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PRINCIPAL INVESTIGATOR: Jessica Lasky-Su

CONTRACTING ORGANIZATION: The Brigham and Women's Hospital, Inc., Boston, MA

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

The overarching hypothesis of this proposal was that exposure to lead (Pb) during active military service is related to the observed high prevalence of poor lung health among veterans. We aimed to explore the mechanisms underlying this relationship utilizing metabolomics; i.e. the systematic profiling of small (<10kDa) metabolites in a biological sample, which will allow us to construct a causal pathway demonstrating the mechanistic and biological connections between Pb exposure and lung health. To achieve this aim we identified participants from the ongoing Normative Ageing Study of Veterans. We selected men with detailed histories on their exposure to Pb, with comprehensive data on long term lung health and with blood samples suitable for metabolomics profiling. We identified 661 plasma samples from 464 veterans, which we shipped to Metabolon Inc. for metabolomic profiling using four LCMS platforms, enabling the broadest coverage of the metabolome possible. We applied QC and data processing pipelines to these data and created the '*Normative Aging Study Metabolomics Cohort'*. To date, we have successfully identified a metabolomic profile associated with Pb exposure and a metabolomic profile associated with poor lung health. Our analyses, encompassing both frequentist and network approaches, suggested that the relationship between Pb and the lung is mediated, in part, by dysregulated xanthine metabolism; leucine, isoleucine and valine Metabolism; lipids and aceylated amino acids. These findings have been presented at both national and international conferences, and have resulted in one published manuscript, one under submission, and one pending submission. In addition, we have initiated two new research avenues utilizing the metabolomics data generated as part of this project one focused on metabolomics and air pollution (three manuscripts currently under review) and the other on metabolomics and mental health, in the Normative Aging Study Metabolomics Cohort (analysis ongoing).

15. SUBJECT TERMS
None listed.

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1. Introduction

Poor respiratory health relating to environmental exposures during active service represents a significant public health burden for Military service personnel and Veterans. Lead (Pb) is commonly found on military bases in the form of fine particulate matter and is thought to adversely affect pulmonary function for many decades after exposure. However, a complete understanding of the effects of Pb exposure on the respiratory system remains to be fully elucidated, and further investigation is paramount. Metabolomics, the systematic profiling of all the small molecules in a biological system, represents a powerful tool to (i) increase mechanistic understanding of the pathogenesis of Pb exposure on the respiratory system, and (ii) identify biomarkers of 'toxic' levels of exposure. The overarching hypothesis of this project is that a metabolomic profile of Pb exposure can be identified and leveraged to understand how Pb exposure and the resulting changes on the metabolome have a downstream impact on respiratory health. Therefore, this projects aims to determine the influence of Pb exposure on respiratory health through the integration of the metabolomic profiles of heavy metal exposure and of respiratory disease. This will allow for the identification of novel blood-based biomarkers associated with long-term toxic Pb exposure and respiratory health. These biomarkers will provide mechanistic insights into the pathogenic effects of Pb on the respiratory system and into the disease pathways involved, supporting the future development of novel therapeutics. This represents the first study to utilize metabolomics in the exploration of both short-term and long-term Pb exposure and its effect on respiratory disease.

2. Keywords

Respiratory disease; Metabolomics; Metabolome; Lead (Pb); Heavy metals; Biomarkers; Metals Toxicology; Omics

3. Accomplishments

3.a Major Goals of the Project

The original major goals of this project, as detailed in the **Statement of work (SOW)** in our proposal are show in **Table 1a-c.** These goals are delineated by aim and subdivided by tasks (major and sub), and the outcome of each goal and completion dates, along with accompanying notes is provided. A detailed description of the methodology and findings to date is provided below, or information on the relevant manuscript, as applicable (all referenced manuscripts are included as Appendices). The corresponding table or figure describing the results is indicated in square brackets. One notable issue was a delay in the HPRO approval that has now been resolved (*see* ***section 5a*** *for details*).

Table 1a: SOW and Progress in Accomplishing the Outline Goals (Specific Aim 1)

Specific Aim 1 To identify metabolomic profiles of lead exposure in a population of Veterans	**Timeline**	**% completion**	**Completion Date**	**Comments**
Major Task 1 Metabolomic profiling of 374 NAS individuals	Months			
Subtask 1: Selection and shipment of blood plasma	1	100%	11/16/17	Additional samples; *total 661,* were profiled following price negotiations with Metabolon [**Table 2**]
Subtask 2: Metabolomic profiling	2-3	100%	1/31/18	
Subtask 3: Data processing and Quality control procedures using Metabolon, Inc internal standards	2-3	100%	1/31/18	
Subtask 4: Data Quality Control pipeline at the Channing Laboratory	3	100%	3/31/18	
Milestone Achieved: Metabolomic dataset received ready for analysis	*3*	100%	3/31/18	[**Table 3 & Figure 1**]
HRPO regulatory review	1-3	100%	2/4/18	IRB concluded the research did not constitute human subjects research
Milestone Achieved: HRPO Approval	*3*	100%	2/4/18	
Major Task 2 Identification of the metabolome of Pb exposure				
Subtask 1: Creation of a composite measure of Pb exposure incorporating duration and level, based on pre-existing measures in bone, toenail, blood and urine	1	100%	12/1/19	We focus primarily on blood levels of Pb Exposure, and utilize the additional measures in other bio samples to further explore longer

				term exposure **[Table 5 & Figure 2]**
Subtask 2: Linear regression models and sensitivity analyses, exploring different confounders, to identify differential metabolites and pathway enrichment analysis to identify the metabolomic pathways mapping to these metabolites associated with exposure	2-3	100%	12/1/19	Analyses have been completed, as detailed in our published manuscript **[Appendix 1**]
Subtask 3: Network approaches, including WGCNA to identify metabolomic networks	3-4	100%	10/30/20	Analyses have been completed, as detailed in our manuscript pending submission [**Appendix 2**]
Milestone Achieved: Publication of a manuscript exploring the metabolome of Pb exposure	*5*	100%	7/29/20	Manuscript published in Environmental Research (Impact Factor (IF): 5.03) **[Appendix 1**] Additional network-based manuscript pending submission to Metabolites (IF: 3.3) [**Appendix 2**]

Table 1b: SOW and Progress in Accomplishing the Outline Goals (Specific Aim 2)

Specific Aim 2 To identify metabolomic profiles of pulmonary Function in a population of Veterans	**Timeline**	**% completion**	**Completion Date**	**Comments**
Major Task 1 To identify a metabolomic profile of pulmonary function within a population of Veterans				
Subtask 1: Linear regression and network analysis as described in Major task 2 applied to the outcome of pulmonary function, as denoted by FEV_1 and FVC	6-8	100%	02/04/2020	Analyses completed
Subtask 2: Development and assessment of a metabolomic score based on the findings from subtask 1 that can be used to discriminate men by their degree of lung function	8-9	100%	10/30/20	ROC curve analyses and sensitivity and specificity are used to assess score. To date we have explored generating scores based on metabolite levels or the first principal component of those that were significant [**Figure 4 - 5 & Table 7-8**] Extensive analyses determined little biomarker potential of metabolites for the explored metrics of lung function, and as such these results will not be included in the published manuscripts
Milestone Achieved: Publication of a manuscript exploring the metabolome pulmonary function	*9*	100%	Under submission date *tbd*	Analyses completed manuscript submitted to the American Journal of Respiratory and Critical Care Medicine (IF: 17.45) [**Appendix 3**]

Table 1c: SOW and Progress in Accomplishing the Outline Goals (Specific Aim 3)

Specific Aim 3 To determine the influence of Pb exposures on respiratory health through the integration of the metabolomics profiles of Pb exposure and pulmonary function	**Timeline**	**% completion**	**Completion Date**	**Comments**
Major Task 1: Identify metabolites, metabolite networks and metabolomics pathways along the causal pathway from Pb exposure to reduced pulmonary function	Months			
Subtask 1: Identify common differential metabolites and metabolomic pathways for Pb exposure and pulmonary lung function	10	100%	06/01/2020	Analyses complete, as detailed in our manuscript pending submission [**Appendix 2**]
Subtask 2: Utilize WGCNA to identify the elements of metabolic networks that are consistent for Pb and pulmonary lung function	10-11	100%	06/01/2020	Analyses complete, as detailed in our manuscript pending submission [**Appendix 2]**
Major Task 2: Construction of a biologically informative causal pathway				
Subtask 1: Utilize the results from Major Task 1 together with mediation analyses and structural equation modelling to construct a causal pathway from Pb exposure to respiratory outcome	12-14	100%	10/30/20	Analysis determined no direct significant association between Pb exposure and lung function, as such it is not possible to conduct formal mediation analyses. The potential causal pathways are now discussed in our

				manuscript pending submission [**Appendix 2**]
Milestone Achieved: Publication of findings	*15*	100%	Pending submission date *tbd*	Manuscript pending submission [**Appendix 2**]
Milestone Achieved: Publication discussing the applicability of metabolomics for constructing causal pathways	*16*	100%	Pending submission date *tbd*	This discussion has been subsumed into our manuscript pending submission [**Appendix 2**]

Major Task 3: Biomarker Development				
Subtask 1: Utilizing the results from Aims 1-3 explore the potential development of biomarkers of exposure, outcome and intermediate biomarkers along the causal pathway that may be suitable for Therapeutic target	15-18	100%	Pending submission date *tbd*	This discussion has been subsumed into our manuscript pending submission [**Appendix 2**]
Subtask 2: Search for suitable replication population(s)	18	100%	12/01/2018	We identified the European Prospective Investigation into Cancer – Norfolk subset (EPIC-Norfolk) as a replication population for our lung function analyses [**Appendix 3**]
Milestone Achieved: Publication of findings	*18*	100%	Pending/under submission date *tbd*	This discussion of biomarkers has been subsumed into our manuscript pending submission [**Appendix 2**] and the identified replication population forms part of our submitted manuscript [**Appendix 3**]
Milestone Achieved: Finalize plan and secure funding for future study to develop findings further	*18*	100%	06/01/2020	We have developed the NAS Metabolomics Cohort, and we are now actively working with several other investigators, who have independent funding to extend our work to consider air pollution and lung function [**Appendix 3-4**]. We are also utilizing this cohort to explore metabolomics and mental health, and we have received independent funding to focus on neurological health, which we will utilize to continue our analysis on this cohort.

3.b. Accomplishments of these goals

There have been no changes to the overall goals of this project as stated in the **SOW** (**Table 1a-c**), with the exception of Specific Aim 3; Major task 2; subtask 2. Despite extensive searches we were unable to identify suitable collaborators with Pb exposure information, pulmonary function data and plasma metabolomic profiling. Consequently, we now limit our replication to our pulmonary function findings. We identified the European Prospective Investigation into Cancer – Norfolk subset (EPIC-Norfolk), as a suitable replication population for this aim, and have now submitted a manuscript to the American Journal of Respiratory and Critical Care Medicine based on the replicated findings. *See **section 3.b.2** and **Appendix 3** for more details*. All planned analyses have been completed, although dependent on findings not all are included in the published/submitted/pending manuscripts. Some work e.g. Specific Aim 3; Major Task 2; Subtask 1, have been subsumed within other manuscripts rather than representing stand along manuscripts, based on their results. The major accomplishments by task are outlined below, methods are described and results and conclusions are presented either as stand along tables/figures, or with reference to the appendices.

3.b.1 AIM ONE: Major Task one: Metabolomic profiling of NAS individuals

There was an advantageous change in the number of samples we were able to profile following price negotiations with Metabolon Inc., who were sub-contracted to perform the metabolomics profiling analyses (*see **section 5.b** for further details*). Rather than the 374 samples we stated in our proposal, we were able to profile 661 samples from 464 men, including a number from the same men over a longer period of time (**Table 2**). This substantially increased our overall power, and specifically our power to explore changes in the metabolome over time. Subject selection was carefully performed to ensure we included both a range of lung function and range of Pb exposure, and we prioritized the selection of men with multiple longitudinal blood samples for profiling.

Metabolomic profiling was performed during the first reporting period, and is described in detail in our first report. In brief relative abundance of 1301 metabolites could be quantified in the 661 plasma samples (**Table 3**). The metabolic super pathways, as defined by metabolon, covered by these profiles are described in **Figure 1**. Differing numbers of metabolites were used in the subsequent analyses depending on the specific QC pipeline used; for example, in our second manuscript, we restricted the tested metabolites to those that could also be measured in our replication population, EPIC-Norfolk. For the majority of analyses the 320 metabolites that could not be named were excluded from analyses. We note that Metabolon is continually updating its library, and we may be able to name these ‘unknown’ metabolites moving forward and utilize them in subsequent analyses.

Table 2: Baseline characteristics of 464 Men from the Normative Ageing Study with Metabolomic of Plasma Samples

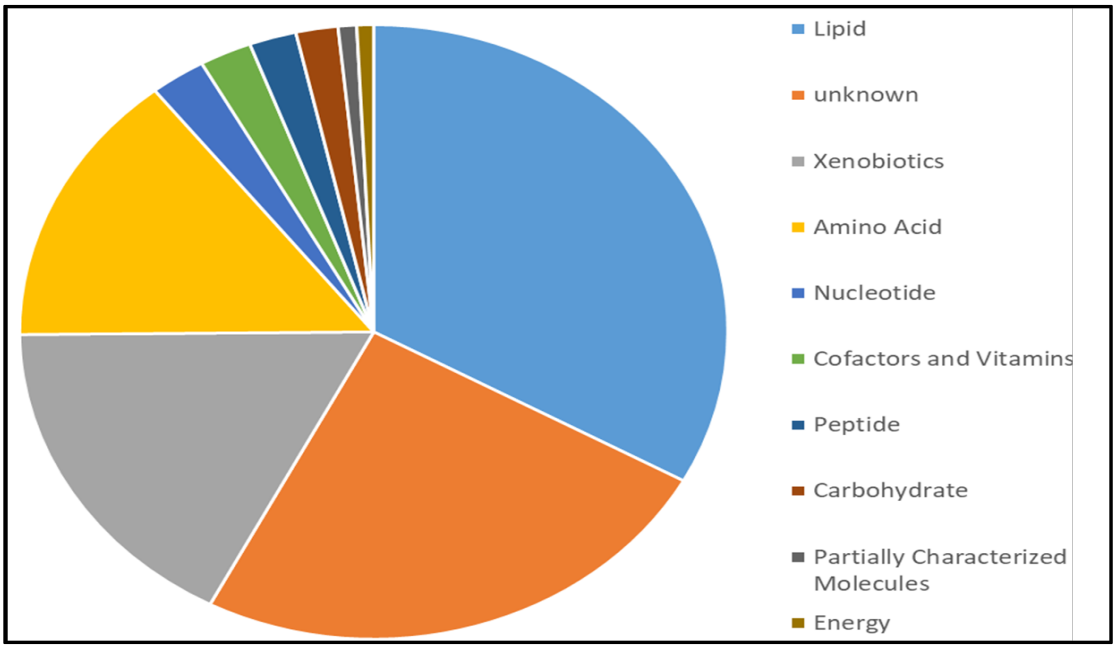
Characteristic		n=464 Men from the Normative Ageing Study
Age (yrs)	**mean [range]**	75 [57, 97]
BMI	**Underweight *n* (%)**	3 (0.7%)
	Normal *n* (%)	122 (26.3%)
	Overweight *n* (%)	249 (53.7%)
	Obese *n* (%)	90 (19.4%)
Race	**White *n* (%)**	456 (98.7%)
	Black *n* (%)	6 (1.3%)
	Other *n* (%)	2 (0.4%)
Smoking Status	**Never *n* (%)**	138 (29.9%)
	Regular Smoker *n* (%)	19 (4.1%)
	Former Smoker *n* (%)	307 (66.5%)
Asthma	**Yes *n* (%)**	15 (3.2%)
	Previous *n* (%)	16 (3.5%)
	No *n* (%)	433 (93.7%)
Forced Expiratory Volume in One Second (FEV_1, L)	**mean [range]**	3.39 [1.42, 5.85]
Forced Vital Capacity (FVC, L)	**mean [range]**	2.51 [0.80, 4.32]
FEV_1/FVC ratio	**mean [range]**	73.7% [36.1%, 92.5%]
Fresh Blood Lead (µg/mL)	**mean [range]**	3.46 [0.00, 29.00]
Second Blood Sample Available	**Yes *n* (%)**	169 (36.4%)
Third Blood Sample Available	**Yes *n* (%)**	28 (6.0%)

Table 3: Four Profiling Platforms Employed by Metabolon to Characterize 1301 Metabolites

Profiling Platform	Metabolites	n
LC/MS Negative	Metabolites that Ionize in the Negative Mode	688
LC/MS Polar	Polar Metabolites	70
LC/MS Positive Early	Metabolites that Ionize in the Positive Mode, elute early	286
LC/MS Positive Late	Metabolites that Ionize in the Positive Mode, elute late	257

LC/MS – Liquid Chromatography Mass Spectrometry

Figure 1: Metabolic Pathways Encompassed by the Metabolomic Profiles



3.b.1 AIM ONE: Major Task 2 Identification of the metabolome of Pb exposure

Data analyses are complete and our first manuscript has now been published in the journal of Environmental Research (Impact Factor (IF): 5.026) **[Appendix 1]**.

3.b.1a Manuscripts

The first manuscript "**Metabolomic Signatures of Lead Exposure in the VA Normative Aging Study**", based on 399 men who met the eligibility criteria for this study, focusses on blood metabolites associated with current and longer term Pb exposure as measured in blood and toenails respectively. This study represented one of the first to explore the metabolome of Pb exposure, and was unique in its focus on population-based individuals (rather than highly exposed populations) and in its large sample size. We identified a number of novel, biologically plausible associations, which can help to lay the groundwork for the development of novel exposure biomarkers. The manuscript is summarized in its abstract as shown below, and further information can be found in the figures and tables of the manuscript [**Appendix 1**], as listed.

Abstract

Background: Lead (Pb) is widespread and exposure to this non-essential heavy metal can cause

multiple negative health effects; however the mechanisms underlying these effects remain
incompletely understood.
Objectives: To identify plasma metabolomic signatures of Pb exposure, as measured in blood
and toenails.
Methods: In a subset of men from the VA Normative Aging Study, mass-spectrometry based
plasma metabolomic profiling was performed. Pb levels were measured in blood samples and
toenail clippings collected concurrently. Multivariable linear regression models, smoothing
splines and Pathway analyses were employed to identify metabolites associated with Pb exposure.
Results: In 399 men, 858 metabolites were measured and passed QC, of which 154 (17.9%)
were significantly associated with blood Pb ($p<0.05$). Eleven of these passed stringent correction
for multiple testing, including pro-hydroxy-pro (β(95%CI): 1.52 (0.93,2.12), p=7.18x10-7), Nacetylglycine (β(95%CI): 1.44 (0.85,2.02), p=1.12x10-6), tartarate (β(95%CI): 0.68 (0.35,1.00),
p=4.84x10-5), vanillylmandelate (β(95%CI): 1.05 (0.47,1.63), p=4.44x10-7), and lysine
(β(95%CI): -1.88 (-2.8,-0.95), p=9.10x10-5). A subset of 48 men had a second blood sample
collected a mean of 6.1 years after their first. Three of the top eleven metabolites were also
significant in this second blood sample. Furthermore, we identified 70 plasma metabolites
associated with Pb as measured in toenails. Twenty-three plasma metabolites were significantly
associated with both blood and toenail measures, while others appeared to be specific to the
biosample in which Pb was measured. For example, benzanoate metabolism appeared to be of
importance with the longer-term exposure assessed by toenails.
Discussion: Pb exposure is responsible for 0.6% of the global burden of disease and
metabolomics is particularly well-suited to explore its pathogenic mechanisms. In this study, we

identified metabolites and metabolomic pathways associated with Pb exposure that suggest that
Pb exposure acts through oxidative stress and immune dysfunction. These findings help us to
better understand the biology of this important public health burden.

Tables and Figures

Table 1: Baseline characteristics of 399 men from NAS, stratified by Blood Pb Level

Table 2: Top Metabolites Significantly Associated with Blood Pb Levels in the total sample
and the subset excluding one outlier
Table includes all metabolites with p value reaching at least the ENT75% threshold in either dataset

Figure 1: Volcano Plot demonstrating the association between continuous Pb blood levels
and 858 metabolites colored according to Metabolon-defined Superpathway

Figure 2: Dose-Response Splines for Eleven Top Metabolites Associated with Blood Pb levels in 399 men at ENT75%
Dose-response models with smoothing splines generated using the R package drsmooth; showing the spline estimated dose-response function with its upper (green) and lower (red) 95 percent confidence bounds. This method does not adjust for confounders.

Figure 3: Blood Pb-metabolite associations in the first sample from 399 men and the second
sample from 48 men for the top 11 hits at a threshold of ENT75%
All estimates are from generalized linear models including Pb and metabolite intensity as continuous variable adjusted for age at blood collection, height, weight, smoking status (regular or former) and race (White or Other)

Figure 4: Seventy blood metabolites associated ($p<0.05$) with Pb exposure levels as measured in concurrently collected toenail clippings

All estimates are from generalized linear models including Pb and metabolite intensity as continuous variables adjusted for age at blood collection, height, weight, smoking status (regular or former) and race (White or Other)

Figure 5: Metscape-derived Pathway based network of 'Glycine serine alanine and threonine metabolism'; 'Histidine metabolism'; 'Purine metabolism'; 'Urea cycle and metabolism of arginine, proline, glutamate, asparatate and asparagine'; with blood Pb associated metabolites in dark red

Metabolites are shown in hexagons; metabolites from these pathways identified as significant in
the metabolite~blood Pb analysis in 399 men are in dark red.
Reaction numbers are shown along the edges between the metabolite nodes

Figure 6: Metscape-derived Pathway based network of 'Glycerophospholipid metabolism';
and 'Glycosphingolipid metabolism' with blood Pb associated metabolites in dark red
Metabolites are shown in hexagons; metabolites from these pathways identified as significant in
the metabolite~blood Pb analysis in 399 men are in dark red.
Reaction numbers are shown along the edges between the metabolite nodes

Supplementary Tables and Figures

Supplementary Table 1: Metrics of Pb burden in Blood and Toenails of participants with available information

Supplementary Table 2: 154 Metabolites Associated ($p<0.05$) with Blood Pb

Supplementary Table 3: Baseline Characteristics of 48 men with a second blood sample, categorized according to blood Pb level in this second sample

Supplementary Table 4: Baseline Characteristics of 310 men with a measure of toenail Pb, categorized according to blood Pb level in their first blood sample

Supplementary Table 5: 70 Metabolites Associated ($p<0.05$) with toenail Pb

Supplementary Figure 1: Study Timeline: Sample collection for Pb measurement (blood and toenail) and metabolomics profiling (blood only)

Supplementary Figure 2: Percentage of imputed data for 858 metabolites included in analysis

Supplementary Figure 3: 858 Metabolites Included in the Analysis According to Super Pathway

Supplementary Figure 4: Score Plot for the First Two Principal Components of 858 metabolites colored according to blood Pb level category

Supplementary Figure 5: Heatmap demonstrating Correlation Between Intensity levels of 154 metabolites significantly associated with blood Pb levels in 399 men

Supplementary Figure 6: Correlation in Pb levels between first and second blood sample from 48 men with two samples suitable for metabolomic profiling

Supplementary Figure 7: Correlation in between blood Pb and toenail Pb levels in 310 men with metabolomic profiling

3.b.1a Metabolomics Methods Development

There are currently no consensus standards for multiple testing correction in metabolomics; methods applied to other 'omic' datatypes such as the Bonferroni correction, and even more liberal corrections such as Benjamini-Hochberg FDR, are considered too stringent for metabolomics data due to the high correlation of metabolites that are closely linked together through biological pathways. Therefore, in this manuscript we pioneered the use of the 'Effective Number of Tests approach' (PMID: 22143225; PMID: 14997420), which was initially developed for genetic data; for use with metabolomic data. This method takes into account the presence of highly correlated metabolites mapping to the same biological pathway, using a principal components analysis (PCA) approach to identify the number of effective tests. We applied PCA to the 858 metabolites and determined the number of components required to explain a given % of the variance in the data (i.e., the number of effective tests). The adjusted *p*-value threshold is then calculated as α/m where α denotes the nominal *p*-value threshold of 0.05, and m denotes the number of effective (i.e., independent) tests. For this analysis, we explored a threshold of 75% and of 90% variance explained and found that five and two metabolites, respectively, retained significance (**Table 4**). We additionally applied this in our subsequent lung function manuscript [**Appendix 3**], and it has now been widely adopted by our wider research lab.

Table 4: Significance Thresholds Based on the 'Effective Number of Tests' approach to Account for Multiple Testing in our manuscript "Metabolomic Signatures of Lead Exposure in the VA Normative Aging Study"

Significance Threshold	% Variance Explained	Number of PCs	P-value	N (%) Significant Metabolites
α	-	-	<0.05	241 (18.5%)
ENT 75	75%	88	<5.68x10-5	2 (0.15%)
ENT 90	90%	187	<2.67x10-4	5 (0.38%)

3.b.1b: Pb Metabolome in other biosamples

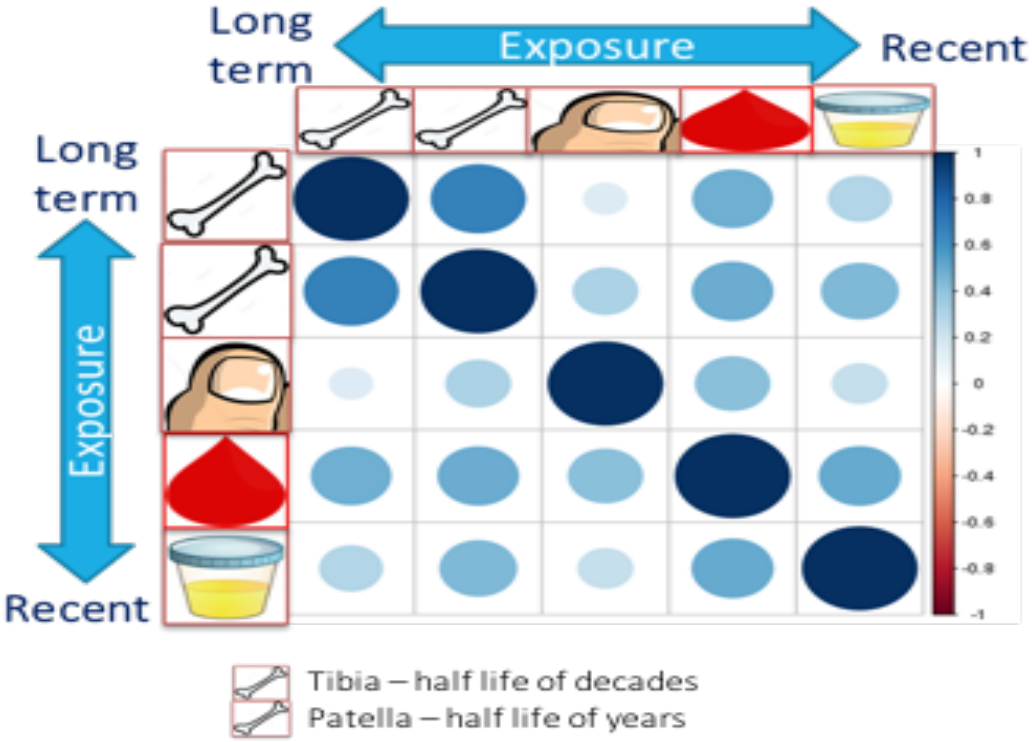
In addition to blood and toenail Pb levels, we had information on Pb in other biosamples which provide a more complete picture of long time Pb exposure (**Table 5**). (i) Long terms exposure measured in mid-tibia shaft (cortical bone: Pb half-life of many decades) and patella (primarily trabecular bone: Pb half-life of a few years) using cadmium-109 K-shell X-ray fluorescence spectroscopy, and (ii) recent exposure measured in urine by inductively coupled plasma mass spectrometry. Due to the rate of missingness for the measures in bone and in urine, despite the interesting results, these biosample exposure measures will not be included in any published manuscripts. However, these findings provide useful exploratory analyses and a more complete understanding of the influence of temporal Pb exposure on the metabolome.

Table 5: Mean and Standard deviation of Pb Levels as measured in blood, bone, tonenail and urine

Measure	Mean	Standard Deviation
Blood (µg/dL)	3.47	*2.61*
Toenail (µg/g)	0.62	*1.28*
Tibia (µg/g)	1.51	*0.95*
Patella (µg/g)	20	*13.47*
Urine (mg/dL)	0.67	*1.09*

Within the available data there was evidence of correlation between temporal measures of Pb (**Figure 2**). Tibia and Patella levels were highly correlated and blood Pb levels were correlated with these longer-term exposures, indicating a consistency in an individual's exposure to Pb over time. This was also observed between blood and toenail Pb which provide short and medium term estimates of Pb exposure respectively as reported in **Appendix 1, Supplementary Table S5**.

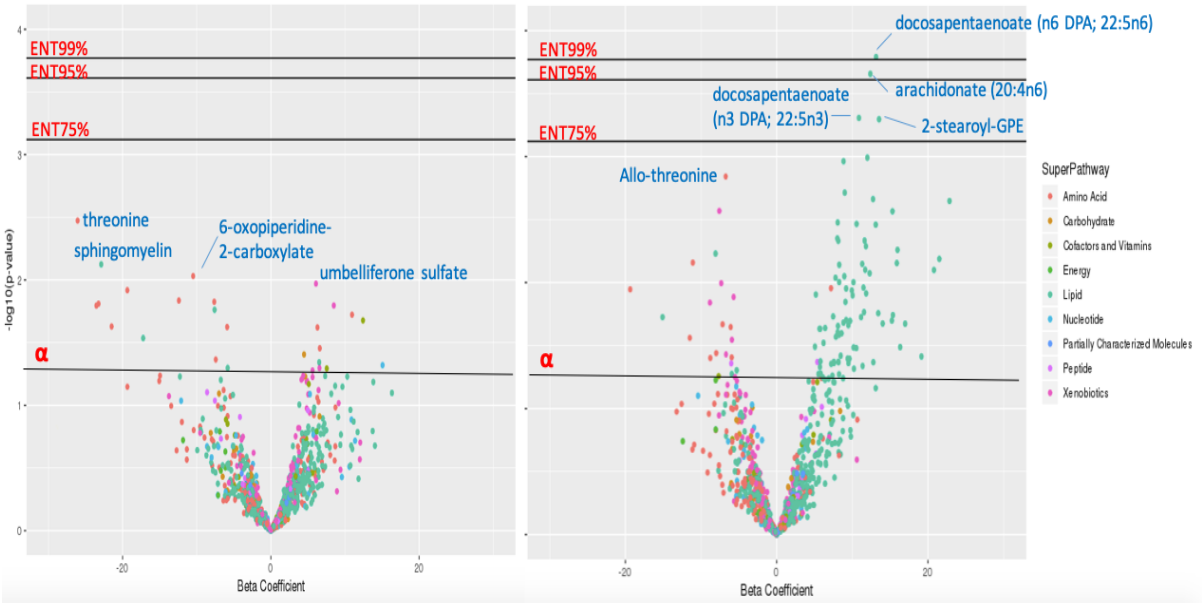
Figure 2: Correlation between temporal measures of Pb exposure



Accordingly, both long term and more short-term measures of Pb exposure primarily affect metabolites involves in lipid and amino acid metabolism; and a number of metabolites, including acylcarnitines, were associated with multiple measures of lead exposure (**Figure 3**). Even when

the exact metabolites identified as significant for the different biosamples differed, as can be shown by the low crossover in **Table 6**, the metabolites tended to be involved in the same biological pathways and processes.

Figure 3: Metabolite- Pb Associations in Different When Measuring Pb in Tibia and Patella Bone



α = 0.05

Table 6: Crossover in Significant (P<0.05) Pb associated metabolites when measuring Pb in different biological samples

	Blood	Toenail	Patella	Tibia
Blood	**154**			
Toenail	23	**70**		
Patella	4	1	**22**	
Tibia	7	5	2	**97**

3.b.1c Network Approaches

We additionally employed a network approach to identify metabolic networks, rather than single metabolites associated with Pb exposure (Specific Aim 1; Major Task 2; Subtask 3). Network approaches move away from reductionist methodologies to combine systems biology and network science, providing a holistic methodology to better understand biology through the identification and investigation of non-linear relationships and networks of interacting components. Weighted Gene Correlation Network Analysis (WGCNA,

horvath.genetics.ucla.edu/html/CoexpressionNetwork/Rpackages/WGCNA/index) is a network method for identifying clusters or 'modules' or highly correlated variables (such as metabolites) that are likely to be co-regulated, or working together in biologically coherent fashion. A module can then be summarized as a single unit, which can be correlated with phenotypes of interest. WGCNA was used to identify metabolomic network modules within the first sample from all men based on correlation patterns. The correlation matrix quantifies interconnectedness between metabolites and assigns them to co-expression modules. Features that do not show high enough co-expression metrics with any module are excluded from further analysis (they are assigned to a redundant grey module). Modules can be summarized by an eigenvector (based on the first principal component of each module) for each participant, then associations between the modules and Pb exposures were explored.

These data analyses are now complete and the associated manuscript is pending submission to the journal Metabolites (IF: 3.3), while awaiting code review from our institution [**Appendix 2**]. The manuscript is summarized in its abstract as shown below, and further information can be found in the figures and tables of the manuscript. Analyses are based on the 396 participants with blood samples used for Pb measurement and metabolomic profiling and concurrent spirometry. A total of 981 named metabolites were included in the analysis.

Abstract:

Evidence suggests a link between Pb exposure and lung function, however the mechanisms underlying this relationship remain poorly understood. We leveraged 396 men from the Normative Aging Study Metabolomics cohort with plasma metabolomic profiling, estimates of Pb exposure as measured in blood and in toenails and spirometry. We determined that 21 of 96 (21.9%) metabolites associated with Pb exposure as measured in blood were also associated with FEV1, while a larger percentage of metabolites associated with Pb measured in toenails, which provides a longer-term measure of exposure, 14 of 43 (32.6%) were associated with FEV1. When using network analyses we further identified two modules of co-regulated metabolites that associated with both Pb exposure and spirometry outcomes. These metabolites and metabolite modules were characterized by oxidative stress which provides a plausible biological mechanisms by which Pb exposure may influence lung health. This represents the first such metabolomic study to focus on a dysregulated metabolome as the causal link between metal exposure and lung function, and provides novel insights into this relationship.

Tables and Figures

Table 1. Baseline characteristics of NAS Study participants according to whole blood lead level

Table 2: Metabolites associated with Pb exposure as measured in either blood or Toenails, and with FEV_1

Table 3: Associations between measures of Pb exposure and metrics of spirometry adjusted for age, height, weight, smoking status and race

Figure 1. Cluster Dendrogram showing assignment of 858 metabolites to six merged dynamic modules

Figure 2. Heatmap describing the relationships between the module eigengenes and the traits of interest

Supplementary Tables and Figures

Supplementary Table S1: Association between whole blood Pb levels and concurrently measured spirometry
Supplementary Table S2: Association between Pb exposure as measured in toenails and concurrently measured spirometry
Supplementary Figure S1 Beta coefficients for 525 metabolites with Blood Pb and FEV1 colored according to significance of association

3.b.2 AIM TWO: Major Task 1: To identify a metabolomic profile of pulmonary function within a population of Veterans

3.b.2a Manuscripts

Data analyses are complete and our manuscript has now been submitted the American Journal of Respiratory and Critical Care Medicine (IF: 17.45) [**Appendix 3**]. Through dissemination of our initial findings to the wider metabolomics community, we identified a suitable replication cohort for our *AIM TWO* analysis; The European Prospective Investigation into Cancer- Norfolk Population (EPIC-Norfolk) led by Claudia Langenberg of the University of Cambridge. EPIC Norfolk includes 10,460 participants (4,868 males [46.5%] and 5,592 females [53.5%]) with a mean age of 59.7 years [SD 9.0] who had both metabolomics profiling and spirometric measures (performed by deeply inhaling and forcefully exhaling into a spirometer) of forced expiratory volume-one second (FEV_1) and the percent of the forced vital capacity or lung size (FVC) that can be exhaled in one second (FEV_1/FVC ratio). Replication of key findings remains a bottle-neck in metabolomics research, and therefore the ability to validate our results in a comparable population represents a significant strength of these analyses. Given the larger sample size of EPIC-Norfolk, it was determined that this would act as the discovery population, and the NAS as the replication population. To be entirely concordant with the analyses run in EPIC-Norfolk, a subset of men from the NAS (n=439) were used for these analyses, analyses were based on a single time point for each man, and only FEV_1 and FEV_1/FVC ratio were considered as endpoints (even though we additionally have FVC for the men in NAS, and focus on this endpoint in other work, see ***section 3.b.1c***). A total of 693 metabolites were measured in both the EPIC-Norfolk and the NAS populations. As NAS is a male-only population while EPIC-Norfolk includes both males and females, we ran the analysis in EPIC-Norfolk both in the full population and in the EPIC-Norfolk males only, and tried to replicate both sets of results. In this study, we identified and replicated blood metabolites that were associated with reduced lung function. Our replicated hits were characterized by metabolites involved in oxidative stress, which is in keeping with evidence suggesting lungs are particularly susceptible to an imbalance of reactive oxidative species and antioxidants influences. We were able to validate 34 FEV_1 associated metabolites and 6 FEV_1/FVC associated metabolites. The manuscript is summarized in its

abstract as shown below, and further information can be found in the figures and tables of the manuscript [**Appendix 3**], as listed:

Abstract

Rationale: The biochemical mechanisms underlying lung function level are not fully understood.
Objectives: This study aims to identify and validate the plasma metabolome of lung function in adulthood utilizing two independent cohorts: Discovery - the European Prospective Investigation into Cancer Norfolk (EPIC-Norfolk, n=10,460) and Replication - a subset of men from the VA Normative Aging Study (NAS, n=437).
Methods: We ran independent linear regression models for 693 metabolites that could be profiled in both cohorts to identify associations with forced expiratory volume in one second (FEV_1) and the ratio of FEV_1 to forced vital capacity (FEV_1/FVC), in EPIC-Norfolk and then replicated the significant findings in NAS.
Measurements and Main Results: Of the 156 metabolites that associated with FEV_1 in EPIC-Norfolk at an effective number of tests threshold of 95% after adjustment for age, sex, BMI, height, smoking and asthma status, 34 (21.8%) were validated in NAS, including a number of metabolites involved in oxidative stress. When restricting the discovery sample to men only, 18 of 79 significant metabolites (22.8%) were validated. A smaller number of metabolites were validated for FEV_1/FVC, 6 of 65 (9.2%) replicated when including all EPIC-Norfolk as the discovery population, and 2 of 34 (5.9%) when restricting to men. These metabolites were characterized by their involvement in respiratory track secretants. Interestingly, there was no crossover in the metabolites validated for FEV_1 and FEV_1/FVC.
Conclusions: The validation of metabolites associated with respiratory function can help to better understand mechanisms of lung health and may assist the development of biomarkers.

Tables and Figures

Table 1. FEV1 associated metabolites validated in the VA Normative Aging Study

Table 2: FEV_1/FVC -associated metabolites validated in the VA Normative Aging Study

Figure 1: Manhattan plot demonstrating the strength of association between FEV1 and 693 metabolites in EPIC-Norfolk, colored according to Metabolon Superpathway

Figure 2: Beta Coefficients for every metabolite association with FEV_1 and FEV_1/FVC in EPIC-Norfolk colored according to significance of effect

Figure 3: Effect estimates and 95% confidence intervals in the validated lung function associated metabolites: (A) EPIC-Norfolk estimates for 34 FEV1 validated metabolites, (B) NAS estimates for 6 FEV_1 validated metabolites, (C) EPIC-Norfolk estimates for 34 FEV_1/FVC validated metabolites, (D) NAS estimates for 6 FEV_1 validated metabolites

Supplementary Tables and Figures

Table E1. Baseline characteristics of included EPIC-Norfolk participants

Table E2. Baseline characteristics of included participants in NAS

Table E3: Pathways identified as significant in the Pathway analysis based on the ENT95% associated metabolites from EPIC-Norfolk for FEV1 and FEV_1/FVC Ratio

Table E4. Metabolites associated with FEV_1 at a threshold of ENT95% in the total sample, in men or in women

Table E5. Metabolites associated with FEV_1/FVC at a threshold of ENT95% in the total sample, in men or in women

Figure E1: Venn Diagram displaying the cross-over in significant metabolites between the Total EPIC-Norfolk population, Men only and Women only at a significance threshold of p<ENT95%

Figure E2. Volcano plot demonstrating the FEV_1-metabolite associations in EPIC Norfolk in (A) the total population and (B) Men only, indicating those which replicated in the NAS

Figure E3: Manhattan plot demonstrating the strength of association between FEV_1/FVC ratio and 693 metabolites in EPIC-Norfolk

Figure E4: Venn Diagram displaying the cross-over in significant metabolites between the Total EPIC-Norfolk population, Men only and Women only at a significance threshold of p<ENT95%

Figure E5. Volcano plot demonstrating the FEV_1/FVC ratio-metabolite associations in EPIC Norfolk in (A) the total population and (B) Men only, indicating those which replicated in the NAS

Figure E6: Effect estimates and 95% confidence intervals in the validated lung function associated metabolites (A) EPIC-Norfolk Male estimates for 18 FEV1 validated metabolites, (B) NAS estimates for 18 FEV_1 validated metabolites, (C) EPIC-Norfolk Male estimates for 2 FEV_1/FVC validated metabolites, (D) NAS estimates for 2 FEV_1 validated metabolites

3.b.2b Development of a predictive score

To determine the translatable utility of these validated metabolites we further generated a metabolomic score that can be used to discriminate men by their degree of lung function (subtask 2), as defined by FEV_1/FVC ratio. In a clinical setting FVC, FEV_1 and FEV_1/FVC ratio are compared to reference values based on healthy individuals with normal lung function, to determine the degree of lung function of the patient. The normal value for the FEV_1/FVC ratio is 70% or above, with a lower measured value corresponding to a more severe lung abnormality. In this population, 144 (30.7%) men had a ratio ≤70%, while 325 (69.3%) had a ratio >70%.We compared models for the discrimination of an FEV_1/FVC ratio above and below 70% using receiver operator characteristic (ROC) cures and the corresponding area under the curves (AUC) to determine whether the validated metabolites improved prediction; Model 1: a baseline model including standard epidemiological characteristics; age, height and smoking status; and Model 2: the baseline model plus levels of the six validated metabolites identified in our submitted manuscript [**Appendix 3**] for FEV_1/FVC. The sensitivity and specificity were computed based on the optimal cut-off to maximize sensitivity and specificity weighting both equally, as determined using the 'ROCR' package in R. Model 1 had moderate discriminatory ability in a Receiver Operator Characteristic (ROC) curve analysis (AUC: 0.606 (95% CI 0.546, 0.665) while the Model 2 based on six metabolites demonstrated a very marginal, and non-significant, improvement in the AUC (0.617 (95% CI 0.581, 0.680) (**Figure 4**). The sensitivity at the optimal cut-off was higher for Model 1(65.8% versus 60.5%) but the specificity was higher for Model 2 (60.6% versus 53.5%) (**Table 7**). Taken together these results suggest that a biomarker FEV_1/FVC based on the validated metabolites would have little clinical predictive ability beyond that of standard epidemiological characteristics. For this reason, we are not including these

results in a published manuscript, but rather we argue the greater utility of metabolomics for lung function may be in the understanding of biology and mechanisms, rather than in the development of biomarkers.

Figure 4: ROC Curve comparing the AUC of Model 1 (blue) to Model 2 (red) for prediction of an FEV_1/FVC ratio below 70%

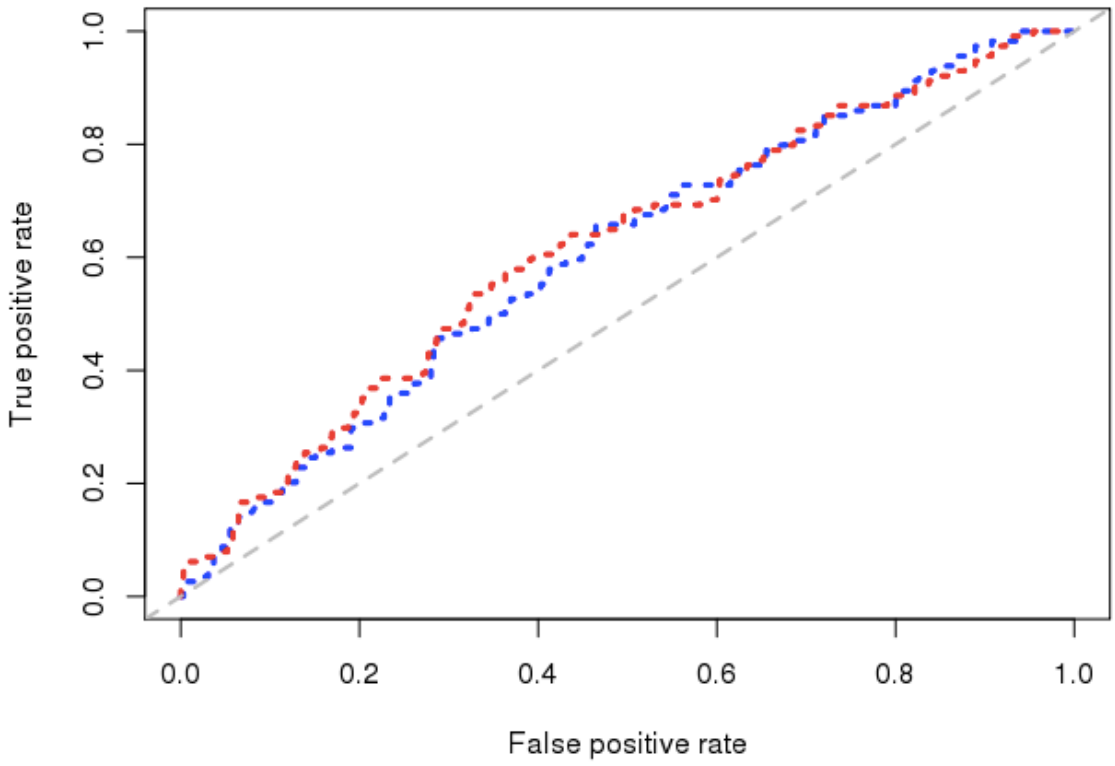


Table 7: AUCs, Sensitivity and Specificity for two models for the prediction of an FEV_1/FVC ratio below 70%

Classifier	AUC (95% CI)	Performance compared to Model 1	Sensitivity	Specificity
Model 1: Baseline Model	0.606 (0.546, 0.665)		65.8%	53.5%
Model 2: Baseline Model + levels of six validated metabolites	0.617 (0.581, 0.680)	*p=0.460*	60.5%	60.6%

Next we compared the predictive ability of a metabolite model for FEV_1. As there is no clinically defined threshold, we compared the ability of a metabolite model to predict whether an individual's FEV_1 is above or below the median for that population. In this analysis the median was 2.5L. For this analysis, Model 1 was the same as for the FEV_1/FVC analysis: a baseline model including standard epidemiological characteristics; age, height and smoking status; Model 2: was the baseline model plus a summary score based on the first five principal components of the 34 validated metabolites for FEV_1. In this analysis the metabolite Model 2 significantly outperformed the baseline Model 1 (**Figure**), with an AUC of 0.804 (0.763, 0.844). Although both models displayed good sensitivity, the specificity was better for the Model 2 (**Table 8**). This is in keeping with other results suggesting FEV1 is more strongly reflected in the metabolomics profile than FEV_1/FVC, and indicates there may be a potential for biomarker development for FEV_1.

Figure 5: ROC Curve comparing the AUC of Model 1 (blue) to Model 2 (red) for prediction of an FEV_1 above the median

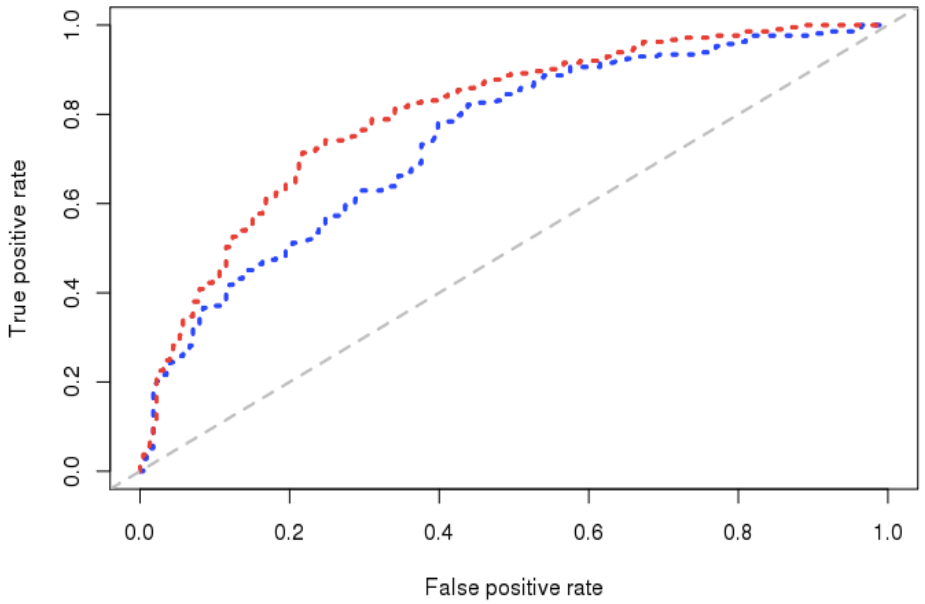


Table 8: AUCs, Sensitivity and Specificity for two models for the prediction of an FEV_1 above the median

Classifier	AUC (95% CI)	Performance compared to Model 1	Sensitivity	Specificity
Model 1: Baseline Model	0.744 (0.698, 0.789)		77.9%	60.2%
Model 2: Baseline Model + levels of six validated metabolites	0.804 (0.763, 0.844)	*p=2.8x10-4*	74.2%	75.2%

AIM THREE: Major Task 1: Identify metabolites, metabolite networks and metabolomic pathways along the causal pathway from Pb exposure to reduced pulmonary function

3.c.1 Individual metabolites associated with lung function and Pb exposure

In our manuscript pending submission Metabolomics as a means to explore the relationship between lead (Pb) exposure and lung function [**Appendix 2**], we first explored the relationship between Pb exposure as measured in both toenail and blood with the two metrics of lung function included in **Appendix 3**; FEV_1 and FEV_1/FVC. Although there was a clear trend of increased Pb levels correlating with decreased lung function, this relationship did not reach significance [Appendix 2, Supplementary Table S1 and Supplementary Table S2]. As such we could no conduct a formal mediation analyses. Therefore, Aim Three is focused on those metabolites that were significantly associated with both Pb exposure and lung function, and network analyses to identify metabolites modules associated with these exposures and outcomes. Full results are reported in **Appendix 3**, see section ***3.b.1c*** for details

Interestingly, it was uniformly observed that metabolites that were inversely associated with lung function – i.e. those which were increased with decreased lung function, were positively associated with blood Pb. In others words, these results supported our hypothesis that increased blood levels lead to decreased lung function. These are novel findings in a human population, which are supported by experimental evidence in the literature. Pathway analysis was performed with MetaboAnalyst v.4.0 (www.metaboanalyst.ca) to identify the KEGG defined (www.genome.jp/kegg/pathway.html) metabolomic pathways that the metabolites associated with each of the four end points of interest were enriched for. Pathway analysis extends and enhances the concept of metabolite set enrichment analysis by incorporating topology analysis. This evaluates the importance of a given metabolite based on its position within a pathway using graph theory, and therefore provides a more meaningful interpretation of list of differential metabolites. In this analysis, the hypergeometric test was specified for the over-representation analysis and relative-betweenness centrality was specified for the pathway topology analysis; all measured metabolites included in a given analysis were input as the reference metabolome.

It can be seen that a number of metabolomic pathways were significantly enriched for each of the endpoints, and that many of these pathways were the same between the endpoints, suggesting they may be involved in the pathological effects of Pb exposure on lung function (**Table 9**). Valine, leucine and isoleucine biosynthesis was associated with all four endpoints, this could be involved in pathogenesis through its role as a mediator of oxidative stress and the immune response. After discussion with co-authors these pathway analyses were not included in the final manuscript, but are included here for information.

Table 9: Metabolomic Pathway analysis of metabolites identified as being significantly dysregulated by degree of lung function and by blood and toenail Pb levels measured concurrently

A red square indicates that a pathway was significantly enriched among the metabolites identified as being significantly associated with the phenotype. It can therefore by hypothesized that this pathway is dysregulated with changes in the relevant phenotype

KEGG Defined Pathway	Blood Pb (µg/mL)	Toenail Pb (µg/g)	FEV1 (L)	FEV1/FVC (%)
Alanine, aspartate and glutamate metabolism	■			
Amino sugar and nucleotide sugar metabolism		■	■	
Aminoacyl-tRNA biosynthesis	■			
Arginine and proline metabolism	■	■		■
Arginine biosynthesis	■	■		■
Ascorbate and aldarate metabolism	■	■		
beta-Alanine metabolism	■			
Caffeine metabolism	■			
D-Arginine and D-ornithine metabolism	■			
D-Glutamine and D-glutamate metabolism	■			
Fatty acid degradation		■	■	
Galactose metabolism		■		■
Glycerophospholipid metabolism	■	■		■
Histidine metabolism	■	■		
Inositol phosphate metabolism		■		
Linoleic acid metabolism	■			
Nicotinate and nicotinamide metabolism	■	■		
Pentose phosphate pathway		■		
Phenylalanine, tyrosine and tryptophan biosynthesis		■	■	
Phosphatidylinositol signaling system		■		
Pyrimidine metabolism	■	■		
Sphingolipid metabolism	■	■		■
Sulfur metabolism	■	■		
Valine, leucine and isoleucine biosynthesis	■	■	■	■

FEV1 – measure of how much air can be exhaled in one second following a deep inhalation
FEV1/FVC – represents the percent of the lung size (FVC) that can be exhaled in one second

3.c. Opportunities for training and professional Development Provided by the Project

Throughout this final reporting period, Dr. Lasky-Su continued to closely mentor Dr. Kelly. This grant has also been instrumental in providing Dr. Kelly with the opportunity, time and appropriate dataset to mentor early-stage investigators. Specifically, Dr. Kelly acted as the primary mentor for Ms. Haley Bayne, a summer intern who is an undergraduate student at Northeastern University, who under the supervision and guidance of Dr. Kelly performed a portion of the statistical analysis for the metabolome of pulmonary function (*AIM TWO*) manuscript [**Appendix 3**]. Ms. Bayne was interning at the Channing though the Northeastern Cooperative Education Program (https://careers.northeastern.edu/cooperative-education/); and her time at the Channing Division of Network Medicine (CDNM) provided her with the

opportunity to experience a real life research environment, foster her analytical, statistical and coding skills and to present her work at an International Conference (*see* ***section 3.d***). Ms. Bayne has since decided to pursue a career in academic scientific research, and has recently been awarded the 2020 Muckenhoupt Scholarship, which is focused on environmental research, largely through her work on the metabolome of Pb.

Furthermore, this grant has helped to propel Dr. Kelly from a postdoc to a junior faculty member (currently Instructor and now in the process of promotion to Assistant Professor) in the Channing Division of Network Medicine, as she utilized the grant writing and project management skills developed throughout this period to be successfully awarded a K01 training grant (NHLBI, K01 HL146980) also focused on metabolomics and respiratory disease. Together Dr. Lasky-Su and Dr. Kelly have additionally successfully been awarded two others R01 grants focused on metabolomics and respiratory health grant (NHLBI, R01 HL141826 and R01HL123915). The results from this current award have been instrumental in providing preliminary data for these grant applications.

Finally, the dissemination of these results, specifically those related to the metabolome of Pb, led to Dr. Kelly being invited to be on the Editorial Board of a special issue of the journal Frontiers in Public Health titled "Metabolomics and the Exposome".

3.d. Dissemination of results to communities of interest

Multiple presentations relating to this proposal, including theoretical study design and preliminary findings have been presented to colleagues in the fields of metabolomics and respiratory health within the Channing Division of Network Medicine. These findings have also been presented to the wider National and International Community as follows:

"Metabolomics of Lead Exposure and Its Role in Respiratory Disease among Veterans"
Research, Innovation, and Scholarship Expo (RISE) 2019;
Boston MA, April 4th 2019
Presenter: Haley Bayne

"Metabolomics of Lead Exposure and Its Role in Reduced Lung Function Among Veterans"
Advances in Clinical Lung Research
Boston MA, May 8th 2019
Presenter: Rachel Kelly

"The Metabolomics of Lead Exposure and Its Role in Respiratory Disease"
American Thoracic Society 2019 International Conference
Dallas TX, May 17-22nd 2019
Presenter: Rachel Kelly

"Metabolomics of Poor Respiratory Health"
15th Annual Conference of the Metabolomics Society
The Hauge, The Netherlands, June 23-27th 2019
Presenter: Haley Bayne

Furthermore, results from this project formed part of Dr. Lasky-Su's invited plenary talk at the first ever Metabolomics Association of North America Conference, in Atlanta in November 2019.

Planned submissions to international conferences in 2020 have been postponed due to the COVID-19 outbreak.

3.e. Future plans

Following the completion of this grant, we shall continue to develop our mental health and metabolomics work within the NAS Metabolomics Cohort, and to work with our collaborators on the metabolome and air pollution. Pending the evolving situation, we also aim to present our novel findings at pertinent conferences in 2021.

Furthermore, throughout this award period we have been furthering our involvement in the International Metabolomics community; Dr. Jessica Lasky-Su is chair of the Consortium of Metabolomics Studies (COMETS) committee and a board member of the Metabolomics Society. Dr. Kelly is a steering committee member of COMETS, an executive committee member of the ATS Genetic and Genomic Section, representing metabolomics, and together they are co-leading the first international metabolomics meta-analysis within COMETS with a focus on body mass index. We will continue to work with the metabolomics community to develop novel methodologies for the analysis of metabolomics data, to ensure can successfully complete our aims and drive forward the field.

4. Impact

4.a. Impact on the development of the principal discipline of the project

The principal discipline of this project is the field of metabolomics. The overarching goal is to construct a casual pathway between Pb exposure and poor respiratory health in a cohort of veterans, with the hypothesis that this pathway is mediated through measurable metabolomic pathways. To do so required the development of novel analytical and statistical techniques with a focus on network methodology. We worked closely with our bioinformatics and network scientist colleagues to develop the appropriate methodologies. These techniques will then be applicable to a wide range of projects in the field of metabolomics, where the construction of causal pathways mediated by metabolites is a key, but as yet unachieved, goal for many.

4.b. Impact on other disciplines

In *Specific aim 1* (**Table 1a**), we proposed to identify the metabolome of past Pb exposure that takes both duration and intensity into account. Our initial findings have been very promising in this area and, as such, support the utility of metabolomics in the disciplines of exposure science and exposure biomarker development. Exploring respiratory disease through metabolomics is more established. Although we did not observe substantial utility in the development of metabolomic biomarkers of lung function, these results also add to the discipline of respiratory health, though (i) the addition of novel literature in an underrepresented population, and (ii) supportive mechanistic evidence for a link between Pb exposure and poor respiratory health.

As described in detail in section **6.e.,** throughout the reporting period we have also built up new collaborations with existing NAS researchers, who are keen to utilize the novel cohort we have generated 'The NAS Metabolomic Cohort' explore research questions within their own disciplines, namely *healthy ageing*, *the health impacts of air pollution* **[Appendix 4&5]** and *the biology of cognitive function and mental health* (analyses ongoing).

4.c. Impact on technology transfer

Nothing to report

4.d. Impact on society beyond science and technology

We have demonstrated evidence to support the role of dysregulated metabolism with both Pb exposure and poor respiratory health, as outlined in our proposal. As such the results of this study will be of great importance to the growing population who were exposed to Pb during active service in the Gulf, Iraq and Afghanistan, as well as to the wider US population in whom Pb exposure still widespread. The findings will support **(i) Prevention:** by supporting improved regulations and safeguards pertaining to Pb exposure in the military **(ii) Early intervention** through the development of markers in the blood that can be used to identify harmful levels of Pb exposure **(iii) Treatment of Pb induced pulmonary dysfunction** through the identification of metabolites that are affected by Pb exposure and influence respiratory health, which can then be targeted by novel drugs and therapies.

5. Changes/Problems

5.a Changes in approach and reasons for change

As outlined in our previous reports, there was a six month delay in the receipt of HRPO approval, and therefore in the start of the project. It was ultimately concluded that "the activities conducted by the Partners investigators do not constitute human subjects research". As such no new IRB approval was required, and the project could proceed. This delay did not result in any changes to our approach or analytical plans, and has not influenced this previous reporting period or the overall success of this project.

5.b. Changes that had a significant impact on expenditures

As outlined in our previous reports, due to price negotiations with Metabolon Inc., who performed the metabolomic profiling, we were able to profile 661 samples, rather than the anticipated 374, for the same cost. This increased the power and impact of our proposal. *Further details in Accomplishments. Section 3.a*

5.c. Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report

6. Products, Inventions, Patent Applications, and/or Licenses

6.a Publications, conference papers, and presentations

Published Manuscripts

Rachel S. Kelly; Haley Bayne; Avron Spiro, II; Pantel Vokonas; David Sparrow; Scott T. Weiss; Joel Schwartz; Feiby L. Nassan; Kathleen Lee-Sarwar; Mengna Huang; Priyadarshini Kachroo; Su H. Chu; Augusto A. Litonjua; Jessica A. Lasky-Su **Metabolomic Signatures of Lead Exposure in the VA Normative Aging Study.** *Environmental Research* (2020) [**Appendix 1**]

Submitted Manuscripts

Rachel S. Kelly*, Haley Bayne*, Isobel Stewart, Priyadarshini Kachroo, Avron Spiro, III; Pantel Vokonas; David Sparrow; Scott T. Weiss; Hanna M. Knihtilä; Augusto A. Litonjua; Nick Wareham, Claudia Langenberg, Jessica A. Lasky-Su **Metabolomic Differences in Lung Function Metrics: Evidence from two Cohorts.** *American Journal of Respiratory and Critical Care Medicine* [**Appendix 3**]

Feiby L. Nassan*, **Rachel S. Kelly***, Petros Koutrakis, Pantel S. Vokonas, Jessica A. Lasky-Su, Joel D. Schwartz **Metabolomics signatures of the short-term exposure to air pollution and temperature** *Environmental Health Perspectives* [**Appendix 4**]

Feiby L. Nassan, **Rachel S. Kelly**, Anna Kosheleva, Petros Koutrakis, Pantel S. Vokonas**,** Jessica A. Lasky-Su, Joel D. Schwartz **Metabolomics signatures of the long-term exposure to air pollution and temperature** *Environmental Health & Risk Assessment* [**Appendix 5**]

Pending Manuscripts

Rachel S. Kelly [1*], Haley Bayne [1], Avron Spiro 2nd [2], Pantel Vokonas [3], David Sparrow [3], Priyadarshini Kachroo[1], Scott T Weiss [1], Joel Schwartz [4], Feiby L Nassan [4], Isobel Stewart[5], Claudia Lagenberg[5], Meryl Stav [1], Margaret Cote [1], Augusto Litonjua[6], Jessica A Lasky-Su [1] Metabolomics as a means to explore the relationship between lead (Pb) exposure and lung function. *Metabolites* **[Appendix** 2**]**

**co-first*

Additional manuscripts within the NAS metabolomics cohort focused on air pollution, aging and cognitive function and planned.

Conferences

This work has been presented at four National and International conferences to date (*see* ***section 3.d****, for details*), as noted planned submission of findings to conferences in 2020 (ATS 2020 and the International Metabolomics Society 2020) have been postponed due to COVID-19. We anticipate we will present some of this work at the 2021 conferences, if these conferences go ahead as planned

6.b. Websites or Other Internet Sites

Nothing to report

6.c. Technologies or techniques

Novel statistical techniques under development (see *Impact* ***section 4.a***)

6.d. Inventions, patent applications, and/or licenses

Nothing to report

6.e Other Products

Comprehensive Metabolomic Dataset. "*The Normative Aging Study Metabolomic Cohort*". The generation of a database of 661 longitudinal plasma samples with metabolomic profiling from 464 military veterans with measures of Pb exposure and lung function, as well a host of other longitudinal phenotypes and data collected on these individuals since the NAS was established in 1961, provides an invaluable research resource beyond the scope of this current project. This database will allow us to address a large number of research questions and provide a valuable validation population for an ongoing project exploring the link between the metabolome and body mass index. Further, we have been working with other collaborators from the Normative Ageing Study regarding a number of projects, exploring the (i) metabolome of Aging; (ii)The metabolome of Pb and its impact on cognitive function and (iii) The impact of air pollution on the metabolome (see manuscripts in ***section 6.a***). We anticipate we will continue develop these collaborations further beyond the timeline of this grant, and that they will result in additional manuscripts and conference presentations, as well as further projects levering the available metabolomics data.

7. Participants & Other Collaborating Organizations

7.a. Individuals who have worked on this project

Name:	*Jessica Lasky-Su*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	*0000-0001-6236-4705*
Nearest person month worked:	*3 person months*
Contribution to Project:	*Dr. Lasky-Su is the PI of this project and has overseen all aspects and in particular the management of the budget and working with Metabolon to negotiate metabolomic profiling prices. Dr. Lasky-Su has worked directly with Dr. Kelly on the data analysis and the manuscripts, and is helped to disseminate the findings via her plenary talk at the Metabolomics Society of North America (MANA) inaugural conference in November 2019*
Funding Support:	*This DOD award*

▪

Name:	*Rachel Kelly*
Project Role:	*Co-PI*
Researcher Identifier (e.g. ORCID ID):	*0000-0003-3023-1822*
Nearest person month worked:	*3 person months*
Contribution to Project:	*Dr. Kelly is the co-PI of this project. She worked with collaborators at the Normative Ageing study to collect the samples, applied the QC and data processing to the data and led the data analysis and manuscript preparation. Dr Kelly was selected by the Environmental and Population Health Assembly of the American Thoracic Society to give an oral presentation of these findings to the to the American Thoracic Society Annual conference in Dallas in May 2019. She is now leading additional analyses of the NAS Metabolomic Cohort focusing on the metabolome and mental health in a population of Veterans*
Funding Support:	*This DOD award*

Name:	*Haley Bayne*
Project Role:	*Intern*
Researcher Identifier (e.g. ORCID ID):	*bayne.h@husky.neu.edu*
Nearest person month worked:	*3 person months*
Contribution to Project:	*Ms. Bayne was a summer intern under the supervision of Dr.'s Kelly and Lasky-Su who performed statistical analyses for AIMs 1 and 2, which formed the basis of the first publication. Additionally, Ms. Bayne assisted with drafting this manuscript and the pending Pb manuscript. Ms. Bayne presented this work at both the RISE Expo, and at the International Metabolomics Society meeting.*
Funding Support:	*Northeastern Cooperative Education Program*

7.b. Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report

7.c. What other organizations were involved as partners?

Nothing to Report