 11.52	100.21 ± 6.94	119.63 ± 9.81	132.61 ± 8.47	113.24 ± 18.63	122.29 ± 10.26	103.44 ± 3.96	108.32 ± 9.97
LV Mass Corr (LV Trace)	mg	84.20 ± 9.22	80.17 ± 5.55	95.70 ± 7.85	106.08 ± 6.78	90.59 ± 14.91	97.84 ± 8.21	82.75 ± 3.17	86.66 ± 7.97
SV (LV Trace)	uL	23.36 ± 3.45	22.24 ± 3.19	24.05 ± 3.43	23.62 ± 3.26	22.69 ± 4.38	24.22 ± 1.46	22.73 ± 4.13	31.49 ± 4.82
V;d (LV Trace)	uL	24.16 ± 3.48	25.13 ± 3.13	25.74 ± 2.43	25.18 ± 2.13	24.09 ± 4.82	25.41 ± 1.66	23.72 ± 4.36	29.66 ± 2.82
V;s (LV Trace)	uL	1.81 ± 0.18	1.45 ± 0.40	1.69 ± 0.52	1.55 ± 0.28	1.40 ± 0.49	1.19 ± 0.31	1.00 ± 0.39	1.68 ± 0.14

Table S2.2: Ultrasound imaging one week prior and following nano-TiO₂ inhalation exposure in the Het and KO groups. Measurements were taken for at least three consecutive systolic and diastolic peaks and troughs, respectively, for each animal. Significance ($P \leq 0.05$) is denoted for a specific category through bold text and reference to the sample number that it is significantly different from. Sample numbers are listed in the column heading. Het = heterozygous for the miRNA-378a allele, KO = knockout for the miRNA-378a allele, Sham = control filtered air exposed, Ex = nano-TiO₂ exposed, Pre = one week prior to inhalation exposure, Post = 24 post inhalation exposure, CO = cardiac output, Diameter;d = diastolic diameter, Diameter;s = systolic diameter, EF = ejection fraction, FS = fractional shortening, LV Mass = left ventricular mass, LV Mass Corr = left ventricular mass corrected, SV = stroke volume, V;d = volume during diastole, V;s = volume during systole.

Table S2.3

Measures	Units	WT Sham ¹	WT Ex ²	Het Sham Pre ³	Het Sham Post ⁴	Het Ex Pre ⁵	Het Ex Post ⁶	KO Sham Pre ⁷	KO Sham Post ⁸	KO Ex Pre ⁹	KO Ex Post ¹⁰
Heart Rate	beats/min	538.22 ± 14.49	545.81 ± 9.99	506.19 ± 25.00	519.79 ± 21.03	548.25 ± 27.64	544.15 ± 24.07	498.40 ± 37.06	530.93 ± 10.26	531.20 ± 38.12	529.08 ± 11.34
Radial Velocity (Endo)	Pk cm/s	-2.05 ± 0.14	-2.21 ± 0.16	-2.01 ± 0.26	-2.16 ± 0.33	-2.37 ± 0.27	-2.13 ± 0.35	-1.61 ± 0.14	-2.20 ± 0.05	-2.24 ± 0.35	-2.05 ± 0.27
Radial Displacement (Endo)	Pk mm	0.00 ± 0.00	-0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	-0.01 ± 0.00	0.00 ± 0.00	-0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Radial Strain (Endo)	Pk %	-0.58 ± 0.27 _{3,4,5,6,7,8,9,10}	-0.48 ± 0.08 _{3,4,5,6,7,8,9,10}	-2.22 ± 1.13	-2.18 ± 0.41	-3.51 ± 1.24	-2.20 ± 0.42	-3.14 ± 2.10	-2.54 ± 0.38	-3.85 ± 1.93	-2.48 ± 0.34
Radial Strain Rate (Endo)	Pk 1/s	-10.54 ± 0.93	-14.36 ± 0.61	-14.98 ± 1.62	-19.81 ± 1.58 _{1,5,6,7,8,9,10}	-13.51 ± 1.53	-12.60 ± 1.69	-7.79 ± 0.94	-12.82 ± 1.21	-11.82 ± 1.76	-10.88 ± 1.02
Circumferential Velocity (Endo)	Pk deg/s	-289.64 ± 37.03	-397.82 ± 59.93	-327.13 ± 78.10	-393.17 ± 47.03	-416.00 ± 63.33	-319.16 ± 46.13	-399.62 ± 101.16	-484.66 ± 103.14	-415.47 ± 83.05	-384.98 ± 57.72
Circumferential Displacement (Endo)	Pk deg	-2.75 ± 0.62	-2.79 ± 0.74	-1.77 ± 0.53	-2.95 ± 1.12	-1.03 ± 0.53	-1.77 ± 0.51	-1.30 ± 0.50	-0.90 ± 0.05	-2.54 ± 0.64	-1.79 ± 0.99
Circumferential Strain (Endo)	Pk %	0.27 ± 0.06	0.31 ± 0.15	0.65 ± 0.43	0.86 ± 0.62	1.02 ± 1.42	0.80 ± 0.70	1.86 ± 1.41	2.11 ± 0.78 _{1,2,3,4,6}	2.24 ± 0.71 _{1,2,3,4,6}	1.50 ± 0.49 ₂
Circumferential Strain Rate (Endo)	Pk 1/s	-19.16 ± 2.11	-14.76 ± 0.76	25.97 ± 5.85	21.67 ± 4.04	32.99 ± 8.35	23.38 ± 8.35	14.75 ± 2.38	14.75 ± 2.38	23.39 ± 6.30	23.39 ± 6.30

Table S2.3: B-Mode images were used in the short axis to examine circumferential and radial stress-strain parameters during diastole. Ultrasound imaging one week prior (Het and KO groups) and following nano-TiO₂ inhalation exposure (WT, Het, and KO). Significance ($P \leq 0.05$) is denoted for a specific category through bold text and reference to the sample number that it is significantly different from. Sample numbers are listed in the column heading. WT = Wild type, Het = heterozygous for the miRNA-378a allele, KO = knockout for the miRNA-378a allele, Sham = control filtered air exposed, Ex = nano-TiO₂ exposed, Pre = one week prior to inhalation exposure, Post = 24 post inhalation exposure.

Table S2.4

Measures	Units	WT Sham ¹	WT Ex ²	Het Sham ³ Pre	Het Sham ⁴ Post	Het Ex ⁵ Pre	Het Ex ⁶ Post	KO Sham ⁷ Pre	KO Sham ⁸ Post	KO Ex ⁹ Pre	KO Ex ¹⁰ Post
Heart Rate	beats/min	538.22 ± 14.49	545.81 ± 9.99	506.19 ± 25.00	519.79 ± 21.03	548.25 ± 27.64	544.15 ± 24.07	498.40 ± 37.06	530.93 ± 10.26	531.20 ± 38.12	529.08 ± 11.34
Radial Velocity (Endo)	Pk cm/s	-1.31 ± 0.18	-1.19 ± 0.17	-1.45 ± 0.13	-1.33 ± 0.20	-1.29 ± 0.13	-1.19 ± 0.20	-0.99 ± 0.12	-1.24 ± 0.20	-1.33 ± 0.22	-0.90 ± 0.09
Radial Displacement (Endo)	Pk mm	-0.01 ± 0.01	-0.02 ± 0.01	-0.01 ± 0.01	-0.01 ± 0.01	-0.01 ± 0.00	-0.01 ± 0.00	-0.01 ± 0.00	0.00 ± 0.00	-0.01 ± 0.01	-0.01 ± 0.00
Radial Strain (Endo)	Pk %	-0.73 ± 0.32	-2.31 ± 0.30 1,3,4,5,6,7,8,9,10	-0.55 ± 0.22	-0.73 ± 0.27	-0.36 ± 0.22	-0.53 ± 0.24	-0.79 ± 0.34	-0.95 ± 0.27	-0.38 ± 0.26	-0.84 ± 0.28
Radial Strain Rate (Endo)	Pk 1/s	-6.52 ± 1.08	-6.30 ± 0.54	-7.34 ± 0.37	-8.85 ± 1.44	-6.56 ± 0.94	-6.76 ± 1.05	-5.03 ± 0.76	-7.15 ± 1.69	-6.68 ± 1.57	-5.90 ± 1.88
Longitudinal Velocity (Endo)	Pk deg/s	-0.81 ± 0.13	-0.77 ± 0.15	-0.71 ± 0.14	-0.75 ± 0.14	-0.55 ± 0.05	-0.66 ± 0.05	-0.92 ± 0.22	-0.68 ± 0.10	-0.77 ± 0.19	-0.64 ± 0.04
Longitudinal Displacement (Endo)	Pk deg	-0.01 ± 0.00	-0.02 ± 0.01	-0.02 ± 0.01	-0.02 ± 0.01	-0.03 ± 0.01	-0.05 ± 0.02	-0.01 ± 0.00	-0.02 ± 0.02	-0.02 ± 0.01	-0.05 ± 0.04
Longitudinal Strain (Endo)	Pk %	0.61 ± 0.20	1.10 ± 0.38	0.45 ± 0.20	1.08 ± 0.36	0.47 ± 0.13	0.59 ± 0.30	1.01 ± 0.30	1.04 ± 0.27	0.96 ± 0.96	0.59 ± 0.26
Longitudinal Strain Rate (Endo)	Pk 1/s	4.86 ± 0.57	2.68 ± 0.31 3,4,5	6.47 ± 0.74	7.19 ± 0.89	5.93 ± 0.38	3.72 ± 0.53 3,4	4.40 ± 0.23	5.13 ± 0.74	4.62 ± 0.80	4.07 ± 0.65

Table S2.4: B-Mode images were used in the long axis to examine circumferential and longitudinal stress-strain parameters during diastole. Ultrasound imaging one week prior (Het and KO groups) and following nano-TiO₂ inhalation exposure (WT, Het, and KO). Significance ($P \leq 0.05$) is denoted for a specific category through bold text and reference to the sample number that it is significantly different from. Sample numbers are listed in the column heading. WT = Wild type, Het = heterozygous for the miRNA-378a allele, KO = knockout for the miRNA-378a allele, Sham = control filtered air exposed, Ex = nano-TiO₂ exposed, Pre = one week prior to inhalation exposure, Post = 24 post inhalation exposure.

Table S2.5

Measures	Units	WT Sham ¹	WT Ex ²	Het Sham Pre ³	Het Sham Post ⁴	Het Ex Pre ⁵	Het Ex Post ⁶	KO Sham Pre ⁷	KO Sham Post ⁸	KO Ex Pre ⁹	KO Ex Post ¹⁰
Heart Rate	beats/min	538.22 ± 14.49	545.81 ± 9.99	506.19 ± 25.00	519.79 ± 21.03	548.25 ± 27.64	544.15 ± 24.07	498.40 ± 37.06	530.93 ± 10.26	531.20 ± 38.12	529.08 ± 11.34
Radial Velocity (Endo)	Pk cm/s	2.38 ± 0.21	1.97 ± 0.06	1.94 ± 0.20	2.22 ± 0.35	2.46 ± 0.21	1.90 ± 0.21	1.78 ± 0.36	2.66 ± 0.11	2.26 ± 0.43	2.22 ± 0.19
Radial Displacement (Endo)	Pk mm	0.77 ± 0.07	0.70 ± 0.03	0.58 ± 0.03	0.63 ± 0.09	0.63 ± 0.03	0.54 ± 0.06	0.50 ± 0.05	0.58 ± 0.04	0.51 ± 0.06	0.57 ± 0.03
Radial Strain (Endo)	Pk %	42.39 ± 4.11	35.24 ± 1.97	41.95 ± 2.73	47.09 ± 1.85 _{1,2,6}	40.55 ± 3.11	27.97 ± 3.31	38.04 ± 2.84	40.12 ± 4.68	39.62 ± 4.96	37.04 ± 2.28
Radial Strain Rate (Endo)	Pk 1/s	11.87 ± 1.13	8.92 ± 0.44 _{1,4,5}	12.15 ± 1.24	12.82 ± 1.08	14.18 ± 1.61	9.35 ± 0.99	11.01 ± 2.69	12.88 ± 2.18	12.38 ± 2.25	11.09 ± 1.67
Circumferential Velocity (Endo)	Pk deg/s	387.12 ± 94.80	310.20 ± 71.63	321.54 ± 55.53	461.01 ± 34.60	635.88 ± 159.07	337.21 ± 61.29	452.42 ± 103.81	476.84 ± 22.23	452.37 ± 105.52	451.66 ± 81.90
Circumferential Displacement (Endo)	Pk deg	4.78 ± 1.92	3.61 ± 1.67	5.94 ± 0.70	7.80 ± 1.01	9.38 ± 3.77	5.36 ± 1.07	6.27 ± 1.61	6.50 ± 1.24	5.81 ± 1.60	6.58 ± 2.08
Circumferential Strain (Endo)	Pk %	-48.51 ± 3.88	-40.36 ± 3.38	-37.61 ± 3.75	-43.61 ± 3.61	-42.24 ± 2.62	-28.78 ± 2.63 _{1,4,5}	-34.46 ± 4.31	-38.65 ± 3.48	-36.35 ± 5.39	-39.32 ± 3.57
Circumferential Strain Rate (Endo)	Pk 1/s	-29.02 ± 3.20	-22.37 ± 1.15	-22.73 ± 4.42	-27.44 ± 6.30	-27.83 ± 5.95	-23.14 ± 4.99	-19.65 ± 6.55	-33.70 ± 3.90	-28.86 ± 11.48	-28.29 ± 4.83

Table S2.5: B-Mode images were used in the short axis to examine circumferential and radial stress-strain parameters during systole. Ultrasound imaging one week prior (Het and KO groups) and following nano-TiO₂ inhalation exposure (WT, Het, and KO). Significance ($P \leq 0.05$) is denoted for a specific category through bold text and reference to the sample number that it is significantly different from. Sample numbers are listed in the column heading. WT = Wild type, Het = heterozygous for the miRNA-378a allele, KO = knockout for the miRNA-378a allele, Sham = control filtered air exposed, Ex = nano-TiO₂ exposed, Pre = one week prior to inhalation exposure, Post = 24 post inhalation exposure.

Table S2.6

Measures	Units	WT Sham ¹	WT Ex ²	Het Sham ³ Pre	Het Sham ⁴ Post	Het Ex ⁵ Pre	Het Ex ⁶ Post	KO Sham ⁷ Pre	KO Sham ⁸ Post	KO Ex ⁹ Pre	KO Ex ¹⁰ Post
Heart Rate	beats/min	538.22 ± 14.49	545.81 ± 9.99	506.19 ± 25.00	526.40 ± 24.25	548.25 ± 27.64	544.15 ± 24.24	498.40 ± 37.06	530.93 ± 10.26	531.20 ± 38.12	529.08 ± 11.34
Radial Velocity (Endo)	Pk cm/s	1.31 ± 0.12	1.25 ± 0.11	1.14 ± 0.08	1.09 ± 0.14	1.18 ± 0.09	1.14 ± 0.13	0.96 ± 0.17	1.03 ± 0.11	1.22 ± 0.21	0.91 ± 0.08
Radial Displacement (Endo)	Pk mm	0.42 ± 0.06	0.37 ± 0.04	0.43 ± 0.02	0.35 ± 0.04	0.37 ± 0.02	0.34 ± 0.04	0.32 ± 0.04	0.35 ± 0.04	0.38 ± 0.04	0.28 ± 0.02
Radial Strain (Endo)	Pk %	18.65 ± 2.94	14.92 ± 3.37	21.46 ± 1.16	16.89 ± 1.25	19.51 ± 1.95	18.46 ± 0.86	13.97 ± 2.69	14.56 ± 0.77	14.87 ± 2.24	13.14 ± 1.12₃
Radial Strain Rate (Endo)	Pk 1/s	5.36 ± 0.60	3.79 ± 0.24	4.65 ± 0.34	6.82 ± 1.46	5.21 ± 0.53	5.27 ± 0.54	3.43 ± 0.51	4.37 ± 0.26	4.23 ± 0.26	3.54 ± 0.35
Longitudinal Velocity (Endo)	Pk deg/s	0.90 ± 0.06	0.53 ± 0.08	0.58 ± 0.08	0.63 ± 0.15	0.61 ± 0.05	0.73 ± 0.19	0.70 ± 0.21	0.67 ± 0.09	0.74 ± 0.25	0.60 ± 0.13
Longitudinal Displacement (Endo)	Pk deg	0.23 ± 0.02	0.17 ± 0.04	0.12 ± 0.03	0.11 ± 0.04	0.10 ± 0.02	0.07 ± 0.01	0.20 ± 0.07	0.10 ± 0.04	0.16 ± 0.08	0.10 ± 0.03
Longitudinal Strain (Endo)	Pk %	-10.58 ± 1.74	-7.10 ± 0.74₃	-11.55 ± 0.59	-9.41 ± 1.31	-9.45 ± 0.69	-8.02 ± 0.66	-8.46 ± 1.78	-9.55 ± 0.60	-9.11 ± 1.39	-8.80 ± 0.61
Longitudinal Strain Rate (Endo)	Pk 1/s	-3.90 ± 0.42	-3.09 ± 0.23	-4.13 ± 0.23	-4.73 ± 0.75	-4.02 ± 0.37	-3.36 ± 0.33	-3.61 ± 0.45	-3.91 ± 0.29	-3.84 ± 0.58	-3.57 ± 0.50

Table S2.6: B-Mode images were used in the long axis to examine circumferential and longitudinal stress-strain parameters during systole. Ultrasound imaging one week prior (Het and KO groups) and following nano-TiO₂ inhalation exposure (WT, Het, and KO). Significance ($P \leq 0.05$) is denoted for a specific category through bold text and reference to the sample number that it is significantly different from. Sample numbers are listed in the column heading. WT = Wild type, Het = heterozygous for the miRNA-378a allele, KO = knockout for the miRNA-378a allele, Sham = control filtered air exposed, Ex = nano-TiO₂ exposed, Pre = one week prior to inhalation exposure, Post = 24 post inhalation exposure.

Figure S2.1

Figure S2.1: The genomic loci of Ppargc1b. **(A)** Total exonic and intronic region of Ppargc1b, with miRNA-378a being transcribed in the first intron. **(B)** Magnified view of the first intron of Ppargc1b. Image was taken from the University of Santa Cruz (UCSC) genome browser from the mouse GRCm38/mm10 assembly. Ppargc1b = Peroxisome proliferator-activated receptor gamma coactivator 1-beta.

Figure S2.2

Figure S2.2

Figure S2.2: Expression of miRNA-378a in other tissues and transgenic animals. **(A)** qPCR of Ppargc1b (PGC-1 β), the miRNA-378a host gene, following nano-TiO₂ exposure. **(B)** qPCR in the heart, lung, and liver for miRNA-378a-3p and miRNA-378a-5p. Using the transgenic animals **(C)** The relative abundance of miRNA-378a-3p versus miRNA-378a-5p was also verified. With the knockdown and knockout transgenic animals, **(D)** qPCR confirmed miRNA-378a-3p and miRNA-378a-5p expression in the whole heart tissue and isolated mitochondria. Groups are considered significantly different if $P \leq 0.05 = *$. All data are presented as the mean $\pm$ standard error of the mean (SEM). WT = wild-type, Het = heterozygous for the miRNA-378a allele, KO = knockout for the miRNA-378a allele, Sham = control filtered air exposed, Ex = nano-TiO₂ exposed, Ppargc1b or PGC-1 β = peroxisome proliferator-activated receptor gamma coactivator 1-beta.

Figure S2.3

Figure S2.3

B

Figure S2.3: Bioenergetic function of cardiac mitochondria following nano-TiO₂ inhalation exposure in the WT. **(A)** Electron transport chain complex activities were measured for complexes I, III, IV, and V in the WT. **(B)** Mitochondrial State 3 and State 4 respiration were measured through glucose mediated pathways and fatty acid mediated pathways in the WT. Groups are considered significantly different if $P \leq 0.05 = *$. All data are presented as the mean $\pm$ standard error of the mean (SEM). WT = Wild type, Sham = control filtered air exposed, Ex = nano-TiO₂ exposed, P-Carnitine = palmitoyl carnitine.

Figure S2.4

Figure S2.4

Figure S2.4: Expression of miRNA-378a and its bioenergetic effects on other tissues. **(A)** qPCR was implemented to assess expression of miRNA-378a-3p and miRNA-378a-5p in the lung and liver. **(B)** Electron transport chain complex activities were measured for complexes I, III, IV, and V in the lung and liver for the Het and KO groups. Groups are considered significantly different if $P \leq 0.05 = *$. All data are presented as the mean $\pm$ standard error of the mean (SEM). WT = Wild type, Het = heterozygous for the miRNA-378a allele, KO = knockout for the miRNA-378a allele, Sham = control filtered air exposed, Ex = nano-TiO₂ exposed.

Figure S2.5

Figure S2.5

Figure S2.5: Mitochondrial structure and function and the regulation of the *Ppargc1b* host gene. (A) Flow cytometry Sphero AccuCount Blank Particles, 2.0 μm sizing beads and MitoTracker™ Deep Red FM/633 staining allowed for measurements of mitochondrial size and structure. (B) Citrate synthase activity was used to measure mitochondrial number/content on isolated mitochondria. Flow cytometry assessed mitochondria through (C) internal complexity and (D) membrane potential implementing MitoTracker™ Deep Red FM/633 and JC-1 staining, respectively. (E) Cardiac tissue was assessed for H_2O_2 concentration, normalized to mitochondrial number. Groups are considered significantly different if $P \leq 0.05 = *$. All data are presented as the mean $\pm$ standard error of the mean (SEM). FSC PMT-H = forward scatter, SSC-H = side scatter, WT = Wild type, Het = heterozygous for the miRNA-378a allele, KO = knockout for the miRNA-378a allele, Sham = control filtered air exposed, Ex = nano-TiO₂ exposed.

Figure S2.6

A

Position 356-363 of MED19 3' UTR
mmu-miR-378a-3p

Predicted consequential pairing of target region (top) and miRNA (bottom)	Site type	Context++ score
5' ...CUCCAGAACCGACAU--AGUCCAGA... 3' GGAAGACUGAGGUUCAGGUCA	8mer	-0.49

	..350.....360.....370
Mouse	accgacauaguccagaccacccaggu
Human	aucgacauaguccagaccacccaggu
Chimp	aucgacauaguccagaccacccaggu
Rhesus	accggcrauaguccagaccacccaggu
Squirrel	accgacauaguccagaccauccaggu
Rat	accgacauaguccagaccacccgggu
Rabbit	gccgacacaguccagaccacccgggu

GGAAGACUGAGGU UCAGGUC A miRNA-378a-3p

Position 1335-1342 of MFN1 3' UTR
mmu-miR-378a-3p

Predicted consequential pairing of target region (top) and miRNA (bottom)	Site type	Context++ score
5' ...CAAGCCAGUAUCUGUAGUCCAGA... 3' GGAAGACUGAGGUUCAGGUCA	8mer	-0.29

	..620.....630.....640
Mouse	..-GAGUCCAG-CAAGG-AGAGCUUUUCU
Human	..-GAUUCCAA-UAAGG-AACGCUUUCCU
Chimp	..-GAUUCCAA-UAAGG-AACGCUUUCCU
Rhesus	..-GAUUACAA-UAAGG-AAAGCUUUCCU
Squirrel	..-GAGUCCAG-UAAGG-AAAGCUUUUCU
Rat	..-GAGUCCUG-CGAGG-AGAACUUCUCU
Rabbit	..-GCUUCCUG-UAAGG-AGAGCUUUUCU

GGAAGACUGAGGU UCAGGUC A miRNA-378a-3p

Figure S2.6

Figure S2.6: Seed sequence homology in the 3'UTR of Med19 and Mfn1. Through TargetScan, miRNA-378a-3p was shown to bind the 3' UTR of (A) Med19 and Mfn1 with seed sequence homology. "Position" refers to the location in the 3'UTR region that binding occurs. The "Site Type" refers to the specific binding of the miRNA to the mRNA strand by the number of bases that share continued homology. The "Context Score" (0 to -0.99) is an index of the confidence of binding, with a more negative score instilling higher confidence. (B) Immunoblotting of additional proteins involved in mitochondrial fusion (Opa1) and fatty acid metabolism (Ppara). (C) Increased transcription of the miRNA-378a host gene, Ppargc1b, occurs following inhalation exposure. (D) Chromatin Immunoprecipitation (ChIP) Sequencing of H3K4me3 in gestational nano-TiO₂ exposed progeny revealed increased H3K4me3 association at the Ppargc1b promoter loci, suggesting increased epigenetic regulation and subsequent expression of the gene. Groups are considered significantly different if $P \leq 0.05 = *$. All data are presented as the mean $\pm$ standard error of the mean (SEM). P -adjusted values = P values derived from DESeq2 and calculated with the False Discovery Rate (FDR) = 0.05. Groups are considered significantly different if $P_{adj} \leq 0.05 = *$. Mfn1 = Mitofusin 1, Med19 = mediator complex subunit 19, 3' UTR = 3' untranslated region, WT = Wild type, Het = heterozygous for the miRNA-378a allele, KO = knockout for the miRNA-378a allele, Sham = control filtered air exposed, Ex = nano-TiO₂ exposed, Ppargc1b = Peroxisome proliferator-activated receptor gamma coactivator 1-beta, H3K4me3 = Histone 3 lysine 3 trimethylation.

Chapter 3: Specific Aim 2

Machine-Learning to Stratify Diabetic Patients using Novel Cardiac

Biomarkers and Integrative Genomics

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Abstract

Background – Diabetes mellitus is a chronic disease that impacts an increasing percentage of people each year. Among its comorbidities, diabetics are two to four times more likely to develop cardiovascular diseases. While HbA1c remains the primary diagnostic for diabetics, its ability to predict long-term, health outcomes across diverse demographics, ethnic groups, and at a personalized level are limited. The purpose of this study was to provide a model for precision medicine through the implementation of machine learning algorithms using multiple cardiac biomarkers as a means for predicting diabetes mellitus development.

Methods – Right atrial appendages from 50 patients, 30 non-diabetic and 20 type 2 diabetic, were procured from the WVU Ruby Memorial Hospital. Machine-learning was applied to physiological, biochemical, and sequencing data for each patient. Supervised learning implementing SHapley Additive exPlanations (SHAP) allowed binary (no diabetes or type 2 diabetes) and multiple classification (no diabetes, prediabetes, and type 2 diabetes) of the patient cohort with and without the inclusion of HbA1c levels. Findings were validated through Logistic Regression (LR), Linear Discriminant Analysis (LDA), Gaussian Naïve Bayes (NB), Support Vector Machine (SVM), and Classification and Regression Tree (CART) models with 10-fold cross validation.

Results – Total nuclear methylation and hydroxymethylation were highly correlated to diabetic status, with nuclear methylation and mitochondrial electron transport chain (ETC) activities achieving superior testing accuracies in the predictive model (~84% testing, binary). Mitochondrial DNA SNPs found in the D-Loop region (SNP-73G, -16126C, and -16362C) were highly associated with diabetes mellitus. The CpG island of transcription factor A, mitochondrial (TFAM) revealed CpG24 (chr10:58385262, $P = 0.003$) and CpG29 (chr10:58385324, $P = 0.001$) as markers correlating with diabetic progression. When combining the most predictive factors from each set, total nuclear methylation and CpG24 methylation were the best diagnostic measures in both binary and multiple classification sets.

Conclusions – Using machine-learning, we were able to identify novel as well as the most relevant biomarkers associated with type 2 diabetes mellitus by integrating physiological, biochemical, and sequencing datasets. Ultimately, this approach may be used as a guideline for future investigations into disease pathogenesis and novel biomarker discovery.

Keywords: Epigenetics, Mitochondria, Heart, Machine-Learning, CART, SHAP

Background

A disconnect continues to persist in the diagnosis and pathogenesis of diabetes-induced cardiovascular dysfunction. While diabetics are at a two to four fold greater risk of developing cardiovascular diseases (7, 22), understanding how the numerous biochemical markers involved in the pathology integrate and influence disease progression has not been fully explicated. In a clinical setting, the ability to better calculate prognostics of a patient's health through the integration of biomarkers facilitates the potential for developing personalized and generalized Medicine, as well as treatment strategies (50). While glycated hemoglobin (HbA1c) remains a hallmark for disease diagnosis (8), other biomarkers may exist that more unequivocally define the severity of the pathology, characterize the mechanisms involved, and/or provide a better predictive tool of future cardiovascular events.

Diabetes mellitus is a multi-faceted disease, consisting of systemic comorbidities which necessitate a variety of treatment modalities and stratify those affected with the disease (12). Before the implementation of machine-learning algorithms in Medicine, linear statistical models have highlighted measures, such as HbA1c, as diagnostic staples for the evaluation of diabetes mellitus onset and progression (40). By exploring these previously published metadata sets, machine-learning has been applied in refining the accuracy of biomarkers used to characterize the pathology as well as to highlight vulnerable populations in need of clinical intervention (27). Machine-learning has also revealed that coupling HbA1c with additional biomarkers, such as 8-hydroxy-2-deoxyguanosine (8-OHdG) and other metabolites, can increase the accuracy of the predictive model and better characterize the severity of the disease (26).

In cardiology, machine-learning approaches have been applied primarily to imaging-based diagnostics, including echocardiography and computed tomography angiography to evaluate cardiovascular health and outcomes (1, 59). It is estimated that machine-learning applications in the field of cardiovascular research will continue to grow at an exponential rate (51). While image-derived deep learning models are increasing in popularity, little is known about the predictive power of machine-learning models on basic genomic, epigenomic, proteomic, and metabolomic profiles of the heart. While the beginning of the "big data" age was characterized by the accumulation and compartmentalization of datasets, the recent advent of combining metadata, deep sequencing, and "omics"-based approaches warrants the union between hierarchical

predictive algorithms and biological processes. As more patients have access to their personal “omics” profiles, machine-learning will be instrumental in providing feedback for the individual patient and the general population of patients impacted by the disease, enhancing health practice by the caregiver.

While demographic information and physical examination data are more easily procured from patients, the genomic and transcriptomic characterization of a tissue or cell type provides a much finer granularity and uniqueness when predicting phenotypic outcomes in patients (36). HbA1c, fasting blood glucose, and BMI are examples of easily accessible, valuable measurements when evaluating diabetes progression and onset (40, 43), but genetic components, including epigenetic, epitranscriptomic, single nucleotide polymorphisms (SNPs), and others, provide a wealth of undiscovered information for disease classification. This genetic component may be specifically important when understanding the pathogenesis of diabetes in ethnic groups, when BMI (2, 44) and HbA1c (14) show distinct differences between ethnicities. Though applying patient-matched, genomic information is currently unrealistic for disease diagnosis, it may hold the key for revealing commonalities across ethnic and demographic groups when classifying diabetic onset, progression, and severity.

In the current study, machine-learning was used as a predictive tool to integrate cardiac physiological, biochemical, genomic, and epigenomic biomarker data in a patient-matched fashion and enable determination of type 2 diabetic status. In 50 patients, machine-learning algorithms revealed the interconnectedness between diabetic classification, mitochondrial function, and methylation status. Our study highlights how novel biomarkers can be used to augment existing diagnostic standards as well as provide new, and more precise, methods for identifying the development and severity of type 2 diabetes mellitus in potentially at-risk populations, such as those with prediabetes. While we examine physiological, biochemical, and molecular datasets using machine-learning algorithms, our goal was to understand which features possessed the best predictive accuracies and if these specific features could be used alone, or in conjunction, with HbA1c. The purpose for the inclusion of models that do not rise above 50% predictive accuracy was to contrast them against those models that do rise above 50% in the absence of HbA1c, to determine which biomarkers are the best overall predictors.

Research Design and Methods

Study Approval

All tissue and patient information was acquired in a double de-identified fashion, and was approved by the West Virginia University Institutional Review Board and Institutional Biosafety Committee (19). Patients were all consented by the Heart and Vascular Institute, J.W. Ruby Memorial Hospital at the West Virginia University School of Medicine. Right atrial appendages were removed during open-heart and/or valvular surgeries. Both groups of patients (non-diabetic and type 2 diabetic) who were receiving open-heart surgery could elect for their tissues to be used for research purposes, with no direct or indirect incentivisation. A total of fifty patients were selected for the study (between August 2016 and May 2018), thirty of which were non-diabetic (ND) and twenty that were type 2 diabetic (T2DM) and existed along a spectrum of measured HbA1c levels. Patient inclusion into the study was determined by (a) election for open heart surgery and release of tissue for research purposes, (b) was not undergoing surgery due to heart failure, and (c) demographic data was provided. Patient characteristics are provided in Table 3.1, listing patients classified as non-diabetic and those who have been clinically diagnosed as type 2 diabetic. Patient information is also provided for separation of the groups into those with no diabetes, prediabetes, and type 2 diabetes (Table S3.1). Table S3.1 contains the matching fifty patient cohort analyzed in Table 3.1 but with the creation of a new non-diabetic cohort ($n = 16$), comprised of those individuals with an $\text{HbA1c} < 5.7$, and prediabetic cohort ($n = 14$), comprised of clinically non-diabetic patients whose HbA1c is between 5.7 and 6.4.

Mitochondrial Isolation

Mitochondria were isolated from a portion of the right atrial appendage as previously described (45), with modifications by our laboratory (4, 5, 20). Mitochondrial subpopulations of subsarcolemmal and interfibrillar mitochondria were extracted and combined to achieve a total mitochondrial population.

Electron Transport Chain (ETC) Complex Activities

A portion of the tissue from all 50 patients was homogenized using a Polytron PowerGen 500 S1 tissue homogenizer (Fisher Scientific, Hampton, NH) in NP-40 buffer (150 mM NaCl, 50 mM, pH 8.0 Tris-Cl, and 1.0% NP-40). Protein homogenates were used to measure electron transport chain complexes I, III, IV, and V (ATP synthase) spectrophotometrically, as previously described (3) and implemented by our laboratory (4, 23, 24, 52, 53). Protein concentrations were normalized using the Bradford method, with standardization to bovine serum albumin (9).

Citrate Synthase Activity

Isolated mitochondria from all 50 patients, was used to measure citrate synthase activity using a colorimetric Citrate Synthase Assay Kit (Sciencell, San Diego, CA), as previously described (25). Citrate synthase activity, normalized to protein content, was used to determine mitochondrial content.

Methyltransferase

Using a colorimetric Methyltransferase Assay Kit (Caymen, Ann Arbor, Michigan), S-adenosylmethionine (SAM)-dependent methyltransferase activity was assessed, per manufacturer's instructions. Briefly, nuclear homogenates were used to assess total SAM-dependent methyltransferase activity in all 50 patients.

DNA 5mC and 5hmC Quantification

Using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany), DNA was isolated from both 10 mg of atrial appendage tissue and mitochondria, per manufacturer's instructions. Levels of 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC) were measured through a 5mC and 5hmC ELISA Kit (Zymo Research, Irvine, CA), per manufacturer's instructions. DNA was quantified using a NanoDrop™ 1000 Spectrophotometer (Thermo Fisher, Waltham, MA). 100 ng of nuclear (tissue extract) and mitochondrial DNA were used to assess total 5mC and 5hmC content spectrophotometrically for all 50 patients.

Western Blotting

Using 4 – 12% gradient gels, immunoblotting was performed through MES SDS-PAGE, as previously described (18, 20, 23, 42, 58). Protein was normalized using the Bradford method. Primary antibodies used in the study included: anti-TFAM, transcription factor A, mitochondrial, 1:500 (SCBT, Dallas, TX), anti-GAPDH 1:1,000 (Abcam, Cambridge, MA). The secondary antibody used in the study was a goat anti-mouse IgG (H&L) horseradish peroxidase (HRP) conjugate 1:10,000 (Thermo Fisher). GAPDH expression was used to normalize protein content. Chemiluminescence was measured through Radiance Chemiluminescent Substrate (Azure Biosystems, Dublin, CA), per manufacturer's instructions and imaged using the G:Box Bioimaging system (Syngene, Frederick, MD). Images were captured through GeneSnap/GeneTools software (Syngene). Densitometry was analyzed using ImageJ and Fiji Software (NIH, Bethesda, MD). Data is represented as optical density with arbitrary units.

Chromatin Immunoprecipitation (ChIP) – qPCR

The SimpleChIP® Plus Sonication Chromatin IP Kit (Cell Signaling Technology, Danvers, MA) was used, per manufacturer's instructions. Briefly, 100 mg of atrial tissue was minced into ~2 mm³ pieces and treated with freshly prepared 37% formaldehyde for 30 minutes. Sonicated DNA was assessed for sheering and further immunoprecipitated with anti-TFAM (SCBT) bound Protein G magnetic beads overnight at 4°C. The beads were washed, DNA reverse cross-linked, and DNA eluted as previously described (52, 56). DNA bound to TFAM was further examined through qPCR. 2% chromatin inputs for each sample were used for normalization of expression. An Applied Biosystems 7900HT Fast Real-Time PCR system (Applied Biosystems, Foster City, CA) was used to assess expression through SYBR Green. Quantification was achieved using the $2^{-\Delta\Delta CT}$ method (49). All primer pairs to assess the mitochondrial D-Loop are provided (Table S3.2).

Overhang-Bisulfite Sequencing

DNA was extracted from patient tissue as described above. DNA was bisulfite-treated using the EZ DNA Methylation-Lightning Kit (Zymo Research), per manufacturer's instructions. Primers were designed for the CpG island of TFAM; primer set 1 amplified the 3' end and primer set 2 amplified the 5' end of the CpG island (Table S3.2). Bisulfite DNA was prepared for sequencing using a 2-Step PCR amplification process (6). Step-1 PCR adapters included a base pairing region (~23 bp) and an overhang Illumina adapter arm (~33 bp). Bisulfite DNA was PCR amplified using Step-1 primers utilizing Platinum™ Taq DNA Polymerase (Thermo Fisher), run on 2% agarose gels, extracted through a QIAquick Gel Extraction Kit (Qiagen), and DNA purified. DNA was then further amplified using Step-2 Illumina barcoded adapters and prepared using a 300-cycle MiSeq Reagent Micro Kit v2 (Illumina, San Diego, CA). PCR amplicons were sequenced on the MiSeq with paired-end (PE) 250 base pair reads. Files were aligned to the bisulfite converted reference genome GRCh38 release 94 implementing Bismark (31, 32). Alignment was obtained through Bismark using the Bowtie2 (33) engine using “non-directional” and “paired-end.” Complete sequencing code is provided (https://github.com/qahathaway/WVU_Machine-Learning-50/tree/master).

Mitochondrial SNP Sequencing

Mitochondrial DNA was isolated as described above and further amplified using the REPLI-g Mitochondrial DNA Kit (Qiagen), per manufacturer's instructions. Libraries for amplified DNA were prepared using the MiSeq Reagent Kit v3 (Illumina). Mitochondrial DNA was sequenced on the MiSeq with paired-end (PE) 300 base pair reads. Files were aligned to the reference genome GRCh38 release 94 through Bowtie2 using “sensitive-local.” BAM files were sorted, run through variant calling, and single-nucleotide polymorphisms (SNPs) were identified in the mitochondria through SAMtools (21, 34, 35). Complete sequencing code is provided (https://github.com/qahathaway/WVU_Machine-Learning-50/tree/master).

Machine-Learning Algorithms

Decision tree classifier algorithms were created in python (v3.6.5) using *scikit-learn* (46) and *pandas* (41) libraries (Figure 3.1A). In binary classification, patient labels were determined

through previous clinical diagnoses, where diabetic status was retained even if current HbA1c levels were below 6.5%. In multiple classification, patients with an HbA1c value in the range of 5.7% to 6.4% were designated as having prediabetes. Due to this, the HbA1c feature was excluded from all tested trials, and the derived accuracies are in comparison to that of the “perfect” accuracy obtained from HbA1c classification. A decision tree classifier model was created using the functions of *scikit-learn tree*. The data file was split into 80% training and 20% testing partitions using a defined seed value. Different seeds were chosen for each set to maintain the training and testing set distributions. Selected seed values maintained a binary classification testing set of 5 diabetics and 5 non-diabetics. In the multi-classification testing set, seed values maintained a distribution of 3 diabetics, 3 non-diabetics, and 4 pre-diabetics. Seeds were only chosen such that the resulting training and testing accuracies were similar; ensuring that the created classification tree did not over fit to the small sample size and remained generalizable to future testing samples.

Within the model, 10-fold cross validation was implemented. CART analysis was then performed on each of the datasets using the *scikit-learn* model, and the features of importance extracted using the *feature importance* parameter. The physiological/biochemical, genomic, and epigenomic datasets were combined into a single file to serve as the “all features” dataset used to extract the best and most influential biomarkers. For each trial, selected combinations of biomarkers from each dataset were utilized, and within each trial CART analysis was performed five times. After each of the five iterations, average feature importance, average training, and average testing accuracies were obtained. Standard deviations were taken over each of the five iterations per trial. For each dataset, the first iteration of CART analysis included all biomarkers of each set. If the average feature importance was less than 1%, the feature was no longer included in subsequent trials. After all iterations, if the average feature importance was less than or equal to 8% it was assigned to a category titled “other.” These same trials were repeated with other default *scikit-learn* machine-learning frameworks (Logistic Regression, Linear Discriminant Analysis, K-Nearest Neighbors Classifier, Gaussian Naïve Bayes, and Support Vector Machine). 10-fold cross validation and the same seed parameters were used in analysis of these 5 models and the resulting training and testing accuracies are provided (Tables S3.3-S3.10). The 6 models tested include few modifications and utilize no additional regularization techniques aside from those default to the *scikit-learn* library models. Only the Support Vector Machine model received modifications, with the probability parameter set to “true” to provide probability estimates for each data point and a

linear kernel used over the default Radial Basis Function ‘rbf’ kernel. Code for analyses is also provided (https://github.com/qahathaway/WVU_Machine-Learning-50/tree/master).

Machine-Learning Feature Extraction and Accuracy

To determine which model(s) would provide the most accurate predictions on the current data, we assessed the 345 total features across all 6 models in binary (Table 3.2) and multiple (Table 3.3) classification of diabetic status. Through assessment of individual datasets (i.e. physiological/biochemical, genetic, and epigenetic), a set of 18 features was further used to classify diabetic status in binary (Table S3.11) and multiple (Table S3.12) classification. Model analysis was enacted for each dataset, and the established 10-fold cross validation and seed parameters for binary and multi-classification were utilized. Each dataset was tested five times per model. Averages were obtained for training accuracy, training standard deviation, testing accuracy, testing standard deviation, model average F1 score, and area under the curve (AUC). AUC values were provided for each of the 6 tested algorithms for the testing data under binary classification using the *roc_auc_score* function of *scikit-learn*, but not for multiple classification. AUC was determined as 1.0 for LR and SVM models when evaluating the 345 total features due to the large sample size of biomarkers. From the available 345 features, 2 predictors were chosen that perfectly distinguished diabetic and nondiabetic status for this particular dataset. As such, these AUC values were removed from Table 3.2 for LR and SVM, as this was not an accurate indicator of the model’s predictive ability. As the feature set was restricted to the 18 “best” features from each dataset, AUC values of 1.0 were no longer observed (Tables S3.11 and S3.12).

Extracted important features and corresponding feature bias within each model, with the exception of KNN, were determined and are provided for binary and multiple classification. CART feature importance was extracted from the trained model using the *feature importance* parameter. For the NB model, feature influence was determined using the *predict_log_proba* function to return the most important biomarker per class in both binary (0,1) and multiple (0,1,2) classification schemes. Feature importance was not determined for the KNN model due to the restrictions of the default model. LDA, LR, and SVM feature influence was determined by taking the magnitude of the model coefficients, *coef_* parameter, times the standard deviation of that biomarker in the testing data. The resulting values are ranked based off magnitude and are reported

with sign under the “Important Feature Bias” (Tables 3.2 and 3.3, Tables S3.11 and S3.12). A positive influence value indicates a biomarker favoring classification towards one label while a negative value favors the opposite classification label. The larger the magnitude, the more strongly that feature shifts classification.

SHapley Additive exPlanations (SHAP)

SHAP framework, from slundberg (<https://github.com/slundberg/shap>), was used to visually explain the classification trees developed for the 50 patient samples using an XGBoost model (Figure 3.1B) (16, 38, 39). Figure 3.1B illustrates how SHAP plots are presented in three dimensions, with the “X” and “Y” dimensions plotted spatially while the “Z” dimension is indicated only through color; allowing for the examination of how variables, “X” and “Z”, can influence the nature of the model and classification of patients, “Y”, into non-diabetic and diabetic cohorts. Through Jupyter Notebook (v0.34.9) (30) with a Python 3 kernel and importing *pandas*, *shap*, and *xgboost* libraries, SHAP plots were derived. Delineation of binary and multiple classification systems are defined above. The entire 50 patient population was utilized during training of the XGBoost model and both the patient biomarker values and patient labels were provided during training. The XGBoost and SHAP tree explainer utilized were left unaltered. The number of influential features shown in the summary plot were selected using the *max display* parameter. Plot generation utilized *force plot*, *dependency plot*, and *summary plot* SHAP functions. Force plots depict the effect of biomarker values on the model’s output. Dependency plots relate specific biomarker values to model prediction and show how the chosen biomarker depends on other tested biomarkers. Summary plots depict the top influential biomarkers and how they influence the model prediction. Code for analyses is also provided (https://github.com/qahathaway/WVU_Machine-Learning-50/tree/master)

Statistics

Significance was determined using a two-tailed Student’s t-test or one-way analysis of variance (ANOVA), where appropriate. Tukey’s multiple comparisons test was implemented following the ANOVA to derive significance between multiple groups. Differences between

groups were considered statistically different if $P \leq 0.05$, denoted by * if statistically different from non-diabetic or # if statistically different from pre-diabetic. All data are presented as the mean $\pm$ standard error of the mean (SEM).

Data Availability

Mitochondrial DNA-Seq: Sequence Read Archive PRJNA520920

<https://dataview.ncbi.nlm.nih.gov/?search=SUB5124294>

TFAM Promoter Methylation Amplicon-Seq: Sequence Read Archive PRJNA520920

<https://dataview.ncbi.nlm.nih.gov/?search=SUB5125264>

Bioinformatics and Machine-learning Scripts: Github

https://github.com/qahathaway/WVU_Machine-Learning-50/tree/master

Results

Alterations to the interaction networks that exist between the nucleus and mitochondrion play a significant role in the development of diabetic cardiomyopathy (11, 17, 47, 60). As a result, we wanted to determine how observed changes in these parameters could predict diabetic status using machine-learning algorithms. All of the machine-learning algorithms in this study implemented to draw conclusions were constructed around tree ensembles, such as Classification and Regression Trees (CART). CART algorithms proved to have the overall highest testing and training accuracies when compared to other models (Tables S3.3-S3.10), while also performing superiorly in multiple classification of prediabetes (Tables 3.2-3.3). When examining the testing, training, and area under the curve (AUC) values that depict model performance, CART performed consistently at, or near, the top of the six models in both the binary (Table 3.2) and multiple (Table 3.3) classification sets when assessing all 345 features. SHapley Additive exPlanations (SHAP) which implement CART functions were used to provide binary (non-diabetic or type 2 diabetic) as well as multiple (non-diabetic, prediabetic, and type 2 diabetic) classification analyses. SHAP analysis maps a particular biomarker's numeric values to a computationally defined SHAP value that represents the degree to which specific biomarker values classify the patient to a particular label (non-diabetic or type 2 diabetic). We wanted to demonstrate how machine-learning algorithms, applied across a variety of health outcome datasets, could be implemented to identify novel biomarkers, with and without HbA1c, to provide better assessment of type 2 diabetes mellitus. By presenting each dataset distinctly, we were able to assess which biomarkers provided the best overall predictive power.

Physiological and Biochemical Analyses

Those with type 2 diabetes mellitus had significantly lower electron transport chain (ETC) complex I and III activities, along with a decreased methyltransferase activity (Table S3.13). Using CART analysis and machine-learning, total nuclear methylation, total mitochondrial hydroxymethylation, and total nuclear hydroxymethylation were shown to be the most important factors influencing the model (Figure 3.2A). Total nuclear methylation was also shown to be significantly increased in type 2 diabetics (Figure 3.2B) with a corresponding decrease in total nuclear hydroxymethylation (Figure 3.2C). Nuclear methylation increased as HbA1c levels

increased (Figure 3.2D) while the rate of hydroxymethylation, generally inversely correlated with methylation levels, decreased as HbA1c increased (Figure 3.2E). Methyltransferase activity, total mitochondrial hydroxymethylation, total nuclear methylation, and total nuclear hydroxymethylation were shown to be important features in predicting type 2 diabetes mellitus in the absence of HbA1c (Figure 3.2F). High s-adenosyl methionine (SAM) methyltransferase activity was also shown to be strongly associated with lower total nuclear methylation levels in the absence of HbA1c (Figure 3.2G).

A decrease in mitochondrial ETC complex III activity was associated with a higher BMI (Figure 3.2H). While those who were considered to be prediabetic (HbA1c 5.7-6.4) did not show significant differences between any of the biochemical measures except total TFAM CpG methylation (Table S3.14), total nuclear methylation was still shown to provide partial classification of patients into non-diabetic, prediabetic, and type 2 diabetic designations (Figure 3.2I-J). CART 10-fold cross validation confirmed findings for binary (testing (0.838), training (0.7448)) and multiple (testing (0.598), training (0.545)) classification (Figure S3.1 A-D).

Genomic Analyses

The complete mitochondrial genomes of all patients were sequenced, and a list of all single nucleotide polymorphisms (SNPs) was compiled. The binary nature of SNPs, i.e. either being converted or not, allowed the dynamic HbA1c levels to influence the machine-learning model much more efficiently (Figure 3.3A). When HbA1c was removed, classification of diabetic or non-diabetic through SNPs was much more apparent, revealing that the 16362 base pair was most significantly impacted (Figure 3.3B). When examining the distribution of SNPs across the mitochondrial genome, the most significant area for base pair alterations to occur was shown to be the D-Loop, or control region (Figure 3.3C).

The D-Loop (mtDNA 16,025bp – 576bp), as compared to all other regions in the mitochondrial genome, contained the highest frequency of SNPs used to predict type 2 diabetes mellitus (Figure 3.3A-B, Figure S3.2A-B). We further investigated how transcription factor binding could be altered at the D-Loop through chromatin immunoprecipitation (ChIP) of mitochondrial transcription factor A, mitochondrial (TFAM). Though protein levels of TFAM

were unchanged (Figure 3.3A), ChIP-qPCR revealed decreased binding of TFAM to the proximal and distal end of the control region in type 2 diabetics (Figure S3.3B). SNPs near the replication of the H strand (Figure 3.3D) or at the end of the D-Loop region (Figure 3.3E) could impact TFAM binding and mitochondrial genome transcription. CART 10-fold cross validation confirmed findings for binary (testing (0.79), training (0.92)) and multiple (testing (0.576), training (0.808)) classification (Figure S3.2 A-D).

Epigenomic Analyses

The cytosine nucleotide followed by a guanine nucleotide (CpG) island of TFAM was examined (Figure 3.4A), using overhang bisulfite PCR to amplify regions of the island for sequencing (Figure 3.4B). Though total methylation of the gene was low (~3%) and showed no significant differences between non-diabetic and type 2 diabetic patients (Table S3.11), site-specific CpG island methylation changes revealed significant differences between groups (Figure 3.4A). Specifically, the 24th (CpG24) and 29th (CpG29) CpGs in the amplified region revealed significant hypomethylation in type 2 diabetic patients (Figure 3.4C-D).

Without using the HbA1c parameter, methylation levels at CpG24, 1, 29, and 35 were shown to be significant contributors to the prediction of diabetic status (Figure 3.4E). When comparing the interactions of CpG24 and CpG29, methylation levels at CpG24 were shown to allow distinct separation of the non-diabetic and type 2 diabetic population (Figure 3.4F). CpG24 methylation remained a primary predictor, even in the presence of HbA1c for multiple classification (Figure 3.4G). Examining total methylation of the TFAM CpG island, prediabetics exhibited an overall increase in methylation, while non-diabetics and type 2 diabetics with similar HbA1c levels showed much lower expression (Figure 3.4H). CART 10-fold cross validation confirmed findings for binary (testing (0.79), training (0.925)) and multiple (testing (0.668), training (0.767)) classification (Figure S3.4 A-D).

Best/Combined Analyses

Those physiological, biochemical, genomic, and/or epigenomic markers that provided the best association within their class for predicting type 2 diabetes mellitus status were used in the final analyses. With the combined list of features, CART algorithms continued to perform consistently at, or near, the top for testing and training accuracies in binary (Table S3.13) and multiple (Table S3.14) classification. Total nuclear hydroxymethylation and total nuclear methylation levels provided the most powerful predictors in delineating between binary (non-diabetic and type 2 diabetic) (Figure 3.5A) and multiple (non-diabetic, prediabetic, type 2 diabetic) (Figure 3.5B) classifications, distinguishing them as potentially suitable biomarkers to accompany diagnostic practices using HbA1c. When using machine-learning to predict diabetic status without HbA1c, CpG24 methylation status and total nuclear methylation proved to be the most powerful predictors in both the binary (Figure 3.5C) and multiple (Figure 3.5D) classification datasets. Ultimately, both in the prediction of type 2 diabetes mellitus (Figure 3.5E) and in assessing the onset (Figure 3.5F), CpG24 hypomethylation was strongly correlated with total nuclear hypermethylation. CART 10-fold cross validation confirmed findings for binary (testing (0.78), training (0.832)) and multiple (testing (0.67), training (0.542)) classification (Figure S3.5 A-D). Within our datasets, CpG24 methylation status and total nuclear methylation provided the best predictive measures for assessing type 2 diabetes mellitus. The incorporation of physiological, biochemical, genetic, and epigenetic features with machine-learning algorithms exemplifies the potential for more informative diagnostics in the future, as well as personalized approaches to generalized treatment modalities (Figure 3.6).

Discussion

Machine-learning can be applied as a systems biology approach, integrating multiple classes of biometric data to assess the importance of specific factors, while also predicting future outcomes. Whereas conventional assessments of disease identification exist, more detailed genomic and epigenomic testing is likely to reveal a comprehensive, systemic valuation of an etiology. To-date, studies have applied machine-learning algorithms in examining the physiological, biochemical, and/or genetic components of disease onset or progression (15). The advantage of our current study is through the assimilation of patient-matched data across a variety of critically impacted systems, providing an archetype for developing novel, descriptive, diagnostic measures through machine-learning algorithms that are specific for each disease type. By individually representing our datasets in Figures 3.2-3.4, we were able to reach more conclusive data in Figure 3.5 by choosing the most predictive features for our final model. For the first time, a multi-omics, machine-learning approach was used to assess the progression and development of type 2 diabetes mellitus in a patient population, identifying potential biomarkers for cardiovascular risk and revealing the fundamental role of genetics in the pathology.

Molecular Pathogenesis and Machine Learning

While clinical practice has recently experienced a surge in deep learning applications used for non-invasive imaging (29), implementing machine-learning algorithms to the fundamental biochemistry and cellular and molecular processes of the body is now only blossoming. Onset and progression of type 2 diabetes has been traditionally measured through blood glucose levels, but, the multifaceted aspects of the disease could create variability in prognosis between vastly different demographic and ethnic groups. Owusu Adjah et al. recently identified BMI as a risk factor for determining ethnic group disposition to type 2 diabetes mellitus (44). Specifically, the relationship between BMI and increased incidence of diabetes mellitus is non-linear; some groups, such as South Asian populations, were more disposed to developing the disease even at lower BMIs. While the current manuscript examines cardiovascular tissue, other less invasive approaches have been used to apply machine-learning algorithms. By retrieving blood from the basilica vein, circulating biomarkers were examined for their role in predicting early recurrence of atrial fibrillation following cryoballoon ablation (10). Support vector machines confirmed that

decreased levels of creatine-kinase (CK-MB) and Troponin T (TnT) were associated with increased early recurrence of atrial fibrillation following cryoballoon ablation. Additionally, a unique, non-invasive approach for potentially diagnosing type 2 diabetes in patients was performed through the examination of toenails. Carter et al., through a variety of machine learning algorithms, focused on 22 elements, including aluminum, cesium, nickel, vanadium, and zinc, and was able to get an AUC of 0.90 when predicting diabetic status using a random forest model (13).

Similar to parts of the aims of this study, other groups have attempted to use machine learning to separate diabetic and non-diabetic patients without the inclusion of blood glucose or HbA1c (62). In a testing set of 13700 patients from the Luzhou, China region, random forest machine learning algorithms provided a 0.7225 accuracy when predicting diabetic status from physical examination data in the absence of blood glucose (62). Also using a random forest model, Tang et al. revealed how CpG island methylation data, combined with miRNA expression profiles, can be instrumental in cancer pathogenesis (57); implementing this two-feature selection process, they were able to identify the best tissue specific features, ultimately allowing for the identification of the originating tissue where tumor progression began. In a similar fashion, the machine learning algorithm HeteSim (54), which examines heterogeneous datasets and calculates their relatedness, was employed in ascribing how gene profiles can be related to phenotypic outcomes, specifically in the validation and prediction of genes classified within major diseases (61).

While understanding how to better form prognoses and treat cardiac dysfunction in patients with type 2 diabetes mellitus remains a critical mission, more than 80 million American adults, most of which are undiagnosed, are prediabetic (48). In the current work, we have implemented predictive algorithms to assess biomarkers likely involved in the onset, as well as prediabetic progression, of type 2 diabetes mellitus. Although multiple classification categories further reduce the predictive power of the model, separation into distinct groupings revealed a unique phenotype for prediabetics (Figure 3.4H). The effects of diabetes mellitus on the body is a high glucose stressed condition, altering substrate metabolism and causing systemic inflammation (55). Due to this environmental change, researchers have shown how epigenetic changes occur across most, if not all, tissues that are impacted by diabetes mellitus (37, 60).

In the cardiovascular system, the heart, circulatory system, and regulating immune system are all transcriptionally regulated through epigenetic alterations (17, 28), resulting in cellular

adaptations to the environmental stress. Examining atrial appendages, the results obtained in this study are a direct reflection of changes within the heart. While blood is more easily acquired in type 2 diabetic patients, cardiac tissue, which is mitochondrially rich, provides a direct connection between physiological dysfunction observed in the heart and the impact of altered genomic profiles in the mitochondrion and nucleus. Machine-learning, which at current has been applied to very few genetic applications, may play a significant role in defining the epigenome of those with diabetes mellitus, likely unveiling genes and molecular pathways first impacted by the pathology.

The Challenges of Machine Learning in the Clinical Setting

Machine-learning algorithms produce generalizations as they are inherently predictive, which means a smaller sample size can occasionally result in increased emphasis on outliers within the patient dataset and determination of the outliers' biomarker features to be most influential in disease diagnosis. With a limited 50 patient dataset, there are concerns of overfitting the model, where the derived classification tree would have branches for each patient sample encountered during training. If this was to occur, the produced tree would fail future test cases while maintaining near perfect training accuracy, which was not observed. 10-fold cross validation ensured that no single developed tree was composed solely of outliers or a group of patient data of one label type, allowing patients of different labels to train the algorithm. Additionally, choosing seed values provided an even patient distribution during model training and testing. Both 10-fold cross validation and setting a seed allowed the derived models to not over fit the training data. With this being said, it should be noted that the small sample size limits the conclusions and predictions made by the machine-learning algorithms within the manuscript, and future investigations will need to validate specific features, including CpG24 of TFAM and global nuclear DNA methylation.

For developed frameworks and the implemented SHAP visualization, the results are inherently regulated by HbA1c since patient HbA1c values were used to assign the labels from which the machine-learning algorithm then proceeded to train. HbA1c is used as a guide in this study to help clarify how clinically assessed progression of diabetes (commonly through HbA1c levels) is related to the biochemical and genetic signatures found in the heart. Although no specific biomarker or biomarker combinations can replace HbA1c due to the apparent diagnostic bias in

this study (essentially ~100% accuracy when included), they can provide predictive accuracies near that of HbA1c. While previous clinical diagnoses determined a patients' diabetic status in this study, some patients diagnosed as type 2 diabetics had HbA1c levels within normal ranges; begging the question of whether sustained, or attenuated, health effects can be accurately assessed through HbA1c levels alone when intervention (lifestyle or medicinally) occurs? Ultimately, this study provides a machine-learning algorithm utilizing the respective advantages of HbA1c in combination with other biomarkers to help circumvent the limitations of modern HbA1c diagnosis, as well as introduces completely novel cardiac risk stratification paradigms for patients with type 2 diabetes mellitus.

The quantity and diversity of omics-based approaches continues to expand. Convenience and increasingly inexpensive options for biometric-based valuations incite a growing demand for the incorporation and meaningful explanation of large and diverse patient datasets. The methodology outlined in this manuscript can serve as an archetype for the development and implementation of machine-learning to other disciplines seeking to evaluate disease progression. By using various health outcomes datasets, we were able to identify, and combine, the most prominent biomarkers into an accurate predictive algorithm engineered around 50 patients. While we have identified specific genetic features that are highly predictive in 50 patients, as a much larger patient population is applied to this model, the prioritization of other features is likely to occur, enhancing the diagnostic potential for the individual diabetic or prediabetic patient. Indeed, this is the advantage of using machine-learning models, in that they continue to learn and develop more accurate predictions as the number of features and sampled population grows.

Conclusions

Our work highlights the importance of identifying biomarkers in systems known to be disturbed during the disease (i.e. the mitochondrion and nucleus), and further applying these biological systems to personalized prognostics. By implementing classification tree, machine-learning algorithms to cardiac tissue from type 2 diabetic patients, we determined that hypermethylation of the nuclear genome was predictive of diabetic status and that it may provide added benefit to diagnostic applications in the future. Additionally, through our machine-learning model, as little as a ~5% change in methylation status of a gene promoter could provide valuable predictive data when determining diabetic status. Defining new diagnostic parameters, better predicting future health outcomes, and specializing modalities of care begins with the integration of “big data” into machine-learning systems; this study reveals how integration of data assists in the determination of diabetic status in the heart.

Abbreviations

5hmC: 5-hydroxymethylcytosine

5mC: 5-methylcytosine

AUC: Area Under the Curve

CART: Classification and Regression Trees

CpG: Cytosine Nucleotide followed by a Guanine Nucleotide

ETC: Electron Transport Chain

HbA1c: Glycated Hemoglobin

LR: Logistic Regression

LDA: Linear Discriminant Analysis

KNN: K-Nearest Neighbors

NB: Naive Bayes

SHAP: SHapley Additive exPlanations

SVM: Support Vector Machine

T2DM: Type 2 Diabetes Mellitus

TFAM: Transcription Factor A, Mitochondrial

Declarations

Ethics approval and consent to participate

All tissue and patient information was acquired in a double de-identified fashion, and was approved by the West Virginia University Institutional Review Board and Institutional Biosafety Committee. Patients were all consented by the Heart and Vascular Institute, J.W. Ruby Memorial Hospital at West Virginia University School of Medicine. Right atrial appendages were removed during open-heart and/or valvular surgeries (Refer to Methods, section *Study Approval*).

Consent for publication

N/A

Availability of data and material

The datasets generated and/or analysed during the current study, including sequencing files and computer code, are available (Refer to Methods, section *Data Availability*). Primary used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

Designing research studies (QAH, SMR, MVP, JMH), conducting experiments (QAH, MVP, AK, AJD, GKF, ADT, APG), acquiring data (QAH, MVP), surgical procedures (CCC), analyzing data (QAH, SMR, MVP, TBC, JHG, GAA, JMH), writing the manuscript (QAH, SMR, MVP, DCS,

CCC, JLA, JMH). The author Quincy A. Hathaway had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors read and approved the final manuscript.

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Additional Material

File name: Additional File 1

File format: DOCX

Title of data: Supplemental Material

Description of data: Supplemental data to the primary manuscript including, patient characteristics, primer design, and 10-fold cross validation for machine learning algorithms. For each specific data set, we applied six different machine-learning models (CART, LR, LDA, KNN, NB, SVM) and determined which model would yield the best predictions on the data sets. CART yielded the best result, the other test/train accuracies are provided for comparison and support of our conclusions.

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Table 3.1

Parameter	Non-Diabetic	Type 2 Diabetic
Age	61.97 ± 2.449	61.16 ± 3.047
Sex	Male = 26, Female = 4	Male = 15, Female = 5
BMI (kg/m ²)	29.13 ± 1.08	29.14 ± 1.448
Coronary Artery Disease	73.33% ± 8.212%	100% ± 0.0%*
Hypertension	86.67% ± 6.312%	94.74% ± 5.263%
Valvular Disease	26.67% ± 8.212%	15.79% ± 8.595%
HbA1c	5.567 ± 0.07898	8.016 ± 0.4024*

Table 3.1: Patient characteristics and demographic information. Groups are considered significantly different if $P \leq 0.05 = *$ compared to non-diabetic. All data are presented as the mean $\pm$ standard error of the mean (SEM). HbA1c = glycated hemoglobin.

Table 3.2

Model	Training	Training (StDev)	Testing	Testing (StDev)	F1 Score	Important Features	Important Feature Bias	AUC
LR	0.608	0.301	0.667	0.0	0.640	Complex III, Complex I, CpG31, CpG28, CpG30, Complex IV, CpG8, CpG4, CpG12, Age	(-2.688), (-1.688), (1.648), (-1.163), (-1.016), (0.982), (0.945), (0.887), (0.882), (0.848)	NA
LDA	0.567	0.203	0.556	0.0	0.400	SNP16245, SNP16344, SNP151, SNP5463, SNP4295, SNP13722, SNP94, SNP15884, SNP9055, SNP477	(-3.896E+15), (-3.896E+15), (-3.896E+15), (-3.896E+15), (-2.719E+15), (-2.719E+15), (3.398E+14), (3.398E+14), (3.398E+14), 0.266	0.700
KNN	0.642	0.239	0.444	0.0	0.430	NA	NA	0.600
NB	0.725	0.227	0.778	0.0	0.780	Mito 5hmC, Methyltransferase	(1.000), (0.000)	0.775
SVM	0.583	0.337	0.667	0.0	0.640	Complex III, CpG31, Complex I, CpG28, CpG8, CpG22, CpG12, CpG29, CpG4, CpG35	(-0.732), (0.488), (-0.443), (-0.372), (0.350), (-0.349), (0.322), (-0.260), (0.259), (0.257)	NA
CART	0.790	0.209	0.711	0.1	0.714	CpG 24, CpG 28, Nuc 5mC, CpG11, CpG23, CpG1, CpG4	(0.587%), (0.213%), (0.040%), (0.040%), (0.040%), (0.040%), (0.040%)	0.715

Table 3.2: Overview of 6 machine-learning model analysis on all 345 features in binary classification. Model analysis was conducted five times and averages are reported for the resulting training accuracy, training standard deviation, testing accuracy, testing standard deviation, F1 score, and *area under the curve* (AUC). Important biomarker features associated with each trained model are provided along with the associated influence value for each feature. Important features are listed in order of influence within the model. LR, LDA, SVM feature bias exists as an influence parameter where magnitude dictates feature influence. A positive influence value indicates the biomarker favors classification towards one label while a negative value indicates favorable classification of the opposite label. The larger the magnitude, the more strongly that feature shifts classification. NB feature influence indicates the most important biomarker per class in binary (0,1) classification schemes. CART feature bias percentages indicate feature influence on the created classification tree. Larger percentages indicate a feature that arises near the beginning of a tree before subsequent branching. Influence is not provided for KNN due to model restrictions.

Table 3.3

Model	Training	Training (StDev)	Testing	Testing (StDev)	F1 Score	Important Features	Important Feature Bias
LR	0.333	0.207	0.444	0.0	0.430	Complex V, CpG35, BMI, CpG38, CpG18, CpG40, CpG19, CpG23, Complex IV, CpG25	(-2.417), (-2.214), (1.942), (-1.541), (-1.313), (-0.994), (-0.881), (-0.824), (-0.812), (0.8071)
LDA	0.433	0.178	0.333	0.0	0.170	SNP11167, SNP10506, SNP16309, SNP16343, SNP2294, SNP14139, SNP16162, SNP3672, SNP8642, SNP143	(-4.623E+14), (-4.623E+14), (-4.623E+14), (-4.623E+14), (-4.623E+14), (-4.623E+14), (-4.623E+14), (-4.623E+14), (-4.623E+14), (5.779E+13)
KNN	0.358	0.239	0.444	0.0	0.450	NA	NA
NB	0.425	0.243	0.778	0.0	0.780	Methyltransferase, Mito 5hmC, Nuc 5 hmC	(0.000), (1.000), (2.000)
SVM	0.442	0.163	0.556	0.0	0.520	Complex V, BMI, Complex III, Complex I, Complex IV, CpG31, Age, CpG19, CpG22, CpG6	(-0.943), (0.754), (0.561), (-0.383), (-0.344), (0.307), (-0.287), (-0.268), (-0.210), (0.198)
CART	0.660	0.257	0.556	0.0	0.558	CpG24, TFAM CpG, TFAM Non-CpG, BMI, SNP94, Complex IV, SNP8557, CpG7, SNP242, SNP13722, Complex III, Mito 5mC	(0.328%), (0.206%), (0.176%), (0.137%), (0.016%), (0.045%), (0.016%), (0.016%), (0.016%), (0.016%), (0.016%), (0.016%)

Table 3.3: Overview of 6 machine-learning model analysis on all 345 features in multiple classification. Model analysis was conducted five times and averages are reported for the resulting training accuracy, training standard deviation, testing accuracy, testing standard deviation, and F1 score. Important biomarker features associated with each trained model are provided along with the associated influence value for each feature. Important features are listed in order of influence within the model. LR, LDA, SVM feature bias exists as an influence parameter where magnitude dictates feature influence. A positive influence value indicates the biomarker favors classification towards one label while a negative value indicates favorable classification of the opposite label. The larger the magnitude, the more strongly that feature shifts classification. NB feature influence indicates the most important biomarker per class in multiple (0,1,2) classification schemes. CART feature bias percentages indicate feature influence on the created classification tree. Larger percentages indicate a feature that arises near the beginning of a tree before subsequent branching. Influence is not provided for KNN due to model restrictions.

Figure 3.1

Figure 3.1

Figure 3.1: Overview of machine-learning using Classification and Regression Trees (CART) and SHapley Additive exPlanations (SHAP). (A) Classification trees begin with a specific parameter that most successfully partitions the samples, such as CpG24 methylation, and determine the probability of correctly delineating a population into classifications, such as non-diabetic and diabetic, through a discrete value of the parameter (e.g. 0.275). The delineation is then given a probability score (i.e. 0.475, or a 47.5% chance of classifying the sample incorrectly), assigned a label, and further passed on to other parameters in the tree (e.g. CpG11 methylation and CpG28 methylation). As the samples progress through the tiers of the tree, the gini impurity gets smaller, more accurately delineating samples that make it to that particular “truth” statement. (B) An example of how SHAP illustrates sample distribution. The “SHAP Value” delineates between a condition being true (value > 0.0 , T2DM) and it being false (value < 0.0 , ND). The more a specific value of a sample influences the composition of the model, the farther the point will migrate away from zero on the y-axis. If the value of a sample does not influence the model, it will reside near or at zero on the y-axis. In the example, a larger value of “X” and lower value of “Z” are highly predictive of the patient being ND, with these values strongly influencing the model “Y”. CpG = cytosine nucleotide followed by a guanine nucleotide, ND = non-diabetic, T2DM = type 2 diabetic.

Figure 3.2

Figure 3.2

Figure 3.2: Feature importance of physiological and biochemical characteristics from patients. (A) Using HbA1c for binary classification representing the factors positively (red) and negatively (blue) impacting the construction of the model, with size of the bars depicting importance. The (B) total nuclear methylation and (C) total nuclear hydroxymethylation of patients. SHAP binary depiction of the interaction between (D) total nuclear methylation and (E) total nuclear hydroxymethylation and HbA1c levels. (F) Not including HbA1c for binary classification representing the factors positively (red) and negatively (blue) impacting the construction of the model, with size of the bars depicting importance. SHAP binary depiction without HbA1c of the interaction between (G) total nuclear methylation and methyltransferase activity and (H) electron transport chain complex III and BMI. Examining the multi classification effects of prediabetes, (I) A modified T-Plot where the main effects of biomarkers on the prediction output are shown along the diagonal axis whereas interaction effects are shown off the diagonal. SHAP depiction of patient separation with the individual and correlated effects of HbA1c and total nuclear methylation. SHAP multi depiction of the interaction between (J) total nuclear methylation and HbA1c. SHAP values > 0.0 are diabetic (T2DM), SHAP values < 0.0 are non-diabetic (ND), SHAP values $= 0$ are either ND or T2DM without influence on the model. Groups are considered significantly different if $P \leq 0.05 = *$ compared to non-diabetic. All data are presented as the mean $\pm$ standard error of the mean (SEM). ND = non-diabetic, T2DM = type 2 diabetic, Nuc = nuclear, Mito = mitochondrial, 5mC = 5-methylcytosine, 5hmC, 5- hydroxymethylcytosine, HbA1c = glycated hemoglobin, binary = no diabetes and diabetes, multi = no diabetes, prediabetes, and type 2 diabetes.

Figure 3.3

Figure 3.3

Figure 3.3

Figure 3.3: Feature importance of mitochondrial DNA SNPs from patients. (A) The most important predictive parameters using binary classification with HbA1c, the absolute value of a feature being high (red) or low (blue) depicting diabetic (right-side) or non-diabetic (left-side) status. (B) The most important predictive parameters using binary classification without HbA1c, the absolute value of a feature being high (red) or low (blue) depicting diabetic (right-side) or non-diabetic (left-side) status. (C) Frequency of mitochondrial DNA SNPs by nucleotide converted in ND and T2DM patients; increasing frequency of SNPs occurring in the patient population are depicted by movement closer to the mitochondrial DNA strand. (D) SHAP binary depiction with HbA1c of the interaction between snp16126 and HbA1c. (E) SHAP binary depiction without HbA1c of the interaction between snp7028 and snp73. SHAP values > 0.0 are diabetic (T2DM), SHAP values < 0.0 are non-diabetic (ND), SHAP values $= 0$ are either ND or T2DM without influence on the model. ND = non-diabetic, T2DM = type 2 diabetic, HbA1c = glycated hemoglobin, binary = no diabetes and diabetes, multi = no diabetes, prediabetes, and type 2 diabetes.

Figure 3.4

Figure 3.4

Figure 3.4: Feature importance of CpG island methylation of TFAM from patients. (A)

Methylation across the promoter CpG region of the TFAM gene was determined using overhang bisulfite sequencing. (B) Experimental paradigm for amplification of the bisulfite-converted DNA for 23 CpG sites proximal (Amplicon 1) and 19 CpG sites distal (Amplicon 2) to the TFAM start site. SHAP binary depiction with HbA1c of the interaction between (C) CpG24 methylation and HbA1c and (D) CpG29 methylation and HbA1c. (E) Not including HbA1c for binary classification representing the factors positively (red) and negatively (blue) impacting the construction of the model, with size of the bars depicting importance. (F) A modified T-Plot where the main effects of biomarkers on the prediction output are shown along the diagonal axis whereas interaction effects are shown off the diagonal. SHAP binary depiction without HbA1c of patient separation with the individual and correlated effects of CpG24 methylation and CpG29 methylation. (G) Using HbA1c for multi classification representing the factors positively (red) and negatively (blue) impacting the construction of the model, with size of the bars depicting importance. (H) SHAP multi depiction with HbA1c of the interaction between TFAM gene total methylation and HbA1c. SHAP values > 0.0 are diabetic (T2DM), SHAP values < 0.0 are non-diabetic (ND), SHAP values $= 0$ are either ND or T2DM without influence on the model. Groups are considered significantly different if $P \leq 0.05 = *$ compared to non-diabetic. All data are presented as the mean $\pm$ standard error of the mean (SEM). ND = non-diabetic, T2DM = type 2 diabetic, HbA1c = glycated hemoglobin, CpG = cytosine nucleotide followed by a guanine nucleotide, TFAM = transcription factor A, mitochondrial, binary = no diabetes and diabetes, multi = no diabetes, prediabetes, and type 2 diabetes.

Figure 3.5

Figure 3.5

Figure 3.5

Figure 3.5: Feature importance of best factors combined from patients. The most important predictive parameters using (A) binary and (B) multi classification with HbA1c, the absolute value of a feature being high (red) or low (blue) depicting diabetic (right-side) or non-diabetic (left-side) status. The most important predictive parameters using (C) binary and (D) multi classification without HbA1c, the absolute value of a feature being high (red) or low (blue) depicting diabetic (right-side) or non-diabetic (left-side) status. SHAP (E) binary and (F) multi depiction without HbA1c of the interaction between total nuclear methylation and CpG24 methylation. SHAP values > 0.0 are diabetic (T2DM), SHAP values < 0.0 are non-diabetic (ND), SHAP values $= 0$ are either ND or T2DM without influence on the model. ND = non-diabetic, T2DM = type 2 diabetic, HbA1c = glycated hemoglobin, CpG = cytosine nucleotide followed by a guanine nucleotide, Nuc = nuclear, 5mC = 5-methylcytosine, binary = no diabetes and diabetes, multi = no diabetes, prediabetes, and type 2 diabetes.

Figure 3.6

Machine Learning: SHAP Visualization & Decision Tree Classifier

Figure 3.6: Overview of machine-learning pipeline implementing biological variables across a spectrum of gathered information. From the patient population undergoing coronary artery bypass graft surgery (CABG), physiological parameters (demographics, health reports, etc.) and atrial tissue were used for subsequent analyses. From cardiac tissue genomic (mitochondrial DNA), epigenomic (TFAM promoter CpG methylation), and biochemical (nuclear and mitochondrial function) were assessed. Cumulatively, the biological data was processed through tree ensembles in SHAP and validated through CART analysis with 10-Fold Cross Validation. Using these machine-learning algorithms, graphical depictions and biomarker feature importance are able to be derived, allowing for prediction of the onset and progression of diabetes. Ultimately, by using biological data at the genomic and epigenomic level, it allows for a precision medicine approaches and more personalized diagnostics and prognostics. TFAM = transcription factor A, mitochondrial, mtDNA = mitochondrial DNA, CpG = cytosine nucleotide followed by a guanine nucleotide, CART = Classification and Regression Trees, SHAP = SHapley Additive exPlanations.

Table S3.1

Parameter	Non-Diabetic	Pre-Diabetic	Type 2 Diabetic
Age	58.06 ± 3.692	66.43 ± 2.799	61.16 ± 3.047
Sex	Male = 15, Female = 1	Male = 11, Female = 3	Male = 14, Female = 5
BMI (kg/m ²)	30.11 ± 1.405	28.01 ± 1.669	29.14 ± 1.448
Coronary Artery Disease	68.75% ± 11.97%	78.57% ± 11.38%	100% ± 0.0%
Hypertension	81.25% ± 10.08%	92.86% ± 7.143%	94.74% ± 5.263%
Valvular Disease	31.25% ± 11.97%	21.43% ± 11.38%	15.79% ± 8.595%
HbA1c	5.263 ± 0.08892	5.914 ± 0.0443	8.016 ± 0.4024*#

Table S3.1: Patient characteristics and demographic information for non-diabetic, pre-diabetic, and type 2 diabetic patients. Groups are considered significantly different if $P \leq 0.05 = *$ compared to non-diabetic or # compared to pre-diabetic. All data are presented as the mean $\pm$ standard error of the mean (SEM). HbA1c = glycated hemoglobin.

Table S3.2

Software	Purpose	Primer Name	Sequence	Product Size
MethPrimer 2.0	Bisulfite Overhang PCR Sequencing	TFAM 1 F1	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGGATAG AGGTGGTTTAATAG	399
		TFAM 1 R1	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGATACAC AACTCTACTCCAAACCTTC	
		TFAM 1 F2	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNNGGATA GAGGTGGTTTAATAG	
		TFAM 1 R2	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNATACA CAACTCTACTCCAAACCTTC	
		TFAM 1 F3	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNNGGGAT AGAGGTGGTTTAATAG	
		TFAM 1 R3	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNNATAC ACAACTCTACTCCAAACCTTC	
MethPrimer 2.0	Bisulfite Overhang PCR Sequencing	TFAM 2 F1	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAAAATGA AGATTAATGGGTTTAA	356
		TFAM 2 R1	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCTATTA AACCACCTCTATCCC	
		TFAM 2 F2	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNAAAATG AAGATTAATGGGTTTAA	
		TFAM 2 R2	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNCTATT AAACCACCTCTATCCC	
		TFAM 2 F3	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNAAAAT GAAGATTAATGGGTTTAA	
		TFAM 2 R3	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNNCTAT TAAACCACCTCTATCCC	
Primer3	Chromatin Immunoprecipitation qPCR	D-Loop F1	CATGGGGAAGCAGATTTGGG	174
		D-Loop R1	TTGTAAGCATGGGGAGGGG	
		D-Loop F2	TACATTACTGCCAGCCACCA	
		D-Loop R2	CATGGGGACGAGAAGGGATT	113
		D-Loop F3	CTTGACCACCATCCTCCGT	
		D-Loop R3	AGAGCTCCCGTGAGTGGTTA	137
		D-Loop F4	GAGCTCTCCATGCATTTGGT	
		D-Loop R4	TACTGCGACATAGGGTGCTC	91
		D-Loop F5	GAGCACCTATGTCGCAGTA	
		D-Loop R5	CTGTGTGGAAAGTGGCTGTG	169
		D-Loop F6	GTCTGCACAGCCACTTTCC	
		D-Loop R6	TAGTATGGGAGTGGGAGGGG	225
		D-Loop F7	CCCCTCCCACTCCATACTA	
		D-Loop R7	GGTGATGTGAGCCCGCTAA	183

Table S3.2: Primer design. Using MethPrimer 2.0 for bisulfite converted DNA and Primer3 for chromatin immunoprecipitation, primers were designed to measure TFAM CpG island methylation and TFAM binding to the D-Loop region of the mitochondrial DNA, respectively. TFAM = transcription factor A, mitochondrial.

Table S3.3

SEED = 2					
	CART Binary				
	Features Implemented in Training and Testing by Trial	Training (Average Accuracy)	Testing (F1 Score)	Train St.D Average	Test St. D
TRIAL 1	Nuc 5mC, Complex I, III, IV	0.73	0.836	0.1778	0.092
TRIAL 2	Methyl, Complex III, IV, V	0.6782	0.74	0.1902	0.055
TRIAL 3	Methyl, Nuc 5hm, Complex I	0.67	0.67	0.2072	0.000
TRIAL 4	Complex I, III, IV	0.573	0.482	0.2218	0.085
TRIAL 5	Methyl, Nuc 5mC, Complex I, Citrate Synthase	0.7448	0.604	0.2012	0.054
TRIAL 6	Methyl, Complex I, III	0.652	0.7	0.2252	0.000
	LR Binary				
	Features Implemented in Training and Testing by Trial	Training (Average Accuracy)	Testing (F1 Score)	Train St.D Average	Test St. D
TRIAL 1	Nuc 5mC, Complex I, III, IV	0.791667	0.7	0.107044	0
TRIAL 2	Methyl, Complex III, IV, V	0.583333	0.4	0.182574	0
TRIAL 3	Methyl, Nuc 5hm, Complex I	0.708333	0.52	0.201556	0
TRIAL 4	Complex I, III, IV	0.608333	0.6	0.186525	0
TRIAL 5	Methyl, Nuc 5mC, Complex I, Citrate Synthase	0.725	0.6	0.175	0
TRIAL 6	Methyl, Complex I, III	0.633333	0.38	0.217945	0
	LDA Binary				
	Features Implemented in Training and Testing by Trial	Training (Average Accuracy)	Testing (F1 Score)	Train St.D Average	Test St. D
TRIAL 1	Nuc 5mC, Complex I, III, IV	0.741667	0.7	0.114564	0
TRIAL 2	Methyl, Complex III, IV, V	0.558333	0.4	0.175	0
TRIAL 3	Methyl, Nuc 5hm, Complex I	0.683333	0.7	0.210159	0
TRIAL 4	Complex I, III, IV	0.608333	0.6	0.149304	0
TRIAL 5	Methyl, Nuc 5mC, Complex I, Citrate Synthase	0.675	0.8	0.195256	0
TRIAL 6	Methyl, Complex I, III	0.633333	0.49	0.187083	0
	KNN Binary				
	Features Implemented in Training and Testing by Trial	Training (Average Accuracy)	Testing (F1 Score)	Train St.D Average	Test St. D
TRIAL 1	Nuc 5mC, Complex I, III, IV	0.675	0.45	0.317214	0
TRIAL 2	Methyl, Complex III, IV, V	0.825	0.2	0.195256	0

TRIAL 3	Methyl, Nuc 5hm, Complex I	0.633333	0.67	0.15	0
TRIAL 4	Complex I, III, IV	0.675	0.45	0.317214	0
TRIAL 5	Methyl, Nuc 5mC, Complex I, Citrate Synthase	0.633333	0.67	0.15	0
TRIAL 6	Methyl, Complex I, III	0.675	0.38	0.275	0
	NB Binary				
	Features Implemented in Training and Testing by Trial	Training (Average Accuracy)	Testing (F1 Score)	Train St.D Average	Test St. D
TRIAL 1	Nuc 5mC, Complex I, III, IV	0.7	0.38	0.217945	0
TRIAL 2	Methyl, Complex III, IV, V	0.625	0.23	0.321131	0
TRIAL 3	Methyl, Nuc 5hm, Complex I	0.65	0.7	0.229129	0
TRIAL 4	Complex I, III, IV	0.6	0.29	0.229129	0
TRIAL 5	Methyl, Nuc 5mC, Complex I, Citrate Synthase	0.675	0.7	0.275	0
TRIAL 6	Methyl, Complex I, III	0.675	0.49	0.296859	0
	SVM Binary				
	Features Implemented in Training and Testing by Trial	Training (Average Accuracy)	Testing (F1 Score)	Train St.D Average	Test St. D
TRIAL 1	Nuc 5mC, Complex I, III, IV	0.633333	0.33	0.187083	0
TRIAL 2	Methyl, Complex III, IV, V	0.633333	0.52	0.187083	0
TRIAL 3	Methyl, Nuc 5hm, Complex I	0.608333	0.52	0.149304	0
TRIAL 4	Complex I, III, IV	0.633333	0.33	0.187083	0
TRIAL 5	Methyl, Nuc 5mC, Complex I, Citrate Synthase	0.658333	0.67	0.184278	0
TRIAL 6	Methyl, Complex I, III	0.608333	0.52	0.149304	0

Table S3.3: Machine learning analyses for physiological/biochemical features using binary classification. CART = Classification and Regression Trees, LR = Logistic Regression, LDA = Linear Discriminant Analysis, KNN = K-Nearest Neighbors, NB = Naïve Bayes, SVC = Support Vector Machines. Nuc = Nuclear, Mito = Mitochondrial, 5mC = 5-methylcytosine, 5hmC = 5-hydroxymethylcytosine, Methyl = S-adenosyl methionine methyltransferase activity, St.D. = standard deviation, binary = non-diabetic and type 2 diabetic, multi = non-diabetic, prediabetic, and type 2 diabetic.

Table S3.4

SEED = 22					
CART Multiple					
	Features Implemented in Training and Testing by Trial	Training (Average Accuracy)	Testing (F1 Score)	Train St.D Average	Test St. D
TRIAL 1	Mito 5hmC, Nuc 5mC, Complex I, III, V	0.49	0.35	0.2968	0.000
TRIAL 2	Nuc 5mC, Complex I, III, IV, V, Citrate Synthase	0.44	0.48	0.2828	0.079
TRIAL 3	Methyl, Complex III, V	0.545	0.598	0.26	0.045
TRIAL 4	Nuc 5mC, Complex III, V	0.52	0.412	0.2266	0.049
TRIAL 5	Methyl, Nuc 5mC, Citrate Synthase	0.4532	0.464	0.2362	0.048
TRIAL 6	Methyl, Nuc 5mC, Complex III, Citrate Synthase	0.4748	0.36	0.1812	0.060
LR Multiple					
	Features Implemented in Training and Testing by Trial	Training (Average Accuracy)	Testing (F1 Score)	Train St.D Average	Test St. D
TRIAL 1	Mito 5hmC ,Nuc 5mC, Complex I, III, V	0.5	0.28	0.33541	0
TRIAL 2	Nuc 5mC, Complex I, III, IV, V, Citrate Synthase	0.5	0.26	0.316228	0
TRIAL 3	Methyl, Complex III, V	0.525	0.1	0.283945	0
TRIAL 4	Nuc 5mC, Complex III, V	0.525	0.27	0.283945	0
TRIAL 5	Methyl, Nuc 5mC, Citrate Synthase	0.45	0.22	0.291548	0
TRIAL 6	Methyl, Nuc 5mC, Complex III, Citrate Synthase	0.5	0.12	0.295804	0
LDA Multiple					
	Features Implemented in Training and Testing by Trial	Training (Average Accuracy)	Testing (F1 Score)	Train St.D Average	Test St. D
TRIAL 1	Mito 5hmC ,Nuc 5mC, Complex I, III, V	0.475	0.15	0.325	0
TRIAL 2	Nuc 5mC, Complex I, III, IV, V, Citrate Synthase	0.525	0.32	0.283945	0
TRIAL 3	Methyl, Complex III, V	0.6	0.11	0.339116	0
TRIAL 4	Nuc 5mC, Complex III, V	0.55	0.28	0.331662	0
TRIAL 5	Methyl, Nuc 5mC, Citrate Synthase	0.5	0.4	0.37081	0
TRIAL 6	Methyl, Nuc 5mC, Complex III, Citrate Synthase	0.5	0.4	0.353553	0
KNN Multiple					
	Features Implemented in Training and Testing by Trial	Training (Average Accuracy)	Testing (F1 Score)	Train St.D Average	Test St. D
TRIAL 1	Mito 5hmC ,Nuc 5mC, Complex I, III, V	0.55	0.1	0.291548	0

TRIAL 2	Nuc 5mC, Complex I, III, IV, V, Citrate Synthase	0.575	0.15	0.296859	0
TRIAL 3	Methyl, Complex III, V	0.516667	0.1	0.229129	0
TRIAL 4	Nuc 5mC, Complex III, V	0.516667	0.1	0.229129	0
TRIAL 5	Methyl, Nuc 5mC, Citrate Synthase	0.425	0.23	0.336341	0
TRIAL 6	Methyl, Nuc 5mC, Complex III, Citrate Synthase	0.491667	0.3	0.215542	0
	NB Multiple				
	Features Implemented in Training and Testing by Trial	Training (Average Accuracy)	Testing (F1 Score)	Train St.D Average	Test St. D
TRIAL 1	Mito 5hmC ,Nuc 5mC, Complex I, III, V	0.4	0.15	0.3	0
TRIAL 2	Nuc 5mC, Complex I, III, IV, V, Citrate Synthase	0.45	0.22	0.269258	0
TRIAL 3	Methyl, Complex III, V	0.6	0.1	0.254951	0
TRIAL 4	Nuc 5mC, Complex III, V	0.475	0.15	0.283945	0
TRIAL 5	Methyl, Nuc 5mC, Citrate Synthase	0.491667	0.48	0.267317	0
TRIAL 6	Methyl, Nuc 5mC, Complex III, Citrate Synthase	0.475	0.22	0.378319	0
	SVM Multiple				
	Features Implemented in Training and Testing by Trial	Training (Average Accuracy)	Testing (F1 Score)	Train St.D Average	Test St. D
TRIAL 1	Mito 5hmC ,Nuc 5mC, Complex I, III, V	0.366667	0.14	0.217945	0
TRIAL 2	Nuc 5mC, Complex I, III, IV, V, Citrate Synthase	0.416667	0.14	0.241523	0
TRIAL 3	Methyl, Complex III, V	0.366667	0.14	0.269258	0
TRIAL 4	Nuc 5mC, Complex III, V	0.366667	0.14	0.269258	0
TRIAL 5	Methyl, Nuc 5mC, Citrate Synthase	0.5	0.23	0.316228	0
TRIAL 6	Methyl, Nuc 5mC, Complex III, Citrate Synthase	0.466667	0.48	0.298608	0

Table S3.4: Machine learning analyses for physiological/biochemical features using multi classification. CART = Classification and Regression Trees, LR = Logistic Regression, LDA = Linear Discriminant Analysis, KNN = K-Nearest Neighbors, NB = Naïve Bayes, SVC = Support Vector Machines. Nuc = Nuclear, Mito = Mitochondrial, 5mC = 5-methylcytosine, 5hmC = 5-hydroxymethylcytosine, Methyl = S-adenosyl methionine methyltransferase activity, St.D. = standard deviation, binary = non-diabetic and type 2 diabetic, multi = non-diabetic, prediabetic, and type 2 diabetic.

Table S3.5

SEED = 16					
CART Binary					
	Features Implemented in Training and Testing by Trial	Training (Average Accuracy)	Testing (F1 Score)	Train St.D Average	Test St. D
TRIAL 1	72, 310, 3010, 4769, 8697, 11419, 11812, 14233, 15904, 16362	0.92	0.70	0.17	0
TRIAL 2	310, 3010, 4769, 11419, 16362	0.92	0.58	0.17	0
TRIAL 3	310, 4769, 16362	0.77	0.58	0.18	0
TRIAL 4	72, 310, 16362	0.72	0.79	0.31	0
LR Binary					
	Features Implemented in Training and Testing by Trial	Training (Average Accuracy)	Testing (F1 Score)	Train St.D Average	Test St. D
TRIAL 1	72, 310, 3010, 4769, 8697, 11419, 11812, 14233, 15904, 16362	0.7833	0.67	0.2506	0
TRIAL 2	310, 3010, 4769, 11419, 16362	0.7833	0.67	0.2506	0
TRIAL 3	310, 4769, 16362	0.7833	0.67	0.2506	0
TRIAL 4	72, 310, 16362	0.7833	0.67	0.2506	0
LDA Binary					
	Features Implemented in Training and Testing by Trial	Training (Average Accuracy)	Testing (F1 Score)	Train St.D Average	Test St. D
TRIAL 1	72, 310, 3010, 4769, 8697, 11419, 11812, 14233, 15904, 16362	0.8083	0.58	0.2983	0
TRIAL 2	310, 3010, 4769, 11419, 16362	0.8917	0.58	0.175	0
TRIAL 3	310, 4769, 16362	0.7083	0.67	0.2083	0
TRIAL 4	72, 310, 16362	0.75	0.67	0.2838	0
KNN Binary					
	Features Implemented in Training and Testing by Trial	Training (Average Accuracy)	Testing (F1 Score)	Train St.D Average	Test St. D
TRIAL 1	72, 310, 3010, 4769, 8697, 11419, 11812, 14233, 15904, 16362	0.8917	0.45	0.175	0
TRIAL 2	310, 3010, 4769, 11419, 16362	0.8917	0.45	0.175	0
TRIAL 3	310, 4769, 16362	0.7583	0.52	0.2399	0
TRIAL 4	72, 310, 16362	0.7333	0.33	0.2261	0
NB Binary					

	Features Implemented in Training and Testing by Trial	Training (Average Accuracy)	Testing (F1 Score)	Train St.D Average	Test St. D
TRIAL 1	72, 310, 3010, 4769, 8697, 11419, 11812, 14233, 15904, 16362	0.775	0.67	0.2473	0
TRIAL 2	310, 3010, 4769, 11419, 16362	0.7833	0.67	0.2506	0
TRIAL 3	310, 4769, 16362	0.7833	0.67	0.2506	0
TRIAL 4	72, 310, 16362	0.7833	0.67	0.2506	0
	SVM Binary				
	Features Implemented in Training and Testing by Trial	Training (Average Accuracy)	Testing (F1 Score)	Train St.D Average	Test St. D
TRIAL 1	72, 310, 3010, 4769, 8697, 11419, 11812, 14233, 15904, 16362	0.6	0.33	0.2409	0
TRIAL 2	310, 3010, 4769, 11419, 16362	0.7833	0.67	0.2506	0
TRIAL 3	310, 4769, 16362	0.7833	0.67	0.2506	0
TRIAL 4	72, 310, 16362	0.7833	0.67	0.2506	0

Table S3.5: Machine learning analyses for mitochondrial SNP features using binary classification.

CART = Classification and Regression Trees, LR = Logistic Regression, LDA = Linear Discriminant Analysis, KNN = K-Nearest Neighbors, NB = Naïve Bayes, SVC = Support Vector Machines. St.D. = standard deviation, binary = non-diabetic and type 2 diabetic, multi = non-diabetic, prediabetic, and type 2 diabetic.

Table S3.6

SEED = 87					
CART Multiple					
	Features Implemented in Training and Testing by Trial	Training (Average Accuracy)	Testing (F1 Score)	Train St.D Average	Test St. D
TRIAL 1	114, 143, 151, 195, 310, 3010, 4295, 4769, 7028, 8860, 9055, 9840, 10398, 11812, 12246, 13401, 14766, 15191, 15223, 16124, 16183, 16189, 16270, 16336, 16344, 16362, 16390, 16519	0.67	0.576	0.23	0.009
TRIAL 2	4295, 7028, 10398, 16362, 16390	0.808	0.5	0.171	0
TRIAL 3	7028, 10398, 16362	0.758	0.5	0.181	0
TRIAL 4	114, 143, 151, 195, 310, 16124, 16183, 16189, 16270, 16336, 16344, 16362, 16390, 16519	0.33	0.12	0.309	0
LR Multiple					
	Features Implemented in Training and Testing by Trial	Training (Average Accuracy)	Testing (F1 Score)	Train St.D Average	Test St. D
TRIAL 1	114, 143, 151, 195, 310, 3010, 4295, 4769, 7028, 8860, 9055, 9840, 10398, 11812, 12246, 13401, 14766, 15191, 15223, 16124, 16183, 16189, 16270, 16336, 16344, 16362, 16390, 16519	0.6667	0.38	0.2789	0
TRIAL 2	4295, 7028, 10398, 16362, 16390	0.6167	0.5	0.2333	0
TRIAL 3	7028, 10398, 16362	0.6417	0.5	0.2329	0
TRIAL 4	114, 143, 151, 195, 310, 16124, 16183, 16189, 16270, 16336, 16344, 16362, 16390, 16519	0.3917	0.15	0.2238	0
LDA Multiple					
	Features Implemented in Training and Testing by Trial	Training (Average Accuracy)	Testing (F1 Score)	Train St.D Average	Test St. D
TRIAL 1	114, 143, 151, 195, 310, 3010, 4295, 4769, 7028, 8860, 9055, 9840, 10398, 11812, 12246, 13401, 14766, 15191, 15223, 16124, 16183, 16189, 16270, 16336, 16344, 16362, 16390, 16519	0.575	0.58	0.2621	0
TRIAL 2	4295, 7028, 10398, 16362, 16390	0.7833	0.5	0.159	0
TRIAL 3	7028, 10398, 16362	0.7583	0.5	0.1805	0
TRIAL 4	114, 143, 151, 195, 310, 16124, 16183, 16189, 16270, 16336, 16344, 16362, 16390, 16519	0.3833	0.15	0.2533	0
KNN Multiple					

	Features Implemented in Training and Testing by Trial	Training (Average Accuracy)	Testing (F1 Score)	Train St.D Average	Test St. D
TRIAL 1	114, 143, 151, 195, 310, 3010, 4295, 4769, 7028, 8860, 9055, 9840, 10398, 11812, 12246, 13401, 14766, 15191, 15223, 16124, 16183, 16189, 16270, 16336, 16344, 16362, 16390, 16519	0.5417	0.25	0.2451	0
TRIAL 2	4295, 7028, 10398, 16362, 16390	0.7583	0.5	0.1805	0
TRIAL 3	7028, 10398, 16362	0.7583	0.5	0.1805	0
TRIAL 4	114, 143, 151, 195, 310, 16124, 16183, 16189, 16270, 16336, 16344, 16362, 16390, 16519	0.35	0.14	0.2784	0
	NB Multiple				
	Features Implemented in Training and Testing by Trial	Training (Average Accuracy)	Testing (F1 Score)	Train St.D Average	Test St. D
TRIAL 1	114, 143, 151, 195, 310, 3010, 4295, 4769, 7028, 8860, 9055, 9840, 10398, 11812, 12246, 13401, 14766, 15191, 15223, 16124, 16183, 16189, 16270, 16336, 16344, 16362, 16390, 16519	0.45	0.35	0.2303	0
TRIAL 2	4295, 7028, 10398, 16362, 16390	0.6167	0.68	0.2048	0
TRIAL 3	7028, 10398, 16362	0.5417	0.68	0.1873	0
TRIAL 4	114, 143, 151, 195, 310, 16124, 16183, 16189, 16270, 16336, 16344, 16362, 16390, 16519	0.3917	0.46	0.3119	0
	SVM Multiple				
	Features Implemented in Training and Testing by Trial	Training (Average Accuracy)	Testing (F1 Score)	Train St.D Average	Test St. D
TRIAL 1	114, 143, 151, 195, 310, 3010, 4295, 4769, 7028, 8860, 9055, 9840, 10398, 11812, 12246, 13401, 14766, 15191, 15223, 16124, 16183, 16189, 16270, 16336, 16344, 16362, 16390, 16519	0.45	0.14	0.2014	0
TRIAL 2	4295, 7028, 10398, 16362, 16390	0.4917	0.14	0.1557	0
TRIAL 3	7028, 10398, 16362	0.5417	0.3	0.1502	0
TRIAL 4	114, 143, 151, 195, 310, 16124, 16183, 16189, 16270, 16336, 16344, 16362, 16390, 16519	0.45	0.14	0.2014	0

Table S3.6: Machine learning analyses for mitochondrial SNP features using multi classification.

CART = Classification and Regression Trees, LR = Logistic Regression, LDA = Linear Discriminant Analysis, KNN = K-Nearest Neighbors, NB = Naïve Bayes, SVC = Support Vector Machines. St.D. = standard deviation, binary = non-diabetic and type 2 diabetic, multi = non-diabetic, prediabetic, and type 2 diabetic.

Table S3.7

SEED = 8					
	CART Binary				
	Features Implemented in Training and Testing by Trial	Training (Average Accuracy)	Testing (F1 Score)	Train St.D Average	Test St. D
TRIAL 1	CpG 24, CpG 26, CpG 28, CpG 29, CpG 39	0.86336	0.742	0.17244	0.06572671
TRIAL 2	CpG 24, CpG 28, CpG 29, CpG 39	0.88336	0.718	0.15724	0.06572671
TRIAL 3	CpG 24, CpG 26, CpG 28, CpG 29	0.89002	0.79	0.12708	0
TRIAL 4	CpG 24, CpG 28, CpG 29	0.925	0.79	0.1146	0
TRIAL 5	CpG 24, CpG 29	0.9	0.67	0.1225	0
	LR Binary				
	Features Implemented in Training and Testing by Trial	Training (Average Accuracy)	Testing (F1 Score)	Train St.D Average	Test St. D
TRIAL 1	CpG 24, CpG 26, CpG 28, CpG 29, CpG 39	0.733333	0.49	0.189297	0
TRIAL 2	CpG 24, CpG 28, CpG 29, CpG 39	0.791667	0.6	0.190941	0
TRIAL 3	CpG 24, CpG 26, CpG 28, CpG 29	0.775	0.7	0.190212	0
TRIAL 4	CpG 24, CpG 28, CpG 29	0.833333	0.58	0.139443	0
TRIAL 5	CpG 24, CpG 29	0.766667	0.8	0.238048	0
	LDA Binary				
	Features Implemented in Training and Testing by Trial	Training (Average Accuracy)	Testing (F1 Score)	Train St.D Average	Test St. D
TRIAL 1	CpG 24, CpG 26, CpG 28, CpG 29, CpG 39	0.758333	0.58	0.141667	0
TRIAL 2	CpG 24, CpG 28, CpG 29, CpG 39	0.808333	0.58	0.129368	0
TRIAL 3	CpG 24, CpG 26, CpG 28, CpG 29	0.758333	0.58	0.087003	0
TRIAL 4	CpG 24, CpG 28, CpG 29	0.808333	0.58	0.129368	0
TRIAL 5	CpG 24, CpG 29	0.733333	0.58	0.226078	0
	KNN Binary				
	Features Implemented in Training and Testing by Trial	Training (Average Accuracy)	Testing (F1 Score)	Train St.D Average	Test St. D
TRIAL 1	CpG 24, CpG 26, CpG 28, CpG 29, CpG 39	0.625	0.45	0.214897	0
TRIAL 2	CpG 24, CpG 28, CpG 29, CpG 39	0.65	0.49	0.181812	0
TRIAL 3	CpG 24, CpG 26, CpG 28, CpG 29	0.683333	0.45	0.177951	0
TRIAL 4	CpG 24, CpG 28, CpG 29	0.783333	0.7	0.113039	0

TRIAL 5	CpG 24, CpG 29	0.858333	0.9	0.182764	0
	NB Binary				
	Features Implemented in Training and Testing by Trial	Training (Average Accuracy)	Testing (F1 Score)	Train St.D Average	Test St. D
TRIAL 1	CpG 24, CpG 26, CpG 28, CpG 29, CpG 39	0.7	0.52	0.201384	0
TRIAL 2	CpG 24, CpG 28, CpG 29, CpG 39	0.7	0.52	0.201384	0
TRIAL 3	CpG 24, CpG 26, CpG 28, CpG 29	0.758333	0.52	0.234076	0
TRIAL 4	CpG 24, CpG 28, CpG 29	0.783333	0.52	0.194365	0
TRIAL 5	CpG 24, CpG 29	0.783333	0.52	0.224227	0
	SVM Binary				
	Features Implemented in Training and Testing by Trial	Training (Average Accuracy)	Testing (F1 Score)	Train St.D Average	Test St. D
TRIAL 1	CpG 24, CpG 26, CpG 28, CpG 29, CpG 39	0.625	0.7	0.289276	0
TRIAL 2	CpG 24, CpG 28, CpG 29, CpG 39	0.716667	0.6	0.236291	0
TRIAL 3	CpG 24, CpG 26, CpG 28, CpG 29	0.783333	0.58	0.217945	0
TRIAL 4	CpG 24, CpG 28, CpG 29	0.783333	0.58	0.15899	0
TRIAL 5	CpG 24, CpG 29	0.783333	0.67	0.194365	0

Table S3.7: Machine learning analyses for Transcription Factor A, Mitochondrial (TFAM) CpG island methylation features using binary classification. CART = Classification and Regression Trees, LR = Logistic Regression, LDA = Linear Discriminant Analysis, KNN = K-Nearest Neighbors, NB = Naïve Bayes, SVC = Support Vector Machines. CpG = cytosine nucleotide followed by a guanine nucleotide, St.D. = standard deviation, binary = non-diabetic and type 2 diabetic, multi = non-diabetic, prediabetic, and type 2 diabetic.

Table S3.8

SEED = 22					
	CART Multiple				
	Features Implemented in Training and Testing by Trial	Training (Average Accuracy)	Testing (F1 Score)	Train St.D Average	Test St. D
TRIAL 1	CpG 1, CpG 5, CpG 14, CpG 20, CpG 23, CpG 24, CpG 33, TFAM CpG	0.71836	0.668	0.19942	0.05761944
TRIAL 2	CpG 1, CpG 14, CpG 20, CpG 23, CpG 24, CpG 33, TFAM CpG	0.69832	0.666	0.20908	0.06024948
TRIAL 3	CpG 1, CpG 20, CpG 23, CpG 24, TFAM CpG	0.73168	0.6	0.19586	0
TRIAL 4	CpG 1, CpG 14, CpG 20, CpG 23, CpG 24, CpG 35, TFAM CpG	0.76668	0.644	0.21556	0.06024948
TRIAL 5	CpG 1, CpG 20, CpG 23, CpG 24, CpG 35, TFAM CpG	0.74834	0.644	0.19818	0.06024948
	LR Multiple				
	Features Implemented in Training and Testing by Trial	Training (Average Accuracy)	Testing (F1 Score)	Train St.D Average	Test St. D
TRIAL 1	CpG 1, CpG 5, CpG 14, CpG 20, CpG 23, CpG 24, CpG 33, TFAM CpG	0.491667	0.15	0.242813	0
TRIAL 2	CpG 1, CpG 14, CpG 20, CpG 23, CpG 24, CpG 33, TFAM CpG	0.491667	0.26	0.215542	0
TRIAL 3	CpG 1, CpG 20, CpG 23, CpG 24, TFAM CpG	0.475	0.28	0.190212	0
TRIAL 4	CpG 1, CpG 14, CpG 20, CpG 23, CpG 24, CpG 35, TFAM CpG	0.491667	0.26	0.242813	0
TRIAL 5	CpG 1, CpG 20, CpG 23, CpG 24, CpG 35, TFAM CpG	0.466667	0.27	0.253311	0
	LDA Multiple				
	Features Implemented in Training and Testing by Trial	Training (Average Accuracy)	Testing (F1 Score)	Train St.D Average	Test St. D
TRIAL 1	CpG 1, CpG 5, CpG 14, CpG 20, CpG 23, CpG 24, CpG 33, TFAM CpG	0.466667	0.22	0.227303	0
TRIAL 2	CpG 1, CpG 14, CpG 20, CpG 23, CpG 24, CpG 33, TFAM CpG	0.466667	0.28	0.227303	0
TRIAL 3	CpG 1, CpG 20, CpG 23, CpG 24, TFAM CpG	0.475	0.28	0.190212	0
TRIAL 4	CpG 1, CpG 14, CpG 20, CpG 23, CpG 24, CpG 35, TFAM CpG	0.441667	0.28	0.283945	0
TRIAL 5	CpG 1, CpG 20, CpG 23, CpG 24, CpG 35, TFAM CpG	0.441667	0.28	0.283945	0
	KNN Multiple				
	Features Implemented in Training and Testing by Trial	Training (Average Accuracy)	Testing (F1 Score)	Train St.D Average	Test St. D

TRIAL 1	CpG 1, CpG 5, CpG 14, CpG 20, CpG 23, CpG 24, CpG 33, TFAM CpG	0.5	0.24	0.235702	0
TRIAL 2	CpG 1, CpG 14, CpG 20, CpG 23, CpG 24, CpG 33, TFAM CpG	0.475	0.31	0.247347	0
TRIAL 3	CpG 1, CpG 20, CpG 23, CpG 24, TFAM CpG	0.45	0.38	0.167498	0
TRIAL 4	CpG 1, CpG 14, CpG 20, CpG 23, CpG 24, CpG 35, TFAM CpG	0.308333	0.38	0.186525	0
TRIAL 5	CpG 1, CpG 20, CpG 23, CpG 24, CpG 35, TFAM CpG	0.416667	0.38	0.247207	0
	NB Multiple				
	Features Implemented in Training and Testing by Trial	Training (Average Accuracy)	Testing (F1 Score)	Train St.D Average	Test St. D
TRIAL 1	CpG 1, CpG 5, CpG 14, CpG 20, CpG 23, CpG 24, CpG 33, TFAM CpG	0.45	0.13	0.124722	0
TRIAL 2	CpG 1, CpG 14, CpG 20, CpG 23, CpG 24, CpG 33, TFAM CpG	0.475	0.26	0.153885	0
TRIAL 3	CpG 1, CpG 20, CpG 23, CpG 24, TFAM CpG	0.55	0.11	0.256038	0
TRIAL 4	CpG 1, CpG 14, CpG 20, CpG 23, CpG 24, CpG 35, TFAM CpG	0.45	0.28	0.201384	0
TRIAL 5	CpG 1, CpG 20, CpG 23, CpG 24, CpG 35, TFAM CpG	0.5	0.28	0.207498	0
	SVM Multiple				
	Features Implemented in Training and Testing by Trial	Training (Average Accuracy)	Testing (F1 Score)	Train St.D Average	Test St. D
TRIAL 1	CpG 1, CpG 5, CpG 14, CpG 20, CpG 23, CpG 24, CpG 33, TFAM CpG	0.5	0.07	0.260875	0
TRIAL 2	CpG 1, CpG 14, CpG 20, CpG 23, CpG 24, CpG 33, TFAM CpG	0.483333	0.16	0.235112	0
TRIAL 3	CpG 1, CpG 20, CpG 23, CpG 24, TFAM CpG	0.425	0.17	0.237024	0
TRIAL 4	CpG 1, CpG 14, CpG 20, CpG 23, CpG 24, CpG 35, TFAM CpG	0.508333	0.07	0.248468	0
TRIAL 5	CpG 1, CpG 20, CpG 23, CpG 24, CpG 35, TFAM CpG	0.508333	0.17	0.272463	0

Table S3.8: Machine learning analyses for Transcription Factor A, Mitochondrial (TFAM) CpG island methylation features using multi classification. CART = Classification and Regression Trees, LR = Logistic Regression, LDA = Linear Discriminant Analysis, KNN = K-Nearest Neighbors, NB = Naïve Bayes, SVC = Support Vector Machines. CpG = cytosine nucleotide followed by a guanine nucleotide, TFAM CpG = total CpG methylation of the TFAM promoter region, St.D. = standard deviation, binary = non-diabetic and type 2 diabetic, multi = non-diabetic, prediabetic, and type 2 diabetic.

Table S3.9

SEED = 15					
CART Binary					
	Features Implemented in Training and Testing by Trial	Training (Average Accuracy)	Testing (F1 Score)	Train St.D Average	Test St. D
TRIAL 1	Methyl, Mito 5hmC, Nuc 5hmC, Mito 5mC, Nuc 5mC, CpG 11, CpG 23, CpG 24, CpG 28	0.786666	0.758	0.2043988	0.0491935
TRIAL 2	Methyl, Mito 5hmC, Nuc 5hmC, Mito 5mC, Nuc 5mC, CpG 1 - 24, TFAM CpG, TFAM Non-CpG	0.8083318	0.714	0.2168344	0.06024948
TRIAL 3	Methyl, Mito 5hmC, Nuc 5hmC, Mito 5mC, Nuc 5mC, CpG 24, TFAM CpG, TFAM Non-CpG	0.831667	0.67	0.2166188	0
TRIAL 4	Methyl, Nuc 5mC, CpG 1 - 24	0.7899998	0.736	0.209388	0.06024948
TRIAL 5	Nuc 5mC, CpG 1,CpG 7,CpG 8,CpG 9,CpG 11,CpG 12,CpG 22,CpG 23,CpG 24,CpG 28	0.743333	0.78	0.1964948	0
LR Binary					
	Features Implemented in Training and Testing by Trial	Training (Average Accuracy)	Testing (F1 Score)	Train St.D Average	Test St. D
TRIAL 1	Methyl, Mito 5hmC, Nuc 5hmC, Mito 5mC, Nuc 5mC, CpG 11, CpG 23, CpG 24, CpG 28	0.7	0.56	0.279384	0
TRIAL 2	Methyl, Mito 5hmC, Nuc 5hmC, Mito 5mC, Nuc 5mC, CpG 1 - 24, TFAM CpG, TFAM Non-CpG	0.633333	0.67	0.208167	0
TRIAL 3	Methyl, Mito 5hmC, Nuc 5hmC, Mito 5mC, Nuc 5mC, CpG 24, TFAM CpG, TFAM Non-CpG	0.758333	0.66	0.18047	0
TRIAL 4	Methyl, Nuc 5mC, CpG 1 - 24	0.683333	0.66	0.2	0
TRIAL 5	Nuc 5mC, CpG 1,CpG 7,CpG 8,CpG 9,CpG 11,CpG 12,CpG 22,CpG 23,CpG 24,CpG 28	0.658333	0.66	0.253996	0
LDA Binary					
	Features Implemented in Training and Testing by Trial	Training (Average Accuracy)	Testing (F1 Score)	Train St.D Average	Test St. D
TRIAL 1	Methyl, Mito 5hmC, Nuc 5hmC, Mito 5mC, Nuc 5mC, CpG 11, CpG 23, CpG 24, CpG 28	0.766667	0.22	0.238048	0
TRIAL 2	Methyl, Mito 5hmC, Nuc 5hmC, Mito 5mC, Nuc 5mC, CpG 1 - 24, TFAM CpG, TFAM Non-CpG	0.541667	0.43	0.250693	0
TRIAL 3	Methyl, Mito 5hmC, Nuc 5hmC, Mito 5mC, Nuc 5mC, CpG 24, TFAM CpG, TFAM Non-CpG	0.775	0.22	0.325	0

TRIAL 4	Methyl, Nuc 5mC, CpG 1 - 24	0.525	0.44	0.276512	0
TRIAL 5	Nuc 5mC, CpG 1,CpG 7,CpG 8,CpG 9,CpG 11,CpG 12,CpG 22,CpG 23,CpG 24,CpG 28	0.683333	0.78	0.306866	0
	KNN Binary				
	Features Implemented in Training and Testing by Trial	Training (Average Accuracy)	Testing (F1 Score)	Train St.D Average	Test St. D
TRIAL 1	Methyl, Mito 5hmC, Nuc 5hmC, Mito 5mC, Nuc 5mC, CpG 11, CpG 23, CpG 24, CpG 28	0.608333	0.66	0.295921	0
TRIAL 2	Methyl, Mito 5hmC, Nuc 5hmC, Mito 5mC, Nuc 5mC, CpG 1 - 24, TFAM CpG, TFAM Non-CpG	0.608333	0.78	0.229885	0
TRIAL 3	Methyl, Mito 5hmC, Nuc 5hmC, Mito 5mC, Nuc 5mC, CpG 24, TFAM CpG, TFAM Non-CpG	0.716667	0.76	0.236291	0
TRIAL 4	Methyl, Nuc 5mC, CpG 1 - 24	0.633333	0.78	0.230338	0
TRIAL 5	Nuc 5mC, CpG 1,CpG 7,CpG 8,CpG 9,CpG 11,CpG 12,CpG 22,CpG 23,CpG 24,CpG 28	0.641667	0.66	0.258333	0
	NB Binary				
	Features Implemented in Training and Testing by Trial	Training (Average Accuracy)	Testing (F1 Score)	Train St.D Average	Test St. D
TRIAL 1	Methyl, Mito 5hmC, Nuc 5hmC, Mito 5mC, Nuc 5mC, CpG 11, CpG 23, CpG 24, CpG 28	0.691667	0.33	0.300578	0
TRIAL 2	Methyl, Mito 5hmC, Nuc 5hmC, Mito 5mC, Nuc 5mC, CpG 1 - 24, TFAM CpG, TFAM Non-CpG	0.566667	0.67	0.355121	0
TRIAL 3	Methyl, Mito 5hmC, Nuc 5hmC, Mito 5mC, Nuc 5mC, CpG 24, TFAM CpG, TFAM Non-CpG	0.691667	0.54	0.255631	0
TRIAL 4	Methyl, Nuc 5mC, CpG 1 - 24	0.508333	0.77	0.366003	0
TRIAL 5	Nuc 5mC, CpG 1,CpG 7,CpG 8,CpG 9,CpG 11,CpG 12,CpG 22,CpG 23,CpG 24,CpG 28	0.6	0.56	0.357071	0
	SVM Binary				
	Features Implemented in Training and Testing by Trial	Training (Average Accuracy)	Testing (F1 Score)	Train St.D Average	Test St. D
TRIAL 1	Methyl, Mito 5hmC, Nuc 5hmC, Mito 5mC, Nuc 5mC, CpG 11, CpG 23, CpG 24, CpG 28	0.625	0.66	0.321131	0
TRIAL 2	Methyl, Mito 5hmC, Nuc 5hmC, Mito 5mC, Nuc 5mC, CpG 1 - 24, TFAM CpG, TFAM Non-CpG	0.591667	0.61	0.208999	0

TRIAL 3	Methyl, Mito 5hmC, Nuc 5hmC, Mito 5mC, Nuc 5mC, CpG 24, TFAM CpG, TFAM Non-CpG	0.766667	0.61	0.238048	0
TRIAL 4	Methyl, Nuc 5mC, CpG 1 - 24	0.591667	0.61	0.208999	0
TRIAL 5	Nuc 5mC, CpG 1,CpG 7,CpG 8,CpG 9,CpG 11,CpG 12,CpG 22,CpG 23,CpG 24,CpG 28	0.708333	0.76	0.230489	0

Table S3.9: Machine learning analyses for all combined features using binary classification. CART = Classification and Regression Trees, LR = Logistic Regression, LDA = Linear Discriminant Analysis, KNN = K-Nearest Neighbors, NB = Naïve Bayes, SVC = Support Vector Machines. Nuc = Nuclear, Mito = Mitochondrial, 5mC = 5-methylcytosine, 5hmC = 5-hydroxymethylcytosine, Methyl = S-adenosyl methionine methyltransferase activity, CpG = cytosine nucleotide followed by a guanine nucleotide, TFAM CpG = total CpG methylation of the TFAM promoter region, TFAM Non-CpG = total methylation of the TFAM promoter region not at CpG sites, St.D. = standard deviation, binary = non-diabetic and type 2 diabetic, multi = non-diabetic, prediabetic, and type 2 diabetic.

Table S3.10

SEED = 86					
	CART Multiple				
	Features Implemented in Training and Testing by Trial	Training (Average Accuracy)	Testing (F1 Score)	Train St.D Avg.	Test St. D
TRIAL 1	Mito 5mC, Nuc 5mC, Complex III, BMI, CpG 7, CpG 24, CpG 28, TFAM CpG, TFAM Non-CpG	0.7916666	0.558	0.224768	0.00447214
TRIAL 2	Nuc 5mC, BMI, CpG 24, CpG 28, TFAM CpG, TFAM Non-CpG	0.8016666	0.56	0.2271884	0
TRIAL 3	BMI, CpG 24, TFAM CpG, TFAM Non-CpG	0.7316668	0.56	0.2568122	0
TRIAL 4	BMI, CpG 24, CpG 28, TFAM CpG, TFAM Non-CpG	0.8366664	0.56	0.234217	0
TRIAL 5	BMI, CpG 24, TFAM CpG	0.6933334	0.594	0.2764508	0.09476286
TRIAL 6	BMI, CpG 24, TFAM Non-CpG	0.63	0.33	0.20036	0
TRIAL 7	BMI, CpG 24	0.6266668	0.258	0.2756018	0.0752994
	LR Multiple				
	Features Implemented in Training and Testing by Trial	Training (Average Accuracy)	Testing (F1 Score)	Train St.D Avg.	Test St. D
TRIAL 1	Mito 5mC, Nuc 5mC, Complex III, BMI, CpG 7, CpG 24, CpG 28, TFAM CpG, TFAM Non-CpG	0.516667	0.55	0.213437	0
TRIAL 2	Nuc 5mC, BMI, CpG 24, CpG 28, TFAM CpG, TFAM Non-CpG	0.55	0.55	0.145297	0
TRIAL 3	BMI, CpG 24, TFAM CpG, TFAM Non-CpG	0.516667	0.18	0.189297	0
TRIAL 4	BMI, CpG 24, CpG 28, TFAM CpG, TFAM Non-CpG	0.516667	0.13	0.189297	0
TRIAL 5	BMI, CpG 24, TFAM CpG	0.575	0.18	0.191667	0
TRIAL 6	BMI, CpG 24, TFAM Non-CpG	0.558333	0.18	0.220637	0
TRIAL 7	BMI, CpG 24	0.533333	0.18	0.211476	0
	LDA Multiple				
	Features Implemented in Training and Testing by Trial	Training (Average Accuracy)	Testing (F1 Score)	Train St.D Avg.	Test St. D
TRIAL 1	Mito 5mC, Nuc 5mC, Complex III, BMI, CpG 7, CpG 24, CpG 28, TFAM CpG, TFAM Non-CpG	0.525	0.67	0.129368	0
TRIAL 2	Nuc 5mC, BMI, CpG 24, CpG 28, TFAM CpG, TFAM Non-CpG	0.533333	0.43	0.15	0
TRIAL 3	BMI, CpG 24, TFAM CpG, TFAM Non-CpG	0.475	0.44	0.232886	0
TRIAL 4	BMI, CpG 24, CpG 28, TFAM CpG, TFAM Non-CpG	0.541667	0.44	0.125	0

TRIAL 5	BMI, CpG 24, TFAM CpG	0.55	0.34	0.214735	0
TRIAL 6	BMI, CpG 24, TFAM Non-CpG	0.475	0.19	0.232886	0
TRIAL 7	BMI, CpG 24	0.525	0.1	0.232886	0
	KNN Multiple				
	Features Implemented in Training and Testing by Trial	Training (Average Accuracy)	Testing (F1 Score)	Train St.D Avg.	Test St. D
TRIAL 1	Mito 5mC, Nuc 5mC, Complex III, BMI, CpG 7, CpG 24, CpG 28, TFAM CpG, TFAM Non-CpG	0.325	0.3	0.215542	0
TRIAL 2	Nuc 5mC, BMI, CpG 24, CpG 28, TFAM CpG, TFAM Non-CpG	0.366667	0.33	0.284312	0
TRIAL 3	BMI, CpG 24, TFAM CpG, TFAM Non-CpG	0.383333	0.31	0.26405	0
TRIAL 4	BMI, CpG 24, CpG 28, TFAM CpG, TFAM Non-CpG	0.375	0.34	0.294038	0
TRIAL 5	BMI, CpG 24, TFAM CpG	0.383333	0.31	0.286744	0
TRIAL 6	BMI, CpG 24, TFAM Non-CpG	0.408333	0.31	0.26207	0
TRIAL 7	BMI, CpG 24	0.433333	0.13	0.23214	0
	NB Multiple				
	Features Implemented in Training and Testing by Trial	Training (Average Accuracy)	Testing (F1 Score)	Train St.D Avg.	Test St. D
TRIAL 1	Mito 5mC, Nuc 5mC, Complex III, BMI, CpG 7, CpG 24, CpG 28, TFAM CpG, TFAM Non-CpG	0.558333	0.4	0.16266	0
TRIAL 2	Nuc 5mC, BMI, CpG 24, CpG 28, TFAM CpG, TFAM Non-CpG	0.608333	0.56	0.229885	0
TRIAL 3	BMI, CpG 24, TFAM CpG, TFAM Non-CpG	0.608333	0.47	0.273988	0
TRIAL 4	BMI, CpG 24, CpG 28, TFAM CpG, TFAM Non-CpG	0.591667	0.47	0.208999	0
TRIAL 5	BMI, CpG 24, TFAM CpG	0.55	0.18	0.183333	0
TRIAL 6	BMI, CpG 24, TFAM Non-CpG	0.566667	0.18	0.203443	0
TRIAL 7	BMI, CpG 24	0.55	0.18	0.214735	0
	SVM Multiple				
	Features Implemented in Training and Testing by Trial	Training (Average Accuracy)	Testing (F1 Score)	Train St.D Avg.	Test St. D
TRIAL 1	Mito 5mC, Nuc 5mC, Complex III, BMI, CpG 7, CpG 24, CpG 28, TFAM CpG, TFAM Non-CpG	0.35	0.17	0.152753	0
TRIAL 2	Nuc 5mC, BMI, CpG 24, CpG 28, TFAM CpG, TFAM Non-CpG	0.491667	0.31	0.29451	0
TRIAL 3	BMI, CpG 24, TFAM CpG, TFAM Non-CpG	0.408333	0.08	0.275	0

TRIAL 4	BMI, CpG 24, CpG 28,TFAM CpG, TFAM Non-CpG	0.341667	0.13	0.29451	0
TRIAL 5	BMI, CpG 24, TFAM CpG	0.383333	0.18	0.298608	0
TRIAL 6	BMI, CpG 24, TFAM Non-CpG	0.491667	0.08	0.248468	0
TRIAL 7	BMI, CpG 24	0.466667	0.18	0.211476	0

Table S3.10: Machine learning analyses for all combined features using multi classification. CART = Classification and Regression Trees, LR = Logistic Regression, LDA = Linear Discriminant Analysis, KNN = K-Nearest Neighbors, NB = Naïve Bayes, SVC = Support Vector Machines. Nuc = Nuclear, Mito = Mitochondrial, 5mC = 5-methylcytosine, 5hmC = 5-hydroxymethylcytosine, CpG = cytosine nucleotide followed by a guanine nucleotide, TFAM CpG = total CpG methylation of the TFAM promoter region, TFAM Non-CpG = total methylation of the TFAM promoter region not at CpG sites, St.D. = standard deviation, binary = non-diabetic and type 2 diabetic, multi = non-diabetic, prediabetic, and type 2 diabetic.

TableS3.11

Model	Training	Training (StDev)	Testing	Testing (StDev)	F1 Score	Important Features	Important Feature Bias	AUC
LR	0.808	0.171	0.667	0.000	0.670	CpG 35, CpG 29, Mito 5mC, CpG 1, Nuc 5mC, 16362, SNP7028, CpG 24, SNP16519, SNP11719	(2.656), (-1.496), (-0.699), (0.583), (0.535), (-0.359), (0.311), (-0.304), (0.099), (-0.066)	0.700
LDA	0.917	0.129	0.778	0.000	0.780	SNP4295, SNP4917, SNP7028, SNP16362, Nuc 5hmC, SNP16519, 72, Nuc 5mC, SNP8860, CpG 29	(-1.050E+16), (-8.170), (7.367), (-6.313), (-3.659), (3.472), (3.113), (2.483), (-2.119), (-1.359)	0.800
KNN	0.750	0.236	0.556	0.000	0.560	NA	NA	0.675
NB	0.683	0.249	0.778	0.000	0.780	Mito 5hmC, Methyltransferase	(1.000), (0.000)	0.725
SVM	0.750	0.242	0.667	0.000	0.670	CpG 35, CpG 29, SNP16362, Mito 5mC, Nuc 5mC, SNP7028, 72, CpG 24, SNP16519, CpG 1	(2.240), (-0.892), (-0.467), (-0.395), (0.313), (0.225), (0.216), (-0.210), (0.204), (0.190)	0.650
CART	0.833	0.212	0.711	0.061	0.692	CpG 24, Nuc 5mC, CpG 1	(58.711%), (22.547%), (18.742%)	0.740

Table S3.11: Overview of 6 machine-learning model analysis on 18 selected features in binary classification. Model analysis was conducted five times and averages are reported for the resulting training accuracy, training standard deviation, testing accuracy, testing standard deviation, F1 score, and *area under the curve* (AUC). Important biomarker features associated with each trained model are provided along with the associated influence value for each feature. Important features are listed in order of influence within the model. LR, LDA, SVM feature bias exists as an influence parameter where magnitude dictates feature influence. A positive influence value indicates the biomarker favors classification towards one label while a negative value indicates favorable classification of the opposite label. The larger the magnitude, the more strongly that feature shifts classification. NB feature influence indicates the most important biomarker per class in binary (0,1) classification schemes. CART feature bias percentages indicate feature influence on the created classification tree. Larger percentages indicate a feature that arises near the beginning of a tree before subsequent branching. Influence is not provided for KNN due to model restrictions. The 18 selected features include: Methyltransferase, Mito 5hmC, Nuc 5hmC, Mito 5mC, Nuc 5mC, CpG1, CpG24, CpG29, CpG35, SNP72, SNP4295, SNP4917, SNP7028, SNP8860, SNP11719, SNP16354, SNP16362, and SNP16519.

Table S3.12

Model	Training	Training (StDev)	Testing	Testing (StDev)	F1 Score	Important Features	Important Feature Bias
LR	0.408	0.209	0.444	0.000	0.380	Nuc 5mC , CpG1, CpG29, SNP16362, SNP7028, CpG35, SNP16519, SNP11719, CpG24, Mito 5mC	(-0.381), (-0.244), (0.200), (0.191), (-0.168), (-0.122), (-0.099), (0.081), (0.047), (0.031)
LDA	0.392	0.291	0.667	0.000	0.640	CpG35, SNP16362, SNP7028, CpG29, SNP4917, SNP11719, Nuc 5mC, Nuc 5hmC, Methyltransferase, Mito 5mC	(-2.932), (1.759), (-1.431), (1.323), (1.232), (1.151), (-1.144), (0.899), (-0.767), (0.640)
KNN	0.400	0.288	0.222	0.000	0.180	NA	NA
NB	0.367	0.312	0.444	0.000	0.350	Nuc 5hmC, Mito 5hmC, Methyltransferase	(2.000), (1.000), (0.000)
SVM	0.467	0.264	0.333	0.000	0.360	CpG35, Nuc 5mC, Mito 5mC, CpG24, SNP7028, CpG29, SNP11719, CpG1, SNP4917, Mito 5hmC	(0.770), (0.645), (-0.560), (-0.348), (-0.195), (-0.165), (0.137), (0.028), (0.026), (0.011)
CART	0.492	0.352	0.556	0.000	0.570	CpG 24, Methyltransferase, CpG 29, Nuc 5mC, CpG 1, Mito 5hmC, Mito 5mC, Nuc 5hmC, SNP11719	(32.762%), (21.973%), (19.800%), (8.501%), (5.598%), (3.357%), (2.975%), (1.678%), (1.678%)

Table S3.12: Overview of 6 machine-learning model analysis on 18 selected features in multiple classification. Model analysis was conducted five times and averages are reported for the resulting training accuracy, training standard deviation, testing accuracy, testing standard deviation, and F1 score. Important biomarker features associated with each trained model are provided along with the associated influence value for each feature. Important features are listed in order of influence within the model. LR, LDA, SVM feature bias exists as an influence parameter where magnitude dictates feature influence. A positive influence value indicates the biomarker favors classification towards one label while a negative value indicates favorable classification of the opposite label. The larger the magnitude, the more strongly that feature shifts classification. NB feature influence indicates the most important biomarker per class in multiple (0,1,2) classification schemes. CART feature bias percentages indicate feature influence on the created classification tree. Larger percentages indicate a feature that arises near the beginning of a tree before subsequent branching. Influence is not provided for KNN due to model restrictions. The 18 selected features include: Methyltransferase, Mito 5hmC, Nuc 5hmC, Mito 5mC, Nuc 5mC, CpG1, CpG24, CpG29, CpG35, SNP72, SNP4295, SNP4917, SNP7028, SNP8860, SNP11719, SNP16354, SNP16362, and SNP16519.

Table S3.13

Parameter	Non-Diabetic	Type 2 Diabetic
Nuclear 5mC (% Methylation)	2.039 ± 0.1063	2.873 ± 0.2445*
Mitochondrial 5mC (% Methylation)	1.776 ± 0.3107	1.644 ± 0.3658
Nuclear 5hmC (% Methylation)	0.178 ± 0.01935	0.1035 ± 0.0171*
Mitochondrial 5hmC (% Methylation)	0.08123 ± 0.02082	0.04837 ± 0.01984
Methyltransferase (μmol/min/mL)	0.005841 ± 0.0004433	0.004895 ± 0.0001608*
Complex I (nmol/min/μg)	45.24 ± 3.302	35.14 ± 3.193*
Complex III (nmol/min/μg)	106.8 ± 9.509	79.7 ± 6.337*
Complex IV (nmol/min/μg)	15.28 ± 1.7	16.52 ± 2.193
Complex V (nmol/min/mg)	134.7 ± 14.34	114.2 ± 10.1
Citrate Synthase (Unit/mg mitochondria)	0.02384 ± 0.00215	0.02021 ± 0.0009824
TFAM CpG (% Methylation)	1.663 ± 0.07412	1.568 ± 0.1121
TFAM Non-CpG (% Methylation)	1.267 ± 0.05839	1.237 ± 0.08055

Table S3.13: Biochemical features assessed in the non-diabetic and type 2 diabetic cohort. Groups are considered significantly different if $P \leq 0.05 = *$ compared to non-diabetic. All data are presented as the mean $\pm$ standard error of the mean (SEM). 5mC = 5-methylcytosine, 5hmC = 5-hydroxymethylcytosine, CpG = cytosine nucleotide followed by a guanine nucleotide, Complex = electron transport chain complex activity, TFAM = transcription factor A, mitochondrial.

Table S3.14

Parameter	Non-Diabetic	Pre-Diabetic	Type 2 Diabetic
Nuclear 5mC (% Methylation)	2.057 ± 0.1247	2.019 ± 0.1829	2.873 ± 0.2445*#
Mitochondrial 5mC (% Methylation)	1.448 ± 0.261	1.432 ± 0.2627	1.166 ± 0.191
Nuclear 5hmC (% Methylation)	0.1839 ± 0.02649	0.1617 ± 0.02873	0.107 ± 0.0169
Mitochondrial 5hmC (% Methylation)	0.07123 ± 0.03244	0.0964 ± 0.02049	0.05024 ± 0.02082
Methyltransferase (μmol/min/mL)	0.006339 ± 0.0006806	0.004878 ± 0.0004467	0.005185 ± 0.0002661
Complex I (nmol/min/μg)	42.69 ± 4.352	48.14 ± 5.091	35.14 ± 3.193
Complex III (nmol/min/μg)	105.2 ± 11.83	108.7 ± 15.72	79.7 ± 6.337
Complex IV (nmol/min/μg)	13.67 ± 1.738	17.12 ± 3.057	16.52 ± 2.193
Complex V (nmol/min/mg)	119.8 ± 3.07	151.7 ± 26.76	114.2 ± 10.1
Citrate Synthase (Unit/mg mitochondria)	0.01961 ± 0.001418	0.02868 ± 0.004009	0.02021 ± 0.0009824
TFAM CpG (% Methylation)	1.45 ± 0.05362	1.907 ± 0.1179*	1.568 ± 0.1121
TFAM Non-CpG (% Methylation)	1.2 ± 0.03096	1.343 ± 0.1192	1.237 ± 0.08055

Table S3.14: Biochemical features assessed in the non-diabetic and type 2 diabetic cohort. Groups are considered significantly different if $P \leq 0.05$ = * compared to non-diabetic or # compared to pre-diabetic. All data are presented as the mean $\pm$ standard error of the mean (SEM). 5mC = 5-methylcytosine, 5hmC = 5-hydroxymethylcytosine, CpG = cytosine nucleotide followed by a guanine nucleotide, Complex = electron transport chain complex activity, TFAM = transcription factor A, mitochondrial.

Figure S3.1

Figure S3.1: Feature importance using CART for physiological and biochemical characteristics from patients. (A) Training and testing accuracies for combinations of parameters that gave the most predictive outcomes in binary classification. (B) Percentage of individual feature importance by trial for binary classification. (C) Training and testing accuracies for combinations of parameters that gave the most predictive outcomes in multi classification. (D) Percentage of individual feature importance by trial for multi classification. Nuc = nuclear, Mito = mitochondrial, 5mC = 5-methylcytosine, 5hmC, 5-hydroxymethylcytosine, Methyl = S-adenosyl methionine methyltransferase activity, binary = non-diabetic and type 2 diabetic, multi = non-diabetic, prediabetic, and type 2 diabetic.

Figure S3.2

Figure S3.2: Feature importance using CART for mitochondrial DNA SNPs from patients. (A) Training and testing accuracies for combinations of parameters that gave the most predictive outcomes in binary classification. (B) Percentage of individual feature importance by trial for binary classification. (C) Training and testing accuracies for combinations of parameters that gave the most predictive outcomes in multi classification. (D) Percentage of individual feature importance by trial for multi classification. Numbers are indicative of the single nucleotide polymorphism (SNP) found in the mitochondrial DNA. Binary = non-diabetic and type 2 diabetic, multi = non-diabetic, prediabetic, and type 2 diabetic.

Figure S3.3

Figure S3.3: Expression and binding affinity of TFAM. (A) Expression of TFAM in isolated mitochondria from non-diabetic (n = 5, left) and type 2 diabetic (n = 5, right) patients. (B) Binding affinity of TFAM to the D-Loop region of mitochondrial DNA using chromatin immunoprecipitation. Groups are considered significantly different if $P \leq 0.05 = *$ compared to non-diabetic. All data are presented as the mean $\pm$ standard error of the mean (SEM). TFAM = transcription factor A, mitochondrial.

Figure S3.4

Figure S3.4: Feature importance using CART for CpG island methylation of TFAM from patients.

(A) Training and testing accuracies for combinations of parameters that gave the most predictive outcomes in binary classification. (B) Percentage of individual feature importance by trial for binary classification. (C) Training and testing accuracies for combinations of parameters that gave the most predictive outcomes in multi classification. (D) Percentage of individual feature importance by trial for multi classification. CpG = cytosine nucleotide followed by a guanine nucleotide, TFAM CpG = total CpG methylation of the TFAM promoter region, TFAM = transcription factor A, mitochondrial, binary = non-diabetic and type 2 diabetic, multi = non-diabetic, prediabetic, and type 2 diabetic.

Figure S3.5

Figure S3.5: Feature importance using CART for all factors combined from patients. (A) Training and testing accuracies for combinations of parameters that gave the most predictive outcomes in binary classification. (B) Percentage of individual feature importance by trial for binary classification. (C) Training and testing accuracies for combinations of parameters that gave the most predictive outcomes in multi classification. (D) Percentage of individual feature importance by trial for multi classification. Nuc = nuclear, Mito = mitochondrial, 5mC = 5-methylcytosine, 5hmC, 5-hydroxymethylcytosine, Methyltransferase = S-adenosyl methionine methyltransferase activity, CpG = cytosine nucleotide followed by a guanine nucleotide, TFAM CpG = total CpG methylation of the TFAM promoter region, TFAM Non-CpG = total methylation of the TFAM promoter region not at CpG sites, TFAM = transcription factor A, mitochondrial, binary = non-diabetic and type 2 diabetic, multi = non-diabetic, prediabetic, and type 2 diabetic.

Chapter 4: Specific Aim 3

The Mitochondrial Regulatory Network: Subcellular Localization of Non-Coding RNA in the Heart

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Abstract

Non-coding RNAs (ncRNAs) mediate processes such as transcription, translation, and other diverse biological functions within the cell. The purpose of the current study is to characterize the subcellular localization of ncRNA within the mitochondrion, and to understand how import/export mechanisms may dictate changes in RNA diversity during disease progression. Human patient and FVB/NJ mouse cardiac tissues were collected from diabetics ($n = 6$) and non-diabetics ($n = 6$). RNA from isolated subcellular compartments and crosslinking immunoprecipitation (CLIP) with Polynucleotide Phosphorylase (PNPase) were sequenced on the Illumina HiSeq and MiSeq. In HL-1 cells, qPCR of PNPase CLIP knockout mutants (KH and S1 knockout) was performed to evaluate mitochondrial/lncRNA interactions. LncRNA targeting sequences and secondary structures were validated through Classification and Regression Trees (CART) and Support Vector Machines (SVM). LncRNAs (MALAT1, NEAT1, and KCNQ1OT1), small nuclear RNA, small nucleolar RNA, and others were discovered in cardiac mitochondria, with diabetic insult significantly reducing expression of ncRNAs. PNPase was shown to bind lncRNA, with a reduced (~80%) binding affinity in the absence of its RNA binding domains. We identified small, stem-loop secondary structures on lncRNA, which could promote mitochondrial/lncRNA interactions. PNPase may function as a lncRNA import/export protein, facilitating cellular regulation in disease states.

Introduction

Long non-coding RNAs (lncRNAs) have emerged as a class of regulatory RNAs that assume multiple responsibilities within the cell. lncRNAs are known to be involved in both nuclear and cytoplasmic cellular processes such as regulating chromatin structure, histone modifications, mRNA transcription, microRNA (miRNA) sponging, and regulation of protein translation and modifications (64, 67). With increasing study of the roles of lncRNA in the cytoplasm, the implications of lncRNAs in other subcellular regions remains largely unexplored. The mitochondrion represents an isolated spatially-dense region within the cell that could significantly change the stoichiometric interactions between lncRNAs and other RNA species. Recent investigations have assessed the involvement of lncRNA in altering mitochondrial function (23), but the direct interactions of lncRNA, and other ncRNA, within the mitochondrion remain largely unknown.

The mitochondrion, which encodes for less than 1% of its own protein components, relies heavily on nuclear transcription and translation of mitochondrial proteins. At the nuclear and cytoplasmic levels, ncRNA can regulate transcription/translation of nuclear-encoded mitochondrial proteins. A lncRNA antisense of PTEN induced putative kinase 1 (naPINK1) was shown to regulate mitochondrial dynamics by inhibiting expression of PTEN mRNA (46). Likewise, miRNAs, such as miRNA-663, have been shown to influence mitochondrial composition by regulating the expression of electron transport chain complex I, II, III, and IV assembly factors (9). While these provide examples of how ncRNAs can impact mitochondrial assembly, ultrastructure, and overall function from outside of the mitochondrion, the regulatory network of ncRNAs within the organelle can also influence function. The mitochondrial genome is comprised of 2 rRNAs, 22 tRNAs, and 13 coding mRNAs (56). Work from our group (26, 48), and others (36), has demonstrated miRNAs imported into the mitochondria regulate the mitochondrial genome, largely by inhibiting translation of mitochondrial-encoded genes. Additionally, miRNAs have been shown to activate mitochondrial DNA transcription (69) and the production of miRNAs (2) and lncRNAs (18, 43) from the mitochondrial genome itself.

With the focus typically centered on a very limited number of imported miRNAs and their effects on mitochondrial transcription and health, aspects of miRNA diversity are often lost entirely. Expression profiles within the mitochondrion reveal large quantities of miRNAs (1, 26,

28, 53), even in the absence of mitochondrial DNA (16). While ongoing research is continuing to expand our understanding of ncRNA regulation of the mitochondrial genome, it begs the question: why would a large ncRNA regulatory network be required for maintaining 13 mitochondrial transcribed genes? Imported miRNAs have been identified to alter expression of the mitochondrial genome, but a large portion of miRNAs have shown little, or no, homology with mitochondrial derived sequences (26, 53). In understanding the abundance of ncRNAs within the mitochondrion, import/export pathways may provide details regarding the criteria necessary for ncRNA to become included in a mitochondrial regulatory network.

Polynucleotide phosphorylase (PNPase) is a ribonuclease conserved across bacterial, archaeal, and eukaryotic domains of life (49, 54). In bacteria, PNPase is a primary regulator of mRNA degradation (25), modifying the cellular response to temperature change (68) and biofilm formation (42), as well as functioning as a processing enzyme for long RNA (45). Progressing through the evolutionary timeline, eukaryotic PNPase is localized specifically to the intermembrane space of the mitochondrion, seemingly lacking its previously defined functional roles. In 2010, Wang et al. first established an RNA import function for PNPase in mammalian systems (60), revealing that tRNA and ribozyme machinery are transported by PNPase into the mitochondrion. Additionally, changes in PNPase expression have resulted in alterations in cellular function and miRNA import (48, 50). PNPase, through its KH and S1 RNA binding domains, may provide functional context for ncRNA regulation in the mitochondrion.

In the current study, we investigated the ncRNA regulatory network in the mitochondrion and its changes during disease progression. Specifically, tested if other nuclear-encoded ncRNAs, such as lncRNAs, are present within the mitochondrion. Through comparison of both cytoplasmic and isolated mitochondrial ncRNA populations, we identified hundreds of ncRNAs in the mitochondrion, including MALAT1, NEAT1, and KCNQ1OT1 lncRNAs, that were significantly decreased in diabetes mellitus. MALAT1, NEAT1, and KCNQ1OT1 were shown to bind to PNPase, which may act as a shuttle for import/export of lncRNA mediated by recognition of specific, RNA secondary structures. With the present data, we suggest that the mitochondrion has a dynamic ncRNA regulatory network that can be negatively impacted during disease progression.

Material & Methods

Study Approval and Patient Population

The West Virginia University Institutional Review Board and Institutional Biosafety Committee approved the studies and data generated from this work, including all tissue and patient information acquired (14). Patients were all consented by the Heart and Vascular Institute, J.W. Ruby Memorial Hospital at West Virginia University School of Medicine. Right atrial appendages were removed during open-heart and/or valvular surgeries and all tissue and data were stored in a double de-identified process. There was no incentive provided for patients. Surgeries were performed on both diabetic and non-diabetic patients, following consultation with the surgical team. Non-diabetic patients ($\text{HbA1c} < 5.7$ with no previous diagnosis of diabetes mellitus) and diabetic patients ($\text{HbA1c} > 5.7$ or with a previous diagnosis of diabetes mellitus) were designated in the study. Patients' tissue was used irrespective of sex, race, or ethnicity.

Murine Model

FVB/NJ male and female mice were housed in the West Virginia University Health Sciences Center Animal Facility and given access to a rodent diet and water *ad libitum*. FVB/NJ wild type mice were bred with FVB/NJ *Lepr^{db+/-}* mice to obtain a homozygous diabetic model. Animals in the wild type and diabetic groups were aged to 25 weeks and were euthanized for molecular assays using sedation through isoflurane and cervical dislocation. We have previously reported on the cardiovascular deficits of this model (15, 47, 48). Both sexes were evaluated indiscriminately.

Mitochondrial Isolations

Mitochondria were isolated from both human right atrial tissue and mouse whole heart. Mitochondrial subpopulations were isolated for analyses as previously described (39), with modifications by our laboratory (3, 4, 15). Differential centrifugation allowed for the compartmentalization of nuclear, cytoplasmic, and mitochondrial fractions. Mitochondrial subpopulations, subsarcolemmal and interfibrillar, were combined to form a total mitochondrial

population. Mitochondria were further purified through use of a sucrose gradient (23%, 15%, 10% and 3% percoll solution), as previously described (26).

PNPase Overexpression Constructs

Express Cloning Vectors were synthesized through GenScript (Piscataway, NJ) using the pcDNA3.1+N-eGFP backbone. Sequences for the full length PNPase protein (FL), KH domain knockout of exon 23 of PNPase (KH), and the S1 domain knockout of the C-terminus of PNPase (S1) were cloned into vectors, complete sequence information is provided (Figure S4.1). Vectors were then transfected into a bacterial cell line (DH5 α), grown through antibiotic selection, and plasmid DNA was isolated for transfection.

Cell culture

Immortalized HL-1 murine cardiomyocytes were cultured at 37°C, 5% CO₂, as previously described (12, 26, 48). Cells were supplemented with Claycomb media (Sigma Aldrich, St. Louis, MO) containing 10% FBS, glutamine (2 mM), penicillin/streptomycin, and norepinephrine (0.1 mM) (12). Cells were seeded and transfected at 70-80% confluence. Lipofectamine 3000 (Thermo Fisher, Waltham, MA) was used to transfect cells, per manufacturer's instructions. Briefly, cells were divided into five groups; no plasmid control (NP), pcDNA3.1+N-eGFP only (GFP), FL, KH, and S1 (n = 10 for each group of independent transfections). In the GFP, FL, KH, and S1 sets, 10 μ g of each respective plasmid was transfected. Media, with the Lipofectamine 3000, remained on the cells for 48 hours followed by cell imaging using the EVOS™ FL Auto Imaging System (Thermo Fisher) on the GFP fluorescent and phase contrast channels. Cells were washed with PBS, dissociated with 0.05% Trypsin, and preserved at -80°C.

Mitochondrial Bioenergetics

The GFP, FL, KH, and S1 groups were plated on a Seahorse XF96 V3 cell culture microplate (n = 8) at a density of 45,000 cells, determined by the Countess II FL Automated Cell Counter (Thermo Fisher). Cells were incubated overnight and the following day analyzed with a

Seahorse XF Cell Mito Stress Test Kit (Agilent Technologies, Santa Clara, CA) on the Seahorse XF96 Analyzer (Agilent Technologies), per manufacturer's instructions. Information regarding performing the assay has been previously described (22).

Crosslinking Immunoprecipitation (CLIP)

Cells in the NP, GFP, FL, KH, and S1 groups (n = 5 each), as well as diabetic and non-diabetic human and mouse homogenized heart tissue (n = 5 each), were re-suspended in PBS and transferred to a 24-well plate on ice. Crosslinking was performed using the CX-2000 Crosslinker (Analytik Jena, Upland, CA) at (400mJ/cm²) 5 times, as previously described (26) with the following modifications. After crosslinking, cells were centrifuged (1,200 g) for 7 minutes and subsequently frozen (-80°C) for further use. 75 µL per sample of Dynabeads™ Protein G (Thermo Fisher) were prepared through 3 washes with NP-40 buffer (20mM Tris (pH 8.0), 137mM NaCl, 10% Glycerol, 1% Triton X100, 2mM EDTA, 0.1mM PMSF). After the third wash, the supernatant on the beads was discarded and 5 µg of anti-GFP was added, along with NP-40 buffer, to reach a total volume of 100 µL. Beads were incubated overnight at 4°C. The next day, each sample was resuspended in NP-40 Buffer to a total volume of 1 mL, and protein concentrations determined through the Bradford Method (6). 100 µg of protein from each sample was added to a new tube followed by RNase I treatment (1:500 dilution, Ambion™ RNase I, Thermo Fisher) for 3 minutes at 37°C, which allowed for partial digestion of RNA bound to PNPase. The reaction was stopped by incubating the samples on ice. The supernatant on the beads solution was then removed and the protein mixture was added and incubated at 4°C for 4 hours. Following the incubation, beads were washed 3 times in NP-40 buffer, final supernatant removed, and a mixture of water/NuPAGE™ LDS Sample Buffer (4X) (Thermo Fisher) was added to the beads. The beads solution was heated at 70°C for 15 minutes and assessed through immunoblotting.

Western Blotting

Using 4 – 12% gradient gels, immunoblotting was performed through MOPS SDS-PAGE, as previously described (13, 21, 22, 38, 57). Using the Bradford Method, protein concentrations were normalized. Primary antibodies implemented in the study: anti-GFP, 1:1000 ((B-2): sc-9996,

SCBT, Dallas, TX), anti-PNPase, ((C-5): sc-271973, SCBT, Dallas, TX), anti-PNPase, ((D-1): sc-271479, SCBT, Dallas, TX), and anti-GAPDH 1:1000 (Abcam, Cambridge, MA). Goat anti-mouse IgG (H&L) horseradish peroxidase (HRP) conjugate 1:10,000 (Thermo Fisher) was used as the secondary antibody. Normalization of protein content was through GAPDH expression. Chemiluminescence quantified with Radiance Chemiluminescent Substrate (Azure Biosystems, Dublin, CA), per manufacturer's instructions and imaged using the G:Box Bioimaging system (Syngene, Frederick, MD). Images were taken with GeneSnap/GeneTools software (Syngene). Densitometry was analyzed using ImageJ and Fiji Software (NIH, Bethesda, MD). For CLIP samples, the area of nitrocellulose membrane containing the GFP (cells) or PNPase (human and mouse) fluorescent region was excised and saved for RNA analysis.

RNA Isolation/Quantitative PCR

Using the miRNeasy Mini Kit (product no.: 217004, Qiagen, Hilden, Germany), per manufacturer's instructions, RNA was isolated from 20 mg of human right atrial tissue and mouse whole heart and from nitrocellulose membrane containing RNA derived from CLIP samples. Each sample was homogenized in QIAzol lysis reagent before proceeding. Total RNA was isolated for each group and RNA to be analyzed through qPCR was converted to cDNA through the High-Capacity RNA-to-cDNA™ Kit (Thermo Fisher), per manufacturer's instructions. Differential gene expression was assessed for MT-ND3 and MT-ATP6 in mouse cytoplasmic and mitochondrial fractions, as well as Malat1-201 and Neat1-201 in mitochondrial fractions. Malat1 expression was also measured in HL-1 cells following PNPase CLIP (as described above). All primer sequences are provided (Table S4.1). Primers were designed through NCBI Primer BLAST and Primer3 (59). Experiments were performed on the Applied Biosystems 7900HT Fast Real-Time PCR system (Applied Biosystems, Foster City, CA), using 2X SYBR Green Master Mix. Quantification was achieved using the $2^{-\Delta\Delta C_T}$ method (32), standardized to U6 and Gapdh expression where appropriate.

Long non-coding RNA (LncRNA) Sequencing

RNA, isolated from human atrial appendages and mouse whole heart (as described above), was sequenced through the West Virginia University Genomics Core Facility. Briefly, RNA was selected for long non-coding RNA analysis using a Ribo-Zero rRNA Removal Kit (Illumina, San Diego, CA). Samples were run on the HiSeq 2500 (Illumina) in 51bp paired end reads achieving ~30 million clustered reads per sample. Generated Fastq files were processed through HISAT2 (27, 41) under standard parameters, with the exception of quality scoring control specified under “--phred33.” The reference genome used in generating BAM files included fasta formatted DNA from Ensemble release 95 for human (GRCh38) and mouse (GRCm38). Differential gene expression was performed in the R environment, through DESeq2 (35). Visualization through ggplot2 (63) and packages vidger, EnhancedVolcano, and others. The complete differential expression profiles of mitochondrial and cytoplasmic groups are included (Additional File 1).

Small ncRNA Sequencing

RNA isolated from human atrial appendages and mouse whole heart (as described above) was sequenced through the West Virginia University Genomics Core Facility. Small RNA was selected using the NEXTFLEX® Small RNA-Seq Kit v3 (Bioo Scientific, Austin, TX). Samples were run on the HiSeq 2500 in Rapid Run (Illumina) in 64bp single end reads on two lanes achieving ~12 million clustered reads per sample. Adapter trimming through cutadapt (37) (5-TGGAATTCTCGGGTGCCAAGG -3) allowed for isolation of genomic reads. Generated Fastq files were processed through Bowtie (29) using the parameters, “-a -m 6 --best --strata --chunkmbs 250.” The reference genome used in generating BAM files included fasta formatted DNA from Ensemble release 94 for human (GRCh38). Differential gene expression was assessed in the R environment, as indicated above. MiRNA sequencing data was analyzed by the use of IPA (QIAGEN Inc., <https://www.qiagenbioinformatics.com/products/ingenuitypathway-analysis>). The complete differential expression profiles are included (Additional File 1).

High-Throughput Sequencing Crosslinking Immunoprecipitation (HITS-CLIP)

RNA isolated from human atrial appendages and mouse whole heart (as described above) was sequenced through the West Virginia University Genomics Core Facility. RNA was selected

for using the NEXTFLEX® Small RNA-Seq Kit v3 (Bioo Scientific). Samples were run on the MiSeq in 36bp single end reads achieving ~1 million clustered reads per sample. Adapter trimming through cutadapt and sequence alignment was performed as indicated above. Generated Fastq files were processed through Bowtie using the parameters, “-a -m 6 --best --strata --chunkmbs 250.” The reference genome used in generating BAM files included fasta formatted DNA from Ensemble release 96 for human (GRCh38) and mouse (GRCm38). Differential gene expression was assessed in the R environment, as indicated above. The raw counts values for each sample are included (Additional File 1).

Mitochondrial Targeting Sequences

To determine if lncRNA that were found in the mitochondrion and associated with PNPase had sequence or secondary structure homology, RNAfold through the ViennaRNA Package was employed (34). RNAfold was used in conjunction with LncFinder (20) and seqinR (10) to evaluate folding parameters of both the full length lncRNA, as well as isolated regions (12-20 nucleotides) shown to bind to PNPase in CLIP analyses. Isolated regions were identified for 50 human and 50 mouse lncRNA labeled “positive” (found in CLIP Seq) and 100 lncRNA labeled “negative” (randomly generated sequences (55)). The isolated regions from genes identified within the PNPase CLIP were flanked by surrounding nucleotides within the genome to achieve 60 nucleotide sequences implemented in the subsequent RNAfold and machine learning applications. The randomly generated sequences also consisted of 60 nucleotides. The sequences directly identified in the CLIP Seq, as well as flanking regions, are provided for each positive control gene (Additional File 1).

Machine Learning for RNA Sequences

Using 10-fold cross validation, classification of “positive” and “negative” 60 nucleotide isolated regions was assessed through Classification and Regression Trees (CART) in randomForest (30) and rfUtilities (17). Machine learning algorithms were performed on 105 features, which included primary sequence and secondary structure information (Additional File 1). Briefly, *rf.crossValidation* was used with a randomForest object to provide 10-fold cross

validation. An unsupervised learning approach was applied to only the positive 60 nucleotide regions through *rf.unsupervised*, with the identification of four specific clusters. As a confirmatory measure, Support Vector Machines (SVM), using the *svm_cv* function of LncFinder, was implemented to determine 10-fold cross validation of full length and isolated regions.

Statistics

A two-sided Student's t-test or one-way analysis of variance (ANOVA) were used to determine statistical differences, where appropriate. Multiple groups were assessed through Tukey's multiple comparisons test following the ANOVA. Differences between groups were considered statistically different if $P \leq 0.05$, denoted by *. Data are presented as the mean $\pm$ standard error of the mean (SEM), when appropriate. For all sequencing data, raw counts > 2 were considered in statistical analyses. The false-discovery rate (FDR) was set to 0.05 and all significance was determined through a P adjusted value (P_{adj}) < 0.05 using Wald testing.

Results

Mitochondrial Isolation Purity

To evaluate the subcellular localization of non-coding RNA (ncRNA), assessments of fractionation purity are needed to confirm subsequent findings and accuracy. Tissue homogenization, differential centrifugation, and sucrose gradient separation allow for the enrichment of pure populations of mitochondria (Figure 4.1A). Mitochondrial contamination was determined through the presence of cytoplasmic ribosomal RNA (rRNA) within mitochondrial RNA fractions (Figure 4.1B). Typically considered a measure for degradation of RNA, proper isolation of mitochondrial RNA retains minimal traces of cytoplasmic rRNA (1, 26). Additionally, qPCR of mitochondrially derived mRNA revealed a significant difference (~40-50 fold) between mitochondrial and cytoplasmic compartments (Figure 4.1C). Sequencing data indicated a reduced expression of mitochondrial transcribed genes within the cytoplasm compared to mitochondria by ~30 – 500 fold (Additional File 1). Mitochondrial populations revealed a clear separation from cytoplasmic portions of the cell.

Mitochondrial MiRNAs

To understand the cellular mechanisms governing mitochondrial import of nuclear-encoded miRNAs, abundance, fold change, and ontology pathways were assessed. In human patient isolated mitochondria, the twenty most abundant miRNAs are provided (Figure 4.2A). In the presence of diabetic insult, 225 miRNAs were shown to be significantly downregulated within mitochondria, with one miRNA revealing a significant upregulation (Figure 4.2B). Of the miRNAs differentially impacted, Ingenuity Pathway Analysis (IPA) suggested that proteins outside of the mitochondrion were those most significantly impacted, with an emphasis on metabolic pathways (Figure 4.2C). When further examining the cellular pathways impacted by nuclear-encoded miRNAs within mitochondria, their key functions included cellular development, survival, and growth (Figure 4.2D). The complete miRNA expression profile is provided (Additional File 1). MiRNAs are both abundantly found within the mitochondrion and have altered expression profiles in disease states, suggesting significant fluctuations in import/export of miRNAs.

Long non-coding RNA (lncRNA) Characterization of the Mitochondrion

While miRNAs have already been identified as ncRNA species in the mitochondrion, we wanted to assess if other ncRNAs interacted with the mitochondrion, and if more advanced mitochondrial regulatory networks exist and are altered during diabetic insult. Using a long non-coding RNA (lncRNA) sequencing approach on sucrose-purified mitochondria, we found that a variety of ncRNA were differentially expressed in both the diabetic human and mouse heart (Figure 4.3A). Of these, the top genes, as delineated by an adjusted P value (P_{adj}), are illustrated for the human (MALAT1) and mouse (Mhrt) sequencing results (Figure 4.3B). PCA plots illustrated the dispersion between cytoplasmic and mitochondrial RNA isolates, with a greater diversity observed between human mitochondria in the disease state, but not the cytoplasm (Figure 4.3C). Differential expression profiles for all ncRNA genes in human and mouse (mitochondrial and cytoplasmic) cohorts are provided (Additional File 1). LncRNAs, as well as other ncRNAs, are present in the mitochondria with diabetic insult significantly altering expression profiles.

Diabetic Mellitus ncRNA Loss of Diversity

To understand how the ncRNA expression profiles are altered between the subcellular compartments and disease states, we assessed the number of differential genes per group (Figure 4.4A). Using these comparisons, we also wanted to understand the general Log_2 fold change between groups. In diabetic compared to non-diabetic mitochondria, the majority of differentially expressed genes were downregulated (Figure 4.4B). Of the two largest lncRNA populations, the top 5 differentially expressed transcripts are displayed for antisense and long-intergenic ncRNA (lincRNA), determined by $P_{adj} < 0.05$ and containing the highest read counts in each group (Figure 4.4C). Because MALAT1, NEAT1, KCNQ1OT1, and Mhrt (no human equivalent) revealed similar expression profiles between human and mouse groups (Figure 4.4C), we wanted to evaluate the expression profiles of these genes when compared between different disease states and subcellular compartments (Figure 4.4D). MALAT1, NEAT1, and KCNQ1OT1 in human and mouse models revealed a marked downregulation in diabetic mitochondria with unchanging or increased expression in diabetic cytoplasm (Additional File 1, Figure 4.4D). Malat1 and Neat1 expression were further confirmed through qPCR (Figure S4.2A). In a disease state, diminished

localization of lncRNA and other ncRNAs in the mitochondrion (Figure S4.2B) mimic the miRNA response, suggesting a decrease in total ncRNA diversity.

Polynucleotide Phosphorylase (PNPase) Interactions with ncRNA

With data suggesting the immense diversity of the mitochondrial ncRNA regulatory network, and that in diabetes mellitus this network becomes impaired, we wanted to evaluate mitochondrial ncRNA import/export mechanisms. Cross-linking immunoprecipitation (CLIP) of PNPase in human and mouse cohorts revealed a propensity for longer ncRNAs to bind to the protein (Additional File 1). A gene comparison between human and mouse PNPase CLIP revealed 31 homologous ncRNA, including MALAT1, NEAT1, and KCNQ1OT1 (Figure 4.5A). The composition of ncRNA bound to PNPase is displayed (Figure 4.5B), with antisense and lincRNA transcripts being the most prevalent. We wanted to further investigate how PNPase may be involved in shuttling mechanics for ncRNA through its KH and S1 RNA binding domains. Using an HL-1 immortalized mouse cardiomyocyte cell line, we transfected cells with overexpression vectors (Figure 4.5C). Overexpression of constructs was demonstrated through immunoblotting, with a shift in size between the full length, KH domain knockout, and S1 domain knockout (Figure 4.5D). Using CLIP for PNPase in each of the cell variants, RNA was purified in each of the overexpression groups through SDS-PAGE gel electrophoresis, with a representative gel illustrated (Figure 4.5E). Examining the RNA bound to PNPase, MALAT1 expression was shown to be significantly downregulated when the S1 domain was removed from PNPase (Figure 4.5F), suggesting its role in RNA-protein interactions. Functionally, basal respiration and ATP production were significantly decreased in both the KH and S1 domain knockout groups, with maximal respiration and proton leak decreased in the S1 domain knockout group (Figure S4.2C). These results suggest that PNPase has binding affinity for lncRNA, most likely through the KH and/or S1 RNA binding domains.

Mitochondrial RNA Targeting Sequence

Examining the CLIP PNPase sequencing data, we wanted to determine if a mitochondrial RNA targeting sequence or secondary structure exists. A 60 nucleotide region, directly centered

on reads acquired from sequencing, was implemented to determine RNA primary sequence and secondary structure homology through RNAfold (34). RNAfold secondary structures for MALAT1, NEAT1, and KCNQ1OT1 illustrate that RNA sequences bound to PNPase are found in stem-loop structures in both the full length (Figure S4.3) and 60 nucleotide isolated regions (Figure 4.6A). Implementing 10-fold cross validation for Support Vector Machines (SVM) and Classification and Regression Trees (CART), machine learning was applied to 50 human and 50 mouse lncRNA 60 nucleotide reads found to bind to PNPase (positives) and 100 randomly generated 60 nucleotide sequences (negatives) (55) (Table S4.2). Both SVM and CART attained close to 75% prediction accuracy on the entire dataset, for classification of either positive or negative 60 nucleotide sequences (Figure 4.6B) (Table S4.2). Of note, due to the random generation of the 60 base-pair regions, it is possible for some of the negative controls to share similar properties with the positive group, limiting the predictive power of the model.

Area under the curve (AUC) averaged ~0.75, with various iterations ranging from 0.65 – 0.85 (Figure 4.6C). To determine the degree of heterogeneity between 60 nucleotide regions identified as positives, unsupervised learning through CART was applied to examine separation into four subtypes (Figure 4.6D). 10-fold cross validation was further applied to each of the subtypes compared to the 100 negative sequences, (Table S4.2), revealing the distinct primary and secondary structure differences between sequences identified to bind to PNPase (Figure 4.6E). Further, parameters of the LncFinder algorithms, including acguD (Figure S4.4A) and acgu-ACGU (Figure S4.4B), suggest that RNA bound to PNPase has a greater propensity for unpaired loop structures, with differences also in the folding energy (Figure S4.4C) and sequence GC content (Figure S4.4D). Both supervised (Additional File 1) and unsupervised CART models suggest that secondary, not primary sequence, is important for RNA identification by the mitochondrion. Predominately, a stem-loop and internal loop structure may be necessary for recognition of lncRNA by PNPase (Figure 4.7).

Discussion

Our knowledge of the mitochondrion has continued to broaden, with the discovery of new regulatory functions of the organelle and how these diverse functions impact tissue and cellular health. For the last ten years in the field of mitochondrial biology, a question has persisted regarding the purpose and function of nuclear-encoded miRNAs in the mitochondrion. In this study, for the first time, we suggest the existence of a novel mitochondrial regulatory network, which helps to clarify the presence, and purpose, of miRNAs in mitochondria. Our data indicate that the primary purpose of ncRNAs in mitochondria are not to regulate the mitochondrial genome, but rather to provide a repository of regulatory RNA, which can be altered during disease states. We suggest that PNPase, through interaction with long non-coding RNA (lncRNA), may play a significant role in regulating this response within the mitochondrion.

The interaction of lncRNA with mitochondrial function has been shown through a variety of alternative pathways. One of the most prominently studied pathways is the interaction of lncRNA with genes and genomic regions responsible for contributing to substrate utilization within the cell. Altering metabolic function has been shown through sponging of miRNAs that inhibit mRNA (52, 62), interactions with proteins directly (44), and through regulation of transcription in the nucleus (19). It has even been proposed, and further validated, that the mitochondrial genome itself produces lncRNAs (18, 43). In all of these investigations, few have assessed how nuclear produced lncRNAs can interact directly with the mitochondrion. The complete lncRNA interaction network that exists as part of the mitochondrial axis could play a pivotal role in both the maintenance of mitochondrial function and the sequestration of other ncRNAs.

Kren et al. was one of the first to postulate the idea of sequestration (28). In a microarray study discovering 15 miRNAs, the authors suggested that due to miRNAs inhibiting multiple mRNA targets, those mRNA may be shuttled into the mitochondria and regulated during specific cellular conditions. While the field has generally moved away from this idea of sequestration, there may be significant biological advantages to operating under those conditions. The data in this manuscript help support the concept of sequestration in four ways. 1) The number of miRNAs, lncRNAs, and other ncRNAs found within and/or directly interacting with the mitochondrion is likely greater than 1,000 different species. The mitochondrial genome, composed of 13 transcribed

genes, and the mitochondrial proteome, composed of ~1,200, lack an inherent diversity for targeting by all of the ncRNAs (8). 2) The stoichiometric ratio of some RNAs, specifically lncRNA and miRNA, could be significantly elevated in a sub-compartment of the cell (33, 58). LncRNA, which may exist in the cytoplasm at a relatively low abundance, can be amplified through localization into the mitochondrion. 3) MALAT1, NEAT1, KCNQ1OT1, and many of the other lncRNAs found in the sequencing data have verified sponging regions for nuclear-encoded miRNAs in the mitochondria (40). 4) The mitochondrial matrix provides an environment promoting lncRNA folding/processing. High concentrations of magnesium and manganese ions could increase the stabilization of lncRNA secondary and tertiary structures (24, 51, 66), along with further processing through small nuclear RNA (snoRNA) (65) (Additional File 1).

In the current study, we found that MALAT1, along with NEAT1, KCNQ1OT1, and others, may be important lncRNAs in mitochondria during diabetic progression. The increase of MALAT1 has been linked to a variety of pathogenic responses in diabetes mellitus (5, 11, 31). Reduction, or complete knockout, of MALAT1 expression contributed to reduced ROS, inflammatory signaling, and negative regulatory effects to the genome. We showed that MALAT1, though not significantly elevated in the cytoplasm, was decreased in diabetic mitochondria. While we depict a movement toward decreased ncRNA diversity in diabetic mitochondria, it could be that under more overt stress, e.g. infection or injury, the mitochondrial regulatory network is less adaptable to attenuating the response. Additionally, reduction of lncRNA in the mitochondria may not necessarily mean that ncRNA is transferred to other locals within the cell; it could suggest that lncRNAs are degraded in the mitochondria, limiting the potential for sequestration of other ncRNAs, such as miRNA.

The bacterial version of PNPase exists as an enzyme with a primary purpose of modifying and degrading RNA species (7). The mammalian homolog, instead of remaining a free-floating enzyme, is localized to the mitochondrial intermembrane space. While not directly accessible to the cytoplasm or mitochondrial matrix, studies have reported the ability of PNPase to interact with and transport RNAs into the mitochondrion (60, 61). The KH and S1 RNA binding domains of PNPase are suggested as the mechanism for channeling ncRNA through the mitochondrial intermembrane space into the matrix (60). Importantly, Wang et al. suggested that a stem-loop sequence on the RNA was necessary for mitochondrial import (60). These findings support a

mechanism whereby PNPase recognition requires RNA secondary structure as opposed to sequence specificity, which is consistent with our work, including the PNPase CLIP sequencing and machine learning approaches. The framework from LncFinder and the machine learning algorithms begins to provide an archetype for designing personalized lncRNA sequences to target to the mitochondrion; specifically, through incorporating stem-loop structures with more unpaired bases within the loop structures.

We have observed that ncRNAs, including lncRNA, are present in the mitochondrion and that overall diversity in the diabetic mitochondrion is lost. While not focused on in this manuscript, we observed the presence of other ncRNAs, such as snoRNA and small nuclear RNA (snRNA), which may prove to have their own unique biological role, import mechanism, and persistence pattern in the mitochondrion. To determine if ncRNA are degraded or further shuttled to other compartments of the cell, future investigations should evaluate the total cellular levels of lncRNA and miRNA that are downregulated in mitochondria following diabetic insult. The current study provides a more complete understanding of the ncRNA regulatory network of the mitochondrion, while revealing changes that occur during pathological insult. The shared features of lncRNA bound to PNPase highlight a novel mitochondrial RNA import mechanism, through the presence of stem-loop secondary structures, and suggest the presence of an interaction network with mitochondria. Within the mitochondrion, lncRNA would have the capacity to interact with other ncRNAs, such as miRNAs, possibly through sponging and regulation of cellular processes. The expanded mitochondrial regulatory network proposed in this study provides a new perspective on mitochondrial biology and continues to expand our conception of mitochondria as the “powerhouse of the cell.”

Data Availability

All source code and sequencing data files have been made freely available:

Source code at Github: https://github.com/qahathaway/Mitochondria_PNPase_LncRNA.

Sequencing data and sample identification at NCBI SRA under BioProject: PRJNA553501

Differential expression analyses (Additional File 1)

Supplementary Data

Supplementary data is included in the study, specifically supporting data (Tables S4.1-4.2, Figure S4.1-4.4) and differential expression analyses for next-generation sequencing experiments (Additional File 1)

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Conflict of Interest

The authors have declared that no conflict of interest exists.

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Figure 4.1

Figure 4.1: Enrichment of mitochondria from cardiac tissue. (A) From human atrial appendages and whole mouse hearts, samples are homogenized and differentially centrifuged to produce subcellular fractionations; mitochondria are further sucrose-gradient purified to remove cytoplasmic contamination. (B) Purity of cytoplasmic and mitochondrial RNA is assessed through the Bioanalyzer (Agilent Technologies), with a low RNA Integrity Number (RIN) indicating limited or no presence of cytoplasmic ribosomal RNA. (C) Real-time PCR of mitochondrial transcribed genes (MT-ND3 and MT-ATP6) from mouse cytoplasmic and mitochondrial isolated RNA. Differences between groups were considered statistically different if $P \leq 0.05$, denoted by *. All data are presented as the mean $\pm$ standard error of the mean (SEM). MT-ND3 = mitochondrially encoded NADH:ubiquinone oxidoreductase core subunit 3, MT-ATP6 = mitochondrially encoded ATP synthase membrane subunit 6, T2DM = type 2 diabetes mellitus, ND = non-diabetic, DbDb = FVB/NJ *Lepr^{db}+/+* mice, WT = FVB/NJ wild type mice, Mito = mitochondrial, Cyto = cytoplasmic.

Figure 4.2

Figure 4.2

Figure 4.2: MiRNA profiles in human atrial mitochondria. (A) The top 20 most highly expressed nuclear-encoded miRNAs found in mitochondria of human atrial tissue from type 2 diabetic (n = 7) and non-diabetic (n = 7) patients. (B) Heat map illustrating the downregulation (red) and upregulation (blue) of all significantly impacted miRNAs following diabetic onset. (C) Ingenuity Pathway Analysis (IPA) of molecules revealing the highest number of suggested targeting interactions by miRNAs found to be differentially regulated in the mitochondrion. (D) IPA heat map revealing cellular pathways most likely impacted by the miRNAs found to be differentially affected in patients with diabetes mellitus. All statistically significant values are considered $P_{adj} < 0.05$ or $-\text{Log}_{10}P_{adj} = 1.30$. Z-scores are considered statistically significant if the value of the pathway affected is $\geq |2|$.

Figure 4.3

Figure 4.3

Figure 4.3: Characterization of long-non coding RNA (lncRNA) in the mitochondrion.

Human atrial tissue (n = 6) and mouse whole heart (n = 6) were assessed for lncRNA in the mitochondrion. (A) Volcano plots for human (top) and mouse (bottom) demonstrate the total lncRNAs differentially expressed in diabetic mitochondria, with the top gene (lowest P_{adj} value) circled. (B) The top most differentially regulated gene is depicted for human (top) and mouse (bottom) with the raw number of counts per each sample displayed. (C) Principle Component Analysis (PCA) for RNA in human (top) and mouse (bottom) of diabetic and non-diabetic samples for the cytoplasmic and mitochondrial portions. All statistically significant values are considered $P_{adj} < 0.05$ or $-\text{Log}_{10}P_{adj} = 1.30$. MALAT1 = metastasis associated lung adenocarcinoma transcript 1, Mhrt = myosin heavy chain associated RNA transcript, T2DM = type 2 diabetes mellitus, ND = non-diabetic, DbDb = FVB/NJ *Lepr^{db-+/+}* mice, WT = FVB/NJ wild type mice, Mito = mitochondrial, Cyto = cytoplasmic.

Figure 4.4

Figure 4.4

Figure 4.4: Expression profiles of long non-coding RNA (lncRNA) in the mitochondrial regulatory network. Human atrial tissue (n = 6) and mouse whole heart (n = 6) were assessed for lncRNA in the mitochondrion. **(A)** Venn diagrams comparing the differential expression of genes between diabetic and non-diabetic cytoplasmic and mitochondrial fractions in human (left) and mouse (right). **(B)** Illustration of genes in human (left) and mouse (right) that are significantly up or downregulated, including if the Log₂ Fold change is greater or less than one. **(C)** Tables containing the 5 top differentially regulated antisense and lncRNA transcripts in human (left) and mouse (right). Genes were chosen that had the highest mean count number as well as having a $P_{adj} < 0.05$. **(D)** Examining expression profiles of MALAT1, NEAT1, KCNQ1OT1, and Mhrt in comparisons of diabetic vs. non-diabetic mitochondria (top), diabetic mitochondria vs. cytoplasm (top middle), diabetic vs. non-diabetic cytoplasm (bottom middle), and non-diabetic mitochondria vs. cytoplasm (bottom). Data are presented as grouped bars, with the human (left) and mouse (right) depicted, with the exception of Mhrt which is only present in mice with no human homologue. All statistically significant values are considered $P_{adj} < 0.05$ or $-\text{Log}_{10}P_{adj} = 1.30$. LincRNA = long intergenic non-coding RNA, MALAT1 = metastasis associated lung adenocarcinoma transcript 1, NEAT1 = nuclear paraspeckle assembly transcript 1, KCNQ1OT1 = KCNQ1 Opposite Strand/Antisense Transcript 1, Mhrt = myosin heavy chain associated RNA transcript, T2DM = type 2 diabetes mellitus, ND = non-diabetic, DbDb = FVB/NJ *Lepr^{db-+/+}* mice, WT = FVB/NJ wild type mice, Mito = mitochondrial, Cyto = cytoplasmic, D = diabetic, N = non-diabetic.

Figure 4.5

Figure 4.5

Figure 4.5

Figure 4.5: High-throughput sequencing crosslinking immunoprecipitation (HITS-CLIP)

for PNPase. (A) Heat map of ncRNA found in the PNPase CLIP for human atrial tissue type 2 diabetic (n = 5) and non-diabetic (n = 5) and whole heart from Db/Db (n = 5) and wild type (n = 5) mice, with high (dark blue) and low (green) count values illustrated. Samples in each group were pooled, in order to achieve sufficient RNA for sequencing, with only the genes found in both human and mouse analyses displayed. (B) NcRNA species richness in human and mouse PNPase CLIP. (C) Representative images for HL-1 cells in each group (n = 10 per group) showing fluorescence induced by pcDNA3.1+N-eGFP overexpression. (D) Immunoblotting depicting the overexpression in HL-1 cells, as well as shift in size, of the constructs. (E) A representative image of SDS-PAGE following crosslinking immunoprecipitation (CLIP) of HL-1 cells to retrieve RNA specifically bound to PNPase. (F) Real-time PCR on isolated RNA to assess binding affinity of lncRNA MALAT1 (n = 3 each group) to PNPase variants. All statistically significant values are considered $P_{adj} < 0.05$ or $-\text{Log}_{10}P_{adj} = 1.30$. Differences between groups were considered statistically different if $P \leq 0.05$, denoted by *. All data are presented as the mean $\pm$ standard error of the mean (SEM). T2DM = type 2 diabetes mellitus, ND = non-diabetic, DbDb = FVB/NJ *Lepr^{db-/+}* mice, WT = FVB/NJ wild type mice, PNPase = polynucleotide phosphorylase, NP = no plasmid control, GFP = pcDNA3.1+N-eGFP backbone only, FL = full length PNPase open reading frame (ORF) in pcDNA3.1+N-eGFP, KH = exon 23 removed from full length PNPase in pcDNA3.1+N-eGFP, S1 = C-terminus removed from full length PNPase in pcDNA3.1+N-eGFP, MALAT1 = metastasis associated lung adenocarcinoma transcript 1.

Figure 4.6

Figure 4.6

Figure 4.6

Figure 4.6: Mitochondrial RNA targeting sequence. (A) RNAfold of a 60 base-pair region of MALAT1, NEAT1, and KCNQT1OT, surrounding the reads (circled) found to bind to PNPase. (B) Supervised machine learning, through Classification and Regression Trees (CART), indicating the Producer's accuracy and Out of Bag (OoB) error for both the model and 10-fold cross validation of positively identified ncRNAs and randomly generated sequences. (C) The average generated area under the curve (AUC) for prediction of ncRNA found in the PNPase CLIP. (D) Unsupervised learning to discover clustering (n = 4) patterns of ncRNAs bound to PNPase. (E) A representative ncRNA, sharing attributes of its respective cluster were selected and RNAfold was implemented to reveal structural and compositional differences. MALAT1 = metastasis associated lung adenocarcinoma transcript 1, NEAT1 = nuclear paraspeckle assembly transcript 1, KCNQT1OT = KCNQT1 Opposite Strand/Antisense Transcript 1, CV = 10-fold cross validation, Positive = 50 human and 50 mouse ncRNAs determined to be bound to PNPase, Negative = 100 randomly generated sequences, Zfp469 = zinc finger protein 469, HELLPAR = HELLP associated long non-coding RNA, MFE = minimum free energy, GC = GC content.

Figure 4.7

Figure 4.7: Illustrative overview mitochondrial regulatory network. PNPase has the capacity to bind lncRNA through stem-loop motifs, which may help to facilitate import of lncRNA into the mitochondrion. Within the mitochondrion, lncRNA could have the capacity to bind and sequester other ncRNA, to prevent regulation in other areas of the cell. Ultimately, the ncRNA diversity is significantly diminished in disease states (diabetes mellitus) likely impacting the cellular response to further insult.

Table S4.1

Assay	Primer Name	Forward (5' – 3')	Reverse (5' – 3')
Figure 1: Mitochondrial Purity	MT-ND3	AATGCGGATTCGACCCTAC	GAATTGCTCATGGTAGTGGAAG
	MT-ATP6	GATTCCCAATCGTTGTAGCC	GGAAAGAATGGAGACGGTTG
Figure S2: LncRNA mitochondrial validation	Malat1-201	TGGCAAGTAACTCCCAATCC	AGCATCACATCATCCCAGTG
	Neat1-201	ATTGGCCAGAAGACAACAGG	TCTCATAGCAAGCGCAAGAC
Figure 6: PNPase HL-1 CLIP	Malat1	GAATTGCGTCATTTAAAGCCTAG TT	GTTTCATCCTACCACTCCCAATTA AT

Table S4.1: Primers designed for experiments. Primers were designed through Primer3 and NCBI Primer-BLAST.

Table S4.2

	All Positives	Cluster 1	Cluster 2	Cluster 3	Cluster 4
Classification and Regression Cross-Validation (CV)					
User's Accuracy (Positives)	70.00	100.00	0	0	0
User's Accuracy (Negatives)	80.00	100.00	100.00	100.00	100.00
Producer's Accuracy (Positives)	70.85	NA	NA	NA	NA
Producer's Accuracy (Negatives)	73.35	100.00	83.30	83.30	83.30
CV Kappa	0.44	0.67	0.30	0.25	0.32
CV OoB Error	0.28	0.09	0.15	0.18	0.18
CV Error Variance	7.88E-4	1.06E-3	2.07E-4	1.04E-4	3.59E-3
Classification and Regression Model					
User's Accuracy (Positives)	71.60	63.45	23.80	20.80	32.00
User's Accuracy (Negatives)	72.60	97.9	98.90	97.90	95.25
Producer's Accuracy (Positives)	72.75	88.90	80.00	71.40	62.55
Producer's Accuracy (Negatives)	71.80	90.7	85.50	83.00	84.10
Model Kappa	0.45	0.67	0.32	0.25	0.32
Model OoB Error	0.28	0.09	0.15	0.18	0.18
Model Error Variance	2.39E-4	8.27E-5	1.85E-4	1.01E-4	9.57E-5
Support Vector Machines Cross-Validation (CV)					
Sensitivity	0.76	0.67	0.23	0.38	0.38
Specificity	0.67	0.93	0.96	0.94	0.89
Accuracy	0.72	0.88	0.83	0.83	0.79
F-Measure	0.73	NA	NA	NA	NA
Kappa	0.43	0.60	0.23	0.37	0.27

Table S4.2: Machine Learning performance for Classification and Regression Trees (CART) and Support Vector Machines (SVM). Performance was determined for entire dataset (100 positives and 100 negatives) as well as for each cluster determined through unsupervised learning (25 positives and 100 negatives). Positives = 50 human and 50 mouse ncRNAs determined to be bound to PNPase, or 25 genes found in each of the four clusters, Negatives = 100 randomly generated sequences, CV = 10-fold cross validation, OoB = out of bag.

Figure S4.1

Figure S4.1

Start **Termination** **Mitochondrial Targeting Sequence** **KH Domain** **S1 Domain**

Pnpt1 – KH Domain KO, Removal of Exon 23 – 1-608 and 636-783 (conversion of E to G at 607)

ATGCGGGCCTGCAGGCTGTGCTGCTTGTGCCCGTGCCTCCGACCGCTGGGCTGCGGCCCCCTCGGCCGGCCCCGGGCGGAATCGGGCACTCAGCTATTTGCAGATGCGAGCGTTGTGGAGCAGCACCGGATCCAGAGCGGTGACTGTAGACCTGGGACACAGAAAATTAGAAATATCCTCTGGGAAACTGGCAAGATTTGCTGATGGCTGTGCTGTGATACAGTCAGGCGATACTGCAGTAATGGTCACGGCAGTCAGCAAAACAAAAGCTTCGCCTTCCCAATTCATGCCGTTGGTGGTGGACTACCGACAGAAGGCTGCTGCAGCAGGCAGAATCCCCACAACTACCTTAGAAGGGAGATTGGCTCCTCTGACAGAGAGGTTCTTACAAGTCGAGTAATAGATCGTTCAATTCGCCCTCTCTTCCAGCCGGCTATTTTTATGATACTCAGGTTCTCTGTAATCTGCTAGCAGTAGATGGCATCAATGAACCTGATATCCTAGCAGTTAATGGTGCTTCTGTAGCCCTCTCCTATCAGATATCCCTTGGAATGGACCTGTTGGGGCAGTACGAATAGGAATGATTGATGGAGAATGTGTCGTTAACCAACAAGGAGAGAAAATGTCTTCTAGCACTTTAAATTTAGTAGTTGCCGGAGCACCTAAAAGCCAAATTGTTATGTTGGAAGCCTCTGCAGAAAATATTCTACAGCAGGACTTTTGCCATGCTATCAAAGTTGGGGTGAAGTATACACAGCAGATAATTCAGGGCATCCAGCAGTTGGTAAAAGAAAATCGGTGTTGCCAAGAGGACACCGCAGAAGATATTACTCCTTCTGCAGAGATTGTGAAGTACACGAAGATAATTGCCATGGAGAACTCTATGCGGTTTTTACAGATTATGAACATGATAAAGTTTCCAGGGATGAAGCTGTTAACAAGATAAGATTAGATACAGAGGAGCATCTAAAGGAAAAATTTCCAGAGGTTGACCAATTTGAAATAATAGAATCCTTCAACATTGTTGCAAAGGAGGTTTTCCGAAGTATTATTGGAATGAATACAAAAGGTGTGATGGAAGAGATCTGACTTCACTTAGGAATATAAGTTGTGAGGTCGATATGTTTAAACACTTCATGGATCAGCATTATTTAGAGAGGACAAACACAGGTAATCTGTACTGTTACATTTGATTCATTAGAAATCCAGTATTAAGTCAGATCAAATTATAACAGCTATAAATGGGGTAAAAGATAAAAAATTTATGCTGCACTATGAGTTCCCTCCTTATGCAACCAATGAAACCGGCAAGTTACTGGTGTGAATCGAAGAGAACTTGGACATGGTGCTCTTGCAGAGAAAGCTTTGTGTCCTGTTATTCCCAAAGATTTTCTTTTACCATAAGAGTTACATCTGAAGTCCTCGAATCAATGGGTCATCTTCTATGGCATCTGCATGTGGTGGAAGTTTGGCATTAAATGGATGCAGGGGTCCCAATTTATCTGTGTGTTGCAGGTGTAGCAGTGGGATTGGTTACCAAAACCAATCCAGAGAAAGGTGAAATAGAAGACTACCGTTTGCTAACAGATATTCTGGGAATTGAAGATTATAATGGTGACATGGATTTCAAATAGCCGGTACAAATAAAGGAATAACATGATTACAGGCTGATATTAAGTTACCTGGAGTACCAATTAATAATGGAAGCCATCCAACAAGCGTCAGTGCAAAGAAGGAGATACTGCAGATAATGAACAAAACGATTTCAAACCTCGAGCATCAAGAAAAGAAAATGGACAGTTGTAGGTGTAACAATTAGTCAGGTTGATGAAGAAACCTTCTCCATATTTGCACCAACACCTACTGCAATGCATGAAGCAAGAGATTTTATTACAGAAATTTGCAGAGATGATCAAGAGCAACAATTAGAATTTGGAGCAGTTTATACCGCGACAATAACTGAAATCAGAGACACTGGAGTGATGGTAAAACCTGTATCCAACATGACTGCAGTGCTGCTTCATAATTACAACCTTGACCAACGAAAGATTAAACATCCCACTGCCCTAGGACTAGAGGTTGGCCAAGAAATTCAGGTCAATACTTTGGCCGTGATCCAGCTGATGGAAGAATGAGGCTTTCTCGTAAAGTACTTCAGTCTCCAGCTACAACCTGCTCTCAAACTCTAAATGATAGAAGCAGCATTGTAATGGGAGAGCCTGTCTCACAGTCATCTAACTCTAACCTTGA

Figure S4.1

Figure S4.1: Constructs designed for transfection into HL-1 cells. Sequences were inserted into a pcDNA3.1+N-eGFP vector and transfected into cells. FL = full length PNPase open reading frame (ORF) in pcDNA3.1+N-eGFP, KH = exon 23 removed from full length PNPase in pcDNA3.1+N-eGFP, S1 = C-terminus removed from full length PNPase in in pcDNA3.1+N-eGFP.

Figure S4.2

Figure S4.2

Figure S4.2

Figure S4.2: Mitochondrial and PNPase CLIP sequencing. (A) In Db/Db (n = 4) and wild type (n = 4) mice, Malat1-201 and Neat1-201 were evaluated for expression through qPCR. (B) Tree map for human (top) and mouse (bottom) of ncRNAs found in the mitochondria (> 10 averaged counts, boxed number indicates number of genes), along with arrows indicating genes within each category shown to be significantly up or downregulated following differential expression analysis. (C) Mitochondrial respiratory capacity of HL-1 cells following transfection (n = 10 per group) of PNPase variants. Differences between groups were considered statistically different if $P \leq 0.05$, denoted by *. All data are presented as the mean $\pm$ standard error of the mean (SEM). DbDb = FVB/NJ *Lepr^{db-+/+}* mice, WT = FVB/NJ wild type mice, MALAT1 = metastasis associated lung adenocarcinoma transcript 1, NEAT1 = nuclear paraspeckle assembly transcript 1, GFP = pcDNA3.1+N-eGFP backbone only, FL = full length PNPase open reading frame (ORF) in pcDNA3.1+N-eGFP, KH = exon 23 removed from full length PNPase in pcDNA3.1+N-eGFP, S1 = C-terminus removed from full length PNPase in pcDNA3.1+N-eGFP, lincRNA = long intergenic non-coding RNA, snRNA = small nuclear RNA, snoRNA = small nucleolar RNA, OCR = oxygen consumption rate.

Figure S4.3

Figure S4.3

Figure S4.3: RNAfold of full length lncRNA. RNAfold was implemented for human (MALAT1-201, NEAT1-201) and mouse (Malat1-204, Neat1-201 (partial)) lncRNA. Circled regions provide a zoomed in area where the reads from the PNPase CLIP (arrow indicating) are found. MALAT1 = metastasis associated lung adenocarcinoma transcript 1, NEAT1 = nuclear paraspeckle assembly transcript 1.

Figure S4.4

Figure S4.4: LncRNA sequence and secondary structure parameters differentially expressed between positive and negative groups. (A) The log distance of nucleotides found in unpaired configurations of dot-bracket secondary structure notation, or *acguD*, between groups. (B) The log distance of unpaired nucleotides (*acgu*) subtracted by paired nucleotides (ACGU) in the RNA secondary structure, or *acgu-ACGU*, between groups. (C) Minimum free energy of folding and (D) GC content between positive and negative groups. Differences between groups were considered statistically different if $P \leq 0.05$, denoted by *. All data are presented as the mean $\pm$ standard error of the mean (SEM). Positive = 50 human and 50 mouse ncRNAs determined to be bound to PNPase, Negative = 100 randomly generated sequences.

Chapter 5: General Discussion

The culmination of the work presented within this dissertation seeks to elucidate the role of the mitochondrion as a target, and effector, of epigenetic and non-coding RNA regulatory pathways in the cell. The research outlined highlights the role of engineered nanomaterials (ENMs) and diabetic insult as examples of extrinsic and intrinsic stimulation, respectively, in order to measure the mitochondrial response. Likewise, ENMs and diabetes mellitus are used as surrogates, allowing an understanding of how severity and length of insult can transiently or permanently alter the epigenetic and regulatory RNA response. Through acute (ENMs) or chronic (diabetes mellitus) activation of these pathways, a more holistic image is produced concerning the innate responses of the cell. Ultimately, the long-term goal of this research performed and data generated is to provide diagnostic or therapeutic value, providing mechanisms for better predicating mitochondrial dysfunction in any pathological state, not only in type 2 diabetes or inhalation exposure to toxicants.

My central hypothesis is that “changes in the epigenome through DNA methylation and histone modifications alter the ncRNA transcriptome subsequently changing the import dynamics of microRNA (miRNA), long non-coding RNA (lncRNA), and other ncRNA into the mitochondrion and affecting mitochondrial function through RNA-RNA and RNA-protein interactions; ultimately, producing a feedback loop where mitochondrial substrate availability shapes nuclear epigenetic machinery.” The central hypothesis stated is strongly associated with the concept of nuclear influence on mitochondrial function (anterograde signaling) and the mitochondrial influence on metabolites distributed to the nucleus (retrograde) (39). While at the most fundamental level this could include direct interactions, such as reduced transcription of electron transport components needed by the mitochondrion to produce ATP, which continues to lead to a decrease in transcription of the genes by ATP dependent enzymes. In a more dynamic view, nuclear and mitochondrial signaling could influence microRNAs (miRNAs), other non-coding RNAs (ncRNAs), and epigenetic factors that could lead to indirect targeting of transcript and proteins in a systemic fashion (18, 58). We are still a long way from understanding which signaling pathway, changes in nuclear transcription or alterations to substrate utilization in the mitochondrion, precedes the other in the initiation of positive feedback loops between the two

organelles. The research provided in this dissertation helps to further understand the processes of nuclear and mitochondrial adaptation to insult, and further entreat exploration into novel interaction networks within the cell.

In Specific Aim 1, we employed a transgenic mouse model with knockout expression for a ncRNA (miRNA-378a). This was the first time a ncRNA transgenic mouse had been used in the evaluation of ENM exposure paradigms, and the cardiovascular consequences seen provided significant insight into modes of ncRNA expression in the heart following exposure. While miRNA-378a has been shown to have a clear link to metabolism (17, 26, 34) and the miRNA-378a knockout murine model has been implemented in diabetes/obesity research (6), the research presented in Specific Aim 1 highlights how perturbation to the nuclear mitochondrial interaction network can occur. ENM exposure was shown to not only decrease metabolic function through miRNA-378a inhibiting fatty acid metabolism genes, but the data suggest a restructuring of mitochondrial fission/fusion dynamics. While miRNA-378a may only be elevated for a few days following inhalation exposure to ENMs, changes to the number of mitochondria and mitochondrial ultrastructure suggests more latent effects, which could contribute to alterations in the nucleus.

While not explored in Specific Aim 1, epigenomic mechanisms could be one of the governing cellular dynamics producing the maladaptive response to ENM inhalation exposure. Epigenomic remodeling could occur in a multiplicity of ways which could disrupt miRNA-378a expression, but the two most probable sources would be 1) manipulation of transcription machinery and 2) regulation of the PGC-1 β gene. A rise in miRNA-378a expression in young adult mice following ENM exposure could be linked to changes in the epigenetic regulation, transcription, and ultimate alteration of transcription factor activation/repression of the genome. In Specific Aim 1, Ppar α , a transcription factor known to activate genes involved in fatty acid and lipid metabolism (62), was found to be increased in miRNA-378a knockout animals. Likewise, ENM inhalation exposure may cause a larger, dysregulated transcription factor profile in the heart, which could include targets directly increasing the expression of PGC-1 β , such as by Ppar γ (29). Specifically, as documented in osteoblasts (25), increasing iron uptake by the cell transferrin receptor 1 (TfR1) promoted the transcriptional activation of PGC-1 β , which reveals the potential for environmental stimulation to alter PGC-1 β expression.

In the altered cellular state of cardiac tissue following ENM inhalation exposure, it may be the case that the epigenomic environment, either through histone alterations or DNA methylation, of promoter and enhancer regions in genomic sites encoding for transcription factors can lead to decreased/increased expression of that specific protein. These broad, genome-wide epigenomic changes are likely occurring to transcription factors in the experimental model outlined in Specific Aim 1, but the use of both broad-scale proteomic and DNA methylation analyses would be needed to verify which transcription factor networks are perturbed and the effects of miRNA expression, such as miRNA-378a. Another mechanism which could be controlling miRNA-378a expression following ENM inhalation exposure could be the direct epigenetic control of the PGC-1 β promoter loci. Methylation of the PGC-1 β promoter region has been previously reported, with detrimental health consequences associated with increased methylation (33). Through modification to the methylation status of the PGC-1 β promoter region, ENM inhalation exposure could modify the expression profiles of PGC-1 β and subsequently miRNA-378a.

As a proxy for understanding ncRNA dynamics, ENM exposure provides an acute, environmental stimulation that allows for tracking a very specific response. Unlike diabetes mellitus, which is generally chronic and has many systemic comorbidities, inhalation exposure can allow for tracking the persistence of specific epigenetic or regulatory networks across generational gaps (21, 52). Supporting Specific Aim 1, Chapter 6 outlines material pertinent to the assessment of ENM exposure on changes to cellular regulatory networks. In the heart, following maternal ENM inhalation exposure, mitochondrial and cardiovascular function are significantly perturbed in the fetal progeny and this continues, to a lesser extent, into adulthood. To investigate this, we examined both transcriptomic and epigenomic reprogramming in the heart of fetal progeny. Interestingly, the most significantly regulated genes through epigenetic alterations were nuclear-encoded mitochondrial proteins involved in oxidative phosphorylation.

Increased histone modifications, specifically of the histone 3 lysine 4 tri-methylation mark (H3K4me3), suggests that these genes should become more transcriptionally activated through the relaxation of the chromatin ultrastructure (22), but the transcriptomic profile in Chapter 6 suggest that the actual expression of these genes are not becoming altered. Following gestational ENM inhalation exposure, the fetal progeny exhibit obvious epigenomic alterations that, while not immediately revealing transcriptional changes, could become preserved during development and

ultimately make the transcription of those genes more susceptible to transcription factor binding and other stimulation in the future. While the transcriptomic analysis only surveyed mRNA within the heart, gene and protein ontology suggested that miRNA-145 was likely downregulated. MiRNA-145 is commonly downregulated in cancers (9, 42) and plays a role in unregulated cell growth. And while a more thorough analysis of ncRNA interaction with the epigenome following exposure is needed, the results obtained in the three studies (20, 21, 52) suggest that dysregulation of miRNAs could be a common response that can alter cell survival (miRNA-145) or metabolism (miRNA-378a), having lasting consequences.

Specific Aim 2 engages the idea of mitochondrial and nuclear dynamics from a diagnostic perspective. While glycosylated hemoglobin (HbA1c) and/or fasting blood glucose remain primary diagnostic identifiers (16), there persists a major gap in understanding of the linear or non-correlative nature of increased glucose in the blood stream on mitochondrial function. Mitochondrial dysfunction during diabetic insult is well known (46, 50), but the question of how mitochondrial function tracts with “severity” of diabetes, as assessed through changes in HbA1c, is not clear. In Specific Aim 2, we wanted to understand if a specific set of molecular markers coincided with progression of diabetes, as determined by HbA1c, and how those markers may be used in a clinical setting for diagnoses. We found that global DNA methylation (5-methylcytosine) was the best predictor of diabetic status. And interestingly, in patients who had been diagnosed as diabetic, even if they had since their diagnosis controlled their diabetes and reduced HbA1c to normal levels, still exhibited higher global methylation. HbA1c is a convenient and easy diagnostic tool, but the cellular damage induced by hyperglycemic conditions may not be easily reversible by lifestyle interventions (as indicated by DNA methylation levels) (14, 43).

The work in Specific Aim 2 also helps to elucidate how new bioinformatic and artificial intelligence tools can be applied to better characterize data. The interaction network between the nucleus and mitochondrion is very multifaceted. While ATP production and metabolism may be primary function of the mitochondrion, other functions including ROS production, calcium regulation, apoptotic responses, inflammation, and production of other metabolites make interpreting how a specific molecule or pathway is truly influencing the disease or pathogenic state (51). Machine learning, deep learning, and algorithms capable of building on data to predict outcomes introduces a new level to biological analyses. Cardiology is among the major fields

utilizing machine learning algorithms (28), though this has been mainly employed in the assessment of imaging from non-invasive procedures, such as echocardiography and tomography (30, 47). As addressed in Specific Aim 2, what is lacking is a practical application to molecular datasets. In order to truly define the networks existing between the mitochondrion and nucleus, integration of data at a large scale is needed. In diabetes research, investigators are beginning to examine how using collected metadata for commonly assessed parameters, including BMI, age, sex, cardiovascular function, HbA1c, and others can predict diabetic onset, progression, and future outcomes (10, 31). While this is certainly helpful, until machine learning is applied at the granularity of the cell, including DNA, RNA and protein modifications, it will be difficult to truly create a spectrum of disease progression and achieve true personalized medicine.

So where is the field when it comes to understanding “big data” and the applications of tools to derive more specific conclusions? Described in a 2013 by Berger et al., high-throughput data was complex, though the cost-to-data generation was decreasing, a fundamental understanding of how to even analyze large datasets was still needed. In the year 2019, analyzing large complex datasets is not the limitation to scientific output, it is understanding how to combine and integrate multiple, heterogeneous datasets (23, 44). As costs associated with acquiring omics-based assessments continue to decrease (37), the accumulation of more and more datasets will rise. As we come to a critical mass of data collection, it is likely that the days of standalone high-throughput datasets is at an end. Through the use of the cloud, or in a shared workspace accessible by people all over the world, data can be collected, uploaded, and integrated into more useful interpretations. While the basic sciences likely will not see these changes for years to come, healthcare systems are currently moving toward this approach (1, 57, 64). In the future, it is likely that through uploading sequencing datasets to a common repository online, such as a machine learning platform for all data generated around epigenetic marks in diabetes, a more universal understanding of disease pathogenesis can be ascertained.

In understanding how to develop diagnostic standards for molecular data, a major obstacle will be the definition of “normative” values within a population. While standards are well established for characterizing broad diseases such as diabetes mellitus (2) and heart disease (24), “normal” values for transcriptomic and epigenomic profiles need to be established in order to detail the nuances of disease progression. For example, while an HbA1c level of 6.8 may classify an

individual as a type 2 diabetic, the expression profile of gene X, Y, and Z has only direct relation to a cohort that it is immediately compared to, with the absolute expression profile for most genes not having significant influence on disease diagnosis. Only recently, through the advent of more powerful statistical modelling and approaches such as co-expression analysis (54), have more concentric groupings of genes involved in major pathologies begun to be clustered. These approaches will be necessary to expand outside of the confines of transcriptome data and begin to compare epigenetic and epitranscriptomic profiles from individual-to-individual. Ultimately, the production and culmination of broad-scale molecular data will start shaping our definitions on molecular diagnoses, but reporting biases of high-throughput data (45) highlight the need for more standardized approaches when sequencing, uploading, and reporting omics data.

Presented in Specific Aim 3 of this dissertation is an old concept surrounding mitochondrial biology that is now present in a new light. In the first discoveries of ncRNAs in the mitochondrion, specifically miRNAs, it was thought that the mitochondrion may act as a “sink,” holding miRNAs to interact with mRNA or to release when needed (4, 32). This theory has faded away as an interest for miRNA-directed mediation of the mitochondrial genome was pursued (15, 38). While evidence has been found of miRNA-directed regulation of mitochondrial transcripts, it is unlikely that this form of regulation is their primary function. A study by Dasgupta et al., reported that in mitochondrial DNA deficient cells, miRNAs are still found to be imported (13). Our group, in this report as well as previous datasets (26), has reported the presence of miRNAs in the mitochondrion, but most of which appear to have no direct mapping correlate to mitochondrial DNA or transcribed products. What could this mean about the larger mitochondrial regulatory network? As summarized by Kren et al., “...to provide an alternate mechanism for control of cellular function” (32).

The diverse nature of miRNAs, lncRNAs, and other ncRNAs found in/or in association with the mitochondrion suggests a larger regulatory role. The regulatory network of ncRNAs that interacts with the mitochondrion is likely another piece in the larger story of nuclear and mitochondrial signaling mechanics. In Specific Aim 3, the data suggests a significant drop in diversity of ncRNA during diabetic disease progression. While canonical mitochondrial miRNA theory has suggested that a rise in miRNAs within the mitochondrion during diabetic onset (26) and heart failure (11, 12) negatively affects the translation of the mitochondrial genome, the current

data would suggest that a loss of miRNAs may be detrimental. The data within this dissertation help to elucidate the makeup of the ncRNA regulatory network, but also give clarity to the interactions during disease. We suggest that through a loss in ncRNAs, the robustness of response to cellular insults is decreased, impairing the capacity to inhibit specific mRNAs related to apoptosis and cellular stress (32).

With a depletion of total ncRNA within the mitochondrion, that also means a decreased diversity of lncRNAs. While Kren et al. suggested that miRNAs may be within the mitochondrion to regulate a sequestered population of mRNA (32), what if miRNAs were sequestered not by the mitochondrion, but by lncRNAs? In a review assessing the stoichiometric concentrations of ncRNAs and the competing endogenous RNA theory (53), it is suggested that for most lncRNAs, their abundance in the cellular milieu is generally too low to pose a significant threat in the sequestration of miRNAs. While studies have shown popular lncRNAs, such as MALAT1 (3, 8, 40), sponging miRNAs in the nucleus or cytoplasm, most lncRNAs are not transcribed in concentrations dense enough to truly impact miRNA activity (53). Though, if the lncRNA was localized to a cellular compartment, such as the mitochondrion, this could change its stoichiometric ratios. The mitochondrion may use the import of lncRNAs in order to sequester miRNAs to be used later in cellular defense. Through a database recording immunoprecipitation experiments validating lncRNA sponging (41), three of the lncRNAs found to be important in the study (MALAT1, NEAT1, and KCNQ1OT1) were found to bind to most of the miRNAs reported within the mitochondrion.

The work included in this dissertation only begins to assess sponging theory in the mitochondrion. More information is needed on the reason and mechanism for these ncRNAs disappearing during disease onset. One explanation could be that during diabetic insult, increases in ROS, changes in calcium handling, and subsequent increased permeability through the mitochondrial permeability transition pore (MPTP) may lead to increased release of sequestered miRNAs (7, 27). While during basal conditions the mitochondrion is able to import and regulate the flux of ncRNAs, changes in metabolism, such as in diabetes, could reduce the selectivity of the mitochondrial regulatory network. Though changes in membrane permeability might explain the efflux of ncRNA in the diabetic state, there still remains the concern of the import and export mechanisms involved. One popular theory is the transport of miRNAs into the mitochondrion

through Ago2, which has been reported to be within the mitochondrion (35). Through a mechanism involving capping by GW182, to avoid degradation (60), Ago2 can transport into the mitochondrial matrix bringing along other miRNAs (63).

Ago2 has been a popular choice for miRNA import due to the miRNA processing and capacity and RISC formation (48). But, even if Ago2 is responsible for importing every miRNA into the mitochondrion, what mechanism could import larger ncRNAs, such as lncRNA and/or antisense transcripts? PNPase, while only shown to interact with tRNA, ribozymes, and other smaller ncRNA (49, 55, 56) its regulation of larger RNAs has not been reported in mammalian systems. In Specific Aim 3, our data suggest that the RNA bound to PNPase are predominately large ncRNAs, with only a few miRNAs shown to have any binding to PNPase. With the CLIP data, as well as data suggesting PNPase has increased expression in diabetes, surmising a role for the protein is difficult; lncRNAs are binding PNPase, the expression of PNPase is elevated in diabetes, and total lncRNA biodiversity declines in diabetic mitochondrion. One logical conclusion to reach is that PNPase has an assortment of functions, ranging from import, export, and/or degradation of RNA. Because the protein is a known ribonuclease in bacterial systems (5, 19), it is plausible that its nuclease capacity is evolutionarily conserved. The mitochondrial ncRNA import pathway remains as elusive as it did in 2006 when miRNAs were first discovered in the mitochondrion (36), but the studies presented in Specific Aim 3 give a new perspective on the role the KH and S1 RNA binding domains of PNPase may have on mitochondrial RNA import.

As the work presented in Specific Aim 3 provide the first perspective of lncRNA and other ncRNAs occupying the mitochondrial space, future investigations should seek to understand the true purpose of this ncRNA regulatory network. While sequestering is proposed between miRNA/lncRNA a plethora of other possibilities could exist. These roles of lncRNA could include the use of the mitochondrion to provide further processing through small nucleolar RNA (snoRNA) (59), a location to avoid RNA degradation through proteins such as XRN1 (61), and the possible site for novel RNA/protein interactions to promote/disrupt mitochondrial health and function. Further, Specific Aim 3 reveals that snoRNA, small nuclear (snRNA), and other ncRNAs exist in the mitochondrion, adding to the complexity of the regulatory network and complicating the spectrum of associations that could be occurring. The ability to manipulate this multifaceted mitochondrial network may provide new and profound insights into the understanding of disease

progression and the targeting of therapeutics; potentially, functional manipulation of the PNPase KH and S1 RNA binding domains may allow for the control of specific ncRNA species to be imported into the mitochondrion.

Limitations

While the included studies provide insight into mitochondrial biology and cardiovascular function following insult, weakness and limitations to the research should be addressed. Specific Aim 1 examines the effects of miRNA-378a on modifying the metabolic response following ENM exposure, but it is likely that other miRNAs play significant roles in the exposure. Though the transgenic model in Specific Aim 1 could only address one miRNA, future experimentations need to evaluate the complete miRNA and ncRNA transcriptomic response to ENM; this includes investigations applying co-expression analysis, ontology software, and other computer-assisted methodologies to describe the interaction networks of ncRNA found in the heart following insult. Specific Aim 2, an archetype for future applications of machine learning to the level of DNA and RNA, suffers from lack of sample size and patient heterogeneity.

With a sample size of fifty patients, CART analysis provided the best overall predicative accuracies, but this may not hold true with larger sample sizes. Though Specific Aim 2 serves as a guideline for future application, until further investigations are made on larger sample sizes with patient-matched sequencing datasets, the determination of the exact algorithms appropriate for application to a variety of disease-states remains unclear. Specific Aim 3 provides the most novel insights within the body of the dissertation, elucidating new, mitochondrial regulatory pathways. With the breadth of novel work, further validation of the targets found in the study are needed. While MALAT1 is confirmed through qPCR, additional experiments, such as CLIP of MALAT1 and RNA pull downs, are truly needed to explain and validate the novel biology.

Conclusions

The inner workings of the mitochondrial and nuclear network have much to be unveiled. My hope is that the work presented in this dissertation has contributed to the understanding of the anterograde and retrograde signaling of the cardiac cell; specifically, in the assessment of acute (ENM) and chronic (diabetes mellitus) exposures on the epigenetic and ncRNA framework. Machine learning at the DNA level hints at the possibility of precision medicine-based diagnostic

modalities in the future, while PNPase remains a potential target for therapeutic intervention. All-in-all, the mitochondrion, its regulatory network, and the nuclear epigenome remain critical areas of research and need for innovative scientific approaches (Figure 5.1).

Figure 5.1: Overview of Dissertation Aims

Figure 5.1: Disruption to the cellular and mitochondrial networks during insult through ENMs (Specific Aim 1) and diabetes mellitus (Specific Aim 2 & 3).

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Chapter 6: Related Material to Specific Aims

Section 1.1: Supporting Documentation for Specific Aim 1

Maternal Engineered Nanomaterial Exposure Disrupts Progeny Cardiac Function and Bioenergetics

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Running Title: Gestational Nano-TiO₂ and Cardiac Dysfunction

Key Words: Nanomaterials, Cardiac dysfunction, Pregnancy, Mitochondria

Abstract

Nanomaterial production is expanding as new industrial and consumer applications are introduced. Nevertheless, the impacts of exposure to these compounds are not fully realized. The current study was designed to determine whether gestational nano-TiO₂ exposure impacts cardiac and metabolic function of developing progeny. Pregnant Sprague Dawley rats were exposed to nano-TiO₂ aerosols (~10 mg/m³, 130 – 150 nm count median aerodynamic diameter) for 7-8 non-consecutive days beginning at gestational day 5-6. Physiological and bioenergetic effects on heart function and cardiomyocytes across three time points: fetal (gestational day 20), neonatal (4-10 days), and young adult (6-12 weeks) were evaluated. Functional analysis utilizing echocardiography, speckle-tracking based strain, and cardiomyocyte contractility coupled with mitochondrial energetics revealed effects of nano-TiO₂ exposure. Maternal exposed progeny demonstrated a decrease in E and A wave velocities with a 15% higher E/A ratio than controls. Myocytes isolated from exposed animals exhibited ~30% decrease in total contractility, departure velocity, and area of contraction. Bioenergetic analysis revealed a significant increase in proton leak across all ages, accompanied by decreases in metabolic function including basal respiration, maximal respiration, and spare capacity. Finally, electron transport chain complex I and IV activities were negatively impacted in the exposed group, which may be linked to a metabolic shift. Molecular data suggests that an increase in fatty acid metabolism, uncoupling, and cellular stress proteins may be associated with functional deficits of the heart. In conclusion, gestational nano-TiO₂ exposure significantly impairs the functional capabilities of the heart through cardiomyocyte impairment, which is associated with mitochondrial dysfunction.

New and Noteworthy

Cardiac function is evaluated, for the first time, in progeny following maternal nanomaterial inhalation. The findings indicate that exposure to nano-sized titanium dioxide (nano-TiO₂) during gestation negatively impacts cardiac function and mitochondrial respiration and bioenergetics. We conclude that maternal nano-TiO₂ inhalation contributes to adverse cardiovascular health effects, lasting into adulthood.

Introduction

Growing production, distribution, and use of engineered nanomaterials (ENMs) necessitates more thorough evaluations of the physiological effects that exposure to these ENMs induce. One of the most widely used ENM in the world is titanium dioxide (nano-TiO₂); in 2006, 40,000 metric tons of nano-TiO₂ were estimated to be produced domestically, with 2015 projections reaching as high as 260,000 metric tons (43). Specifically, it is well-accepted that inhaled ENMs can interact in one of three ways: an acute inflammatory response, direct translocation, and through the autonomous nervous system in the lungs (19); the link between pulmonary exposure and extrapulmonary effects provides a picturesque example of the complications that may arise when trying to decipher a prominent mechanism for nano-TiO₂ toxicity.

In the heart, it has been shown that through pulmonary exposure (15, 36, 57), intravenous administration (30), and orally (16), ENMs can affect cardiac function, though, this is not without disagreement (8, 20). Nano-TiO₂ accumulation in cardiac tissue has been demonstrated to increase edema and apoptosis during embryonic development (37, 64). In adult exposures, nano-TiO₂ causes a decrease in bioenergetics, increase in inflammatory signaling, and DNA damage (15, 16, 67). While measures of direct interaction between an organism and nano-TiO₂ have revealed much about cardiac dysregulation, very little has been shown regarding the mechanisms affecting cardiac tissue of gestational exposed progeny (22). Recent studies have demonstrated a role for diesel exhaust particulate matter air pollution in causing negative cardiac implications in progeny from maternal exposures (18, 58, 59), but no study has focused specifically on the longitudinal effects of a single, characterized ENM on cardiac function.

Though many extrapulmonary effects are well recognized, *in vivo* cardiac functional impacts are less commonly identified as toxicological end-points, making investigation into the effects essential for expanding our knowledge. Studies have examined the effects of directly placing TiO₂ nanoparticles on cardiomyocytes *in vitro*. These studies have concluded that high enough concentrations of nano-TiO₂ (>10 µg/mL) can directly affect the functionality of cardiomyocytes, including measures of shortening, re-lengthening, and amplitude of contraction (26, 46); decreased functionality remained consistent across varying frequencies of contractions. Previous work has examined the cardiac bioenergetics from young adult Sprague Dawley rats exposed during gestation to nano-TiO₂; this study demonstrated that state 3 and 4 respiration were

significantly altered (54), providing the rationale for defining the specific impacts on cardiomyocyte bioenergetics more closely.

Recent literature has examined how ENM exposure can affect the *in utero* environment. Maternal nano-TiO₂ inhalation disrupts uterine microvascular function, and creates a hostile gestational environment for development (51), further, intravenous injection of carbon nanotubes has been shown to translocate to the placenta, resulting in fetal deformities and lower progeny yields (7, 23, 42). With known physiological impacts to the developing fetus following ENM exposure, the need for an initial understanding of the cardiac consequences resulting from changes within the *in utero* environment is crucial. Ultimately this understanding could have profound effects, including: ENM exposure standards for pregnant women, defining kinetics of nanoparticles across the placental barrier, and outlining mechanisms affecting cardiac/mitochondrial development in exposed progeny. To determine how nano-TiO₂ could affect progeny, we subjected pregnant Sprague Dawley rats to pulmonary exposure of nano-TiO₂ throughout gestation. These exposures allowed for measurement across three critical developmental points: fetal, neonatal, and young adult. To date, no study has examined cardiomyocyte function following gestational exposure to ENMs. We hypothesized that maternal nano-TiO₂ exposure would negatively impact cardiac function and cardiomyocyte bioenergetics as compared to air exposed progeny. Our results provide evidence that gestational exposure to nano-TiO₂ significantly affects the heart and cardiomyocyte function of progeny. Further, changes in mitochondrial respiratory capacity, coupled with decreasing mitochondrial complex activity, provide a potential mechanism for the observed functional deficits.

Methods

Animal Model

The experiments in this study conformed to the *NIH Guide for the Care and Use of Laboratory Animals (8th Edition)* and were approved by the West Virginia University Animal Care and Use Committee. Sprague-Dawley rats (250–275 g female; 300–325 g male) were purchased from Hilltop Laboratories (Scottsdale, PA). Rats were housed at the West Virginia University Health Sciences Center with food and water provided *ad libitum*. Rats were acclimated for at least 72-hours prior to mating, as previously described (53). At gestational day ~6 (5.85, mean of exposure times), pregnant rats were exposed to nano-TiO₂ as described below.

Engineered Nanomaterial

Nano-TiO₂ P25 powder, containing anatase (80%) and rutile (20%) TiO₂, was purchased from Evonik (Aeroxide TiO₂, Parsippany, NJ). Primary particle size was 21 nm with a surface area of 48.08 m²/g (35, 44, 45). Aerosolization by drying, sieving, and storing are described previously for nano-TiO₂ (27, 35).

Inhalation Exposure

The design and use of the nanoparticle exposure system has been previously described (65). Briefly, aerosol size was measured through both electrical low pressure impactor (ELPI) (136.47 ± 1.44 nm) (Figure 6.1.1A) and scanning particle mobility sizer (SPMS) (134.80 ± 1.24 nm) (Figure 6.1.1B). Aerosol concentration of engineered TiO₂ was determined to be (10.35 ± 0.13 mg/m³) (Figure 6.1.1C). Average aerosol concentration was measured over a total of ~8 exposures (7.79 ± 0.26 days) to each of the 13 animals in the gestational nano-TiO₂ exposure group. The first exposure to nano-TiO₂ was given on gestational day ~6, with the last exposure given 24 hours prior to birth. Exposures consisted of 5 hours per day treatments, with a calculated lung daily deposition of 43.8 ± 1.2 µg. Control animals were exposed to filtered air only. Calculation of total exposure volume has been previously described (29, 35, 41, 52). To calculate the dose required to match the appropriate lung deposition paradigm for Sprague Dawley rats, the formula $D = F \times V$

$\times C \times T$ was used, where F is the deposition fraction (10%), V is the minute ventilation based on body weight, C equals the mass concentration (mg/m³) and T equals the exposure duration (minutes).

Echocardiography – B-mode, M-mode, and Doppler Imaging

Nano-TiO₂ exposed and control filtered air young adult (6-12 weeks) Sprague Dawley rats were used in analysis and, subsequently, for cardiomyocyte isolation. Anesthetization was performed similarly to James et al. (25); by slowly reducing the total isoflurane given, measurements were taken as the animal awakens, reducing the anesthetic-induced effect on cardiac function. B-mode, M-mode echocardiograph, as well as Doppler imaging procedures have been described previously in mice (48), but with some adaptations: including the use of a lower frequency transducer (13-24 MHz instead of 32-55 MHz) and 3% isoflurane induction with sustained isoflurane at 1.5% or higher. Ultrasound images were taken using the Vevo 2100 Imaging System (Visual Sonics, Toronto, Canada). Briefly, ultrasound slightly left of the sternum, at the mid-papillary layer, were used for long-axis B-mode imaging. Rotation 90°, perpendicular to the animal, allowed for short-axis B-mode images. B-mode procurement provided detailed information such as area and ejection volume of the left ventricle (LV). Gating midway between the short-axis B-mode images allowed for acquisition of M-mode parameters. In M-mode, measures to determine the left ventricular thickness (interventricular septal, inner, and posterior wall) were conducted on adjacent end-systolic and end-diastolic peaks in relation to LV trace analysis. Doppler echocardiography measured mitral valve function, E and A wave velocity, deceleration time, E/A ratio, mitral valve area, etc. All ultrasound procedures were carried out by one imaging specialist, taken at a frame rate of 109-284 frames/s. M-mode and Doppler echocardiography were examined using averaged values from at least three replicate analyses from each animal. All measures were completed by one analyst.

Stress-Strain – Speckle Tracking

B-mode images, in both the short and long axis, were used for speckle-tracking based strain analyses. Peak strain and strain rates were measured across radial, longitudinal, and

circumferential dimensions. Speckle-tracking based strain analysis has been described previously in mice (48), with caveats for rat analyses mentioned above. Briefly, during each cardiac cycle, strain was measured as a function of total deformation length divided by the original length of a segment (3, 10, 38). Displacement length, velocity, strain, and strain rate were measured in the endocardium over a minimum of three cycles. As previously described, short-axis images in the LV were split into anterior free (AF), lateral (L), posterior (P), inferior free (IF), posterior septum (PS), and anterior septum (AS), while long-axis was split into anterior base (AB), anterior mid (AM), anterior apex (AA), posterior apex (PA), posterior mid (PM), and posterior base (PB) as previously described (48).

Cardiomyocyte Isolation

Fetal/Neonatal

For the fetal time point, pregnant dams were euthanized and pups were removed at gestational day 20 from the exposed and control rat uterus. For the neonatal time point, rats were sacrificed at 4-10 days post-birth. Animals at both time points were kept on ice during dissection. During cardiomyocyte isolation, pregnant rats were anesthetized using 5% isoflurane, with maintenance isoflurane levels sustained at 2.5%. Hearts were removed through a midsagittal cut in the thoracic cavity. Hearts were collected in groups of 4-8, per litter, and atrial and ventricular appendages trimmed. Digestive solution included 2 mg Pancreatin (Sigma Adrich, St. Louis, MO) and 2 mg of Collagen Type II (Worthington Biochemical, Lakewood, NJ), mixed with 2mL of physiological 1X ADS buffer (0.1 M NaCl, 1.2 mM NaH₂PO₄, 0.8 mM MgSO₄, 5.4 mM KCl, and 5 mM glucose at pH 7.4) per heart digested. Digestive solution was used to initially wash the hearts (15 minutes and discarded) and to begin separating single cells from the tissue (3-4 washes at 22 minutes each which were saved). After each saved digestion, the supernatant was removed from the tissue debris and centrifuged at 1000 RPMs for 7 minutes. The supernatant was discarded and 2 mL of newborn calf serum (NCS) was added to the cell suspension and transferred to a 37°C incubator.

In a 15 mL conical tube, a percoll gradient was made for separation of cardiomyocytes from other cells of the heart. The percoll gradient (density = 1.130 g/mL) was made of two layers: clear (top) and red (bottom) percoll solutions. 4 mL of top percoll (density = 1.059 g/mL, 90 mL

percoll stock + 110 mL 1X Ads buffer) were first added, followed by pipetting bottom percoll (density = 1.082 g/mL, 130 mL percoll stock + 70 mL 1X ADS buffer with phenyl red) below the first solution. After all cell collection steps, cells were centrifuged at 1000 RPMs and re-suspended in 2 mL of 1X ADS buffer, per 5 hearts, which was added to the top of the percoll gradient. Cells, on top of the percoll gradient were centrifuged at 3000 RPMs for 30 minutes, with 9-minute acceleration and deceleration time. Cells were then extracted from the middle layer, washed with NCS, and placed in plating media (with fetal bovine serum (FBS)). Two hours after placing in plating media, cells were exchanged to maintenance media (no FBS) and media changed every two days. Cells were counted using a hemocytometer.

Adult

Young adult rats were anesthetized using 5% isoflurane. The isolation procedure was adapted from Xu and Colecraft (61). Briefly, hearts were removed, rinsed in physiological salt solution (PSS), and suspended on a Langendorff perfusion system, with retrograde flow through the aorta. Enzymatic digestion with 20 mg Collagen Type II (Worthington Biochemical, Lakewood, NJ), 10 mg Neutral Protease (Worthington Biochemical, Lakewood, NJ), 20 mg Carnitine (Sigma Aldrich, St. Louis, MO), 25 mg 2,3-Butanedione monoxime (BDM) (Sigma Aldrich, St. Louis, MO), 31 mg Taurine (Sigma Aldrich, St. Louis, MO), 20 mg Glutamine (Sigma Aldrich, St. Louis, MO), and 1.25 μ L of 1M CaCl_2 in 50 mL of Buffer A. After digestion (total calcium concentration added 0.6 mM), gravimetric separation of cells was performed with continual calcium loading during the three wash steps. Cells were counted using hemocytometer.

Cardiomyocyte Contractility

A video-based edge detection system (Ionoptix, Westwood, MA) was used to assess contractility of cardiomyocytes. Phase-contrast microscopy coupled with Ionoptix software, were used to track cardiomyocyte dimensions. Cells were placed in contractility buffer (50.55 mg BDM, 8.82 mg CaCl_2 , 119.5 mg HEPES, 10.16 mg MgCl_2 , 394.47 mg NaCl, 90.075 mg glucose, 14.91 mg KCl, and 2.276 mg NaH_2PO_4) and then diluted 10:1 with contractility buffer from the original cell suspension. Field stimulation at 0.5 Hz at 10 volts was used for all measures. Dynamic measures

taken included departure velocity, peak height, peak height % to baseline, return velocity, 10 and 90% time to shortening, 10 and 90% time to relengthening, area of departure, area of return, and retention of baseline. Cells included into the study matched the specific criteria: 1) chosen based on the best morphology when compared to others within the field (striations, no blebbing) and 2) cells sustain consistent contractions for at least 60 seconds. For reference, Ye et al. provides more detailed parameters of the contractile apparatus (63)

Cardiomyocyte Mitochondrial Bioenergetics

Isolated cardiomyocytes were plated on XF96 V3 cell culture microplates, using the Seahorse XF96 for analysis (Agilent Technologies, Santa Clara, CA). Measurements were taken 24-48 hours after plating cardiomyocytes. Concentrations of fetal and neonatal cells ranged from 35,000 – 85,000 per well. Adult cardiomyocytes, due to slight variation in cell viability and time before analysis for each sample, were not normalized based on cell number, but rather on basal respiration between groups. Replicates were generally performed in sets of 5-6. Yoshida et al provides a comprehensive overview of the Seahorse applications (66). Briefly, oxygen consumption rate (OCR), extracellular acidification rate (ECAR), and proton production rate (PPR) were measured using oligomycin, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), antimycin A, and rotenone. Measures of mitochondrial respiration included ATP production, proton leak, basal respiration, maximal respiration, and spare capacity.

Electron Transport Chain (ETC) Complex Activities

ETC complexes I, III, IV (11, 12) and V (ATP synthase) activities were measured as previously described (9), with minor modifications. Briefly, cardiomyocytes from young adult animals were treated with RIPA Buffer (Life Technologies, Grand Island, NY). A Bradford assay was carried out on each sample to provide a basis for normalization to protein content. Activity was measured in complex I (reduction of decylubiquinone), complex III (reduction of cytochrome *c*), complex IV (oxidation of reduced cytochrome *c*), and complex V (pyruvate kinase and phosphoenolpyruvate and ATP production).

ETC Complex Protein Expression

Blue native polyacrylamide gel electrophoresis (BN-PAGE) was performed as previously described (56), with standardization to protein content through the Bradford method (4). Briefly, young adult cardiomyocytes were digested with RIPA Buffer (Life Technologies, Grand Island, NY) and 40 µg of protein were added to 1% digitonin on ice. Coomassie G-250 was added and samples were run on 4–16% NativePAGE gels. Gels were placed in GelCode® Blue Stain Reagent (Thermo Fisher, Rockford, IL) to fix bands for representation, followed by washes until the desired background staining was removed. Densitometry was measured using Image J Software (National Institutes of Health, Bethesda, MD). Complexes were identified using a 480 KDa ladder with criteria for identifying the molecular weight of each complex matching Wittig et al. (60).

Quantitative PCR

RNA was isolated from fetal progeny hearts using the Vantage™ Total RNA Purification Kit (Origene), following the manufacturer's instructions. RNA was then converted to cDNA for qPCR analysis using the cDNA Synthesis Kit for miRNA (product number: HP100042, Origene), following the manufacturer's instructions. cDNA was used to assess three markers: PPAR γ (product number: Rn00440945_m1, Thermo Fisher, Rockford, IL), CPT1A (product number: Rn00580702_m1, Thermo Fisher, Rockford, IL), and UCP2 (product number: Rn01754856_m1, Thermo Fisher, Rockford, IL). Samples were standardized to GAPDH (product number: Rn01775763_g1, Thermo Fisher, Rockford, IL). Experiments were conducted on the Applied Biosystems 7900HT Fast Real-Time PCR system (Applied Biosystems, Foster City, CA), in a total reaction volume of 20 µL with TaqMan® 2X Universal PCR Master Mix (Applied Biosystems, Foster City, CA).

Western Blot Analyses

12-well, 4-12% gradient gels were used in SDS-PAGE, as previously described [3, 4, 6, 11, 12, 28, 29] with modifications. Briefly, cardiomyocytes from the young adult group were used in all

protein quantification, with the Bradford method being employed to standardize protein concentration using bovine serum albumin (4). Primary antibodies used in the study included: anti-ANT (product number: sc-9300, Santa Cruz Biotechnology Inc., Dallas, TX), anti-APAF-1 (product number: ab2000, Abcam Inc., Cambridge, MA), anti-BAX (product number: ab32503, Abcam Inc., Cambridge, MA), anti-BCL-2 (product number: sc-7382, Santa Cruz Biotechnology Inc., Dallas, TX), anti-H3K4me3 (product number: G.532.8, Thermo Fisher, Rockford, IL), anti-H3K27me3 (product number: G.299.10, Thermo Fisher, Rockford, IL), anti-GAPDH (product number: ab8245, Abcam Inc., Cambridge, MA), anti-HDAC6 (product number: ab61058, Abcam Inc., Cambridge, MA), anti-OPA1 (product number: ab42364, Abcam Inc., Cambridge, MA), anti-PDH (product no. 2784; Cell Signaling Technology, Danvers, MA), anti-PDH E1-alpha subunit (phospho S293) (product number: ab177461, Abcam Inc., Cambridge, MA), anti-PPAR α (product number: ab2779, Abcam Inc., Cambridge, MA), anti-UCP3 (product number: ab3477, Abcam Inc., Cambridge, MA), anti-VDAC (product no. 4866; Cell Signaling Technology, Danvers, MA). Secondary antibodies used included: goat anti-mouse IgG horseradish peroxidase (HRP) conjugate (product no. 31430; Pierce Biotechnology, Rockford, IL), goat anti-rabbit IgG HRP conjugate (product No. 10004301; Cayman Chemical, Ann Arbor, MI), and rabbit anti-goat IgG horseradish peroxidase (HRP) conjugate (product no. 31402; Pierce Biotechnology, Rockford, IL). Standardization of protein levels for each blot were performed with both Ponceau S Solution (Sigma Adrich, St. Louis, MO) and the anti-GAPDH antibody. Pierce Enhanced Chemiluminescence Western Blotting substrate (Pierce Biotechnology; Rockford, IL) was used to detect signal per manufacturer's instructions. G:Box Bioimaging system (Syngene, Frederick, MD) was used to detect signals and data were captured using GeneSnap/GeneTools software (Syngene, Frederick, MD). Densitometry was analyzed using Image J Software (National Institutes of Health, Bethesda, MD). All values were expressed as optical density with arbitrary units (AU).

Statistics

A two-tailed student's *t*-test was used to determine statistical significance between control and gestational nano-TiO₂ exposed groups. Differences between control and exposed groups were considered statistically trending ($P = 0.075 - 0.055$, denoted by †), or statistically different ($P \leq$

0.05, denoted by *, and $P \leq 0.01$, denoted by **) unless explicitly stated otherwise. All data are presented as means $\pm$ standard error (SEM).

Results

Echocardiography and Speckle-Tracking Based Strain Analysis

Young adult animals (6-12 weeks) were assessed using a variety of ultrasound techniques to evaluate *in vivo* cardiac function. M-Mode, measuring end-systolic and end-diastolic diameters and volumes, and B-Mode, measuring the short and long axis diameter of the left ventricle (LV), are provided in Table 6.1.1 and Table 6.1.2, respectively. M-mode was found to have significant changes in the total mass of the LV between groups ($P = 0.033$) and a trending difference in stroke volume ($P = 0.072$). LV thickness was measured across end-diastole and end-systole for changes in interventricular septal, inner, and posterior wall thickness. Interventricular end-systole septal thickness showed a significant decrease ($P = 0.015$) in the experimental group (Table 6.1.3). B-mode showed no changes between groups. Doppler echocardiography revealed differences in LV filling between the control and gestational nano-TiO₂ exposed group (Figure 6.1.2); this included differences in the velocities of both the E wave ($P = 0.025$) (Figure 6.1.2A) and the A wave ($P = 0.040$) (Figure 6.1.2B), with the experimental group showing a significant decrease in both velocities.

Also, even though both the E and A wave velocities were altered, the E/A ratio between groups was found to be higher in the experimental group ($P = 0.052$). Control animals exhibited a normal E/A ratio (1.505) while the experimental group exhibited a higher ratio (1.813) (Figure 6.1.2C). This is represented by Figure 6.1.2D which depicts a control (left) and gestational exposed (right) animal with E/A ratios similar to that of the average for each group. Other parameters were also measured, including mitral valve area (Figure 6.1.2E), and were not found to be significant. For the stress-strain analysis, only two parameters appeared to be altered during contraction of the LV; the long axis systolic displacement and strain (Figure 6.1.2F and 6.1.2G) showed a significant ($P = 0.049$) and trending ($P = 0.065$) difference, respectively. These results demonstrate that maternal exposure can produce adverse effects in cardiac systolic and diastolic function, while also interfering with cardiac development.

Cardiomyocyte Contractility

To further delineate the cardiac outcomes of progeny predisposed to gestational nano-TiO₂, cardiomyocyte contractile function was assessed. Cells were isolated from young adult rats in the control and experimental groups. Cardiomyocyte contractile function was decreased in the gestational exposed compared to the control (Figure 6.1.3). An assessment of decreasing function was demonstrated through changes in departure velocity, peak height, baseline % to peak height, and area under the contractile curve. Departure velocity was significantly decreased in the young adult group ($P = 0.0008$) after gestational exposure (Figure 6.1.3A). In addition, the peak height of contraction was significantly decreased ($P = 0.0001$) (Figure 6.1.3B), with no differences observed in the resting baseline ($P = 0.73$) (Figure 6.1.3C). The exposed group also exhibited a decreased baseline % to peak height ($P = 0.0003$) (Figure 6.1.3D). Lastly, total area of the contraction was shown to be significantly reduced in the exposed group compared to the control, both in area of departure ($P = 0.0001$) and area of return ($P = 0.002$) (Figures 6.1.3E and 6.1.3F). Changes in return velocity, time to 90% peak, and time to 90% baseline, were not significant. Two initial transients, one from both the control and experimental contractile data, are overlaid for a visual representation of differences in the contractile curves (Figure 6.1.3G); the departure velocity and peak height measurements are similar to the averages for each group. All measures were performed on the first contraction after stimulation, referred to further as the first transient. Second transient, or the second contractile peak, measures displayed a similar trend (data not shown). At least three cardiomyocytes were used per control or exposed litter. Attenuated responsiveness during stimulation in the maternal exposure model establish modifications to cardiomyocytes function, which remain into adulthood.

Mitochondrial Bioenergetics

To determine whether changes in contractility resulted from changes in cellular energetics that drive contractile function, the Seahorse XF96 was implemented to determine the effects of gestational nano-TiO₂ exposure on the bioenergetics of the growing progeny. Data from the Seahorse XF96 was compiled at three time points: fetal (gestational day 28), neonatal (4-10 days post birth), and young adult (6-12 weeks). The results indicated mitochondrial respiration dysfunction, with varying degrees of impairment across developmental stages (Figure 6.1.4). Fetal cardiomyocytes, seeded at a 65,000 cell density, showed a decrease in basal respiration ($P =$

0.0001) (Figure 6.1.4A) and an increase in proton leak ($P = 0.003$) in the experimental group. When cardiomyocytes were extracted from the neonatal hearts and seeded at a 55,000 cell density, effects on basal respiration diminished, but significant decreases in maximal respiration ($P = 0.0031$) (Figure 6.1.4B) and spare capacity ($P = 0.003$) were observed within the nano-TiO₂ exposed groups. Also, proton leak continued to show a significant increase in the exposed group ($P = 0.044$). Because of slight variations in the recovery percentage of viable adult cardiomyocytes and limited cell viability in culture, normalization of the young adult group was processed differently than the fetal and neonatal groups.

Due to changes in basal respiration diminishing in the neonatal group, this parameter was used to normalize the young adult age group. Basal respiration normalization resulted in a significant increase in the proton leak in the experimental group ($P = 0.0072$) (Figure 6.1.4C). Normalization to both maximal respiration and spare capacity also showed a significant increase in proton leak in the experimental group (data not shown). A depiction of the oxygen consumption rate (OCR), extracellular acidification rate (ECAR), and proposed bioenergetics capacity for neonatal cardiomyocytes in the control and experimental groups are depicted in Figure 6.1.4D to provide an example of the overall procedure. This experiment validates the sustained, longitudinal effects of gestational exposure to ENMs, highlighting changes occurring in the bioenergetics of the cardiomyocytes.

ETC Complex I, III, IV, and V Activities and Expression

To further target the molecular effects of gestational nano-TiO₂ exposure on bioenergetics, mitochondrial complex activities was measured on young adult cardiomyocytes. Trends were shown in both complex I ($P = 0.067$) (Figure 6.1.5A) and complex IV ($P = 0.056$) (Figure 6.1.5B), with as much as a 20% decrease in activity in the gestational exposed group compared to the controls. Complex III and complex V (ATP Synthase) showed no significant differences in activity between groups (Figures 6.1.5C and 6.1.5D). Though trending differences, complex activity changes indicate potential lasting effects to the quantity, or function, of the mitochondrial electron transport chain constituents. To determine if a reduction in complex activity was due to a decrease in the expression level of the complexes, Blue Native PAGE was performed. No significant changes were found in any of the major ETC complex expressions (Table 6.1.4).

Metabolism and Cell Stress

In order to understand some of the molecular changes resulting from gestational nano-TiO₂ exposure to progeny, qPCR and Western blot analysis were used to probe changing mRNA and protein levels, respectively. At the fetal time point, mRNA of proteins contributing to proton leak and fatty acid metabolism were examined. For proton leak, qPCR of uncoupling protein 2 (UCP2) revealed increased ($P = 0.006$) mRNA levels in the experimental group compared to the control (Figure 6.1.6A), with qPCR of fatty acid metabolism constituents peroxisome proliferator-activated receptor gamma (PPAR γ) ($P = 0.039$) and carnitine palmitoyltransferase I (CPT1A) ($P = 0.023$) exhibiting a similar increase (Figure 6.1.6B and 6.1.6C). Because molecular markers involving proton leak and fatty acid metabolism were changed in progeny at the fetal level of development, the next step was to evaluate protein expression in the young adult cardiomyocytes.

Subsequent experiments to measure changes in metabolism involved assessing pyruvate dehydrogenase (PDH), which was found to be trending toward reduced expression ($P = 0.061$) in the experimental group (Figure 6.1.6D), with activity of PDH, evaluated through phosphorylation at serine 293 (p-S293), found to be statistically insignificant. When comparing the fluorescence ratio of PDH p-S293 to PDH, the amount of p-S293 per PDH expression was higher in the experimental group, but not significantly ($P = 0.082$). Peroxisome proliferator-activated receptor alpha (PPAR α) (Figure 6.1.6E) was found to be significantly increased ($P = 0.017$). As an epigenetic evaluation of the proteome, histone 3 lysine 4 tri-methylation (H3K4me3) and histone 3 lysine 27 tri-methylation (H3K7me3) were used to assess global histone activation/deactivation patterns in the cells, respectively. H3K4me3 expression remained similar between groups (data not shown) while H3K27me3 (Figure 6.1.6F) showed a significant, increased expression ($P = 0.045$) in the experimental animals. Signals for cell stress were also evaluated. Bcl-2-like protein 4 (BAX), B-cell lymphoma 2 (BCL2), and optic atrophy 1 (OPA1) were found to be similarly expressed between groups (data not shown), while apoptotic protease activating factor 1 (APAF-1) (Figure 6.1.6G) was found to be significantly increased ($P = 0.037$) in the experimental group. Lastly, proton leak was further evaluated through voltage-dependent anion channel (VDAC), adenine nucleotide translocase (ANT), and uncoupling protein 3 (UCP3), showing no significant alterations between groups (data not shown). Changes in the epigenetic profile, metabolism, and

mitochondrial stress of the experimental progeny demonstrate the implications of initial, as well as prolonged, effects of gestational nano-TiO₂ exposure.

Discussion

Nanotechnology holds the potential to significantly improve the lives of people around the world through a wide range of applications, including tumor abolition (2) or delivery of medication (31); but, the risks associated with nanomaterial use must first be properly identified. The most widely manufactured nanomaterial in the world is TiO₂, with up to 80% of its uses being involved in cosmetics and other personal care products (40). The ratio of nano-TiO₂/TiO₂ production continues to increase, with some predictions speculating total conversion of the market to nano-TiO₂ by 2025 (43). With the growing interest in nano-TiO₂ applications in the consumer market, nanotoxicological work is needed to fully define the extent to which this ENM contributes to human health and safety. Nano-TiO₂ has demonstrated the potential to cause adverse health outcomes after exposure; what remains poorly understood, is how nano-TiO₂ can interact through indirect, gestational exposure on growing progeny. More specifically, it is unclear as to how nano-TiO₂ impacts cardiac development and function.

The results of our study provide evidence that gestational nano-TiO₂ exposure can significantly affect the cardiac function of progeny, leading to a decreased functional capacity in both the whole heart and the cardiomyocyte. A mechanism for this dysfunction was illustrated through changes to cardiomyocyte bioenergetics after gestational exposure, which was then validated more specifically through mitochondrial complex activity, suggesting a role of nano-TiO₂ in decreasing cardiac function by the disruption of mitochondrial oxidative phosphorylation and respiration rates. The dose of nano-TiO₂ administered was calculated as a conservative estimate of ENM occupational exposure projected as 2.3 – 8.5 years of human exposure in a manufacturing setting. This calculation represents the maximum estimated dose deposited in the lungs. Because variables such as age, mass, clearance, and health states are not part of the calculation; we consider this dose very relevant to occupational settings. The exposures were also carried out over multiple, nonconsecutive days to more appropriately simulate human exposures. Low nanomaterial lung deposition, extended time-courses for dosing, and the use of an inhalation facility all support study observations that have a much higher potential of being attributable to true, physiological outcomes in humans and possessing repeatability across studies.

In vivo measurements, including M-Mode, B-Mode, Doppler echocardiography, and speckle-tracking based strain analysis were taken to assess changes in contraction mechanics of

the LV and the flow of blood through the mitral valve. In systole, radial strain and displacement of the LV walls were decreased in the experimental group, demonstrating initial signs of dysfunction in LV contraction mechanics. Decreasing radial strain and displacement during contraction of the LV is coupled with more overt measures of LV systolic function presented in the study, including a decreased interventricular end-systolic septal thickness and decreased total mass of the LV in the experimental group (with total mass falling into the lower percentile of normative values for Sprague Dawley rats (39)). From this data, we conclude that gestational nano-TiO₂ exposure weakens the contractile properties of the heart, potentially leading to increased risk factors of heart disease in older age. This is also supported by the fact that a decreased stroke volume is also seen to be trending in the experimental group. By decreasing the size of the LV, the thickness of its contractile walls, and measures of systolic radial strain and displacement, the function of the heart becomes more susceptible to perturbation and may indicate early predictive signs of heart complications.

In diastole, a decrease in E and A wave velocity and an increase in total E/A ratio was observed in the experimental group. Studies have suggested that E/A ratios greater than 1.5 can indicate diastolic dysfunction (1, 21), potentially leading to restrictive filling. Further, the total decrease in both the E and A wave velocities seems to indicate that diastole is not only dysregulated through atrial pumping abnormalities (restrictive filling) but potentially through asymmetrical, long axis filling (33). Changes in systolic and diastolic parameters demonstrate that the heart of gestational nano-TiO₂ exposed progeny develops a maladaptive phenotype. These findings raised questions as to the link between the observed cardiac dysfunction and the potential contribution of the cellular contractile unit, the cardiomyocyte.

In cardiomyocytes, changes in the departure velocity, baseline % to peak height, and both the area of departure and return under the curve were detected (Figure 6.1.3F). Though, no changes were observed in other common parameters of cardiomyocyte contractility (time to peak 90% and time to baseline 90%). The changes found with cardiomyocyte contractility in the experimental group further support the hypothesis that gestational nano-TiO₂ exposure weakens the contractile properties of the heart. A reduced baseline % to peak height and length of contraction in cardiomyocytes could be responsible for the attenuation in systolic radial strain and displacement, with reduced contractility also supporting the presentation of restrictive, diastolic filling. The

decreased area of contraction in the experimental group (ultimately leading to a diminished cardiac output) could also be linked to the decreasing stroke volume.

Changes in cardiomyocyte contractility have been observed in the presence of nano-TiO₂, but these experiments have been through direct incubation with the nanoparticle. Exceeding 10 µg/mL nano-TiO₂ concentrations, changes in the departure velocity, return velocity, and peak shortening were observed in the cardiomyocytes (26, 46). Differences between the two approaches (direct application and gestational inhalation exposure) are that return velocity parameters remained unchanged in the present study. The speed of the return velocity is indicative of the effectiveness of calcium shuttling in the cytoplasm (5). Primarily, calcium return to the endoplasmic reticulum from the cytoplasm is accomplished through SERCa pumps and Na⁺/Ca²⁺ transporters. Because return velocity remained constant across both groups, this provides an initial indication that cardiomyocyte dysfunction may not be the result of calcium handling, but rather a mechanism of bioenergetics and mitochondrial function.

Examining fetal/neonatal cardiomyocytes for changes in bioenergetics provided information on the longitudinal effects that gestational nano-TiO₂ exposure may produce on the development of cardiomyocytes; illuminating time points during development which may be more fragile to perturbation. During fetal exposure to nano-TiO₂, substrate unavailability/increased cytoplasmic oxidation could provide the reasoning for decreased basal respiration of cells and ultimately compromised bioenergetics, resulting from either direct or indirect effects of nano-TiO₂ exposure. As observed, this decrease in basal respiration was also coupled with increased proton leak. Proton leak is a factor controlled by both environment (mitochondrial membrane potential and pH) as well as protein constituents of the mitochondrion (14, 47). What is significant about these findings is that, following normal conditions, increases in basal respiration should coincide with increases in proton leak. But, as indicated in the results, a significant decrease in basal respiration was followed by an increase in proton leak.

Examining the data from the neonatal cardiomyocytes, it becomes evident that basal respiration is restored; depicting that at some point between the fetal and neonatal time point, basal respiration levels in the exposed group recovers, matching those found in the controls. This may be an indication that disturbances of basal respiration in the fetal cardiomyocytes were due to environmental conditions (likely through limitations of substrate availability (5, 55) promoted by

nano-TiO₂ interactions with the mother or placenta). Restoration of substrate supplies, no longer limited by nano-TiO₂ interactions, restored basal respiration to that of controls in the neonatal group. Though, the neonatal group exhibited a decreased maximal respiration and spare respiratory capacity, with a similar pattern of increased proton leak in the experimental group. We conclude that changes in maximal respiration and spare capacity may reflect persistent effects of nano-TiO₂ exposure, limiting mitochondrial health and function as the animal matures.

Normalization to basal respiration for the young adult group was performed for two reasons: 1) no significant change in basal respiration were observed in the neonatal group and 2) cell number for adult cardiomyocytes will vary depending on preparation and time before recording oxygen consumption. After normalizing to basal respiration, as well as maximal respiration and spare capacity, proton leak was seen to be increased in the exposed group over the control. With proton leak increasing over all three time points, unbalancing of the proton motive force, proton shuttling protein dysfunction/underexpression, and/or increased total reactive oxygen species could all be mechanisms contributing to the process.

To more specifically pinpoint the mechanisms behind changes in the respiration rates of cardiomyocytes from progeny exposed to nano-TiO₂, ETC complex activities were examined. Complex I, III, IV, and V activities were measured in the young adult group, to determine if changes in mitochondrial ETC complex activities provide support for the longitudinal, detrimental effects of gestational nano-TiO₂ on energy production. Our data revealed no changes in complex III and complex V (ATP Synthase), but strong trends for a decrease in complex I and complex IV as indicated by an average of 20% decrease in activity for both. Because overall complex expression is not changing, a decrease in ETC complex activities, whether through transcriptional, translational, or post-translational mechanisms, demonstrates that mitochondrial function, disturbed at both the fetal and neonatal time points, may still have an impact later in life.

Molecular changes at both the mRNA and protein level suggest that alterations in the metabolic profile of the heart becomes disrupted after gestational nano-TiO₂ exposure, with variations also seen in the epigenetic profile and mitochondrial stability. Research suggests that increasing levels of fatty acid oxidation in the heart, with decreasing glucose metabolism, can be indicative of the initial stages of insulin-insensitivity, hyperglycemia, and/or heart complications (6, 17, 28). In this study, PPAR α , PPAR γ , and CPT1A are seen to increase in the experimental group while PDH,

though trending, is reduced; these molecular changes, coupled with the observed diastolic dysfunction, are supportive of the hypothesis that increasing utilization of fatty acids could be coupled to a decrease in diastolic function resulting from an insult (32). Increases in PPAR α protein expression may also be linked to proton leak and ROS production.

PPAR α acts as a transcriptional regulator of UCPs by activating expression of the proteins (17, 62). This study found increasing mRNA levels of UCP2 in gestational nano-TiO₂ exposed progeny together with increased proton leak. Because increasing fatty acid metabolism leads to higher levels of incomplete β -oxidation, changes in ATP utilization, and changing initial substrates for the ETC, ROS production is likely to be increased (34, 68). With an increase in ROS, a greater proton leak could help to ameliorate damaging effects. The molecular data also reveals increasing APAF-1 expression in the experimental group, potentially pointing to decreased cell viability due to changing metabolic profiles. Because BAX and BCL2 levels were similar between groups (data not shown), APAF-1 may be activated independently of the canonical apoptotic pathway, which has been shown to be possible in certain tissue types (24).

Limitations/Future Directions:

This study focused specifically on the changing bioenergetics of gestational nano-TiO₂ exposed cardiac tissue and cardiomyocytes, but future experimentation could examine alterations in calcium handling. Data presented in this paper, through molecular markers of metabolism and complex activity, suggest that bioenergetics plays a role in contractile dysfunction and reduced diastolic function; though, measures of complex activity are likely underpowered due to variability in progeny birth and cohort sizes. Fluctuations in calcium availability or shuttling may also contribute to some of the phenotype, but would need further exploration. The study was limited by not directly assessing mitochondrial membrane potential and measures of ROS. This study demonstrated that UCP2 could be contributing to proton leak and should indicate a lower mitochondrial membrane potential but, without direct measures of mitochondrial membrane potential, it is unclear if molecular changes resulted in functional changes, which we acknowledge as a limitation. With changing levels of proton leak, examining ROS production is also important in understanding if proton leak is a compensatory mechanism for preserving function, or a result of mitochondrial disease and dysregulation.

Molecularly, changes in constituents involved in fatty acid metabolism suggests an increase of β -oxidation in the gestational nano-TiO₂ exposed group. To validate these conclusions, analysis of mitochondrial respiration in the presence of fatty acids, instead of glucose metabolites, would have been insightful. Lastly, a more in-depth understanding of epigenetic changes could provide greater insight into how function is regulated in gestational nano-TiO₂ exposed progeny. This study showed that transcriptional activation marks on histones (H3K4me3) were not changed between groups (data not shown), but global transcriptional repression on histones (H3K27me3) was upregulated in the experimental group, suggesting decreased global gene expression. Further evaluating epigenetic consequences through whole genome bisulfite sequencing or chromatin immunoprecipitation sequencing could greatly enhance our understanding of nano-TiO₂ effects on growing progeny.

Conclusions

In summary, gestational nano-TiO₂ exposure impacts cardiac function of the progeny. Manifestations of cardiac impairment in the maturing adult can be seen functionally through systolic and diastolic abnormalities and cardiomyocyte contractile attenuation. Mechanisms driving these deficiencies were found to include changes in electron transport of the mitochondria, including decreasing basal and maximal respiration, decreases in complex I and IV activities, and increased proton leak. Our study demonstrates that exposure to nano-TiO₂ indirectly, either through translocation across the placental barrier, change of the *in utero* environment, or through other mechanisms of interaction with the nanomaterial, induces significant cardiac deficiencies in development and adulthood.

This study has begun to define the molecular and physiological health outcomes related to progeny development after maternal ENM exposures, building upon previous work that has examined uterine and developing progeny microvascular function (51, 54). These studies may provide valuable information regarding the effects of ENMs during gestational exposure, but insight into how the progeny heart is adversely affected has yet to be fully elucidated; though, it is likely that inflammatory signaling in the placenta could be linked to improper development of the progeny (13, 49), without even the need for direct translocation of the nanomaterial (50). The purpose of this paper is to better characterize the effects resulting from changes that can alter the

uterine environment, providing insight into the possible mediators involved in nanomaterial exposures. The physiological changes observed in this paper through nano-TiO₂ also begin to raise questions regarding other engineered metal oxides which may share similar physiochemical characteristics and pathologies. Though nanomaterials continue to be a vital part of our industrial society, the effects of prenatal nanomaterial exposure are important to consider as their use continues to increase.

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Table 6.1.1

TABLE 1

Calculation	Units	Control Average and SEM	Exposed Average and SEM
Area;d (LV Trace)	mm ²	60.39 ± 3.87	56.39 ± 2.49
Area;s (LV Trace)	mm ²	25.64 ± 2.69	25.59± 1.97
CO (LV Trace)	mL/min	196.01 ± 18.54	177.18 ± 12.10
EF (LV Trace)	%	77.29 ± 2.69	75.70 ± 2.27
FS (LV Trace)	%	20.64 ± 2.07	16.26 ± 1.64
SV (LV Trace)	μL	200.21 ± 18.21	179.19 ± 12.07
V;d (LV Trace)	μL	262.54 ± 24.54	238.86 ± 16.45
V;s (LV Trace)	μL	62.33 ± 9.68	59.67 ± 7.022

Table 6.1.1: B-Mode echocardiography for control versus gestational TiO₂ exposed animals at the young adult (6-12 weeks) stage. Average and SEM for each measure. Area;d = diastolic area, Area;s = systolic area, CO = cardiac output, EF = ejection fraction, FS = fractional shortening, SV = stroke volume, V;d = volume at diastole, V;s = volume at systole. † ($P = 0.09 - 0.055$), * ($P \leq 0.05$), ** ($P \leq 0.01$).

Table 6.1.2

TABLE 2

Calculation	Units	Control Average and SEM	Exposed Average and SEM
CO (LV Trace)	mL/min	70.86 ± 1.79	65.14 ± 3.05
Diameter;d (LV Trace)	mm	6.72 ± 0.12	6.38 ± 0.16
Diameter;s (LV Trace)	mm	2.90 ± 0.14	2.83 ± 0.11
EF	%	79.41 ± 1.48	80.07 ± 1.05
EF (LV Trace)	%	85.56 ± 1.23	84.96 ± 0.72
FS	%	49.54 ± 1.54	49.84 ± 1.09
FS (LV Trace)	%	57.33 ± 1.63	56.18 ± 0.99
LV Mass (LV Trace)	mg	841.82* ± 51.34	696.89* ± 40.93
LV Mass Corr (LV Trace)	mg	673.45* ± 41.07	557.51* ± 32.75
LV Vol;d	μL	214.37 ± 10.19	195.27 ± 11.32
LV Vol;s	μL	45.78 ± 5.05	38.90 ± 3.11
SV (LV Trace)	μL	200.09† ± 6.95	178.31† ± 9.19
V;d (LV Trace)	μL	236.10 ± 9.63	211.59 ± 11.45
V;s (LV Trace)	μL	36.01 ± 4.02	33.28 ± 2.73

Table 6.1.2: M-Mode echocardiography for control versus gestational TiO₂ exposed animals at the young adult (6-12 weeks) stage. Average and SEM for each measure CO = cardiac output, Diameter;d = diameter at diastole, Diameter;s = diameter at systole, EF = ejection fraction, FS = fractional shortening, LV Mass = left ventricular mass, LV Vol;d = left ventricular volume at diastole, LV Vol;s = left ventricular volume at systole, SV = stroke volume, V;d = volume at diastole, V;s = volume at systole. † ($P = 0.09 - 0.055$), * ($P \leq 0.05$), ** ($P \leq 0.01$).

Table 6.1.3

TABLE 3

Measurement	Units	Control Average and SEM	Exposed Average and SEM
IVS;d	mm	1.92 ± 0.06	1.77 ± 0.06
IVS;s	mm	3.21* ± 0.06	2.91* ± 0.07
LVID;d	mm	6.45 ± 0.13	6.14 ± 0.17
LVID;s	mm	3.28 ± 0.15	3.07 ± 0.10
LVPW;d	mm	1.88 ± 0.10	1.77 ± 0.07
LVPW;s	mm	2.87 ± 0.09	2.69 ± 0.08

Table 6.1.3: Left ventricle thickness, using M-Mode echocardiography for control versus gestational TiO₂ exposed animals at the young adult (6-12 weeks) stage. Average and SEM for each measure IVS;d = Interventricular end-diastolic septal thickness, IVS;s = Interventricular end-systolic septal thickness, LVID;d = left ventricular end-diastolic internal thickness, LVID;s = left ventricular end-systolic thickness, LVPW;d = left ventricular end-diastolic posterior wall thickness, LVPW;s = left ventricular end-systolic posterior wall thickness. † ($P = 0.09 - 0.055$), * ($P \leq 0.05$), ** ($P \leq 0.01$).

Table 6.1.4

TABLE 4

Electron Transport Chain	Optical Density	Control Average and SEM	Exposed Average and SEM
Complex I	AU	8.69 ± 1.66	8.61 ± 2.26
Complex II	AU	7.02 ± 1.07	6.58 ± 1.98
Complex III	AU	9.80 ± 1.10	10.2 ± 2.45
Complex IV	AU	10.6 ± 0.27	9.83 ± 1.82
Complex V	AU	8.59 ± 0.76	8.66 ± 2.00

Table 6.1.4: Blue native polyacrylamide gel electrophoresis (BN-PAGE) for control (n = 3) versus gestational TiO₂ exposed (n = 4) animals at the young adult (6-12 weeks) stage. Complex I ($P = 0.94$), Complex II ($P = 0.74$), Complex III ($P = 0.82$), Complex IV ($P = 0.5$), and Complex V ($P = 0.96$) were evaluated and found to be statistically insignificant. † ($P = 0.09 - 0.055$), * ($P \leq 0.05$), ** ($P \leq 0.01$).

Figure 6.1.1

Figure 6.1.1

Figure 6.1.1: Characterization of nano-TiO₂ used in aerosols exposure paradigm. Total daily deposition = $43.8 \pm 1.2 \text{ } \mu\text{g}$. (A) Electrical Low Pressure Impactor (ELPI) size distribution (aerodynamic diameter) of nano-TiO₂ particles for n = 89 individual measurements ($136.465 \pm 1.436 \text{ nm}$). (B) Scanning Mobility Particle Sizer (SMPS) size distribution (mobility diameter) of nano-TiO₂ particles for n = 89 individual measurements ($134.802 \pm 1.241 \text{ nm}$). (C) Aerosol concentration of engineered nano-TiO₂ ($10.352 \pm 0.134 \text{ mg/m}^3$) during exposures.

Figure 6.1.2

Figure 6.1.2

Figure 6.1.2: Echocardiography and speckle-tracking analysis, for control (white, $n = 17$) versus gestational TiO_2 exposed (black, $n = 20$) animals at the young adult (6-12 weeks) stage. (A) Velocity of E wave ($P = 0.025$), (B) velocity of A wave ($P = 0.04$), and (C) E/A ratio ($P = 0.052$). (D) Representative image of Doppler echocardiography for control (left) and exposed (right) animals. Images were chosen to represent the average E/A ratio for control (1.506 ± 0.043) and exposed (1.813 ± 0.140) animals during ultrasound. Other parameters of echocardiography were found to be insignificantly different (E) mitral valve area ($P = 0.16$). Stress-strain analysis of systolic long axis (F) radial displacement ($P = 0.05$) and (G) radial strain ($P = 0.065$). † ($P = 0.075 - 0.055$), * ($P \leq 0.05$), ** ($P \leq 0.01$).

Figure 6.1.3

Figure 6.1.3

Figure 6.1.3

Figure 6.1.3: All first transient contractions (measured as the first contraction after stimulation) at 10 volts and 0.5 Hz, control (white, n = 39 cardiomyocytes from n = 8 animals) versus gestational TiO₂ exposed (black, n = 42 cardiomyocytes from n = 9 animals) cardiomyocytes at the young adult (6-12 weeks) stage. (A) departure velocity ($P = 0.0008$), (B) peak height ($P = 0.0001$), (C) baseline ($P = 0.73$), (D) baseline % to peak height ($P = 0.0003$), (E) area of departure ($P = 0.0001$), and (F) area of return ($P = 0.0018$). (G) Illustration of contractile properties from the control and exposed cardiomyocytes. Specific contractions were chosen to match the average departure velocity and peak height found in the controls ($-315.446 \pm 18.611 \mu\text{m/s}$, $22.920 \pm 1.174 \mu\text{m}$) and the exposed ($-227.054 \pm 17.268 \mu\text{m/s}$, $16.275 \pm 1.158 \mu\text{m}$) groups. † ($P = 0.075 - 0.055$), * ($P \leq 0.05$), ** ($P \leq 0.01$).

Figure 6.1.4

Figure 6.1.4

Figure 6.1.4: Seahorse XFe96 measurement for oxygen consumption rate (OCR) at pMoles/min, for controls (white) versus gestational TiO₂ exposed (black) cardiomyocytes at the fetal (gestational day 28), neonatal (4-10 days), and young adult (6-12 weeks) stage. For all measures, and average of 65,000 cells were used for fetal measurements, 55,000 cells for neonatal, and the young adult group was normalized to basal respiration between the control and exposed groups. (A) Basal respiration rates, with a change observed between the fetal groups ($P = 0.013$). (B) Proton leak across all three groups, changing in the fetal ($P = 0.0003$), neonatal ($P = 0.045$), and young adult ($P = 0.0072$) groups. (C) Maximal respiration across all three groups, with changes in the neonatal group ($P = 0.003$). (D) Neonatal cardiomyocyte oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in the control and experimental animals. Also an illustration of how values from the Seahorse analysis (basal respiration, maximal respiration, spare capacity, proton leak, etc.) are obtained using inhibitor agents such as oligomycin, FCCP, antimycin A, and rotenone. Fetal $n = 5$ control and $n = 3$ exposed, neonatal $n = 4$ control and $n = 5$ exposed, and young adult $n = 5$ control and $n = 5$ exposed animals used for analysis. Figure 6.1.4C: FC = fetal control, FE = fetal exposed, NC = neonatal control, NE = neonatal exposed, AC = adult control, AE = adult exposed. † ($P = 0.075 - 0.055$), * ($P \leq 0.05$), ** ($P \leq 0.01$).

Figure 6.1.5

Figure 6.1.5: Electron transport chain complex activity, control (white, n = 7) versus gestational TiO₂ exposed (black, n = 8) cardiomyocytes at the young adult (6-12 weeks) stage. (A) Complex I activity ($P = 0.067$), (B) Complex III activity, (C) Complex IV activity ($P = 0.056$), and (D) ATP Synthase activity. Activity was measured through the mass of cardiomyocyte cell lysates, not isolated mitochondria. † ($P = 0.075 - 0.055$), * ($P \leq 0.05$), ** ($P \leq 0.01$).

Figure 6.1.6

Figure 6.1.6

Figure 6.1.6: qPCR and western blot data from fetal and young adult progeny. Fetal heart tissue was analyzed using qPCR for mRNA levels while young adult cardiomyocytes were evaluated using western blotting for protein content. Control (n = 5) versus exposed (n = 5) mRNA fold change levels for (A) UCP2, (B) PPAR γ , and (C) CPT1A. mRNA levels were normalized to GAPDH. Control (n = 4) versus exposed (n = 4) optical density for (D) PDH and PDH p-S293, (E) PPAR α , (F) H3K27me3, and (G) APAF-1. Blots are organized as follows: (Top) sample, (Middle) Ponceau S staining, and (Bottom) GAPDH. One representative image from both the control (left, labeled “con”) and gestational exposed (right, labeled “ex”) groups were chosen to display. † ($P = 0.075 - 0.055$), * ($P \leq 0.05$), ** ($P \leq 0.01$). UCP2 = uncoupling protein 2, PPAR γ = Peroxisome proliferator-activated receptor gamma, CPT1A = Carnitine palmitoyltransferase I, PDH = pyruvate dehydrogenase, PDH p-S293 = pyruvate dehydrogenase E1-alpha subunit (phospho S293), PPAR α = Peroxisome proliferator-activated receptor alpha, H3K27me3 = histone 3 lysine 27 tri-methylation, APAF-1 = Apoptotic protease activating factor 1.

Section 1.2: Supporting Documentation for Specific Aim 1

Maternal Engineered Nanomaterial Inhalation During Gestation Alters the Fetal Transcriptome

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Keywords: nanotechnology, toxicology, nanomaterial, inhalation, epigenetics, maternal, fetal

Abstract

Background: The integration of engineered nanomaterials (ENM) is well-established and widespread in clinical, commercial, and domestic applications. Cardiovascular dysfunctions have been reported in adult populations after exposure to a variety of ENM. As the diversity of these exposures continues to increase, the fetal ramifications of maternal exposures have yet to be determined. We, and others, have explored the consequences of ENM inhalation during gestation and identified many cardiovascular and metabolic outcomes in the F1 generation. The purpose of these studies was to identify genetic alterations in the F1 generation of Sprague-Dawley rats that result from maternal ENM inhalation during gestation. Pregnant dams were exposed to nano-titanium dioxide (nano-TiO₂) aerosols (10 ± 0.5 mg/m³) for 7-8 days (calculated, cumulative lung deposition = 217 ± 1 µg) and on GD (gestational day) 20 fetal hearts were isolated. DNA was extracted and immunoprecipitated with modified chromatin marks histone 3 lysine 4 trimethylation (H3K4me3) and histone 3 lysine 27 trimethylation (H3K27me3). Following chromatin immunoprecipitation (ChIP), DNA fragments were sequenced. RNA from fetal hearts was purified and prepared for RNA sequencing and transcriptomic analysis. Ingenuity Pathway Analysis (IPA) was then used to identify pathways most modified by gestational ENM exposure.

Results: The results of the sequencing experiments provide initial evidence that significant epigenetic and transcriptomic changes occur in the cardiac tissue of maternal nano-TiO₂ exposed progeny. The most notable alterations in major biologic systems included immune adaptation and organismal growth. Changes in normal physiology were linked with other tissues, including liver and kidneys.

Conclusions: These results are the first evidence that maternal ENM inhalation impacts the fetal epigenome.

Introduction

The Barker Hypothesis [1], Developmental Origins of Health and Disease (DOHaD) [2], and fetal programming [3], all explore the relationship between the health of the gestational environment and fetal development and how this predisposes to future disease or sensitivities. Maternal prenatal health challenges such as nutrient deficiency, undernourishment, gestational diabetes and hypertension have been linked to an elevated risk for postnatal cardiovascular diseases [4]. Recently, maternal environmental toxicant exposures have become of prominent interest in relation to the impact of exposure on the fetal milieu and subsequent progeny health [5]. We have reported that maternal ENM inhalation impairs the ability of uterine arterioles to properly dilate, and this impacts litter health in the form of pup weight, number and gender distribution; as well as impaired microvascular function [21]. While these studies have focused on the maternal development of a hostile gestational environment and subsequent reduction in fetal nutrients, fetal epigenetic modifications may also occur. Conceptually, this relationship is not novel, but applications of environmental toxicants to the maternal-fetal models are. For example, bisphenol A [6] and air pollution [7] have been shown to negatively impact fetal outcomes. However, the impact of maternal ENM on fetal health and/or epigenetic modification are poorly understood.

Despite the ubiquitous inclusion of engineered nanomaterials in widespread applications, and their projected proliferation in human endeavors, the consequences of maternal ENM inhalation on the developing fetus and their impacts on future health are at best, vague, yet they are increasingly becoming a health concern. The prevalence of ENM covers an immense spectrum: surface coatings and additives in common consumer products (electronics, food, cosmetics), additives in industrial processes (advanced building materials, synthetic fuels), and components of clinical applications (diagnostics, drug delivery, implantable devices). It is widely recognized that throughout the ENM life cycle, the greatest risk for human exposure and subsequent health consequences begins with ENM inhalation, and is typically followed by systemic injuries. We have reported that pulmonary and systemic microvascular inflammation [29, 32] follow ENM inhalation exposure. Consistent with this, other systemic morbidities known to follow pulmonary ENM exposures include: inflammation/apoptosis [8, 9], macrovascular and microvascular dysfunction [10], atherogenesis [11], and organ level ischemia [12]. The developing fetus is equally a systemic target of numerous anthropogenic toxicants [13].

The impact of gestational ENM exposures on maternal and fetal health have been increasingly studied in the past decade. Adverse impact of ENM exposures on maternal health [14] and pregnancy [15, 16] have been reported in animal models. Teratogenic and embryo-lethal effects associated with ENM exposure have been shown [17]. The outcomes from several studies also highlight post-natal behavioral deficits [18, 19], cardiovascular [20, 21], renal [15], immune [22], reproductive [23, 24], pulmonary, and metabolic [20] [25] abnormalities.

Epigenetics, or the transient control of genes through DNA methylation or histone modification, is a recent area of intense focus by governmental agencies recognizing mechanistic links between environmental toxicants and gene expression [26]. These adverse maternal and fetal outcomes strongly reflect the potential risk of ENM exposure during pregnancy that may be linked. However, given the inherent physiological dependencies and complexities of developing and maintaining a healthy pregnancy, linking the mechanisms of pulmonary exposure and gestational effects remains very challenging. Given the magnitude of and the complexity these transgenerational effects, the most effective approach may be to initiate studies from the fetal epigenome and/or transcriptome. This is largely because fetal epigenetic outcomes resulting from maternal ENM exposure consequences may be caused by the creation of a hostile gestational environment [27], and/or the direct impact of ENM interacting with the developing embryo [13]. Because either of these possibilities would compromise health, the purpose of these studies was to identify epigenetic changes in cardiac gene expression within the maternally exposed F1 generations. We hypothesized that because maternal ENM inhalation lead to uterine microvascular dysfunction [21], this contributes to a hostile gestational environment, and altered fetal gene expression results. To test this, pregnant dams were intermittently exposed to nano-TiO₂ aerosols during gestational days 5-19, and their litters were studied on GD 20.

Materials and Methods

Animal Model

Sprague Dawley rats were purchased from Hilltop Laboratories (250-275 g female; 300-325 g male). All experiments were approved by the West Virginia University Animal Care and Use Committee and experiments adhered to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (8th Ed.). Rats were provided food and water *ad libitum* and housed in an AAALAC approved animal facility at the West Virginia University Health Sciences Center. Before mating, rats were acclimated for a minimum of 72 hours, as previously described [20]. Pregnancy was verified by identification of the vaginal plug, after which, rats were randomly placed into one of two nano-TiO₂ exposure groups. These two exposure groups were virtually identical and were created to generate a discrete tissue bank for RNA sequencing, or ChIP sequencing.

Engineered Nanomaterial

Nano-TiO₂ P25 powder was purchased from Evonik (Aeroxide TiO₂, Parsippany, NJ), containing anatase (80%) and rutile (20%) TiO₂. Nano-TiO₂ was prepared by drying, sieving, and storing, as previously described [28, 29]. Nano-TiO₂ aerosols were created with our aerosol generator (US Patent No. 8,881,997) [30]. Particle characteristics have been determined including the primary particle size (21 nm), the specific surface area (48.08 m²/g) [29, 31], and the Zeta potential (-56.6 mV) [32].

Nano-TiO₂ Inhalation Exposures

The nano-particle aerosol generator (US Patent No. 8,881,997) and whole-body inhalation exposure system used for the current study have been described extensively in previous studies [29, 31]. This collective exposure system consists of a vibrating fluidized bed, a Venturi vacuum pump, cyclone separator, impactor and mixing device, an animal housing chamber, and real-time monitoring devices with feedback control. Nano-TiO₂ was aerosolized via a high velocity air

stream passing through the vibrating fluidized bed and into the Venturi vacuum pump. The generated aerosols then entered the cyclone separator, which is designed to remove agglomerates >400 nm at an input flow rate of 60 l/min of clean dry air before entering the exposure chamber.

Size distribution, mean aerodynamic diameter, and relative mass concentration of the aerosols were monitored in real time (Electrical Low Pressure Impactor (ELPI), Dekati, Tampere, Finland) while the particle size distribution was also measured in real-time with a Scanning Mobility Particle Sizer device (SMPS; TSI Inc., St. Paul, MN). These measurements were verified throughout a given exposure by collecting nanoparticle samples on filters, and making hourly gravimetric measurements with a microbalance. This approach was also used to collect samples for transmission electron microscopy.

Inhalation exposures were initiated on GD 5.78 ± 0.11 and lasted for 7.79 ± 0.26 days of gestation. Exposure days were not consecutive to decrease animal stress. Once the steady state nano-TiO₂ aerosol concentration was achieved, exposure duration was adjusted to produce a daily calculated lung deposition of 31 ± 1.1 µg per day, and the cumulative, calculated dose was therefore 217 ± 1.0 µg. Lung deposition was calculated based on previously described mouse methodology, and normalized to rat weight and to pregnant rat minute ventilation using the equation: $D = F \cdot V \cdot C \cdot T$, where F is the deposition fraction (14%), V is the minute ventilation based on body weight, C equals the mass concentration (mg/m³), and T equals the exposure duration (minutes) [29, 33]. The target concentration was 10 mg/m³ and the duration was 4-6 hours/exposure (depending on the steady state concentration, as this was used to calculate the lung burden). The last exposure was conducted 24 hours prior to sacrifice and experimentation. Control animals were exposed to HEPA filtered air only.

Chromatin Immunoprecipitation (ChIP) Sequencing

Isolation:

Cardiac tissue was isolated from GD 20 pups in both the nano-TiO₂ exposure and control groups. Each litter was considered an n = 1, with cardiac tissue from 5-6 pups within each litter being pooled together to collect enough tissue (~25 mg). Chromatin Immunoprecipitation (ChIP) was carried out using the MAGnify™ Chromatin Immunoprecipitation System (Thermo Fisher, Rockford, IL) per manufacturer's instructions. Briefly, hearts were homogenized and treated with

37% formaldehyde, which was prepared fresh. Cross-linking was stopped with 1.25 M glycine. Samples were pelleted through centrifugation and washed in D-PBS before sonication. Using a Sonicator Ultrasonic Processor XL2015 (Misonix Sonicator, Farmingdale, NY) chromatin was sheared to a size of 500-700 base pairs, determined using gel electrophoresis (Figure 6.2.1A). Chromatin was then isolated through ultracentrifugation (20,000 g) and diluted to ~60 uL of chromatin per immunoprecipitation reaction. Samples from both the control and nano-TiO₂ cohorts were incubated with histone 3 lysine 4 tri-methylation (H3K4me3, product number: G.532.8, Thermo Fisher, Rockford, IL) or histone 3 lysine 27 tri-methylation (H3K27me3, product number: G.299.10, Thermo Fisher, Rockford, IL) antibody bound beads. These are two of the most prominently studied and classically applied for activation/repression analysis of gene activity. After incubation, samples were treated to reverse cross-linking solution and Proteinase K to remove bound proteins. DNA was then eluted from beads, using heat, and quantified using a Qubit (Thermo Fisher, Rockford, IL). The TruSeq ChIP Library Preparation Kit (Illumina, Inc., San Diego, CA) was implemented to build the libraries.

ChIP Bioinformatics:

Samples were processed using the Illumina MiSeq (Illumina, Inc., San Diego, CA) at the West Virginia University Genomics Core, ran as paired-end reads. Fastq files were assessed for quality using FastQC (Babraham Bioinformatics) (Figure 6.2.1B), where it was determined that partial trimming was needed. Trimming of fastq files was accomplished through Trimmomatic [34] (Figure 6.2.1C). Reads were then mapped to the rat genome (rn6) using the default parameters in bowtie2. To perform differential binding analysis on reads while distinguishing peaks, diffReps was used [35]. Bedtools functions were used to delineate upstream promoter regions of genes (bedtools slop) and evaluate the promoter/gene overlay (bedtools intersect). Genes were defined to include 1000 bases upstream from the start of the gene, indicative of our selected “promoter region.”

RNA Sequencing

Isolation:

Cardiac tissue was procured through the same methods as listed above in the ChIP Sequencing section. RNA was then isolated from heart tissue using the Vantage™ Total RNA Purification Kit (Origene, Rockville, MD) per manufacturer’s instructions. Briefly, tissue was

homogenized and lysis buffer was added to the sample. Sample RNA was spin-column purified and measured for RNA concentration using the Qubit (Thermo Fisher, Rockford, IL). Library preparation was performed using TruSeq RNA Library Prep Kit v2 (Illumina, Inc., San Diego, CA). Quality of RNA was determined using the Agilent 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA); degradation of cytosolic ribosomal RNAs (28S and 18S) are used as a measure of the total RNA Integrity Number (RIN) (Figure 6.2.2A-B).

RNA Bioinformatics:

Samples were processed using the Illumina HiSeq (illumina, Inc., San Diego, CA) at Marshall University. Samples were run as paired-end reads. Paired-end, fastq files were aligned with HISAT2 [36] to the rat genome (rn6) without trimming. Samtools 1.2 [37] was used for the conversion of SAM to BAM format. Counts data was prepared using Subread 1.5.2 [38], specifically featureCounts [39]. Differential expression analysis was accomplished using DESeq2 [40] in R.

Ingenuity Pathway Analysis (IPA)

Protein ontology and pathway analysis were completed through QIAGEN's IPA (www.qiagen.com/ingenuity) software. Core analyses and comparative analyses were run on individual and combined ChIP and RNA data sets, respectively. Z-scores are representative of fold change between groups.

RNA IPA Protein Ontology

The intensity of the color, moving toward blue or red, indicates the degree to which a specific pathway is being decreased or increased, respectively. The change in color, reflective of the z-score, is a quantitative measure of confidence (defined as the cumulative *P*-value of molecules in a specific pathway). This measure of confidence, defined on a color scale, indicates the propensity of all the molecules within that pathway to move in a certain direction, toward either increasing or decreasing the likelihood of developing the listed pathology or condition.

Quantitative PCR

As described above, RNA was isolated from fetal heart tissue. Using the First-strand cDNA Synthesis kit for miRNA (Origene, Rockville, MD, Catalog #: HP100042), per manufacturer's

instructions, RNA was converted to cDNA. The cDNA was used for differential quantification of mRNA transcripts Fibroblast Growth Factor Receptor 1 (Fgfr1), Interleukin-18 (Il-18), and Transforming Growth Factor Beta Receptor 2 (Tgfr2). ChIP-qPCR was used to assess the Tgfr2 promoter loci. As described above, chromatin was immunoprecipitated with H3K4me3. DNA was then probed at multiple locations along the Tgfr2 promoter region in order to construct a histone peak profile. Primer design for both the mRNA and ChIP-qPCR are provided (Table S6.2.4). MRNA was normalized to Beta-Actin (β -Actin), while immunoprecipitated DNA was normalized to its respective input control. Experiments were performed on the Applied Biosystems 7900HT Fast Real-Time PCR system (Applied Biosystems, Foster City, CA), using 2X SYBR Green Master Mix. Quantification was achieved using the $2^{-\Delta\Delta CT}$ method.

Statistics

All measures of significance between the control and maternal nano-TiO₂ exposure groups for the sequencing data are presented as adjusted *P*-values. Adjusted *P*-values are a composition of standard, unadjusted *P*-values and the stringency of the False Discovery Rate (FDR). Differential expression analysis through DESeq2 implements the Wald Test, using multiple testing against the null hypothesis that *P*-values are uniformly distributed across a data set, known as the Benjamini-Hochberg procedure. The FDR for this study was set at 0.05. Z-score significance is determined as greater than the absolute value of 2. The z-score is computed as $z = \frac{x}{\sigma_x} = \frac{\sum_i x_i}{\sqrt{n}} = \frac{N_+ - N_-}{\sqrt{N}}$, where N_+ = the number of molecules following a consistent trend, N_- = the number of molecules following an inconsistent trend, and N = the number of interactions within a given pathway. In this way, the z-score, using only values with a significant change ($P \leq 0.05$) can infer direction of a specific pathway while accounting for relationship and data bias and properly weighting the statistical findings (<https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis/>). A consistency score is the non-statistical assignment of confidence to a specific pathway. Where appropriate, a Student's t-test was used with all data presented as $\pm$ the standard error mean (SEM). Significance is determined as $P \leq 0.05$.

Results

Animal and Nano-TiO₂ Aerosol Characteristics

Animal number, age, body weight, and exposure conditions are provided (Table 6.2.1). Separate, but similar, inhalation exposures were used for the ChIP and RNA sequencing experiments. No statistical differences were noted between nano-TiO₂ exposure in Experimental Group 1 (ChIP Seq) and Experimental Group 2 (RNA Seq). No statistical differences were noted in either progeny weight or total number of pups between maternal nano-TiO₂ exposed or control groups. Representative nano-TiO₂ aerosol characterization data are presented in Figure 6.2.3. The target particle concentration was 10 mg/m³ (Figure 6.2.3A). The real-time nano-TiO₂ mobility diameter was 129 nm (Figure 6.2.3B), and the aerodynamic diameter was 143 nm (Figure 6.2.3C). Nanoparticles were collected on filters, and a representative transmission electron microscopy image is presented in Figure 6.2.3D.

ChIP Sequencing

ChIP Sample Metrics:

To better understand the quality and sample dispersion within our cohort for the ChIP sequencing experiment, a series of statistical models were used. To assess the distribution of subpeaks present within the forward and reverse strands of the H3K4me3 and H3K27me3 immunoprecipitations, the average fragment length was determined for each event using the R package csaw [41]. The cross-correlation graph measures the delay distance, or number of base pairs, which separate distinctive subpeaks, also evaluating the consistency of fragment lengths within the data set (Figure 6.2.4A and 6.2.4B). Multi-dimensional scaling (MDS) plots were used to evaluate individual library homology between both the H3K4me3 and H3K27me3 groups with the R package edgeR [42]. Log fold change (LogFC) determined the differences between libraries (control, red and maternal nano-TiO₂ exposed, blue) within the MDS plots (Figure 6.2.4C and 6.2.4D). To visualize read coverage, the R packages ChIPpeakAnno and Gviz were installed [43]. Complex, differential binding was assessed for both the H3K4me3 (Figure 6.2.4E) and H3K27me3 (Figure 6.2.4F) binding loci. Together, these results suggest that the immunoprecipitation and chromatin fragmentation were successful, and that differential binding is observed between groups.

ChIP IPA Protein Ontology

Differential Binding data for both the H3K4me3 and H3K27me3 marks were uploaded and analyzed in QIAGEN's IPA; all changes are shown as maternal nano-TiO₂ exposed condition relative to the control. Diseases and biological functions ($z\text{-score} \geq 2$) for H3K4me3 and H3K27me3 are provided in Table S6.2.1 and S6.2.2, respectively. Of the diseases and biological functions listed, one of the most prominent pathways for H3K4me3 involved infectious disease (Figure 6.2.5A). The heat map reveals how changes in molecular signaling could provide an increase susceptibility to infection in maternal nano-TiO₂ exposed offspring. The top canonical pathways ($z\text{-score} \geq 2$) altered during maternal nano-TiO₂ exposure are presented (Figure 6.2.5B). In general, the canonical pathways altered after exposure involve regulation of growth and cell cycle/apoptosis signaling.

For H3K27me3, the top 10 canonical pathways which are altered are provided (Figure 6.2.5C). For the promoter regions associated with H3K27me3, the majority of signaling changes involve cancer and immunity. A heat map for the toxicological functions of the data representing H3K4me3 is also presented (Figure 6.2.5D). The size and distribution of each major category is proportional to the $z\text{-score}$, which revealed three major organs affected: the heart, kidney and liver. Toxicological pathways associated with the heart, including congenital heart anomaly, heart failure, cardiac hypertrophy (not shown), and cardiac dysfunction (not shown), were found to be significantly decreased in the maternal nano-TiO₂ exposed group. Conversely, toxicological pathways associated with the liver and kidney including, renal necrosis and cell death, liver necrosis and cell death, renal damage, and liver damage (not shown) were found to be increased. Also, an increase in red blood cells, and subsequently the hematocrit, were observed. Increases in H3K4me3 at promoter regions for infection capacity and growth signaling as well as loci involving kidney and liver dysfunction, suggests epigenetic regulation which could significantly alter an organism's susceptibility to disease and potential pre-disposition to future insult. The lack of changes shown for H3K27me3 may suggest an alternative repressive mark implemented as the bivalent companion of H3K4me3.

RNA Sequencing

RNA Sample Metrics:

The raw and normalized counts from the RNA sequencing experiment were subjected to a variety of statistical modelling, using the DESeq2 package in R [44], in order to better understand

sample parameters. To visualize the variance of the normalized counts data means, the rlog function was used (Figure 6.2.6A). For low-count genes, transformation using rlog, a log₂ scale which normalizes data in reference to the library size, helps to better visualize variance-means. Figure 6.2.6A shows limited outliers within the data set for the control vs. control, but increasing variance in the control vs. maternal nano-TiO₂ exposed. Sample-to-sample distance was measured using the PoiClaClu package in R. Sample dissimilarity is depicted as a heat map (Figure 6.2.6B), calculated from the original, not normalized count data. The heat map shows general dissimilarity between the maternal nano-TiO₂ exposed and control groups, with the exception of one of the control samples. Another measure implemented for determining sample distance was a multi-dimensional scaling (MDS) plot based on the rlog-normalized counts (Figure 6.2.6C). Again, the plot shows a general dissimilarity between the maternal nano-TiO₂ exposed and control cohorts. After performing differential expression analysis with DESeq2, we examined the gene with the lowest associated *P*-value (Figure 6.2.6D). The plot illustrates the similar expression of the gene within each group, while showing the disparities across groups. In Figure 6.2.6E, a MA-plot is used to illustrate the number of genes (red) that fall below the *P*-value of 0.05. The statistical models used to assess the RNA sequencing samples indicate that normalized count values between groups are similar and that sample homology is close within groups, but not across groups.

RNA IPA Protein Ontology:

After differential expression analysis processing in R, data was uploaded and analyzed in QIAGEN's IPA; all changes are shown as maternal nano-TiO₂ exposed condition relative to the control. Diseases and biological functions (*z*-score ≥ 2) for the RNA are provided in Table S6.2.3. Again, a prominent pathway that was found to be increased in the maternal nano-TiO₂ exposed animals involved infectious diseases (Figure 6.2.7A). Both the open promoter conformation (H3K4me₃) and the RNA transcript expression reveal an increased propensity for infection. The top canonical pathways (*z*-score ≥ 3.45) altered during maternal nano-TiO₂ exposure are presented (Figure 6.2.7B). The canonical pathways altered primarily involve inflammatory signaling and organismal development. Examining what factors could be causing differential regulation after maternal nano-TiO₂ exposure, we wanted to evaluate molecular regulator effects. The top molecule (consistency score ≥ 10.453) suggested to play a role in differential regulation of pathways was microRNA-145 (Figure 6.2.7C).

In Figure 6.2.7C, it reveals how decreased expression of microRNA-145 can lead to increased expression of pathways involving cell growth and proliferation. A heat map for the toxicological functions of the data representing the RNA is also shown (Figure 6.2.7D). The size and distribution of each major category is proportional to the z-score and, again consistent with the H3K4me3 mark, three major organs were shown to be affected: the heart, kidney and liver. Toxicological pathways associated with the heart, including congenital heart anomaly, cardiac hypoplasia, heart failure, cardiac fibrosis, and cardiac damage, were found to be significantly decreased in the maternal nano-TiO₂ exposed group. Alternatively, toxicological pathways associated with the liver and kidney including, renal necrosis and cell death, liver hyperplasia/hyperproliferation, renal proliferation, renal damage, and renal autophagy were found to be increased. As reported for the H3K4me3 promoter regions, increased RNA transcription of genes involving red blood production are shown. Similar to the epigenetic modification H3K4me3, the differential expression of transcripts follows a similar pattern of increased infection and growth of the organism, with increased molecular markers of dysfunction in the liver and kidney.

Epigenetic Regulation of Transcription

In order to examine how changes between the H3K4me3 mark and RNA transcript data aligned, we performed a comparative analysis through QIAGEN's IPA, all changes are shown as maternal nano-TiO₂ exposed condition relative to the control. The top canonical pathways (z-score ≥ 4.5) for both the transcript and ChIP data are shown (Figure 6.2.8A). The combined data sets illustrate the common pathways involving both inflammation and organismal growth signaling. For toxicological functions, the molecular profile for cardiac dysfunction is significantly decreased compared to the controls, while kidney dysfunction is increased (Figure 6.2.8B). A heat map for the cumulative diseases and biological functions is shown (Figure 6.2.8C). The heat map depicts two major molecular changes that could impact the phenotype: increased survival and increased susceptibility to infection. In Figure 6.2.8D, canonical pathways are sorted by *P*-value, depicting pathways with large sets of molecules having significantly altered expression levels. Although, the mitochondrial dysfunction and oxidative phosphorylation pathways do not have significant z-scores and a very small contribution of changes coming from the transcript data, Figure 6.2.8B demonstrates the epigenetic changes occurring at these loci to a large segment of genes. Figure 6.2.8E illustrates the NF- κ B (Nuclear Factor kappa-light-chain-enhancer of activated B cells)

signaling pathway for the RNA (right) and H3K4me3 (left) sequencing experiments. The comparative analysis suggests that maternal nano-TiO₂ exposure can cause significant changes to how the development of the progeny takes place, changing the epigenetic landscape, which can directly affect transcript abundance.

Molecular Validation of Sequencing

To further confirm the reliability of the sequencing data, we implemented qPCR to examine molecules involved in the NF-κB Pathway, which are not shown in the illustrative Figures 6.2.8D-E. The mRNA levels of *Fgfr1*, *Il-18*, and *Tgfr2* are reported, and coincide with similar expression profiles seen in the sequencing data (Figure 6.2.9A). In Figure 6.2.9A, the data obtained from RNA sequencing (grey bars) are used as a reference to validate the expression profile of the maternal nano-TiO₂ group when running qPCR. Likewise, we also wanted to use ChIP-qPCR to validate that histone modifications were also reliably reported, with the ChIP-Seq revealing epigenetic changes at the *Tgfr2* promoter region. We confirmed the H3K4me3 histone modifications for *Tgfr2*, showing higher H3K4me3 association at its promoter region (Figure 6.2.9B). The increased magnitude of the histone peak of the maternal nano-TiO₂ group, Figure 6.2.9B, suggests the increased abundance of H3K4me3 and active transcription of the *Tgfr2* gene. *Tgfr2* provides an explicit example of how genes reported to be epigenetically altered (ChIP-Seq, through H3K4me3 localization at the *Tgfr2* promoter region) with subsequent changes in transcription (RNA-Seq, reporting increased expression of *Tgfr2* transcripts) can be further validated using other molecular techniques, such as qPCR. An overview of the experimental design is illustrated in Figure 6.2.9C. Briefly, the figure provides an example of suggested functional outcomes related to maternal nano-TiO₂ exposure, with the link between the exposure paradigm and end function being fetal, epigenetic consequences.

Discussion

The gene expression and epigenetic analyses performed in this study provide the first evidence that maternal ENM inhalation may result in significant pathway alterations in the fetus. The two most prominently impacted mechanisms are: inflammatory signaling, and cardiac-renal-hepatic pathology/toxicity. The nano-TiO₂ exposure paradigm used herein (10 mg/m³, 4-6 hours) resulted in a calculated lung deposition of approximately 217 µg. This lung burden, achieved over 7 days of exposure in the second half of gestation, has been previously shown to impair uterine arteriolar reactivity by almost 50% [25]. To estimate how this lung burden compares to what a human may experience, alveolar surface areas must be known [32]. The rat alveolar surface area is 0.4 m²/lung. Therefore, the rat burden of 217 µg/lung would result in 542.5 µg/m². Given that the human alveolar surface area is 102 m², the equivalent human burden of this exposure paradigm would be 55.3 mg. The next logical question is how long would it take to achieve this burden in humans. In this regard, lung burden may be calculated as:

nano-TiO₂ aerosol concentration · minute ventilation · exposure duration · deposition fraction,
with the following values:

55.3 mg = nano-TiO₂ aerosol concentration · 7600 ml/min · (8 hr/day · 60 min/hr) · 14%,
and therefore:

$$55.3 \text{ mg} = \text{nano-TiO}_2 \text{ aerosol concentration} \cdot 0.51 \text{ m}^3/\text{day}.$$

The National Institute for Occupational Safety and Health (NIOSH) Recommended Exposure Limit (REL), or aerosol concentration for nano-TiO₂ is 0.3 mg/m³ (DHHS, 2011). This would result in a lung burden of 0.15 mg/day. Whereas, the Occupational Safety and Health Administration (OSHA) Permissible Exposure Limit is 5 mg/m³ (DHHS 2011). This would result in a lung burden of 2.55 mg/day. Considering the NIOSH REL and OSHA PEL together, it would require 1.45 working years or 21.7 working days (respectively) for a human to achieve comparable lung burdens with the exposure paradigm used herein. Because the human gestational period is 9 months, we consider our exposure paradigm highly relevant to the worker population.

Contrary to the functional deficits seen in the young adult [20, 25] we found that both the transcriptomic and epigenetic data support increased cardiac function (Figures 6.2.5D and 6.2.7D). Though this seems paradoxical, we suggest that the interplay between the heart, liver, and kidneys is vital in understanding the pathology associated with maternal nano-TiO₂ exposure. It is equally plausible that as hematocrit increases, viscosity of the blood also increases, requiring an elevation

in contractile force or a drop in peripheral resistance. Alternatively, it is possible that disruptions in maternal-fetal perfusion balances occur. The pulmonary exposure of the mother is well described, but the secondary effect(s) on the developing progeny is/are likely to come through impacts on the maternal/fetal circulation. Maternal nutrients are delivered to the placenta via the arterial circuit, if blood flow is inadequate, then fetal compensation must occur to support proper nutrient delivery via the umbilical vein to the fetal portal circulation.

At the fetal stage, the heart plays a less significant role in energetics [46]. Whereas, the liver and kidneys play pivotal roles in blood conditioning at this stage of development, and these signaling pathways are altered by maternal ENM inhalation during gestation (Figure 6.2.5). We hypothesize that potential liver and kidney damage from either inflammation, direct ENM translocation or a combination may result in an increased hematocrit, and or maternal-fetal perfusion balance. Together, this may suggest that in maternal nano-TiO₂ exposed progeny, the functional deficits seen later in development may be a result of this initial hepatic and renal insult, with subsequent cardiac overcompensation which may represent a protective mechanism. These findings correspond to reports of hepatic DNA damage in newborn murine offspring after maternal nano-TiO₂ inhalation [47]. Impairments in renal function may have profound effects on tubuloglomerular feedback, the renin angiotensin system, and/or osmotic regulation. These impairments may collectively or individually directly influence cardiovascular health throughout prenatal and postnatal development.

MicroRNA (miRNA) are well known to be altered by transcriptomic and epigenetic regulators. When expressed, miRNA broadly regulate cellular function [48] and have been implicated in numerous epigenetic pathways [49]. In Figure 6.2.7C transcriptomic data is provided that reflects the most consistently altered regulator after maternal nano-TiO₂ inhalation. Decreased expression of miRNA-145 has been suggested to increase protein synthesis of targets directly involved in signaling events that promote organism growth and development. The role of altered miRNAs in progeny after maternal ENM inhalation is poorly understood, and may provide a better understanding of the relationship between ENM toxicities, epigenetics, and gene expression.

Figure 6.2.8C presents an overview of the two primary cell signaling pathways that are altered during gestational exposure: immunity and development. Parameters of organismal health and development are presented largely as molecular markers for cardiac signaling and function. The increased gene expression of molecular markers associated with infection and immunity may

indicate the likelihood of autoimmune disorders associated with an overactive immune system. This is most evident when considering the inflammatory pathways indicated in Figure 6.2.8A and the target organ (kidney) indicated in Figure 6.2.8B reflected by an increased susceptibility as shown in Figure 6.2.8C. These molecular markers may also represent the consequence presented in Figure 6.2.8A of a proinflammatory environment; such an environment has been associated with chronic conditions including cardiovascular disease and cancer [50]. Pulmonary exposure to carbon black nanoparticles has also been identified to contribute to the development of immunotoxicity, particularly in lymphoid organs [22]. Interestingly, organismal death and morbidity/mortality appears to be decreased in maternal nano-TiO₂ offspring, which may again seem counterintuitive. However, we speculate this may reflect a greater systemic response to compensate for the numerous other mechanisms disturbed by ENM inhalation during gestation.

To better identify the future consequences of ENM exposure, the significance of the pathways was represented as the change in *P*-value (Figure 6.2.8D). Mitochondrial dysfunction and oxidative phosphorylation appeared to have the greatest changes in methylation, indicating that future complications in these pathways may occur. Given their widespread involvement, this epigenetic predisposition may manifest in any tissue. In other words, the epigenetic changes associated with energetics may reflect significant alterations that occur during fetal development. It is important to indicate that these changes may not be manifested in functional transcriptomic or proteomic changes until postnatal development or even later into adulthood. If correct, this would be consistent with the Barker Hypothesis and DOHaD.

Maternal nano-TiO₂ exposure is also associated with a pronounced effect on key inflammatory pathways in the exposed progeny. In Figure 6.2.8E, protein kinase B (AKT) signaling is decreased, potentially resulting in an impairment in calcium-independent nitric oxide signaling which would likely result in dysfunctional endothelium-dependent responses. Indeed, calcium dependent and independent mechanisms, as well as endothelial arteriolar dilation are significantly impaired at 3-4 weeks of age [51]. Furthermore, augmented NF- κ B signaling via both alternate and canonical pathways [52] has been reported. Maternal nano-TiO₂ exposure significantly activated the expression of the Lymphotoxin Beta Receptor (LTBR) gene, while suppressing the expression of the regulating enzyme Inhibitor of NF- κ B Kinase Subunit Alpha (IKK α) Figure 6.2.8E. This is important in the negative feedback of the NF- κ B canonical signaling that limits inflammatory gene activation and suggests that more robust inflammatory responses are

possible as evidenced in Figure 6.2.8A. Furthermore, NF- κ B plays a central role in the development of inflammation through further regulation of genes encoding not only pro-inflammatory cytokines, but also adhesion molecules such as E-selectin, VCAM-1 (vascular cell adhesion molecule-1) and ICAM-1 (intercellular adhesion molecule-1), chemokines, and inducible nitric oxide synthase (iNOS) [53, 54]. Figure 6.2.8E also reflects a significant increase in interleukin-8 (IL-8) signaling, a major chemokine associated with neutrophil chemotaxis and degranulation secreted by macrophages and endothelial cells during acute inflammatory responses [55]. Considered jointly, uncontrolled activation of NF- κ B and IL-8 pathways in maternally exposed progeny may predispose towards endothelial-dependent dysfunction and leukocyte adhesion.

Conclusions

The pathway analyses reported herein indicate dysfunction in many physiologic systems. As it is not possible to functionally verify each of these functional implications, the primary goal of the manuscript is to identify those systems as a priority for future study. Systemic impairments associated with acute and chronic nanomaterial exposures is an evolving field as nanotechnology continues to expand. Maternal and fetal outcomes following gestational exposures have recently been considered. While initial functional microvascular assessments have begun, little is known regarding epigenetic alterations within the F1 generation. The findings from this study describe epigenetic changes in the progeny of mothers exposed to nano-TiO₂ aerosols during gestation. The evidence of the study is strengthened by the use of two separate cohorts to separately probe the transcriptomic and epigenetic alterations, suggesting that even in separate discrete experimental populations, changes to the epigenome and RNA transcript levels align and similar exposure paradigms yield consistent results. Changes in the RNA transcripts and histone modifications on DNA suggest that maternal nano-TiO₂ progeny exhibit a propensity toward hepatic and renal disease, increased inflammatory signaling, and growth/survival while showing decreased cardiac dysfunction. What remains to be understood is if and/or how far these epigenetic changes persistent into adulthood, the dose-response relationships, and what stage of development is most sensitive to maternal ENM exposure.

Declarations

Ethics approval and consent to participate: not applicable.

Consent for publication: the authors consent to publication.

Availability of data and material: The datasets supporting the conclusions of this article are included within the article and its Supplemental file.

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Table 6.2.1

Exposure Group	Technique	Number of Animals (N)	Age (days)	Body Weight (grams)	Litter Size	Pup Weight (grams)	Aerosol Concentration (mg/m ³)	Electrical Low-Pressure Impactor (nm)	Scanning Particle Mobility Sizer (nm)
Sham Control	RNA Sequencing	7	109 ± 7	402 ± 8.84	13 ± 2	4.06 ± 0.16	0	0	0
Nano-TiO ₂	RNA Sequencing	4	113 ± 2	422 ± 13.34	14 ± 1	3.99 ± 0.22	10.35 ± 0.13	136.80 ± 1.44	134.80 ± 1.24
Sham Control	ChIP Sequencing	5	104 ± 2	407 ± 8.09	12 ± 2	5.19 ± 1.02	0	0	0
Nano-TiO ₂	ChIP Sequencing	6	98 ± 1	376 ± 19.99	9 ± 5	4.88 ± 1.53	10.5 ± 0.5	143.75 ± 2.32	129.43 ± 3.21

Figure 6.2.1

Figure 6.2.1

Figure 6.2.1: Evaluating DNA fragmentation and read quality for chromatin immunoprecipitation (ChIP) sequencing. (A) Using gel electrophoresis, DNA fragments were evaluated to determine size and distribution (average size of fragments = 654.3 bp). Two controls and two maternal nano-TiO₂ exposed representative samples are shown. Sample quality was assessed using FastQC for both forward and reverse reads (B) before and (C) after using Trimmomatic. Con = control, Exp = maternal nano-TiO₂ exposed, H3K4me3 and K4 = histone 3 lysine 4 tri-methylation, K27 = histone 3 lysine 27 tri-methylation.

Figure 6.2.2

Figure 6.2.2

Figure 6.2.2: Assessing RNA quality for transcriptomic data. (A) Gel electrophoresis was implemented to visualize 28S and 18S ribosomal RNA quality. (B) Cytoplasmic, ribosomal RNA degradation was measured using the Agilent Bioanalyzer 2100. As determined by the RNA Integrity Number (RIN) (left of sample name) the five least degraded samples were chosen for the control ($\text{RIN} = 5.88 \pm 1.22$) and exposed ($\text{RIN} = 6.18 \pm 0.92$) groups. Exposed = maternal nano-TiO₂ exposed.

Figure 6.2.3

Figure 6.2.3: Maternal nano-TiO₂ exposure particle characterization for RNA sequencing experiments. (A) Total aerosol concentration (10 mg/m³) of engineered nano-TiO₂ during maternal exposures. (B) Nano-TiO₂ size distribution (mobility diameter, 129.4 nm) using a scanning mobility particle sizer (SMPS). (C) Nano-TiO₂ size distribution (aerodynamic diameter, 143.3 nm) using an electrical low-pressure impactor (ELPI). (D) Transmission electron microscopy image of aerosolized nano-TiO₂ collected via a sampling filter during an exposure.

Figure 6.2.4

Figure 6.2.4

Figure 6.2.4: Chromatin immunoprecipitation (ChIP) sequencing fragment analysis and sample distribution. To measure the distance between subpeaks and find the maximum correlation, the cross-correlation function (CCF) was used to assess (A) H3K4me3 (248 bp) and (B) H3K27me3 (247 bp). Multi-dimensional scaling (MDS) plots indicate the log fold change (logFC) between samples within the (C) H3K4me3 and (D) H3K27me3 groups, describing sample-to-sample distances. Representative histone peaks are shown for differential binding regions ($P \leq 0.05$) for both (E) H3K4me3 and (F) H3K27me3. Con = control, Exp = maternal nano-TiO₂ exposed, H3K4me3 = histone 3 lysine 4 tri-methylation, H3K27me3 = histone 3 lysine 27 tri-methylation, Wnt5a = Wnt Family Member 5A, Rn5-8s = 5.8S ribosomal RNA for *Rattus norvegicus*.

Figure 6.2.5

Figure 6.2.5

Figure 6.2.5

D

Figure 6.2.5: Assessment of disease and signaling pathways altered epigenetically during maternal nano-TiO₂ exposure. (A) One of the primary disease pathways (z-score = 9.35 ± 1.89) altered epigenetically during exposure was the increased susceptibility to infection in the H3K4me3 group. Disease and toxicological pathways are constructed from specific, individual canonical signaling pathways. (B) Depicts the top canonical pathways for H3K4me3 (z-score $\geq \pm 2.0$) that are significantly ($P \leq 0.05$) impacted, as indicated by the threshold line. (C) The top canonical pathways for H3K27me3 ($P \leq 0.05$) are also shown following exposure (smaller P -values are associated with increasing red intensity for pathways). (D) Toxicological functions predicted for genes mapped to H3K4me3 marks.

Figure 6.2.6

Figure 6.2.6

Figure 6.2.6: Sample-to-sample distribution and differential expression analysis for transcriptomic analysis. (A) Assessment of normalized counts between control vs. control (left) and control vs. maternal nano-TiO₂ exposed (right) using a log₂ transformed scale. (B) Measure of raw count matrices and (C) normalized count matrices to determine variance between samples. (D) The top differentially regulated gene between groups was determined through the normalized counts for each sample. (E) The MA-plot reveals the differentially expressed genes (red, $P \leq 0.05$) in comparison to genes with non-significant change between groups (grey). The top differentially regulated gene is highlighted (blue). Exposed and Exp = maternal nano-TiO₂ exposed, Car1 = carbonic anhydrase 1.

Figure 6.2.7

Figure 6.2.7

Figure 6.2.7: Assessment of disease and signaling pathways altered transcriptionally during maternal nano-TiO₂ exposure. (A) Similar to the activation by H3K4me3, transcriptional upregulation of genes associated with increased susceptibility to infection (z-score = 2.02 ± 0.96) was found. (B) The top canonical pathways (z-score $\geq \pm 3.45$) that are significantly ($P \leq 0.05$) impacted transcriptionally, as indicated by the threshold line. The canonical pathways for the RNA sequencing reveal a significant increase in inflammatory and growth signaling. (C) The top regulator (consistency score = 10.453) determined through pathway analysis of gene expression (arrows = activation, bars = repression). Increasing gene activation (red) and suppression (blue) reveal targeting of multiple cell functions. (D) Toxicological functions predicted for transcript abundance in the RNA sequencing experiment.

Figure 6.2.8

Figure 6.2.8

Figure 6.2.8: Comparison of epigenetic regulation (H3K4me3 and H3K27me3) and transcriptional changes. (A) Top canonical pathways, ranked by z-score, which are changed between groups. (B) Top toxicological functions, ranked by z-score, which are changed between groups. (C) Top diseases and biological functions, ranked by z-score, which are changed between groups. (D) Top canonical pathways, ranked by cumulative *P*-value, which are changed between groups. (E) Example of one of the top canonical pathways altered during maternal nano-TiO₂ exposure. NF-κB signaling changes transcriptionally (right) and epigenetically through H3K4me3 (left) (green = decreased expression, red = increased expression). NF-κB= nuclear factor kappa-light-chain-enhancer of activated B cells.

Figure 6.2.9

Figure 6.2.9: Validation of sequencing and model overview. (A) The mRNA of Fgfr1, Il-18, and Tgfbr2 were assessed in the sham (green, Sham-Control) and maternal nano-TiO₂ (red, Nano-TiO₂ Exposed) exposed progeny, reference to the RNA sequencing observed change (grey, Sequence). Expression was normalized to the β -Actin reporter gene. (B) Tgfbr2 was further characterized through ChIP-qPCR of H3K4me3 to measure the binding affinity of the modified histone at the Tgfbr2 promoter loci in the Sham-Control (green) and maternal nano-TiO₂ (red) exposed progeny. Values were normalized to each sample's input control. Tick marks represent the chromosomal location of each qPCR measurement, ranging from 124,318,034 to 124,319,434 on chromosome 8. (C) Schematic overview of the experimental model for nano-TiO₂ maternal exposure and examination of the fetal progeny. As an example, the changes in Tgfbr2 are used to illustrate how epigenetic alterations through modification of chromatin can lead to increased expression of the mRNA transcript. Finally, the results of the study suggest that the gestational exposure paradigm impacts the heart, through increased function, while the liver and kidney have a detriment in function. Values are expressed as means $\pm$ SE. * = $P \leq 0.05$. Fgfr1 = Fibroblast Growth Factor Receptor 1, Il-18 = Interleukin-18, Tgfbr2 = Transforming Growth Factor Beta Receptor 2, H3K4me3 = histone 3 lysine 4 tri-methylation, ChIP = Chromatin Immunoprecipitation.

Table S6.2.1

H3K4me3 Disease and Biological Functions List			
Diseases or Functions Annotation	<i>P</i>-Value	Predicted Activation State	Activation z-score
infection of cells	0.000000955	Increased	11.372
infection by RNA virus	0.00000358	Increased	11.184
Viral Infection	0.00334	Increased	11.109
HIV infection	0.000494	Increased	10.705
infection by HIV-1	0.0000125	Increased	10.623
infection by lentivirus	0.000392	Increased	10.613
cell viability	0.0094	Increased	9.722
infection of tumor cell lines	0.00000277	Increased	9.561
infection of cervical cancer cell lines	0.0000142	Increased	9.312
cell viability of tumor cell lines	0.0000388	Increased	8.467
infection of kidney cell lines	0.000339	Increased	7.151
infection of embryonic cell lines	0.00018	Increased	7.151
infection of epithelial cell lines	0.00018	Increased	7.151
productive infection of cervical cancer cell lines	0.00461	Increased	6.321
cell proliferation of breast cancer cell lines	0.0000852	Increased	5.014
cell transformation	0.00024	Increased	5.01
cell proliferation of tumor cell lines	0.00425	Increased	4.34
homologous recombination of cells	0.00141	Increased	4.252
cell proliferation of fibroblasts	0.00419	Increased	4.061
cell viability of colorectal cancer cell lines	0.00106	Increased	4.03

cytokinesis	0.00193	Increased	3.855
repair of DNA	8.61E-09	Increased	3.853
metabolism of protein	0.000352	Increased	3.587
Clathrin mediated endocytosis	0.00762	Increased	3.57
development of cytoplasm	0.00782	Increased	3.567
transformation of fibroblast cell lines	0.0133	Increased	3.548
cell viability of myeloma cell lines	0.00392	Increased	3.529
recombination	0.0101	Increased	3.475
cell death of kidney cell lines	0.000989	Increased	3.289
formation of cytoskeleton	0.0134	Increased	3.216
apoptosis of kidney cell lines	0.000769	Increased	3.21
cell viability of breast cancer cell lines	0.00257	Increased	3.181
cytokinesis of tumor cell lines	0.000305	Increased	3.148
initiation of transcription	0.0107	Increased	3.123
excision repair	0.00117	Increased	3.106
proliferation of connective tissue cells	0.00546	Increased	3.106
interphase of fibroblasts	0.00457	Increased	3.073
interphase of connective tissue cells	0.0121	Increased	3.066
cell death of epithelial cell lines	0.00656	Increased	2.992
cell death of embryonic cell lines	0.00184	Increased	2.99
closure of embryonic tissue	0.00665	Increased	2.97
initiation of transcription of RNA	0.0038	Increased	2.964
apoptosis of embryonic cell lines	0.00178	Increased	2.919
apoptosis of epithelial cell lines	0.011	Increased	2.87
M phase	0.000365	Increased	2.855
growth of connective tissue	0.013	Increased	2.852
cytokinesis of cervical cancer cell lines	0.000366	Increased	2.813

expression of RNA	0.00861	Increased	2.801
transport of protein	0.000134	Increased	2.743
entry into interphase	0.00904	Increased	2.704
S phase	0.00427	Increased	2.686
cell death of kidney cells	0.00148	Increased	2.681
association of chromatin	0.00108	Increased	2.606
association of chromosome components	0.0071	Increased	2.606
modification of reactive oxygen species	0.00717	Increased	2.502
proliferation of fibroblast cell lines	0.000267	Increased	2.465
repair of cells	0.00125	Increased	2.403
necrosis of kidney	0.00153	Increased	2.377
initiation of expression of RNA	0.00283	Increased	2.376
apoptosis of nervous tissue cell lines	0.00718	Increased	2.359
M phase of cervical cancer cell lines	0.00107	Increased	2.345
cell cycle progression	0.000099	Increased	2.268
growth of yeast	0.00836	Increased	2.266
cell cycle progression of tumor cell lines	0.0105	Increased	2.25
checkpoint control	0.000101	Increased	2.25
infection of rhabdomyosarcoma cell lines	0.000352	Increased	2.236
modification of hydrogen peroxide	0.00406	Increased	2.232
catabolism of protein	0.00263	Increased	2.231
decay of RNA	0.000808	Increased	2.213
closure of neural tube	0.00866	Increased	2.213
maintenance of cells	0.00741	Increased	2.209
processing of rRNA	0.0068	Increased	2.176
ubiquitination	0.0000226	Increased	2.15

molecular cleavage of DNA	0.00265	Increased	2.114
condensation of chromatin	0.0106	Increased	2.038
cell viability of melanoma cell lines	0.00287	Increased	2.034
beta-oxidation of lipid	0.014	Increased	2.028
cell death of double-positive thymocyte	0.00535	Increased	2
hippocampal learning	0.011	Increased	2

Diseases or Functions Annotation	p-Value	Predicted Activation State	Activation z-score
organismal death	0.000074	Decreased	-20.188
morbidity or mortality	0.000068	Decreased	-19.932
Growth Failure	0.00277	Decreased	-11.033
incidence of malignant tumor	0.00892	Decreased	-6.336
cell death	0.0000327	Decreased	-6.293
cell death of osteosarcoma cells	0.000000464	Decreased	-6
Movement Disorders	0.00954	Decreased	-5.805
midline defect	0.00194	Decreased	-5.446
congenital anomaly of cardiovascular system	0.00365	Decreased	-5.027
congenital malformation of brain	0.00639	Decreased	-4.978
congenital anomaly of central nervous system	0.00731	Decreased	-4.978
congenital heart disease	0.012	Decreased	-4.875
incidence of lymphoma	0.014	Decreased	-4.829
neural tube defect	0.00998	Decreased	-4.788
kyphosis	0.00517	Decreased	-4.737
apoptosis	0.000114	Decreased	-4.721
exencephaly	0.00568	Decreased	-4.577

ventricular septal defect	0.000324	Decreased	-4.231
incidence of lung tumor	0.0143	Decreased	-4.042
necrosis	0.0069	Decreased	-3.81
non-melanoma solid tumor	0.00000221	Decreased	-3.115
familial cardiovascular disease	0.0142	Decreased	-3.087
damage of chromosomes	0.00129	Decreased	-2.874
neoplasia of epithelial tissue	0.00000394	Decreased	-2.827
perimembranous ventricular septal defect	0.00492	Decreased	-2.813
cancer	1.42E-08	Decreased	-2.794
Gastrointestinal Tract Cancer and Tumors	0.0101	Decreased	-2.745
malignant solid tumor	0.000000204	Decreased	-2.697
breakage of chromosomes	0.00146	Decreased	-2.65
cell death of cancer cells	0.0000006	Decreased	-2.631
necrosis of malignant tumor	0.000000676	Decreased	-2.631
DNA damage response of cells	0.0000451	Decreased	-2.54
tumorigenesis of tissue	0.00000215	Decreased	-2.507
quantity of hydrogen peroxide	0.00615	Decreased	-2.483
digestive organ tumor	0.0000111	Decreased	-2.482
adenocarcinoma	0.000662	Decreased	-2.448
liver carcinoma	0.00825	Decreased	-2.34
hepatocellular carcinoma	0.00872	Decreased	-2.34
epithelial cancer	0.0000026	Decreased	-2.255
entry into interphase of cervical cancer cell lines	0.00502	Decreased	-2.236
damage of protein	0.00000992	Decreased	-2.219

abdominal neoplasm	1.76E-08	Decreased	-2.204
cell death of tumor cells	0.000000374	Decreased	-2.195
necrosis of tumor	0.000000616	Decreased	-2.195
DNA damage response of tumor cell lines	0.00247	Decreased	-2.137
digestive system cancer	0.0000244	Decreased	-2.084
hepatobiliary system cancer	0.0141	Decreased	-2.056
necrosis of brain cancer cell lines	0.00298	Decreased	-2.042

Table S6.2.1: Listing of all disease and biological pathways with a positive or negative z-score greater than the absolute value of 2 for H3K4me3.

Table S6.2.2

H3K27me3 Disease and Biological Functions List

Diseases or Functions Annotation	<i>P</i>-Value	Predicted Activation State	Activation z-score
transcription of DNA	8.38E-08	Increased	2.656
activation of DNA endogenous promoter	6.94E-09	Increased	2.572
transcription of RNA	0.0000023	Increased	2.483
Diseases or Functions Annotation	<i>P</i>-Value	Predicted Activation State	Activation z-score
organismal death	0.000264	Decreased	-4.076
morbidity or mortality	0.000106	Decreased	-3.87
Growth Failure	0.0000695	Decreased	-3.132
weight loss	0.00158	Decreased	-2.236

Table S6.2.2: Listing of all disease and biological pathways with a positive or negative z-score greater than the absolute value of 2 for H3K27me3.

Table S6.2.3**RNA Transcript Disease and Biological Functions List**

Diseases or Functions Annotation	P-Value	Predicted Activation State	Activation z-score
size of body	0.000000139	Increased	7.093
migration of tumor cell lines	5.74E-08	Increased	4.915
organization of cytoplasm	6.32E-08	Increased	4.865
organization of cytoskeleton	0.0000006	Increased	4.865
microtubule dynamics	0.00000924	Increased	4.513
cell movement of tumor cell lines	7.56E-08	Increased	4.504
cell death of osteosarcoma cells	1.99E-09	Increased	4.32
colony formation of cells	0.0000099	Increased	3.903
development of body trunk	1.01E-10	Increased	3.896
cell proliferation of tumor cell lines	2.08E-10	Increased	3.77
cell movement	9.27E-15	Increased	3.71
quantity of hematopoietic progenitor cells	0.000000765	Increased	3.694
quantity of cells	5.04E-08	Increased	3.634
migration of cells	8E-14	Increased	3.563
cell viability	7.15E-12	Increased	3.526
cell survival	1.63E-12	Increased	3.513
outgrowth of cells	0.000041	Increased	3.435
colony formation	0.00000244	Increased	3.394
cancer	1.7E-41	Increased	3.346
size of animal	1.71E-08	Increased	3.342

branching of cells	0.0000051	Increased	3.193
quantity of erythroid precursor cells	0.0000343	Increased	3.146
migration of endothelial cells	2.65E-09	Increased	3.103
cell movement of endothelial cells	1.27E-09	Increased	3
cell viability of tumor cell lines	0.000000024	Increased	2.975
quantity of heavy metal	0.000000869	Increased	2.954
synthesis of reactive oxygen species	0.0000329	Increased	2.939
transport of molecule	0.0000013	Increased	2.898
growth of neurites	0.0000144	Increased	2.89
cell proliferation of carcinoma cell lines	0.0000533	Increased	2.873
quantity of blood cells	5.96E-10	Increased	2.837
infection by HIV-1	0.0000122	Increased	2.82
cell transformation	2.87E-08	Increased	2.819
HIV infection	0.0000097	Increased	2.819
hydrolysis of nucleotide	0.0000349	Increased	2.778
activation of DNA endogenous promoter	1.13E-14	Increased	2.776
development of head	0.0000181	Increased	2.76
movement of vascular endothelial cells	0.00000163	Increased	2.749
infection of cells	0.00000573	Increased	2.74
migration of vascular endothelial cells	0.00000714	Increased	2.699
transcription of RNA	6.69E-19	Increased	2.678
transport of metal	0.0000371	Increased	2.662

infection by RNA virus	0.00000199	Increased	2.626
invasion of cells	0.000012	Increased	2.624
sprouting	3.93E-08	Increased	2.568
transcription	5.36E-17	Increased	2.559
metabolism of reactive oxygen species	0.00000685	Increased	2.524
respiratory system development	0.000000988	Increased	2.523
formation of lung	0.0000032	Increased	2.514
infection of cervical cancer cell lines	0.00000589	Increased	2.505
transport of heavy metal	9.84E-09	Increased	2.491
development of body axis	0.0000275	Increased	2.474
differentiation of embryonic cells	8.82E-08	Increased	2.468
advanced malignant tumor	0.0000156	Increased	2.459
Viral Infection	1.81E-13	Increased	2.455
metastasis	0.0000307	Increased	2.369
necrosis of malignant tumor	2.54E-10	Increased	2.307
cell death of cancer cells	7.85E-10	Increased	2.307
quantity of connective tissue	0.00000002	Increased	2.265
quantity of red blood cells	8.08E-08	Increased	2.198
transcription of DNA	7.81E-18	Increased	2.184
development of vasculature	1.79E-18	Increased	2.18
thrombocytosis	0.0000028	Increased	2.178
angiogenesis	1.06E-16	Increased	2.164
spherocytosis	3.06E-08	Increased	2.159

proliferation of connective tissue cells	4.28E-11	Increased	2.136
necrosis of tumor	9.88E-11	Increased	2.121
cell death of tumor cells	6.86E-10	Increased	2.121
growth of connective tissue	2.76E-10	Increased	2.118
cell proliferation of fibroblasts	4.96E-11	Increased	2.116
expression of RNA	1.9E-16	Increased	2.085
transactivation	2.22E-08	Increased	2.016
transactivation of RNA	5.26E-08	Increased	2.002

Diseases or Functions Annotation	P-Value	Predicted Activation State	Activation z-score
organismal death	5.59E-27	Decreased	-10.051
morbidity or mortality	4.69E-27	Decreased	-9.991
perinatal death	0.000000536	Decreased	-6.598
Bleeding	1.41E-09	Decreased	-5.689
neonatal death	0.00000164	Decreased	-5.583
Growth Failure	0.00000029	Decreased	-5.542
Edema	0.0000237	Decreased	-5.48
motor dysfunction or movement disorder	0.000000629	Decreased	-4.374
Movement Disorders	0.000000347	Decreased	-3.935
death of perinatal stage organism	0.0000385	Decreased	-3.328
familial vascular disease	0.0000265	Decreased	-2.789
hypoalbuminemia	0.0000374	Decreased	-2.449
adhesion of cell-associated matrix	0.0000211	Decreased	-2.414

senescence of cells	0.000000606	Decreased	-2.314
adhesion of extracellular matrix	0.0000059	Decreased	-2.17
congenital anomaly of central nervous system	0.0000163	Decreased	-2.144
congenital encephalopathy	0.0000293	Decreased	-2.144
congenital malformation of brain	0.0000402	Decreased	-2.144
intestinal cancer	3.74E-29	Decreased	-2.066
familial cardiovascular disease	0.0000235	Decreased	-2.035
apoptosis	7.86E-19	Decreased	-2.012
diffuse lymphoma	0.00000118	Decreased	-2

Table S6.2.3: Listing of all disease and biological pathways with a positive or negative z-score greater than the absolute value of 2 for RNA.

Table S6.2.4

Primer Sequences			
Experiment	Primer Name	Orientation	Sequence
mRNA-qPCR	Fgfr1	F	GAAAGCAACCGTACACGCA
	Fgfr1	R	CGGTTTGGTTTGGTGTGTCT
	Il-18	F	TCAGACCACTTTGGCAGACTT
	Il-18	R	CACAGGCGGGTTTCTTTTGTC
	Tgfr2	F	ATCCTAGTAAGAAGCGATCTAACC
	Tgfr2	R	GTCTGCTTGAAGGACTCCATGT
ChIP-qPCR	Tgfr2 8043		GTGTTTCACCCCAAATCCAC
	Tgfr2 8043		ATCACTGGCTTTCCATGACC
	Tgfr2 8243	F	GGACTGTCCTGCTCTTTTGC
	Tgfr2 8243	R	CGGAAACGGGAAGTTTGTAG
	Tgfr2 8443	F	AGCGAGGAGCCCTAGTGAAC
	Tgfr2 8443	R	GTTCCCAAGTCGGGTGAGT
	Tgfr2 8643	F	CCTTTATTCCTCGCCCTCTC
	Tgfr2 8643	R	CTCCTGCGCAGCTCTCCT
	Tgfr2 8843	F	GGGAGGGAAACAGGAAACTC
	Tgfr2 8843	R	AGGAGAGGACCGGAGCTG
	Tgfr2 9043	F	GGATTGGACCTTGAGGGACT
	Tgfr2 9043	R	CGTGCGCTTAGTCACTTCTG
	Tgfr2 9243	F	CAGGAGGGAGCTCTGAAATG
	Tgfr2 9243	R	CGCATGCATGTCCATAAAAC
	Tgfr2 9443	F	GGATTGGAAGGACCATTTC
	Tgfr2 9443	R	CGATCACAGGGCCAACACTATT

Table S6.2.4: Primer design for qPCR experiments. Primers for the mRNA-qPCR experiments were designed using Primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). mRNA primers were designed to produced amplicons 100 – 250 bp in length. ChIP-qPCR primers were designed spanning a 1,400 bp loci in the promoter region of the *Tgfr2* gene. Primers were designed to measure chromatin H3K4me3 modifications every 200 bp. Primer sequences were constrained to 60-100 bp amplicon lengths, in order to appropriately span sheered chromatin. All primer designs were performed against the *Rattus norvegicus* July 2014 (RGSC 6.0/rn6) genome build. ChIP = Chromatin Immunoprecipitation.

List of Abbreviations

ENM	Engineered Nanomaterials
ChIP	Chromatin Immunoprecipitation
GD	Gestational Day
H3K4me3	3 lysine 4 tri-methylation
H3K27me3	3 lysine 27 tri-methylation
IPA	Ingenuity Pathway Analysis
DOHad	Developmental Origin of Health and Disease
LogFC	Log Fold Change
IACUC	Institutional Animal Care and Use Committee
RIN	RNA Integrity Number
FDR	False Discovery Rate
NF- κ B	Nuclear Factor kappa-light-chain-enhancer of activated B cells
AKT	Protein Kinase B
VCAM-1	Vascular Cell Adhesion Molecule-1
ICAM-1	Intercellular Adhesion Molecule-1
LTBR	Lymphotoxin Beta Receptor
IKK α	Inhibitor of NF- κ B Kinase Subunit Alpha
IL-8	Interleukin 8
NOS	Nitric Oxide Synthase
Fgfr1	Fibroblast Growth Factor Receptor 1
Il-18	Interleukin-18
Tgfr2	Transforming Growth Factor Beta Receptor 2

Car1	Carbonic anhydrase 1
qPCR	quantitative Polymerase Chain Reaction
REL	Recommended Exposure Limit
PEL	Permissible Exposure Limit
Wnt5a	Wnt Family Member 5A
Rn5-8s	5.8S ribosomal RNA for <i>Rattus norvegicus</i>

Section 2: Supporting Documentation for Specific Aim 2

Repository for Cardiovascular Studies

Primary design and execution of IRB Surgical Tissue Banking protocol # 1812394926A001
(Figure 6.3.1)

Figure 6.3.1

Figure 6.3.1: Samples collected include right atrial appendages, mitral valve tissue, ascending aorta, and other tissues routinely discarded during open heart and/or valvular surgeries. Blood (10 mL preoperatively and postoperatively from standard of care venipuncture), noninvasive cardiovascular imaging data, and/or other relevant medical records.

Section 3: Supporting Documentation for Specific Aim 3

Transgenic Animal Models

Because we are interested in better understanding the *in vivo* consequences of PNPase manipulation to alterations in mitochondrial function and regulatory networks, we are begun the production of a transgenic murine line. Through a single-stranded ssDNA donor CRISPR/Cas9 approach (3, 4) we have been successful in targeting both the upstream (Figure 6.4.1A) and downstream (Figure 6.4.1B) sgRNA region in the introns surrounding Exon 23 of PNPT1. The single-stranded DNA oligonucleotide (ssDNA) (Figure 6.4.2) was created through Genewiz (South Plainfield, NJ) and the gRNA were designed through CHOPCHOP (1, 2) (Figure 6.4.3). Currently, we have found animals heterozygous for the homology direct repair ssDNA insert (Figure 6.4.4). Embryo microinjections were performed by the Genetically Engineered Murine Model (GEMM) Core at The University of Virginia. After the validation of the KH knockout, we plan to continue with the design and development of the S1 knockout mouse model.

CRISPR/Cas9 Cell Models

Using a CRISPR/Cas9 approach, we have designed gRNA (Figure 6.3.5), using CHOPCHOP, to be incorporated into plasmids purchased from Addgene (Watertown, MA) (pSpCas9(BB)-2A-GFP (PX458) was a gift from Feng Zhang (Addgene plasmid # 48138 ; <http://n2t.net/addgene:48138> ; RRID:Addgene_48138), pSpCas9(BB)-2A-Puro (PX459) V2.0 was a gift from Feng Zhang (Addgene plasmid # 62988 ; <http://n2t.net/addgene:62988> ; RRID:Addgene_62988)). The sgRNA was cloned using the guidelines from Ran et al. (5).

References

1. **Labun K, Guo X, Chavez A, Church G, Gagnon JA, and Valen E.** Accurate analysis of genuine CRISPR editing events with ampliCan. *Genome Res*, 2019.
2. **Labun K, Montague TG, Gagnon JA, Thyme SB, and Valen E.** CHOPCHOP v2: a web tool for the next generation of CRISPR genome engineering. *Nucleic Acids Res* 44: W272-276, 2016.
3. **Miura H, Quadros RM, Gurumurthy CB, and Ohtsuka M.** Easi-CRISPR for creating knock-in and conditional knockout mouse models using long ssDNA donors. *Nat Protoc* 13: 195-215, 2018.
4. **Quadros RM, Miura H, Harms DW, Akatsuka H, Sato T, Aida T, Redder R, Richardson GP, Inagaki Y, Sakai D, Buckley SM, Seshacharyulu P, Batra SK, Behlke MA, Zeiner SA, Jacobi AM, Izu Y, Thoreson WB, Urness LD, Mansour SL, Ohtsuka M, and Gurumurthy CB.** Easi-CRISPR: a robust method for one-step generation of mice carrying conditional and insertion alleles using long ssDNA donors and CRISPR ribonucleoproteins. *Genome Biol* 18: 92, 2017.
5. **Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, and Zhang F.** Genome engineering using the CRISPR-Cas9 system. *Nat Protoc* 8: 2281-2308, 2013.

Figure 6.4.1

Figure 6.4.1: Sanger sequencing displaying regions surrounding PNPT1 exon 23 where the sgRNA were designed to target. (A) The upstream site (intron 22) showing both the wild type (top) and targeted (bottom) sites. (B) The upstream site (intron 23) showing both the wild type (top) and targeted (bottom) sites. Mutations, due to non-homologous end-joining, were produced in the resulting DNA strand. KH KO = KH RNA binding domain (exon 23, GxxG motif) removed from full length PNPase.

Figure 6.4.2

Pnpt1 – KH Domain – Exon 23 – Removal of GxxG repeat

Amplicons for genotyping are highlighted in black. The underlined light blue region is the gRNA and separation where the loxP would theoretically be inserted.

Figure 6.4.2: Bioinformatic approach for the creation of a conditional knockout animal. Through the homologous recombination of the single-stranded DNA (ssDNA), LoxP sites are inserted into the PNPT1 introns 22 and 23. Genotyping of animals was accomplished through designing primers that flanked the 5' and 3' LoxP sites, in order to determine if the sgRNA targeted the sequence and successful recombination occurred.

Figure 6.4.3

Gene: Polynucleotide phosphorylase (Pnpt1 or PNPase)

Location: chr11:29,130,751-29,161,828 (UCSC Genome Browser GRCm38/mm10)

Interest: Remove exon 23 of PNPase

CHOP CHOP:

Intron 22: chr11:29154899-29155713: ATGTGCCATATTGTATGTCC**CGG**

Intron 23: chr11:29155796-29156835: TATATTGGATAGCAGCATGG**AGG**

PAM Sequence = **RED**

Figure 6.4.3: Design of sgRNA primers for targeting introns 22 and 23 of PNPT1. Using CHOPCHOP, sgRNA with the highest ranking in each of the locations was chosen. UCSC Genome Browser GRCh38/mm10 was implemented to provide the genomic location, as well as SNPs that may occur in FVB/NJ mice.

Figure 6.4.4

Figure 6.4.4: Genotyping for the KH RNA binding domain knockout using 2% agarose gel electrophoresis. Amplification of the intronic insertion site of the single-stranded DNA (ssDNA) allowed for identification of animals with targeted DNA. The analysis revealed at least one animal containing heterozygous regions for both the upstream and downstream LoxP insertion site. Het = heterozygous bands, Mut = mutations, likely the result from non-homologous end-joining.

Figure 6.4.5

Cloning Primers

FL Pnpt1 KO 1 F: CACCGGCTGAGTGCCCGATTCCGCC

FL Pnpt1 KO 1 R: AAACCCGCCTTAGCCCGTGAGTCGC

FL Pnpt1 KO 2 F: CACCGGTTTTAGGGGCAGTACGAAT

FL Pnpt1 KO 2 R: AAATAAGCATGACGGGGATTTTGC

RNA Pnpt1 KO 1 F: CACCGCGAGCAAAATTCGTTGGGCC

RNA Pnpt1 KO 1 R: AAACCCGGGTTGCTTAAAACGAGCC

RNA Pnpt1 KO 2 F: CACCGCTTACCTGACGCTTGTTGGA

RNA Pnpt1 KO 2 R: AAACAGGTTGTTTCGCAGTCCATTCC

Sanger Sequencing Primers

hU6-F: GAGGGCCTATTTCCCATGATT **Region:** 1 .. 21

LKO.1 5': GACTATCATATGCTTACCGT**Region:** 172 .. 191

Figure 6.4.5: CRISPR/Cas9 approach for HL-1 cell line knockout. Using all-in-one Cas9/sgRNA plasmids, sgRNA was designed to target exon 1 of PNPT1 (likely resulting in complete knockout of the protein as well as exon 23 (KH RNA binding domain, GxxG motif). In order to determine the proper cloning and orientation of Cas9/sgRNA plasmids, Sanger sequencing primers targeting the hU6 and LKO.1 5' region were designed for pSpCas9(BB)-2A-GFP (PX458) and pSpCas9(BB)-2A-Puro (PX459). FL = full length PNase knockout targeting exon 1, KH = KH RNA binding domain knockout targeting exon 23.

Quincy A. Hathaway – Curriculum Vitae

179 Hathaway Road, Jefferson, PA 15344 | 724-255-4637 | qahathaway@mix.wvu.edu

Education

MEDICAL DOCTOR | MAY 2023 | WEST VIRGINIA UNIVERSITY

- Medical student in the Class of 2023

DOCTOR OF PHILOSOPHY | MAY 2019 | WEST VIRGINIA UNIVERSITY

- GPA – 4.0
- Graduate Degree: Applied and Molecular Pathophysiology (Division of Exercise Physiology)

BACHELOR OF SCIENCE | MAY 2015 | WAYNESBURG UNIVERSITY

- Valedictorian: GPA – 4.0
- Major: B.S. Biology (Summa Cum Laude), B.S. Environmental Science (Summa Cum Laude)
- Minor: Chemistry
- Clubs/Organizations: ACS member, member of the Ecological Stewards and Biology clubs, and Habitat for Humanity Greene County Chapter member

HIGH SCHOOL DIPLOMA | JUNE 2011 | CARMICHAELS AREA HIGH SCHOOL

- Salutatorian: GPA – 3.98
- President of the Recycling Committee, Member of The National Honor Society, Newspaper Staff, Yearbook Staff, Envirothon Team, and Ralph K. Bell Bird Club Member

Publications in Review

Dysfunctional mitochondrial respiration can be targeted to induce death in drug resistant B-cell acute lymphoblastic leukemia. Nair RR, Piktel D, Thomas P, **Hathaway QA**, Rellick SL, Sarkar P, Martin K, Geldenhuys WJ, Hollander JM, Gibson GF. *J Biol Chem*. Submitted: December 14th, 2018.

Accepted Publications

ROS promote epigenetic remodeling and cardiac dysfunction in offspring following maternal engineered nanomaterial (ENM) exposure.

Kunovac A, **Hathaway QA**, Pinti MV, Goldsmith WT, Durr AJ, Fink GK, Nurkiewicz TR, Hollander JM. Part Fibre Toxicol. 2019 Jun 18;16(1):24. doi: 10.1186/s12989-019-0310-8. PMID: 31215478

Machine-learning to stratify diabetic patients using novel cardiac biomarkers and integrative genomics.

Hathaway QA, Roth SM, Pinti MV, Sprando DC, Kunovac A, Durr AJ, Cook CC, Fink GK, Cheuvront TB, Grossman JH, Aljahli GA, Taylor AD, Giromini AP, Allen JL, Hollander JM. Cardiovasc Diabetol. 2019 Jun 11;18(1):78. doi: 10.1186/s12933-019-0879-0. PMID: 31185988

miRNA-378a as a key regulator of cardiovascular health following engineered nanomaterial inhalation exposure.

Hathaway QA, Durr AJ, Shepherd DL, Pinti MV, Brandebura AN, Nichols CE, Kunovac A, Goldsmith WT, Friend SA, Abukabda AB, Fink GK, Nurkiewicz TR, Hollander JM.

Nanotoxicology. 2019 Jun;13(5):644-663. doi: 10.1080/17435390.2019.1570372. Epub 2019 Feb 1. PMID: 30704319

Mitochondrial Dysfunction in Type 2 Diabetes Mellitus: An Organ-Based Analysis.

Pinti MV, Fink GK, **Hathaway QA**, Durr AJ, Kunovac A, Hollander JM.

Am J Physiol Endocrinol Metab. 2019 Feb 1;316(2):E268-E285. doi: 10.1152/ajpendo.00314.2018. Epub 2019 Jan 2. PMID: 30601700

Mitochondrial proteome disruption in the diabetic heart through targeted epigenetic regulation at the mitochondrial heat shock protein 70 (mtHsp70) nuclear locus.

Shepherd DL*, **Hathaway QA***, Nichols CE, Durr AJ, Pinti MV, Hughes KM, Kunovac A, Stine SM, Hollander JM.

J Mol Cell Cardiol. 2018 Jun;119:104-115. doi: 10.1016/j.yjmcc.2018.04.016. Epub 2018 May 4. PMID: 29733819

Maternal engineered nanomaterial inhalation during gestation alters the fetal transcriptome.

Stapleton PA*, **Hathaway QA***, Nichols CE, Abukabda AB, Pinti MV, Shepherd DL, McBride CR, Yi J, Castranova VC, Hollander JM, Nurkiewicz TR.

Part Fibre Toxicol. 2018 Jan 10;15(1):3. doi: 10.1186/s12989-017-0239-8. PMID: 29321036

Reactive oxygen species damage drives cardiac and mitochondrial dysfunction following acute nano-titanium dioxide inhalation exposure.

Nichols CE, Shepherd DL, **Hathaway QA**, Durr AJ, Thapa D, Abukabda A, Yi J, Nurkiewicz TR, Hollander JM.

Nanotoxicology. 2018 Feb;12(1):32-48. doi: 10.1080/17435390.2017.1416202. Epub 2017 Dec 15. PMID: 29243970

Regulating microRNA expression: at the heart of diabetes mellitus and the mitochondrion.

Hathaway QA, Pinti MV, Durr AJ, Waris S, Shepherd DL, Hollander JM.

Am J Physiol Heart Circ Physiol. 2018 Feb 1;314(2):H293-H310. doi: 10.1152/ajpheart.00520.2017. Epub 2017 Oct 6. Review. PMID: 28986361

Exploring the mitochondrial microRNA import pathway through Polynucleotide Phosphorylase (PNPase).

Shepherd DL*, **Hathaway QA***, Pinti MV, Nichols CE, Durr AJ, Sreekumar S, Hughes KM, Stine SM, Martinez I, Hollander JM.

J Mol Cell Cardiol. 2017 Sep;110:15-25. doi: 10.1016/j.yjmcc.2017.06.012. Epub 2017 Jul 11. PMID: 28709769

Maternal-engineered nanomaterial exposure disrupts progeny cardiac function and bioenergetics.

Hathaway QA, Nichols CE, Shepherd DL, Stapleton PA, McLaughlin SL, Stricker JC, Rellick SL, Pinti MV, Abukabda AB, McBride CR, Yi J, Stine SM, Nurkiewicz TR, Hollander JM.
Am J Physiol Heart Circ Physiol. 2017 Mar 1;312(3):H446-H458. doi: 10.1152/ajpheart.00634.2016. Epub 2016 Dec 23. PMID: 28011589

Role of microRNA in metabolic shift during heart failure.

Pinti MV, **Hathaway QA**, Hollander JM.
Am J Physiol Heart Circ Physiol. 2017 Jan 1;312(1):H33-H45. doi: 10.1152/ajpheart.00341.2016. Epub 2016 Oct 14. Review. PMID: 27742689

****These authors contributed equally***

Fellowship Awards

AMERICAN HEART ASSOCIATION – PREDOCTORAL FELLOWSHIP

Project Title: “Mitochondrial microRNA Import and Regulation”

Awarded: July 1st, 2017 – July 1st, 2019 – ACCEPTED

NATIONAL SCIENCE FOUNDATION- IGERT (INTEGRATIVE GRADUATE EDUCATION AND RESEARCH TRAINING)

Research and Education in Nanotoxicology at West Virginia University

Project Title: “How pathological conditions within the heart shape the epigenetic landscape of the tissue, specifically in the control of factors important to bioenergetics”

Awarded: August 15th, 2015 – August 15th, 2017 – ACCEPTED

Awarded: August 15th, 2017 – August 15th, 2018 – DECLINED

Research Awards

OUTSTANDING DOCTORAL STUDENT AWARD – EXERCISE PHYSIOLOGY

Awarded: April 5th, 2019

Award Amount: N/A

3-MINUTE THESIS FINALIST – WVU 2019

Award Type: Selected as a finalist for the 3-minute thesis competition at WVU, presenting “Mitochondria, at the Heart of Diabetes”.

Awarded: April 5th, 2019

Award Amount: \$50

TOXICOLOGY WORKING GROUP 2018 TRAVEL AWARD – SOT 2018

Award Type: To present a poster on – “Engineered Nanomaterial Exposure: Effects on Epigenetic and MiRNA Regulation of Cardiac Mitochondria”

Awarded: February 19th, 2018

Award Amount: \$700

APS PG GROUP TRAINEE AWARD – EB 2018

Award Type: Oral presentation on – “Nuclear-Mitochondrial Crosstalk in the Heart during Diabetes Mellitus – The Impact on RNA in Mitochondrial Subpopulations”

Awarded: April 23rd, 2018 for 2nd place

Award Amount: \$200

AMERICAN HEART ASSOCIATION TRAVEL STIPEND

Award Type: Funds for travel allocated from the Predoctoral fellowship entitled – “Mitochondrial microRNA Import and Regulation”

Awarded: July 1st, 2017

Award Amount: \$2000

RISING STAR AWARD – APPLIED AND MOLECULAR PATHOPHYSIOLOGY (DIVISION OF EXERCISE PHYSIOLOGY)

Award Type: One recipient per program, recognizing excellence in research for early career Graduate Research Assistants

Awarded: April 5th, 2017

Award Amount: N/A

Research Presentations

EXPERIMENTAL BIOLOGY (EB), SAN DIEGO, CA: 2018

Oral and poster presentation, abstract entitled “Nuclear-Mitochondrial Crosstalk in the Heart during Diabetes Mellitus – The Impact on RNA in Mitochondrial Subpopulations” (April 23rd, 2018)

WEST VIRGINIA UNIVERSITY VAN LIERE DAY, MORGANTOWN, WV 2018

Poster presentation, abstract entitled “Non-coding RNA and the Mitochondrion – Function and Pathology in the Heart” (March 23rd, 2018)

SOCIETY OF TOXICOLOGY (SOT), SAN ANTONIO, TX: 2018

Poster presentation, abstract entitled “Engineered Nanomaterial Exposure: Effects on Epigenetic and MiRNA Regulation of Cardiac Mitochondria” (March 14th, 2018)

EXPERIMENTAL BIOLOGY (EB), CHICAGO, IL: 2017

Poster presentation, abstract entitled “Developing a Link between Cardiac Function and Maternal Engineered Nanomaterial Exposure in Growing Progeny” (April 24th, 2017)

WEST VIRGINIA UNIVERSITY TOXICOLOGY WORKING GROUP, MORGANTOWN, WV: 2017

Oral Presentation, talk entitled “Gestational Exposure to Nanomaterials: The Interplay between Bioenergetics and the Epigenome in Progeny Cardiac Tissue” (March 30th, 2017). 10-minutes talk.

WEST VIRGINIA UNIVERSITY VAN LIERE DAY, MORGANTOWN, WV: 2017

Poster presentation, abstract entitled “Polynucleotide Phosphorylase (PNPase) and Mitochondrial microRNA import” (March 24th, 2017)

WEST VIRGINIA UNIVERSITY METABOLISM, MITOCHONDRIAL FUNCTION, AND BIOENERGETICS (MMB) WORKING GROUP, MORGANTOWN, WV: 2016

Oral presentation, talk entitled “Mitochondrial Protein Regulation – CpG Methylation” (September 12th, 2016). 10-minute talk.

WEST VIRGINIA UNIVERSITY IGERT ORIENTATION SYMPOSIUM, MORGANTOWN, WV: 2016

Poster presentation, abstract entitled “Gestational Nano-TiO₂ Exposure and Cardiac Implications” (August 11th, 2016).

NATIONAL SCIENCE FOUNDATION-REU INTERNSHIP ON NANOTOXICOLOGY

Poster presentation during the West Virginia University summer undergraduate research symposium (July 25th, 2014)

Poster presentation for the Waynesburg University undergraduate research symposium (April 24th, 2015)

GREENE COUNTY CONSERVATION DISTRICT – 2014 MEETING

Oral presentation, talk entitled “Environmental impacts of the Oil and Gas Industry on Local Streams and Rivers” (November 18th, 2014). 15-minute talk.

[Accepted Abstracts](#)**EXPERIMENTAL BIOLOGY (EB), ORLANDO, FL: 2019**

Hathaway QA, Pinti MV, Roth SM, Sprando DC, Kunovac A, Durr AJ, Cook CC, Fink GK, Cheuvront TB, Grossman JH, Aljahli GA, Roberts HG, Salman M, Giromini AP, and Hollander JM. “Using Machine Learning to Predict the Development of Diabetes and Potential Biomarkers Linked to Cardiac Risk”

EXPERIMENTAL BIOLOGY (EB), ORLANDO, FL: 2019

Kunovac A, **Hathaway QA**, Pinti MV, Durr AJ, Fink GK, Goldsmith WT, Nurkiewicz TR, and Hollander JM. "Elevated ROS and Epigenetic Remodeling Disrupt Cardiac Function in Offspring Following Maternal Engineered Nanomaterial (ENM) Exposure"

EXPERIMENTAL BIOLOGY (EB), ORLANDO, FL: 2019

Pinti MV, **Hathaway QA**, Kunovac A, Durr AJ, Cook CC, Roberts HG, Salman M, and Hollander JM. "MicroRNA Changes in Diabetic Cardiac Mitochondria: What are they doing there?"

EXPERIMENTAL BIOLOGY (EB), ORLANDO, FL: 2019

Durr AJ, **Hathaway QA**, Pinti MV, Shepherd DL, Kunovac A, and Hollander JM. "Stress Strain Speckle-Tacking Segmental Analysis Reveals Early Indications of Diastolic Dysfunction in a Type 2 Mouse Model of Diabetes Mellitus"

EXPERIMENTAL BIOLOGY (EB), SAN DIEGO, CA: 2018

Hathaway QA, Shepherd DL, Durr AJ, and Hollander JM. "Nuclear-Mitochondrial Crosstalk in the Heart during Diabetes Mellitus – The Impact on RNA in Mitochondrial Subpopulations"

EXPERIMENTAL BIOLOGY (EB), SAN DIEGO, CA: 2018

Durr AJ, Shepherd DL, **Hathaway QA**, and Hollander JM "Mitochondrial Bioenergetics in the Diabetic Heart and the Influence of Cortactin"

WEST VIRGINIA UNIVERSITY VAN LIERE DAY, MORGANTOWN, WV 2018

Hathaway QA, Pinti, MV, Durr AJ, Shepherd DL, and Hollander JM. "Non-coding RNA and the Mitochondrion – Function and Pathology in the Heart"

WEST VIRGINIA UNIVERSITY VAN LIERE DAY, MORGANTOWN, WV 2018

Durr AJ, Shepherd DL, **Hathaway QA**, and Hollander JM. "Cortactin's Influence on Mitochondrial Bioenergetics in the Diabetic Heart: A Novel Viewpoint"

WEST VIRGINIA UNIVERSITY VAN LIERE DAY, MORGANTOWN, WV 2018

Noble B, **Hathaway QA**, Durr AJ, and Shepherd DL. "Epigenetics in Diabetes"

SOCIETY OF TOXICOLOGY (SOT) 57TH ANNUAL MEETING, SAN ANTONIO, TX: 2018

Hathaway QA, Nichols CE, Shepherd DL, Durr AJ, Stapleton PA, Abukabda AB, Nurkiewicz TR, and Hollander JM. "Engineered Nanomaterial Exposure: Effects on Epigenetic and MiRNA Regulation of Cardiac Mitochondria"

TRANSLATIONAL RESEARCH CANCER CENTER CONSORTIUM 21ST ANNUAL MEETING, SEVEN SPRINGS, PA 2018

Nair RR, Piktel D, Thomas P, **Hathaway QA**, Geldenhuys WJ, Hollander JM, and Gibson LF. “Pyrvinium Pamoate mediates cell death by disrupting mitochondrial respiration and inhibiting beta-catenin signaling in B-cell acute lymphoblastic leukemia”

EXPERIMENTAL BIOLOGY (EB), CHICAGO, IL 2017

Hathaway QA, Nichols CE, Shepherd DL, Stapleton PA, McLaughlin SL, Stricker JC, Rellick SL, Pinti MV, Abukabda AB, McBride CR, Yi J, Stine SM, Nurkiewicz TR, and Hollander JM. “Developing a Link between Cardiac Function and Maternal Engineered Nanomaterial Exposure in Growing Progeny”

WEST VIRGINIA UNIVERSITY VAN LIERE DAY, MORGANTOWN, WV 2017

Hathaway QA, Shepherd DL, Pinti MV, Nichols CE, Sreekumar S, Hughes KM, Stine SM, Martinez I, and Hollander JM. “Polynucleotide Phosphorylase (PNPase) and Mitochondrial microRNA import”

SOCIETY OF TOXICOLOGY (SOT) 56TH ANNUAL MEETING, BALTIMORE, MD 2017

Stapleton PA, Abukabda AB, Nichols CE, **Hathaway QA**, Rellick SL, McBride CR, Yi J, Hollander JM, Nurkiewicz TR. “Temporal microvascular and mitochondrial implications of prenatal nano-TiO₂ exposure”

Patents

OMNI-CLIPZ | IGERT-WVU | MAY 2016 –MAY 2018

- Omni-ClipZ provisional patent filed March 16, 2017: Application #: 62472157. Filed under “Holster for Electronic Device”. In summary, the device is a holster for insulin pumps, primarily used by Type I Diabetics. The innovation of the product is to include a more durable design as well as incorporating the tubing, which transmits the insulin to the host.
- I am one of four co-founders of the company
- Current Perspectives: conception of product design, patent searching identifying the legal boundaries of the product, preliminary budget and business model for the first 3 years of development, initial surveys gathered on the product design, market identified and channels for production/transportation of the product identified, materials characterized for use, and the first 3D-printed model build.
- Batch testing and additional surveys characterizing the product are currently underway