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EXAMINATION OF URINARY β-NAPHTHOL AS A BIOMARKER INDICATIVE OF JET FUEL EXPOSURES

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Air Force Research Laboratory
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14. ABSTRACT A joint US Air Force/Japan Air Self-Defense Force (JASDF) study was conducted to examine molecular responses in a human cohort occupationally exposed to either JP-4 or JP-8. In this sub-study, we examined the utility of urinary beta-naphthol (uβNph) as a biomarker of exposure to jet fuels. Beta-naphthol is a metabolic product of naphthalene, a poly aromatic hydrocarbon found in jet fuel. Published studies using human occupational exposure samples indicated that urinary increases in βNph correlate well with jet fuel exposure levels. This study used a β-naphthol competitive enzyme-linked immunosorbent assay (ELISA) to quantitate urine samples taken from subjects (18-50 yrs old) prior to shift or immediately post-shift. Exposed group subjects worked in occupations (crew chief, flight line personnel) with likely jet fuel exposures, while the control group was matched for location but in occupations with no expected exposure (office workers). Both JP-4 and JP-8 exposure/control group urine samples were collected and analyzed for β–Nph as well as uβNph - urinary βNph normalized with creatinine levels to account for urine volume dilution. Statistical analyses of the meta data and uβ–Nph included standard analysis of variance, Mann-Whitney Rank Sum test, factor analysis, dimensionality assessment, and correlation figures. Unlike other studies, we did not see any correlation to exposure group, nor did we see indications that smoking was a confounding factor in our analyses. However, our data suggested that age does correlate with β–Nph levels. Further studies using a larger cohort and accurate quantitation of jet fuel dose should clarify issues seen in this study with the use of uβ–Nph as a urinary biomarker of jet fuel exposure.

15. SUBJECT TERMS
Jet fuel exposure, urinary, biomarker, βNph, uβNph, beta naphthol, naphthalene, aromatic hydrocarbon, PAH

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PREFACE

This research was accomplished at the Molecular Bioeffects Branch, Human Effectiveness Directorate of the 711th Human Performance Wing (711 HPW/RHDJ) of the Air Force Research Laboratory (AFRL), Wright-Patterson AFB, OH, Dr. John J. Schlager, Branch Chief. This interim technical report was written for the Mixtures Toxicology Work Unit 7757HD05 from the Aerospace Toxicology program at 711 HPW/RHDJ.

The research reported here is part of a study which completed phase two of a cooperative research project conducted under a Memorandum of Understanding between the Department of Defense of the United States of America and the Ministry of Defense of Japan. The program managers for the Memorandum of Understanding are Asao Kobayashi, PhD for JASDF/AML and David Mattie, PhD for 711 HPW/RHDJ. This international agreement "*The Human Effects of Exposure to Aviation Jet Fuels, JP-4 and JP-8, and Their Engine Exhaust*," was a scientific collaboration between the Molecular Bioeffects Branch (711 HPW/RHDJ) and Japan Air Self-Defense Force, Aeromedical Laboratory (JASDF/AML). Funding for this project was equally provided by JASDF and USAF.

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The authors would like to acknowledge and thank the military members of JASDF and the USAF who served as subjects and who assisted in the completion of this study.

SUMMARY

A joint US Air Force/Japan Air Self-Defense Force (JASDF) study was conducted to examine molecular responses in a human cohort occupationally exposed to either JP-4 or JP-8. In this sub-study, we examined the utility of urinary beta-naphthol (uβ-Nph) as a biomarker of exposure to jet fuels. Beta-naphthol (β-Nph) is a metabolic product of naphthalene, a poly aromatic hydrocarbon found in jet fuel. Published studies using human occupational exposure samples indicated that urinary increases in β-Nph correlate well with jet fuel exposure levels. This study used a β-Nph competitive enzyme-linked immunosorbent assay (ELISA) to quantitate urine samples taken from subjects (18-50 yrs old) prior to shift or immediately post-shift. Exposed group subjects worked in occupations (crew chief, flight line personnel) with likely jet fuel exposures, while the control group was matched for regional work locations but in occupations expected to have no exposure (office workers). Both JP-4 and JP-8 exposure/control group urine samples were collected and analyzed for β-Nph as well as uβ-Nph - urinary β-Nph normalized with creatinine levels to account for urine volume dilution. Statistical analyses of meta data and values acquired for uβ-Nph included standard analysis of variance, Mann-Whitney Rank Sum test, factor analysis, dimensionality assessment, and correlation figures. Unlike other studies, we did not see any correlation to exposure group, nor did we see indications that smoking was a confounding factor in our analyses. However, our data suggested that age does correlate with β-Nph levels. Further studies using a larger cohort and accurate quantitation of jet fuel dose should clarify issues seen in this study with the use of uβ-Nph as a urinary biomarker of jet fuel exposure.

Key Words: JP-8; JP-4; jet fuel; beta-naphthol; β-naphthol; 1-naphthol; 2-naphthol; naphthalene; biomarker; fuel exposure; urine

Interim report February 2014 to February 2015

1. INTRODUCTION

Naphthalene Metabolism

Naphthalene is one of many chemicals which comprise JP-8 jet fuel, and is present at about 0.175%.[1] With JP-8 exposure, naphthalene can enter the body by inhalation and by contact with the skin, where it easily enters the bloodstream.[2] Once inside the body naphthalene is oxidized to naphthalene 1,2-oxide by the cytochrome P450 monooxygenase system (**Fig. 1**). This reaction occurs primarily in the liver, although oxidation can also occur in extrahepatic organs such as the brain, lung, and kidney.[3] Naphthalene 1,2-oxides can reaction directly thiol such as glutathione or in protein, by conjugated enzymatically to glutathione-S-transferase, or spontaneously convert to either 1-naphthol (α-naphthol) or 2-naphthol (β-naphthol). 1 Naphthol can be converted to further to 1,4-naphthoquinone (structure not shown) or, like 2-naphthol, enzymatically conjugated with either sulfate or glucuronide, and excreted into the urine as either free compound or as a glucuronide or sulfate conjugate (structures not shown).

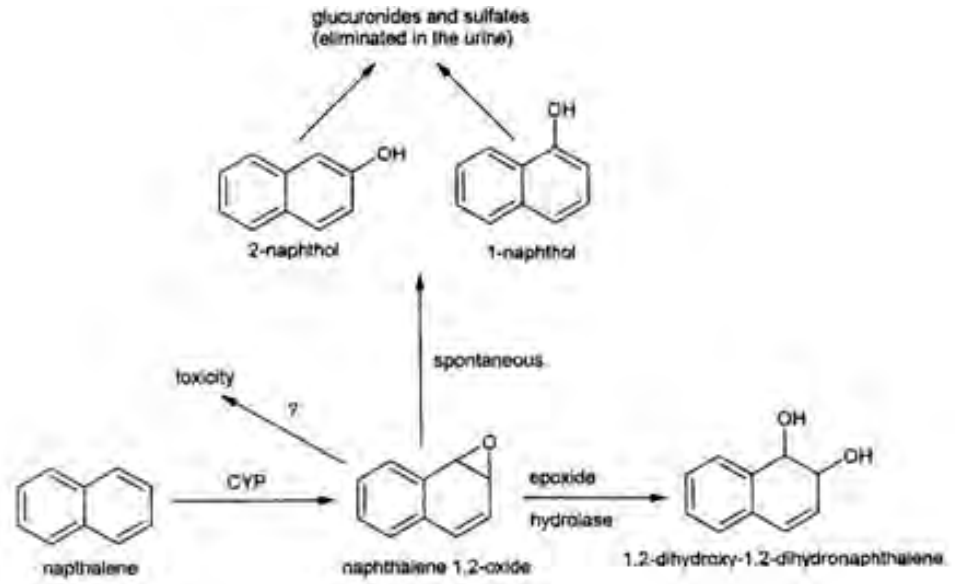

Figure 1. Metabolic conversion of naphthalene. *Figure adapted from ATSDR 2003***.**

Naphthols in Urine

Urinary 1- and 2- naphthols are currently studied for use in individual PAH exposure biomonitoring as biomarkers of exposure to polyaromatic hydrocarbons (PAHs). As such, the reference value has been defined for 1-naphthol as 23 ug/L and for 2-naphthol as 28 ug/L,

exhibiting from a 95% confidence interval for the 95th percentile of population.[4] For the reference value, only data generated from samples exhibiting normal urinary dilution as defined by creatinine levels between 0.5-2.5 g/l, were used in the calculations.[5]

Urinary Naphthol Biomarkers of Poly Aromatic Hydrocarbons

PAHs are found in jet fuels in various concentrations and of these, naphthalene is present in the highest concentration.[6] The use of 2-napththol, also called beta-naphthol (β-Nph) has been examined as a urinary surrogate biomarker of jet fuel exposure as well as PAH exposure from smoking.[7-9] Strong correlations have been found between levels of JP-8 exposure and urinary β-Nph levels, and have found to track to self-reported job exposure categories.[9] β-Nph levels were also examined with meta data and separated according to cigarette smoking status, as the PAHs in cigarette smoke[10] are a confounder for data interpretation. As predicted, smokers were found to have higher β-Nph levels than nonsmokers within the same exposure group; [9] although, urinary beta-naphthol (uβ-Nph) levels were predominantly dependent on JP-8 exposure rather than smoking status. Yang et al. examined β-Nph levels in a cohort of Japanese male workers using high-resolution capillary gas chromatography/mass spectrometry/selected ion monitoring.[11] Yang found that the levels of urinary 1- and 2-naphthol were 3- and 7-fold higher in smokers versus nonsmokers, respectively. They also examined other factors (age, alcohol consumption, or specific types of food) but did not find any significant correlation of urinary 1- or 2-naphthol concentration to these meta parameters. Yang et al. also examined correlations between naphthol levels and cytochrome P450 (CYP) polymorphisms. It was found that cytochrome P450 (CYP) 1A1 exon 7 polymorphism did not correlate to urinary β-Nph levels. However, smokers with the c1/c2 or c2/c2 type of CYP2E1 demonstrated higher levels of urinary 2-naphthol than subjects homozygous for c1. In addition, subjects with the glutathione S-transferase (GST) M1 deficient type demonstrated higher levels of both 1- and 2- naphthol than those homozygous normal. Yang et al. concluded that when using urinary naphthols as biomarkers, both CYP2E1 and GSTM1 genetic types should be determined to get correct correlates to exposure.

2. MATERIALS AND METHODS

2.1 Study Design and Sample Collection

2.1.1 Approvals for Study. All human use research was reviewed and approved by the AFRL IRB under the protocol "*Human Operational Exposure to JP-4 and JP-8 Fuel (Exhaust)*" FWR20110047H under Principal Investigator David R. Mattie, Ph.D. DABT, and as 22-01-01 by the Aeromedical Laboratory Ethical Committee.

2.1.2 Human Study Design and Testing. Subjects who were potentially exposed to either JP-8 or JP-4 as part of their everyday duties were recruited, as well as matched controls (**Table 1**).[12] Control subjects were office or hospital personnel were matched to location of flight line personnel number at U.S. and JASDF air bases, and were sampled in an identical manner as flight line personnel. Subjects were volunteers who were active duty (USAF and JASDF) crew chiefs or other flight line personnel, male or female, and age range of 18-50 years.

Table 1. Cohort Tested for uβNph levels.

Airbase	Group	Potential Fuel Exposure	Number of Subjects Tested	Nationality
U.S. F-15	Exposed	JP-8	10	U.S.
	Control	none	15	U.S.
U.S. C-130	Exposed	JP-8	10	U.S.
	Control	none	10	U.S.
JASDF F-15	Exposed	JP-4	10	Japanese
	Control	none	15	Japanese

Table 2. Meta data collected on subjects.

Exposure Related	Other Possible Confounders
Career field	Age
Rank	Gender
Years of service	Smoker or nonsmoker
Work experience	Caffeine Intake
Hobbies (other fuel or solvent exposures)	
Last time fueled a government or personal vehicle and what type of fuel.	
Flight line time	
Exposure to spills (fuel)	
Exposure to skin (fuel)	
Inhalation exposure (type)	
Shift length	

2.1.3 Urine Collection and Preparation. Prior to shift and post-shift each subject provided a urine sample (entire void). Urine was aliquoted and stored at -20 ^{o}C until shipped overseas to 711 HPW/RHDJ. Samples were shipped on dry-ice in an insulated shipping container. Upon arrival at RHDJ, urine samples were stored at -80 °C until assayed. Samples were thawed on ice prior to analysis. For each subject, the pre-shift samples were used to

determine baseline β-Nph levels and the 4-6 pm post-shift samples examined for correlative increases in jet fuel exposure and β-Nph levels.

Table 3. Urine Sample collection timelines. Tested samples indicated in red.

Prior to Shift (6-8 am)	During Shift (8 am-4 pm)	Post Shift (4-6 pm)	12-16 Post Shift (6-8 am)
• First urine sample collected • personal environmental monitor attached to subject	• work duties performed as usual	• personal environmental monitor removed • 2nd urine sample collected	• 3rd urine sample collected

2.2 Quantitation of urinary β-Naphthol Levels

2.2.1 Beta-Naphthol (βNph) ELISA kit protocol. Competitive enzyme-linked immunosorbent assays (ELISA) were performed as described in the Elabscience® β-Nph (Beta-Naphthol) ELISA kit protocol (Antibodies-Online Cat. No. ABIN1113736). The Elabscience® β-Nph ELISA has a sensitivity of 0.5 ng/mL, with a range of 1.563-100 ng/mL. Urine samples were centrifuged for 20 minutes at 1000 x g and 4 °C. Fifty microliters of supernatant were added to the wells of 96-well microtiter plates pre-coated with β-Nph. Fifty microliters of biotinylated detection antibody against β-Nph were immediately added to each well and incubated for 45 minutes at 37 °C. Plates were washed 3 times with 350 uL of wash buffer. One hundred microliters of HRP-avidin conjugate were added and incubated for 30 minutes at 37 °C. Wells were washed five times with 350 uL of wash buffer. Ninety microliters of TMB substrate (3,3',5,5'-tetramethylbenzidine) were added, and plates were incubated 15 minutes at 37 °C in the dark. Reactions were stopped by the addition of 50 uL of a sulfuric acid stop solution. The absorbance (optical density [OD]) was measured at 450 nm with a microplate reader (Molecular Devices SpectraMax M2e).

2.2.2. Urinary Creatinine Assay. In order to control for variations in urine flow rate between subjects, β-Nph concentrations were reported as a normalized ratio to urinary creatinine concentration. Creatinine concentrations were determined using Eagle Biosciences, Inc. Creatinine Microplate Assays (Eagle Biosciences Cat. No. CRE34-K01), and assays were performed as described in the kit instructions. Urine samples were diluted between 1:3 and 1:175 with 18 mΩ water. Twenty-five microliters of standards or diluted sample were added to the wells of 96-well microplates provided with the kit. One hundred

and eighty microliters of alkaline picrate solution were added to each well and incubated for 10 minutes at room temperature with shaking at 75 RPM. The absorbance (optical density [OD]) was measured at 490 nm with a microplate reader (Molecular Devices SpectraMax M2[e]). After the first reading, 15 uL of acetic acid solution from the kit were added to each well. The contents of the plate were mixed thoroughly by tapping and the plate was incubated at RT for a minimum of 5 minutes. The absorbance was measured a second time at 490 nm. The OD_{490} values of the second reading were subtracted from the OD_{490} values of the first reading. The difference in absorbance is directly proportional to creatinine concentration.

2.2.3 Urinary Biomarker Baseline Normalization. In order to present urinary biomarker concentration data, a standardization method is used to adjust for variation in urine volumes. We utilized a well-known method of normalizing to urinary creatinine values, a common method used for urine volume changes due to water excretion.[13] Beta-naphthol concentrations (ng/mL) were divided by creatinine concentrations (mg/mL) and results were reported as creatinine-normalized β-naphthol, designated as uβ-Nph (ng of uβ-Nph per mg of creatinine).

2.3 Statistical Analyses

Statistical analysis of uβ-Nph data was conducted using SigmaPlot Software (Systat Software, San Jose,CA). The Shapiro-Wilk test was used to determine normality of distribution of the data set. If normality was seen, a standard analysis of variance (ANOVA) was conducted. If normality failed, Kruskal-Wallis ANOVA by ranks was used to access statistical significance of differences between the groups. Addition post-hoc tests (Dunn's) were conducted if significant differences among means were seen. For comparisons between pre-shift and post-shift samples in a single group, paired t-tests were conducted. The Shapiro-Wilk test was used to determine normality of distribution of the data set. If normality failed, the Mann-Whitney Rank Sum test was conducted to determine statistical significance. Statistical correlations (factor analysis, dimensionality assessment, and correlation figures) on meta data and uβ-Nph concentration data (Section 3.2) were conducted using MatLab R2011a (Mathworks Corporation).

3. RESULTS

3.1 Effect of Jet Fuel Exposure on uβ-Nph Levels

3.1.1 Distribution of uβ-Nph Concentration in Cohort. Distribution of uβ-Nph concentrations was graphed for all cohort Control subjects (**Fig. 2**). A slight overall increase in post-shift uβ-Nph concentrations was seen in this group. An examination of all Exposed subjects from the cohort (**Fig. 3**) indicated a tighter distribution of uβ-Nph levels with no indication of higher uβ-Nph levels at the post-shift time point. When the cohort is sorted by jet fuel exposure, the subjects in the JP-8 group seem to be fairly equivalent, with the exception of two outliers **(Fig. 4).** However, we observed an unexpected increase in the distribution of uβ-Nph within the control subjects in the JP-4 group (**Fig. 5**). To examine this increase further, additional analyses were conducted to determine the effects of age and smoking on β-Nph levels (see below).

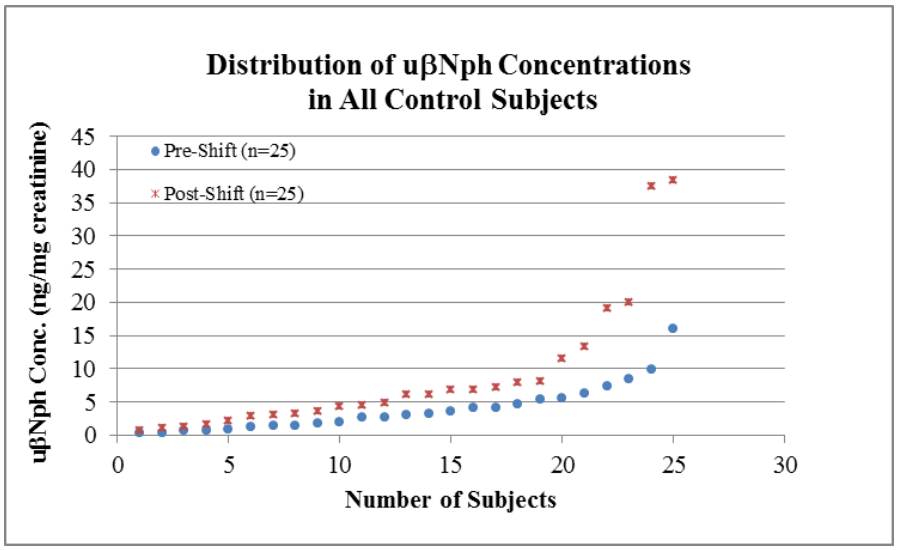

Figure 2. Distribution of pre- and post-shift uβ-Nph concentrations from all control subjects (JP-8 and JP-4).

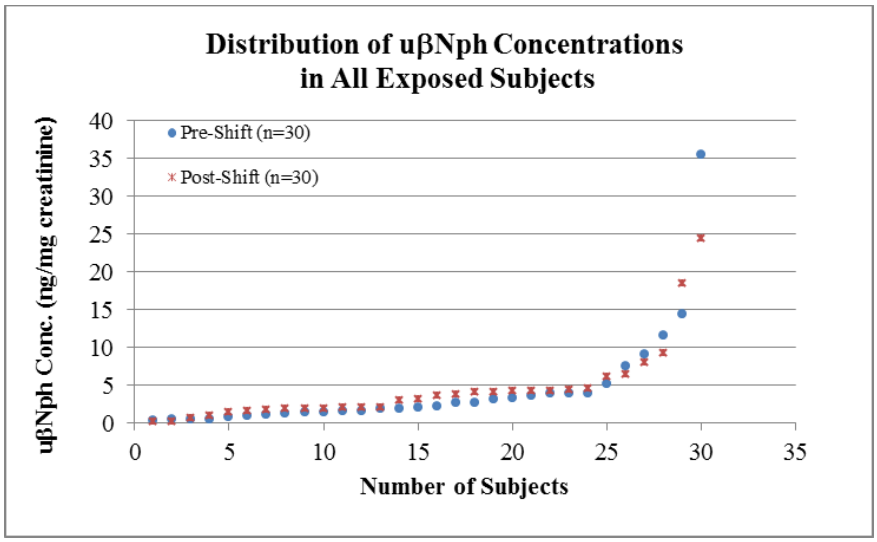

Figure 3. Distribution of pre- and post-shift uβ-Nph concentrations from all subjects (JP-8 and JP-4).

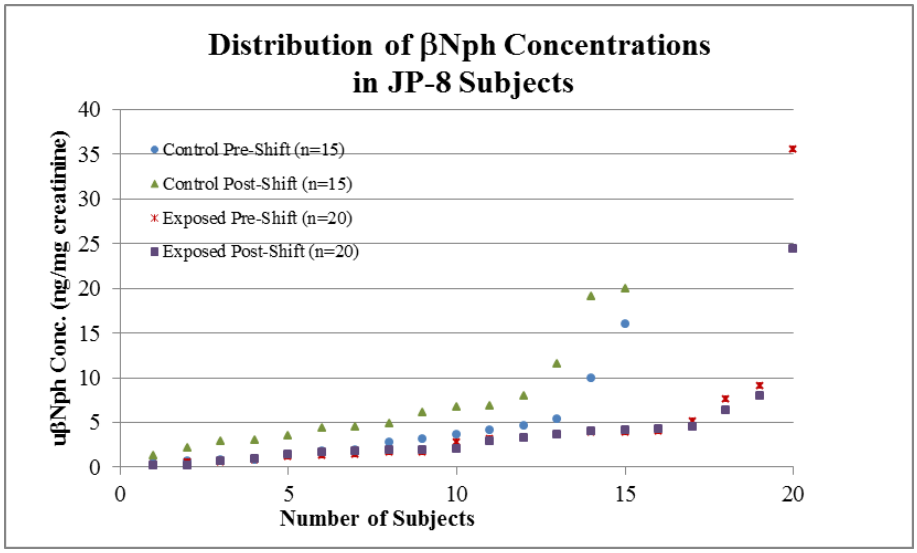

Figure 4. Distribution of pre- and post-shift uβ-Nph concentrations from all JP-8 control and Exposed subjects.

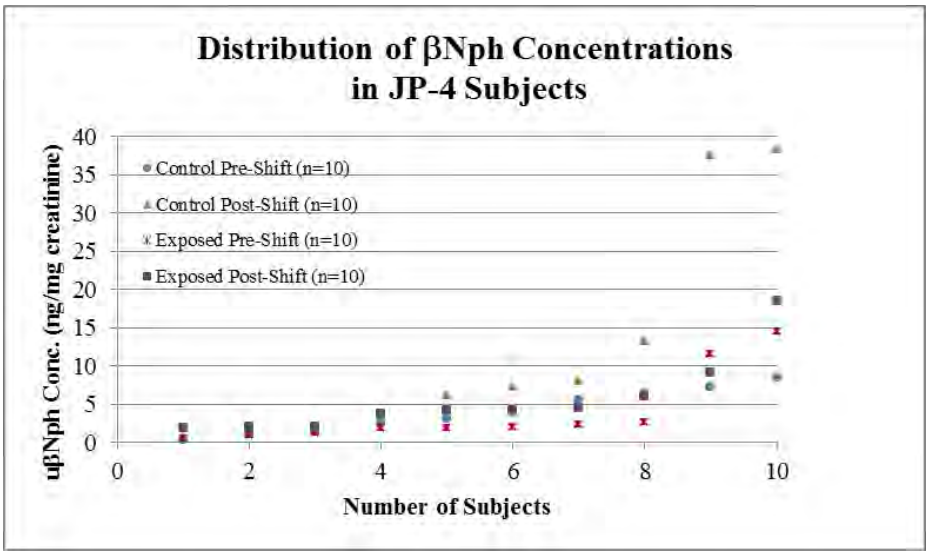

Figure 5. Distribution of pre- and post-shift uβ-Nph concentrations from all JP-4 control and exposed subjects.

3.1.2 Urinary β-Nph Concentrations in Cohort. The means of all test groups were calculated for both β-Nph and uβ-Nph (creatinine-normalized uβ-Nph) (**Table 1**). There were no obvious post-shift increases in β-Nph levels from exposure groups for either JP-8 or JP-4 using means data. Unexpectedly, the largest increases in uβ-Nph levels are seen in the post-shift JP-4 group.

Table 4. Mean of β-Nph and uβ-Nph levels in pre- and post-shift samples from control and exposed groups.

Analyte	JP-4				JP-8			
	Control		Exposed		Control		Exposed	
	Pre-Shift (n=10)	Post-Shift (n=10)	Pre-Shift (n=10)	Post-Shift (n=10)	Pre-Shift (n=15)	Post-Shift (n=15)	Pre-Shift (n=20)	Post-Shift (n=20)
β-Nph (ng/mL)	2.39	2.41	1.72	2.50	2.51	2.59	2.55	2.66
uβ-Nph (ng/mg Creatinine)	4.05	11.72	3.96	5.64	3.81	6.99	4.56	3.91

3.1.3 Statistical Analyses for Data Sets. Statistical analyses were conducted for each group (JP-8 and JP-4) to confirm or refute differences seen for each group and sub-group. For each fuel, we examined each control group for differences in pre- and post-shift uβ-Nph levels using a simple paired t-test if normal distribution was seen, or using Wilcoxon Signed Rank Test if not. This analysis was also conducted with the exposed sample set. In addition, we examined differences between uβNph levels in control and exposed using Kruskal-Wallis One Way Analysis of Variance on Ranks and pairwise multiple comparison using Dunn's method.

JP-8 Pre- and Post-shift, Controls Only

All data sets were analyzed for statistical significant differences as described in Section 2.3. An examination of pre- and post-shift uβ-Nph levels in JP-8 control subjects using the paired t-test gave a two-tailed ***p* = 0.105** (Normality passed) with an $\alpha = 0.050$:0.364. The analysis indicated no statistical difference pre/post shift levels of uβ-Nph in the JP-8 control group, which was expected.

JP-8 Pre- and Post-shift, Exposed Only

An examination of pre- and post-shift uβ-Nph levels in JP-8 exposed subjects using the paired t-test gave a two-tailed ***p* = 0.454** (Normality passed) with an alpha = 0.050: 0.112. Statistical analyses of uβ-Nph data indicate there is no difference between the pre/post-shift levels in the JP-8 Exposed subgroup.

JP-8 Control verses Exposed

An examination of pre- and post-shift uβ-Nph levels in JP-8 indicated a non-normal distribution (as determined by the Shapiro-Wilk Normality test). Further analysis using a Kruskal-Wallis One ANOVA on Ranks produced a ***p* = 0.046**, indicating that there was a statistically significant difference between control and exposed groups.

JP-4 Pre- and Post-shift, Controls Only

An examination of pre- and post-shift uβ-Nph levels in JP-4 control subjects using Shapiro-Wilk normality test indicated a non-normal distribution of the data (Normality failed). Examination of the data set using the Wilcoxon Signed Rank Test produced a ***p* = 0.037**, indicating that the pre- and post-shift uβ-Nph levels in the JP-4 Control subjects are significantly different.

JP-4 Pre- and Post-shift, Exposed Only

An examination of pre- and post-shift uβ-Nph levels in JP-8 subjects using the paired t-test gave a two-tailed ***p* = 0.249** (Normality passed) with an $\alpha = 0.050$: 0.307. The analyses indicate that there is no statistically significant difference between the pre- and post-shift levels of uβ-Nph.

JP-4 Control verses Exposed

An examination of pre- and post-shift uβ-Nph levels in JP-8 indicated a non-normal distribution (as determined by the Shapiro-Wilk Normality test). Kruskal-Wallis One ANOVA on Ranks analysis

produced a ***p* = 0.357**, indicating that there were no statistically significant differences between the control and exposed groups within the JP-4 data set.

3.1.4 Box plot Graphs of JP-8 and JP-4 Data sets. Box plots indicate both the full range of variation, likely range of variation, and median of the data set, which provides a more appropriate visual indicator of outlier data points. Both outliers and suspected outliers can be visualized by this method.[14,15] Outliers can be defined as 3 times the interquartile range (IQR) (above/below), whereas suspected outliers fall within 1.5 times IQR (above/below) (**Fig. 6**). To further visualize any potential differences between groups and sub-groups, Box plot depictions of each set were completed (**Fig 7, Fig. 8**).

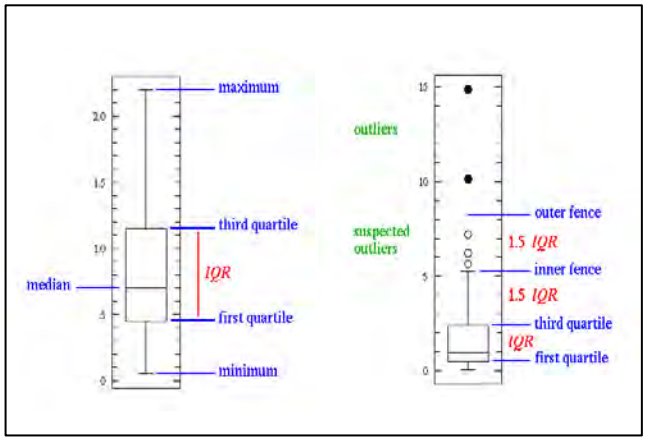

Figure 6. Interpretation of Boxplot indices. *Figure taken from Kirkman 1996.*[16]

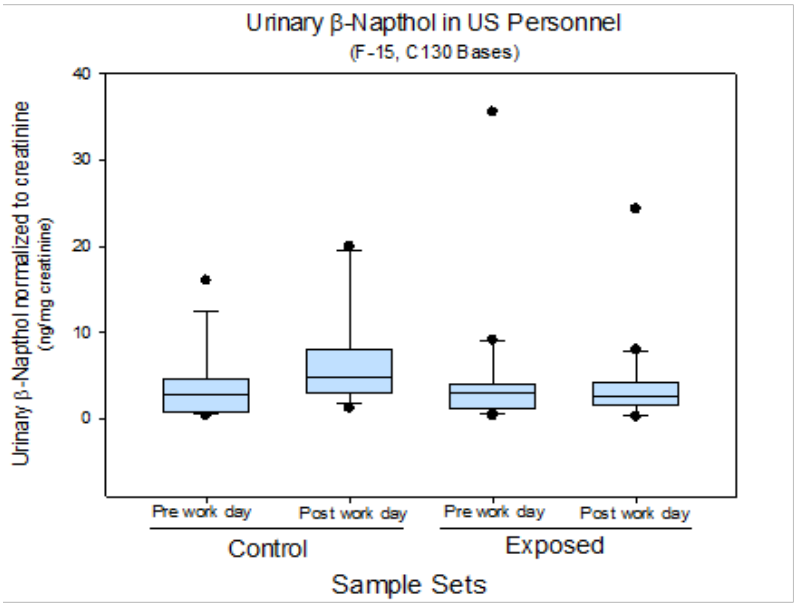

Figure 7. Boxplot of uβ-Nph Concentrations from JP-8 Cohort. Pre- and Post-shift samples Controls and Exposed Group.

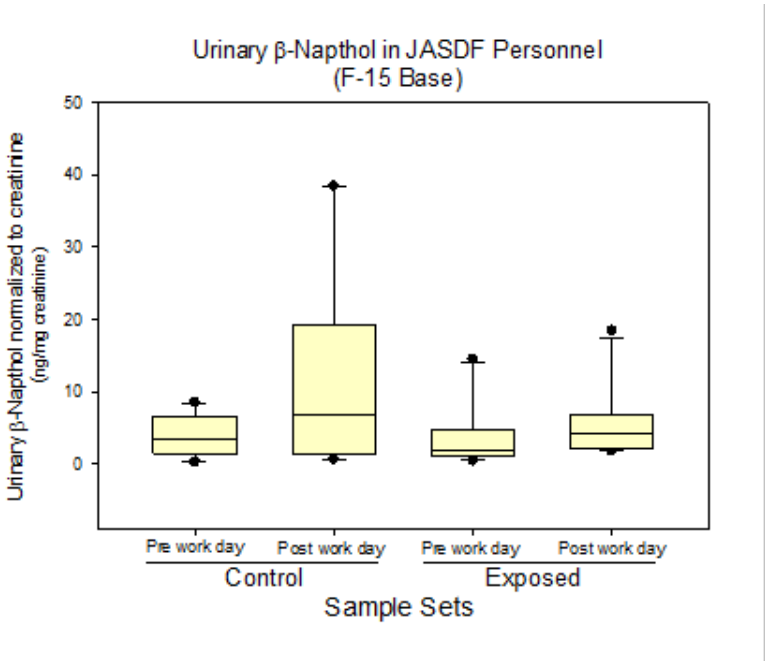

Figure 8. Boxplot of uβ-Nph Concentrations from JP-4 Cohort. Pre- and Post-shift samples from Control and Exposed groups.

3.1.5 Effects of Relative Exposure Levels on uβ-Nph Concentrations. To further examine the cohort data set for possible association of uβ-Nph levels to fuel exposure, the exposure set was binned according to predicted level of exposure as determined by the meta data associated with the sample sets. Supervised separation into bins used the following score set: 1 = Controls; 2 = LOW - Crew Chiefs; 3 = MEDIUM - Engines/Propulsion, 4 = HIGH - Fuels, Fuel Cell Maintenance. A distribution of the data (**Fig. 9**) indicated that no increase was seen in the 'high exposure' subgroup, with either pre- or post-shift samples. Statistical analysis using a Kruskal-Wallis One Way ANOVA failed to find significant differences between exposure groups using the pre-shift ($p = 0.564$) or post-shift ($p = 0.067$) data. Indeed, the distribution further supported the finding that post-shift controls seemed to have an overall higher level of uβ-Nph, a very different conclusion expected for the hypothesis that uβ-Nph levels are linked to jet fuel exposure. To examine possible confounding variables responsible we examined correlations of meta data, especially smoking and age, on uβ-Nph levels.

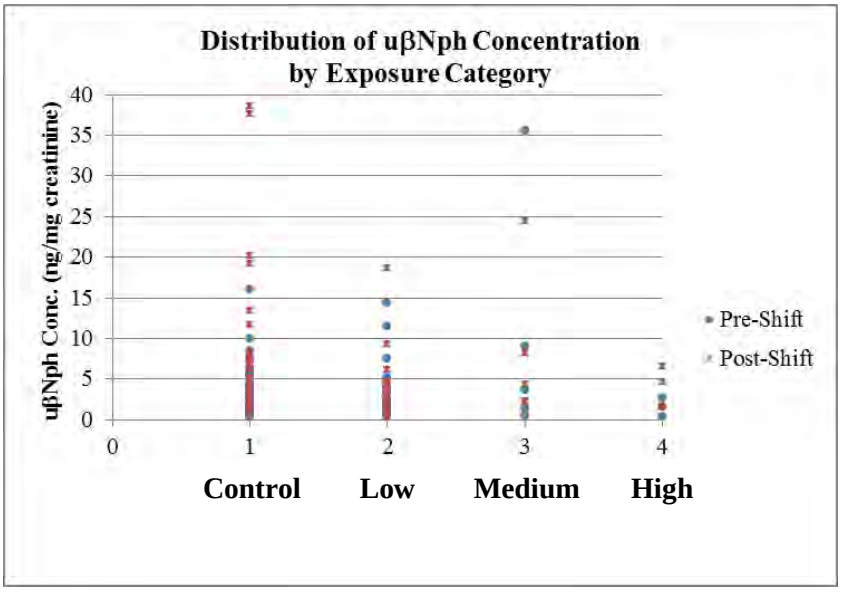

Figure 9. Distribution of Pre- and Post-shift uβ-Nph concentrations from binned exposure groups.

3.2 Correlations of Meta Data and uβ-NPH Concentrations.

Heat maps, consistent with Wilkinson et al.[17] were produced using MATLAB R2011a to visualize the Pearson correlations of the data feature. As seen in this data set (**Fig. 10)**, the data is not highly correlated except between years of service, age, and work experience; a logical result since these should necessarily be related to some degree. While group, exposure, inhalation, and time on flight line are also highly correlated, these are categorical features and thus the correlation is not directly interpretable.

For dimensionality reduction and data organization, principal component analysis (PCA) was considered. Although some of the data is categorical, many features are continuous and thus PCA can be considered. PCA is a statistically optimal method that transforms the data via the eigenvectors of the data covariance matrix,

$$PC = X^{S}_{n\times p} V_{p\times k}$$

where PC is a matrix of PCA scores for k retained PCs,

$$X^{S}_{n\times p} = \left(X_{n\times p} - 1_{n\times 1}\mu^{T}_{1\times p}\right)D^{-1/2}$$

with $X^S_{n\times p}$ being a standardized data matrix, $X_{n\times p}$ being the original data matrix, and $V_{p\times k}$being the eigenvectors of the data covariance matrix.[18] By definition, PCs are thus uncorrelated and organized via eigenvalue magnitude with the first PC explaining the most variance in the data and subsequent PCs explaining sequentially less variation.[19] PCA also permits a dimensionality assessment of the data through examining the covariance matrix eigenvalues (**Fig. 11A**). Kaiser's Criterion, an estimation of data dimensionality based on the mean eigenvalue[19] was used to estimate that there are 4 PCs that explain much of the underlying dimensionality in the data. PCA loadings, the Pearson correlation of the PCs[19] with $X_{n\times p}$, were computed to understand how each data feature relates to the PCs. As seen in **Fig. 11B**, the pre- and post- uβ-Nph load heavily on the first PC along with age, years of service, and work experience. When considering subsequent PCs, time on the flight line and inhalation then load on the second PC, the third and fourth PCs then have much smaller loadings.

Factor analysis, using data covariance matrix eigenvalues, was further considered to understand the data. Factor analysis differs from PCA in that it seeks a correlation explaining representation of features, while PCA seeks a variance explaining representation of features.[20] When considering the data covariance matrix, the initial formulation for factor analysis is identical for PCA.[20] However, one of the key components of Factor Analysis is a rotation of the factors; therefore, a varimax rotation was considered which involves an orthogonal rotation to maximize the variance of the squared loadings on the factors.[19,20] When considering a factor rotation, one selects the number of factors to rotate, consistent with the dimensionality assessment and results of PCA, three factors were rotated in an attempt to reduce and organize the dimensionality further.

The resultant factor loadings, Pearson correlation between factor scores and the original data matrix,[19] are presented in **Fig. 12**. The factor loadings show an age and experience related factor in factor one, an exposure factor in factor two, and factor three which considers pre- and post- uβ-Nph measurements.

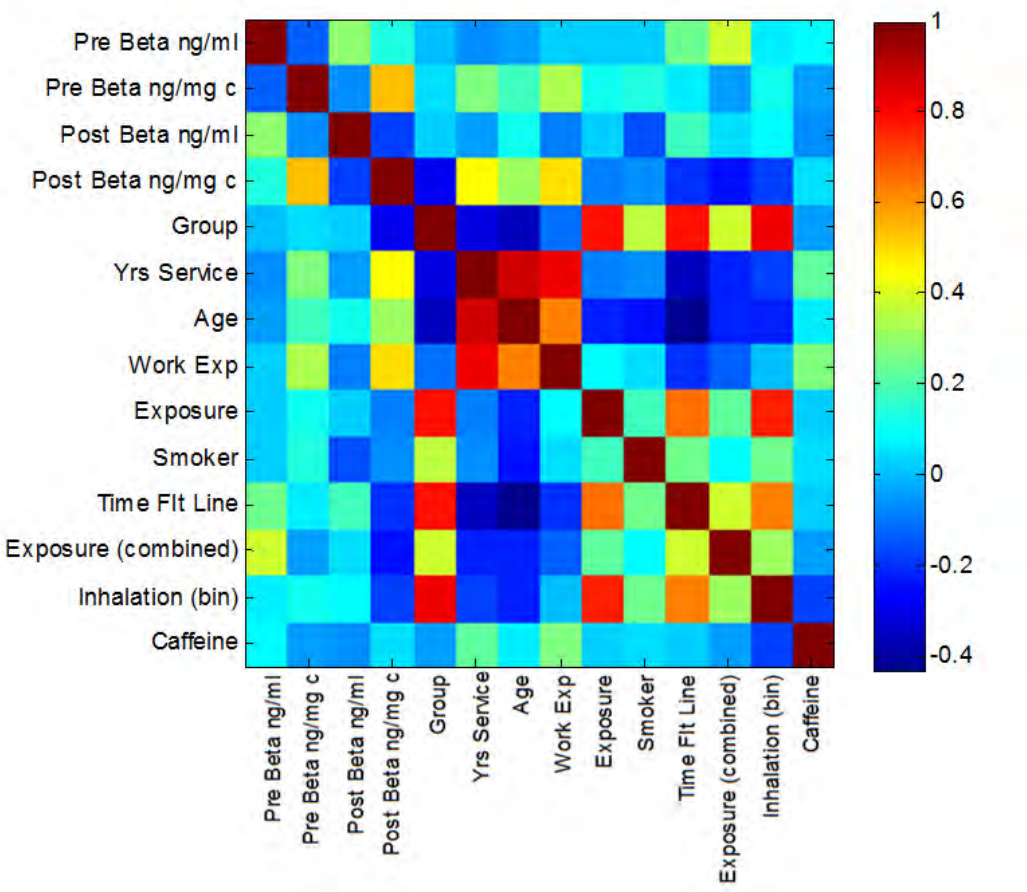

Figure 10. Heat map correlations between meta data, and β-Nph concentrations. Higher correlations are indicated as red, lesser correlations as blue. Pre Beta ng/ml = β-Nph; Pre Beta ng/mg c = pre-exposure uβ-Nph; Post Beta ng/mg c = post exposure uβ-Nph

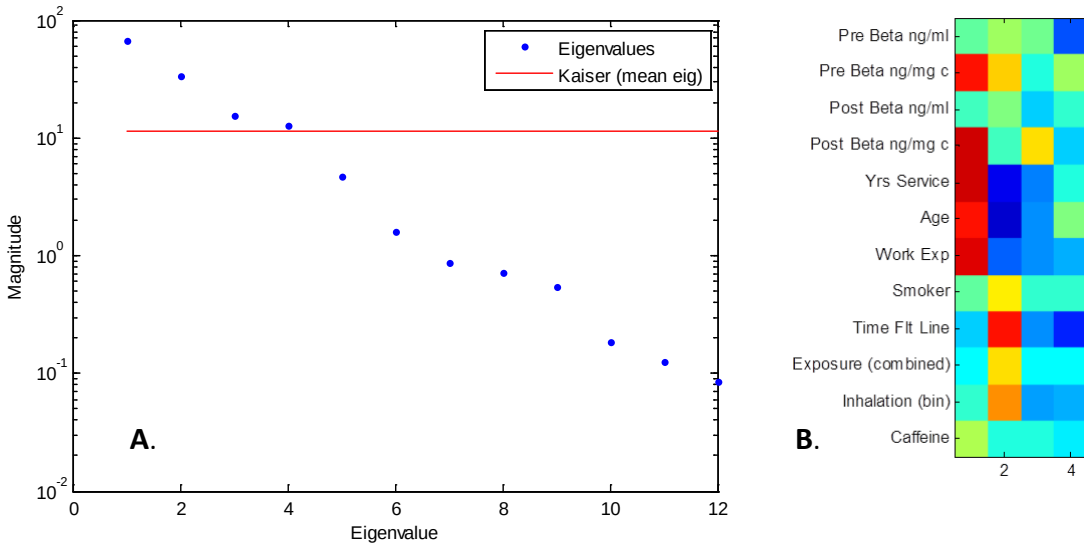

Figure 11. Dimensionality assessment of meta data **(A).** Retaining the first four variables appeal reasonable based on multiple methods **(B**). Higher correlations are indicated as red, lesser correlations as blue. Pre Beta ng/ml = β-Nph; Pre Beta ng/mg c = pre-exposure uβ-Nph; Post Beta ng/mg c = post exposure uβ-Nph

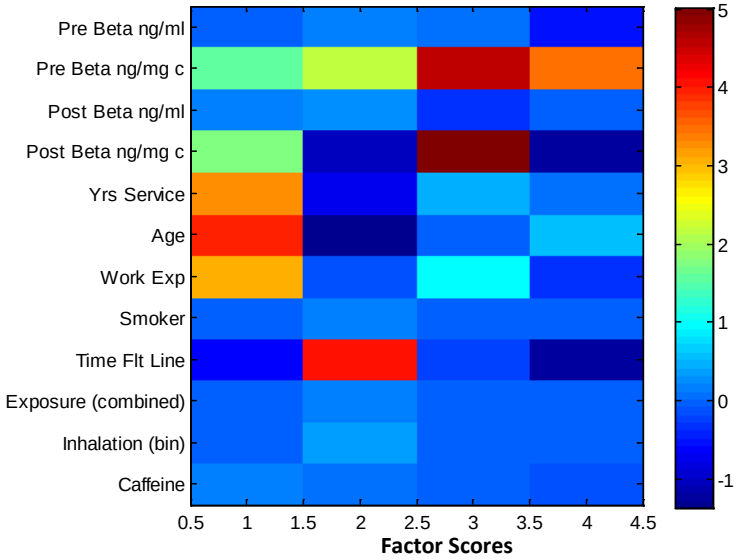

Figure 12. Factor Analysis Loadings. Coefficient of variation based after varimax rotation. Higher correlations are indicated as red, lesser correlations as blue.

3.3 Effects of Smoking on uβ-Nph Levels in Cohort

3.3.1 Distribution of uβ-Nph Concentration in Smokers and Nonsmokers. The data sets were separated into smokers and nonsmokers as determined by self-reported meta data associated with the samples. Examination of the distribution of uβ-Nph in the pre-shift from all controls and all exposure samples indicated a low number of smokers in the control samples (**Fig. 9**), making statistical comparisons difficult. No obvious differences were seen in examining pre-shift samples from all nonsmoking subjects (**Fig. 11**). An examination of the pre-shift samples from the control subjects (smokers/nonsmokers) did not identify any differences, again likely due to the low amount of control subject smokers (**Fig. 12**). The distribution of uβ-Nph in pre-shift samples from the exposed subjects (smoking/nonsmoking) indicates slightly higher levels of uβ-Nph in smokers (**Fig. 13**).

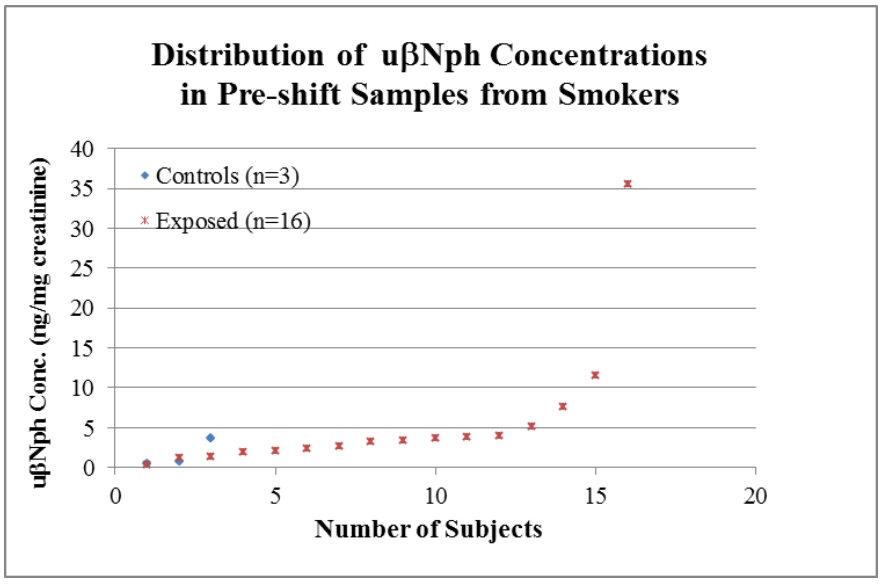

Figure 13. Distribution of uβ-Nph concentrations in control and exposed samples from smokers, pre-shift samples only. Data includes smokers from both JP-8 and JP-4 cohorts.

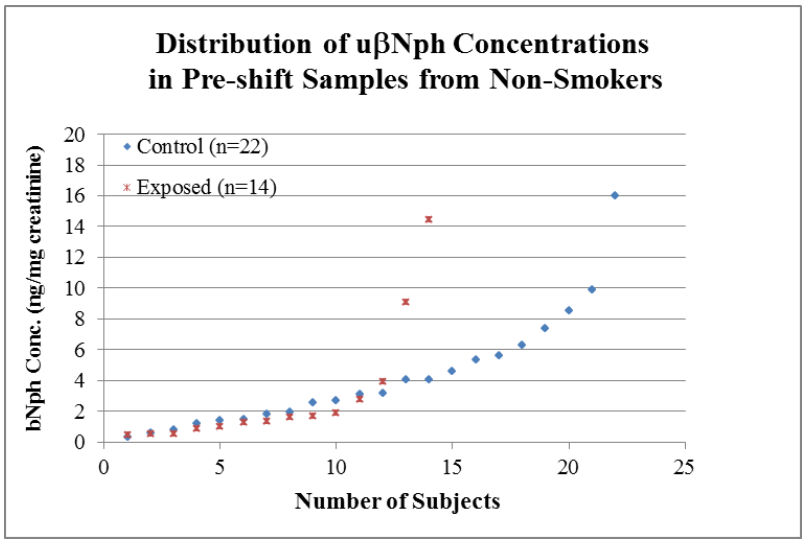

Figure 14. Distribution of uβ-Nph concentrations in nonsmokers from the control and exposed groups, pre-shift samples only. Data includes nonsmokers from both JP-8 and JP-4 cohorts.

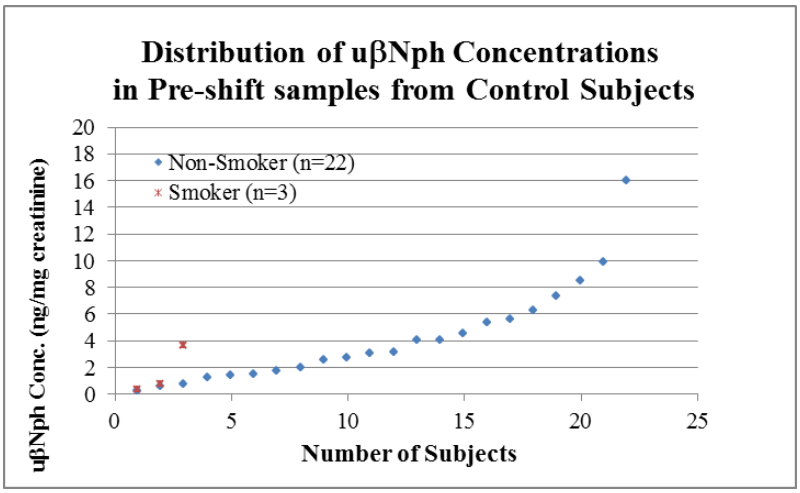

Figure 15. Distribution of uβ-Nph concentrations in pre-shift samples from JP-8 and JP-4 control groups segregated into smokers and nonsmokers.

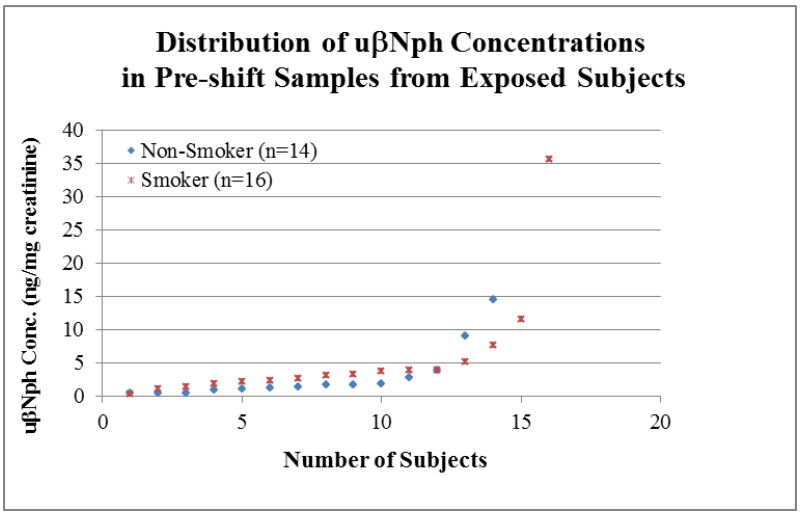

Figure 16. Distribution of uβ-Nph concentrations in pre-shift samples from JP-8 and JP-4 exposure groups segregated into smokers and nonsmokers.

3.3.2 β-Nph Concentration in Smokers and Nonsmokers. The mean levels of β-Nph and uβ-Nph were calculated using pre-shift, post-shift, and combined data (**Table 2**).

Table 5. Average β-Nph and uβ-Nph levels in pre-shift samples from smokers/non-smokers. A) Pre-shift data only; B) Post-shift data only; C) Combined Pre- and Post-shift data. S = smoker, NS = nonsmoker.

A. Pre-Shift	**JP-4**				**JP-8**			
	Control		**Exposed**		**Control**		**Exposed**	
Analyte	S (n=0)	NS (n=10)	S (n=5)	NS (n=5)	S (n=3)	NS (n=12)	S (n=11)	NS (n=9)
β-Nph (ng/mL)	---	2.39	1.69	1.74	2.82	2.44	2.49	2.62
β-Nph (ng/mg Creatinine)	---	4.05	4.09	3.83	1.61	4.36	6.28	2.47

B. Post-Shift	**JP-4**				**JP-8**			
	Control		**Exposed**		**Control**		**Exposed**	
Analyte	S (n=0)	NS (n=10)	S (n=5)	NS (n=5)	S (n=3)	NS (n=12)	S (n=11)	NS (n=9)

β-Nph (ng/mL)	---	2.41	2.20	2.81	2.47	2.62	2.35	3.03
β-Nph (ng/mg Creatinine)	---	11.72	7.65	3.63	4.71	7.56	4.87	2.73

C. Combined	**JP-4**				**JP-8**			
	Control		**Exposed**		**Control**		**Exposed**	
Analyte	S (n=0)	NS (n=20)	S (n=10)	NS (n=10)	S (n=6)	NS (n=24)	S (n=22)	NS (n=18)
β-Nph (ng/mL)	---	2.40	1.95	2.28	2.64	2.53	2.42	2.82
β-Nph (ng/mg Creatinine)	---	7.88	5.87	3.73	3.16	5.96	5.58	2.60

3.3.3 Statistical Analysis of uβ-Nph Levels in Smokers/Nonsmokers. Using previously described statistical methods, the uβ-Nph levels in smokers and nonsmokers were examined for significant differences.

All Smokers (Control and Exposed), Pre-shift Samples **(Fig. 10)**
Analysis using a Normality test (Shapiro-Wilk) failed, indicating a non-normal data distribution of the data sets. Comparisons using a Mann-Whitney Rank Sum Test calculated a ***p* = 0.162**, indicating that there was no statistically significant differences in uβ-Nph levels between control and exposed subjects who smoke.

All Nonsmokers (Control and Exposed), Pre-shift Samples **(Fig. 11)**
Analysis using a Normality test (Shapiro-Wilk) failed, indicating a non-normal data distribution of the data sets. Comparisons using a Mann-Whitney Rank Sum Test calculated a ***p* = 0.077**, indicating that there was no statistically significant differences in uβ-Nph levels between control and exposed subjects who are nonsmokers in the pre-shift samples.

Control Subjects (Smokers/nonsmokers), Pre-shift Samples **(Fig. 12)**
Analysis using a Normality test (Shapiro-Wilk) failed, indicating a non-normal data distribution of the data sets. Comparisons using a Mann-Whitney Rank Sum Test calculated a ***p* = 0.220**, indicating that there was no statistically significant differences in uβ-Nph levels between smokers and nonsmokers in all control subjects in the pre-shift samples.

Exposed Subjects (Smokers/nonsmokers), Pre-shift Samples **(Fig.13)**
Analysis using a Normality test (Shapiro-Wilk) failed, indicating a non-normal data distribution of the data sets. Comparisons using a Mann-Whitney Rank Sum Test calculated a ***p* = 0.059**, indicating that there was no statistically significant differences in uβ-Nph levels between smokers and nonsmokers in all exposed subjects in the pre-shift samples.

3.3.4 Boxplot Graphs of JP-8 and JP-4 Data sets. Box plot depiction of each set described above (in Section 3.2) was completed. As seen with the statistical analyses, there were no statistically significant differences in the data sets when segregated into the various groups as described (**Fig. 14**). The data set most approaching significance is that of exposed subjects (smokers/nonsmokers, $p = 0.059$).

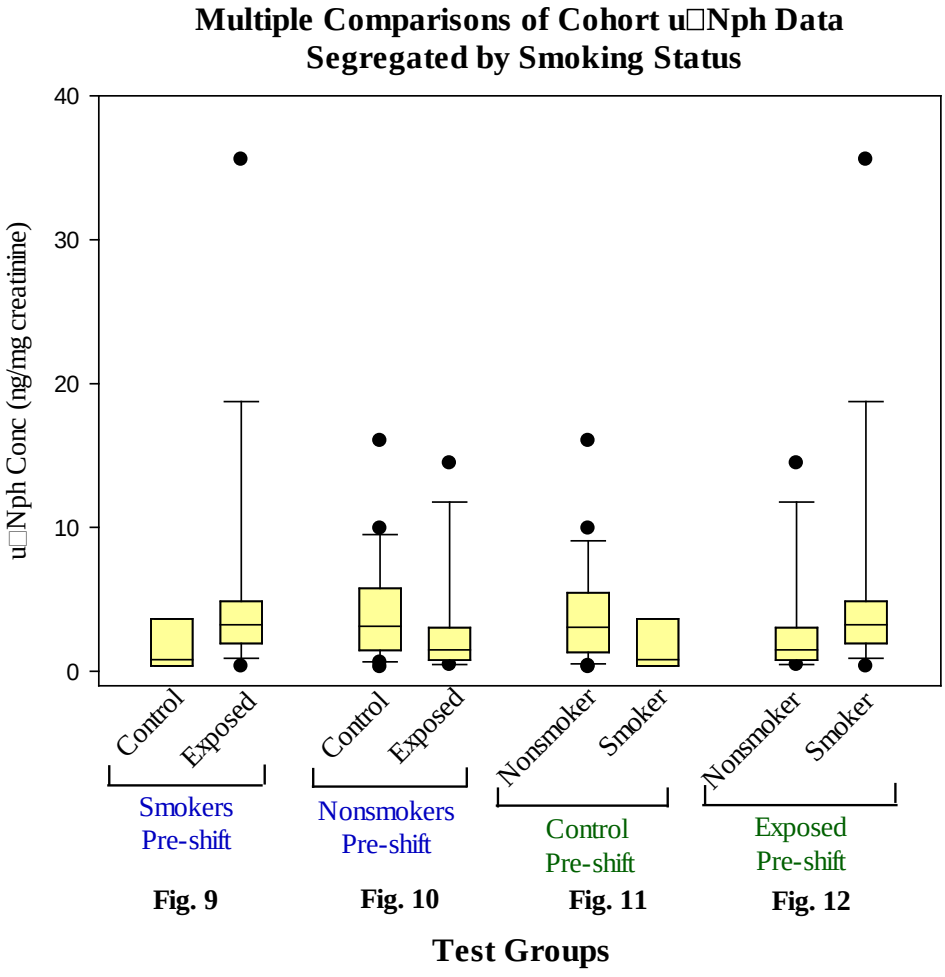

Figure 17. Boxplots of uβ-Nph concentrations in data sets described in Figs 8-11.

3.4 Effect of

Age on uβNph Levels in Cohort.

To examine the possible effects of age on uβ-Nph levels, the cohort data was binned according to age. The age data were separated into four groups: 25 yrs and under; 26-35 yrs; 36-40 yrs; and over 40 yrs.

3.4.1 Distribution of uβ-Nph Concentration by Age Group. An examination of distribution in pre-shift samples indicated no obvious separation of uβ-Nph levels based on age (**Fig. 15**).

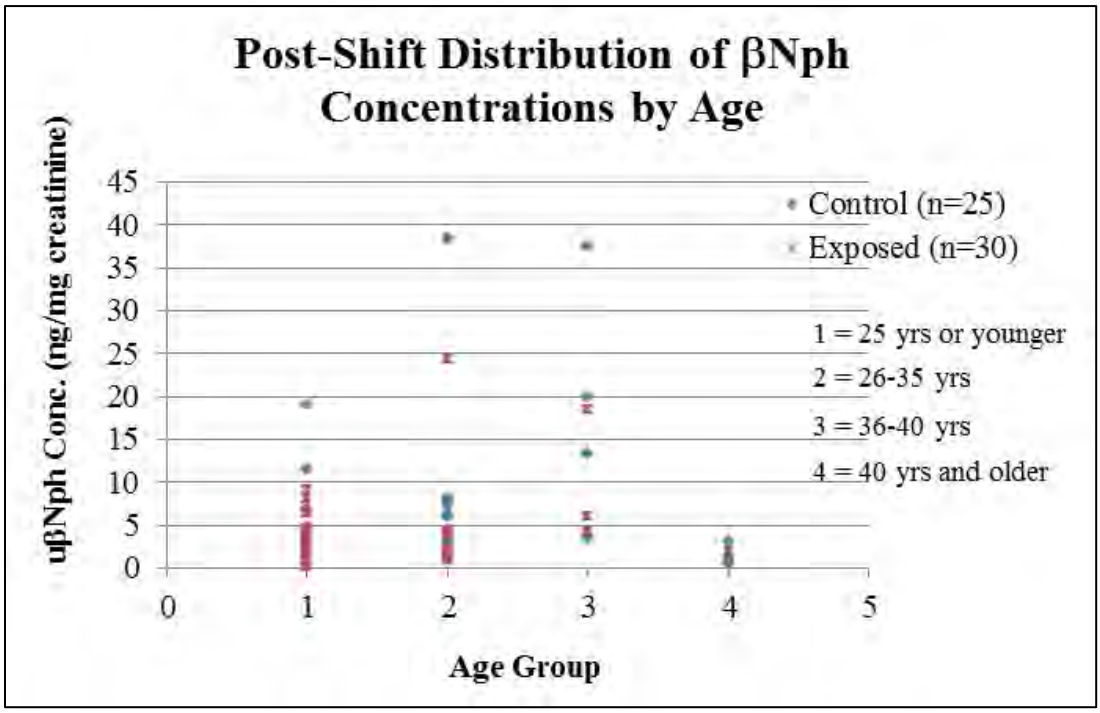

Figure 18. Distribution of uβ-Nph concentrations in post-shift samples from JP-8 and JP-4 groups as binned by age. **1** = 25 yrs and under; **2** = 26-35 yrs; **3** = 36- 40 yrs; **4** = over 40 yrs.

3.4.2 Statistical Analysis of uβ-Nph Levels by Age Group. Both pre- and post-shift sample data binned by age were examined for significant differences in uβ-Nph. Statistical analysis using Kruskal-Wallis One Way ANOVA on Ranks of pre-shift samples of all subjects binned by age indicated that age may play a role in modulating uβ-Nph levels (***p*** =

0.012). Examination of post-shift analyzed in the same manner generated a ***p* = 0.022,** supporting the premise that age may play a role in determining β-Nph levels in the urine.

3.4.3 Boxplot Graphs of JP-8 and JP-4 Data sets by Age Group. Box plot analysis suggests that uβ-Nph may increase with age (**Fig. 16**). Pairwise comparison of this data using Dunn's method indicated that the levels of β-Nph within the age groups were significantly different (p = 0.012).

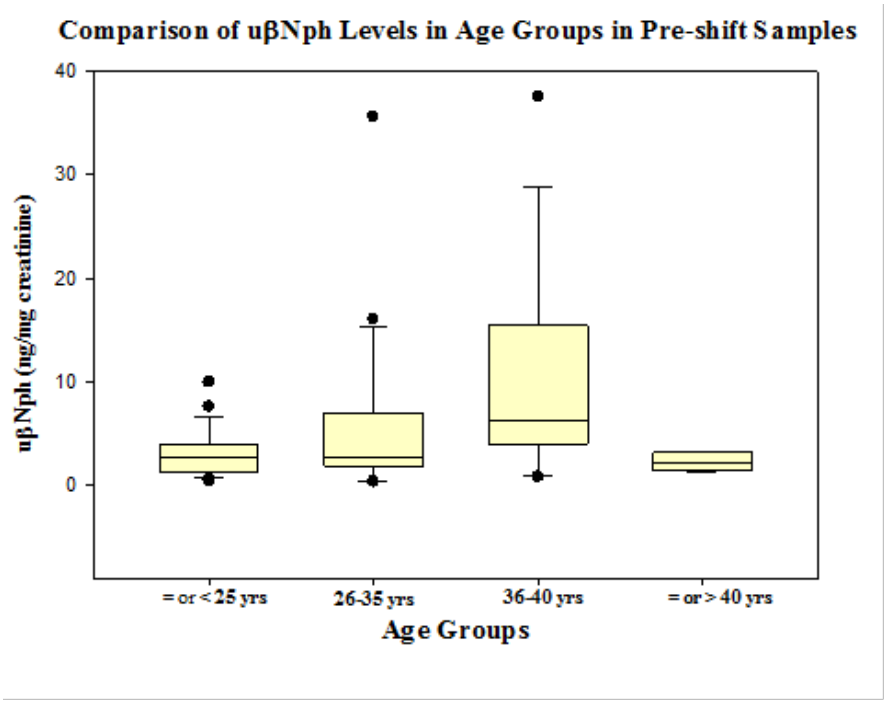

Figure 19. Box plot uβ-Nph concentrations in post-shift samples from exposed/control subjects from both JP-8 and JP-4 groups as binned by age.

4. DISCUSSION

uβ-Nph as an Exposure Biomarker

In this study, statistically significant differences were only found within the JP-8 group (control vs. exposed). However, statistical analysis indicated that while the two groups were different, uβ-Nph levels in the control set were unexpectedly higher, not lower, than the exposed subjects. It is not clear why differences in β-Nph levels were not seen in our study but seen in previously published research.[7-9] In published studies, uβ-Nph was quantified in samples from Air Force personnel by means of gas chromatography-mass spectrometry (GC-MS). We utilized a β-Nph ELISA with a lowest detectable limit (LDL) of 0.94 ng/ml with a detection range of 1.56-100 ng/ml. Unlike Sedar *et al*. or Chao *et al.* who sampled after 4 hrs in the workplace, this study collected a post-dose sample after a full 8 hr workday. Therefore, it was expected that the β-Nph levels in our exposure groups should be at even higher levels than published. Given the level of detection seen in the ELISA kit and the predicted β-Nph levels, the ELISA should have permitted detection at similar levels as previously published. It is possible that the urinary naphthol degraded during storage; however, if such decreases occurred it is presumed that equivalent degradation would have occurred throughout all samples. In addition, past studies indicate that urinary naphthols are relative stable even under variable storage conditions,[21] so the likelihood of introduction of sample variation due to inappropriate handling conditions is small.

uβ-Nph levels within exposure groups

In a more robust examination of the exposure subjects, the exposed data was binned into four sets based on self-reported fuel exposure levels. No differences in control versus exposure groups were seen using multiple statistical methods.

Smoking as a confounder

Besides increasing exposure to PAHs, smoking is known to alter the metabolism of naphthalene.[9] An unexpected significant difference was also seen in the comparison of pre/post-shift samples from the JP-4 Control group (see **Fig. 8**). To examine this difference further, and to scrutinize smoking as a possible confounder, we analyzed the data by separating it into smokers/nonsmokers as self-reported. However, unlike previous studies, our analyses of the data failed to find any significant impact of smoking on β-Nph levels within this binned cohort set.

Age as a confounder

Finally, we also analyzed the effect of age on uβ-Nph levels within this cohort, irrespective of smoking or exposure status. Interestingly, the analyses of these data do suggest that age seems to play a role in modulating uβ-Nph levels. Age was not examined as a variable in previous published studies. The metabolism of naphthalene adduct 1,4-naphthoquinone (1,4-NPQ) by cytochrome P450 has been shown to alter with age, diminishing at a rate of ~ 3% per year.[22] Subject age effects on cytochrome P450 enzymes has been noted previously, and occurs primarily through post-translational or transcriptional modifications.[23] Therefore, our research indicates that age should be considered when evaluating PAH exposure based on uβ-Nph concentrations.

Future use of uβ-Nph as a Biomarker of Exposure

The use of biomarkers for biomonitoring require careful validation studies to identify confounding factors to aid in adjusting and interpreting concentration changes. Such studies are ongoing to assess the usefulness of naphthols as biomarkers of exposure to PAHs.[5] Smoking status has been examined in several studies previously mentioned and certainly must be identified within the test cohort. Data indicate that cytochrome P450 and GST mutations may be confounding variables, but further research is needed to establish a firm link to the CYP2E1 and GSTM1 mutations. Additionally, gender may be a confounder, as the estrus cycle has been seen to affect the metabolism of naphthalene.[24,25] Our study did not see an effect of smoking on uβ-Nph levels – an unexpected result - possibly due to the low sample size created when the cohort was binned into smoking/nonsmoking groups. However, this study suggests that age may also alter uβ-Nph concentrations, possibly due to the aging of cytochrome P450. We did not examine gender differences, and did not have enough female subjects enrolled to allow statistical analysis of this as a variable. For future examination of uβ-Nph as a biomarker to jet fuel exposures, we suggest that data from these possible confounders should be examined (**Table 3**).

Table 6. Possible cofounding variables affecting uβ-Nph biomarker concentrations

Trait	Confounder?	Include as metadata?
Smoking	Yes	**Yes**
Gender	Possibly	**Yes**
CYP2E1	Possibly	**Yes**
GSTM1	Possibly	**Yes**
Age	Possibly	**Yes**
Alcohol Consumption	No	No
Food intake (types)	No	No

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6. LIST OF SYMBOLS, ABBREVIATIONS, AND ACROYNMS

ANOVA	standard analysis of variance
β-Nph	beta-naphthol, concentrations not corrected for urine volume
JP-8	Jet Propellant 8
Nph	naphthols
OD	optical density
1,4-NPQ	1,4-naphthoquinone
PAH	poly aromatic hydrocarbons
PCA	principal component analysis
RPM	rotations per minute
RT	room temperature
uβ-Nph	urinary β-Nph normalized to urine creatinine concentration; [β-Nph] corrected for urine volume