

The Utility of Naphthyl-Keratin Adducts as Biomarkers for Jet-Fuel Exposure

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Abstract

We investigated the association between biomarkers of dermal exposure, naphthyl-keratin adducts (NKA), and urine naphthalene biomarker levels in 105 workers routinely exposed to jet-fuel. A moderate correlation was observed between NKA and urine naphthalene levels ($p=0.061$). NKA, post-exposure breath naphthalene, and male gender were associated with an increase while *CYP2E1*6* DD and *GSTT1*-plus (++) genotypes were associated with a decrease in urine naphthalene level ($p<0.0001$). NKA show great promise as biomarkers for dermal exposure to naphthalene. Further studies are warranted to characterize the relationship between NKA, other exposure biomarkers, and/or biomarkers of biological effects due to naphthalene and/or PAH exposure.

Introduction

Jet propulsion fuels (kerosene-based fuel mixtures) are standard fuels for commercial aviation as well as for military aircrafts and vehicles. Jet propulsion fuel type 8 (JP-8) is the battle field fuel for all U.S. military operations and was chosen to replace its predecessor JP-4 in 1972 by North Atlantic Treaty Organization (NATO) due to its higher flashpoint (ATSDR 1998; ATSDR 1998; Zeiger and Smith 1998; Carlton and Smith 2000; NRC 2003). In addition, JP-8 is used as a degreaser, heat source, and cooking fuel by the U.S. military. It is estimated that 5 billion gallons of JP-8 is used by the U.S. military and NATO each year (NRC 2003). It has been recognized as the single largest source of chemical exposure for the military personnel (Carlton and Smith 2000). Dermal exposure to jet fuel, along with inhalation exposure, was observed to contribute significantly to body burden in the U.S. Air Force (USAF) fuel-cell maintenance workers (Chao, Kupper et al. 2006; Kim, Andersen et al. 2007). However, due to its low vapor pressure and slower evaporation rate in comparison to JP-4, JP-8 presents the possibility of increased duration of dermal contact, therefore, increased dermal exposure (Chao, Gibson et al. 2005). Although JP-8 contains hundreds of aliphatic and aromatic hydrocarbons, naphthalene and its metabolites (urine naphthols) have been established as effective surrogate biomarkers for exposure to JP-8 (Egeghy, Hauf-Cabalo et al. 2003; Serdar, Egeghy et al. 2003).

Accurate quantification of the contribution of dermal exposure to the systemic dose is required in order to complement and improve current exposure assessment models for long-term health effects of jet fuel exposure, a facet that is missing from current epidemiological research. Quantitation of the individual dermal dose and determination of the mechanism of action hinges upon development of biomarkers that can encompass both acute and chronic exposure and upon development of specific analytical procedures to quantitate these biomarkers. The only method for quantitation of dermal exposure to jet fuel (JP-8) reported to date measures the

unmetabolized naphthalene (surrogate for JP-8) on the skin surface, which can only be attributed to recent exposure because of rapid absorption and biotransformation of naphthalene (Chao et al., 2005; Chao et al., 2006). Naphthalene metabolites bound to skin keratins would be appropriate and specific biomarkers for dermal exposure, as the bioactivation required for naphthalene metabolite adduction to keratin only occurs in the suprabasal layer of the epidermis (Kang-Sickel et al., 2008; Kang-Sickel et al., 2010), where keratin is synthesized *de novo* and the non-living squame formed (Watt, 1988). Approximately 20-28 days are required for differentiation and maturation of suprabasal keratinocytes (Junqueira et al., 2005; Furukawa et al., 2008) to migrate from the basal layer to the surface to form the stratum corneum, which requires continuous replacement. Therefore, non-invasive sampling of stratum corneum by sequential tape-stripping can be used to monitor individual's past (<28 days) exposure to naphthalene-containing mixtures and characterize individual variation in dermal metabolic capacity and keratin adduction in human populations.

The potential contribution of dermal exposure to total JP-8 exposure is suggested based on the correlation between the metabolite levels in urine (e.g., 1- and 2-naphthol) and various surrogates of dermal exposure, including skin irritation, work inside the fuel cell, removing and cleaning fuel puddles (Egeghy et al., 2003; Serdar et al., 2004). However, these surrogate indicators provide only qualitative, not quantitative, information. We previously established that naphthalene (as a model polycyclic aromatic hydrocarbon) and its metabolites 1-naphthol (1NAP) and 2-naphthol (2NAP) in urine can be used as quantitative biomarkers of a complex jet fuel mixture exposure to the skin (Chao et al., 2006). We further established that dermal exposure to jet fuel, along with inhalation exposure, contributes significantly to the total body burden in the exposed U.S. Air Force (USAF) fuel-cell maintenance workers (Chao et al., 2005; Chao et al., 2006; Kim et al., 2007). We have also shown that highly specific polyclonal

antibodies to naphthalene metabolites can be used for a sensitive enzyme-linked immunosorbent assay (ELISA) for quantification of naphthyl-keratin adducts as biomarkers of dermal exposure to jet fuel (Kang-Sickel et al., 2008; Kang-Sickel et al., 2010). In dermal tape-strip samples collected from 105 individuals exposed to JP-8, naphthyl-conjugated keratin peptides were detected at levels from 0.004 to 6.104 pmol adduct/ μ g keratin, but were undetectable in unexposed volunteers (Kang-Sickel et al., 2008; Kang-Sickel et al., 2010).

Our first objective was to characterize the association between biomarkers of dermal exposure (i.e., naphthyl-keratin adducts) and urine naphthalene and naphthalene metabolite levels in 105 fuel cell maintenance workers along with inhalation exposure as well as individual and work place factors that may contribute to these biomarker levels using multiple linear regression models. However, naphthalene and its metabolites are substrates for biotransformation enzymes, including cytochrome P-450s (CYPs), glutathione-*S*-transferases (GSTs), and NAD(P)H:quinone oxidoreductases (NQO1) (Buckpitt et al., 2002; Preuss et al., 2003; Waidyanatha et al., 2004). Genetic polymorphisms account for interindividual differences in the expression of these enzymes and, thus, may contribute to the differences in biomarker levels and exposure-related toxicity. In the skin, as in the liver, metabolizing enzymes convert naphthalene to more polar metabolites but also catalyze the synthesis of reactive metabolites that bind to nucleophilic macromolecule (protein or DNA) to form adducts (Ross et al., 1993; Isbell et al., 2005; Shimada, 2006; Saeed et al., 2009). Therefore, our second objective was to examine the potential contribution of genetic variants of four genes (*CYP2E1*, *GSTM1*, *GSTT1*, *NQO1*), which code for biotransformation enzymes intrinsic to naphthalene metabolism, to the observed levels of naphthyl-keratin adducts and urine biomarkers in this worker population.

Materials and Methods

Study Population

Exposure data was available from the broad exposure assessment and health effects study of the personnel exposed to JP-8 at six USAF bases in the continental United States (described in (Egeghy, Hauf-Cabalo et al. 2003; Chao, Gibson et al. 2005; Chao, Kupper et al. 2006). While 339 USAF personnel were enrolled in the overall study, 105 fuel-cell maintenance workers from five bases who were monitored for both dermal and systemic levels were included in this study. These fuel-cell maintenance personnel serviced primarily F-series fighter jets or C-130 transporter jets. The dermal tape strip samples were collected in the aircraft hanger to minimize any possible confounding factors, such as ambient temperature and humidity. The workers comprised of 98 males (93.3%) and 7 females (6.7%), 92 Caucasians (87.6%), and 13 non-Caucasians (African-American, Hispanic, or Asian; 12.4%), and 46 were smokers (43.8%). The average age of the workers was 24.7 ± 5.0 years and ranged from 18 – 40 years. Workers were recruited with informed consent from active duty USAF personnel who routinely worked with and were exposed to JP-8. This study was approved by the institutional review board of each of the participating investigators and for the USAF, and the study complied with all applicable U.S. requirements and regulations.

Questionnaires and work diaries were recorded after the work shift to obtain detailed information on demographic factors including age, ethnicity, gender, smoking status, job tasks and durations, use of personal protective equipment (PPE), and other work-related characteristics. Each of the workers had been assigned *a priori* into one of three exposure categories (high, moderate, low) based on the primary job tasks (Chao et al., 2005). Workers who entered fuel tanks (entrants) on the sampling day were classified as high-exposure group. The medium-exposure group consisted of workers who were attendants and/or runners assisting

the entrants. Other field workers who had occasional contact with JP-8 were classified as the low-exposure group (Chao et al., 2005).

Collection and Analyses for Skin, Inhalation, and Urine Samples

The collection of dermal tape-strip samples and analysis for naphthalene (Chao et al., 2005) and four naphthyl-keratin adduct (NKA) levels (Kang-Sickel et al., 2008; Kang-Sickel et al., 2010) have been described previously. Briefly, tape-strip samples were collected post-exposure using three sequential adhesive tape-strips in each site (2.5 cm × 4.0 cm, surface area 10 cm²; Cover-RollTM tape, Beiersdorf AG, Germany) from three exposed body regions with potentially the greatest JP-8 exposure, as identified by the subject and confirmed via visual inspection by the investigators. In each site, side-by-side samples were collected, one set for determination of naphthalene level and the other set for determination of NKA level. Each tape was applied onto the skin surface with a constant pressure and removed at an approximately 45-degree angle after one minute. For determination of naphthalene level, tape was folded and placed into a labeled scintillation vial containing 5 ml acetone and 20 µl of 25 µg/ml naphthalene-d₈ (internal standard) and stored at -80°C until analysis by gas chromatography-mass spectrometry (GC-MS). For determination of NKAs, tape was rolled with the adhesive side facing out and placed into a 2-ml cryovial and stored at -80°C until the keratin from the tape sample was extracted and quantified by Bradford assay and four NKAs (1-naphthyl-keratin-1, 2-naphthyl-keratin-1, 1-naphthyl-keratin-10, and 2-naphthyl-keratin-10) quantified by ELISA (Kang-Sickel et al., 2008).

Inhalation exposure to naphthalene was monitored during the 4-h work shift using passive monitors attached to the workers' collars. End-exhaled breath samples were collected before and immediately after the work shift using 75-cm³ glass bulbs. The full-face supplied-air respirator was worn by 69 (100%) of the *a priori* high-exposure group workers, while 10 (59%) of

medium-exposure and none of the low-exposure workers wore a respirator. Both breathing-zone air and breath samples were analyzed by thermal desorption followed by GC with photoionization detection, as described elsewhere (Egeghy et al., 2003). The collection of urine samples was performed before and after the work shift and the concentrations of the urine 1NAP and 2NAP were determined by GC-MS, as described elsewhere (Serdar et al., 2003).

Genotyping for Candidate Genes

A sample of whole blood was collected into a 10 ml EDTA tube (Vacutainer[®]; Beckton-Dickinson and Company, Franklin Lakes, NJ) for each worker at the beginning and at the end of the work shift. The samples were packed with Blue Ice[®] (Rubbermaid, Atlanta, GA) and shipped to arrive at the lab within 24 h at a temperature of 4°C. Genomic DNA was isolated from nucleated blood cells for genotyping from 0.4 ml of whole blood using InstaGene Genomic DNA Kit (Cat #732-6028, BioRad, Hercules, CA). When additional DNA was needed for genotyping, genomic DNA was isolated from 0.3 ml of whole blood that had been stored at –70°C using Gentra Puregene Blood Kit (Qiagen Sciences, Inc., Germantown, MD) following the manufacturers procedures.

The *CYP2E1 DraI* (mutant allele: *CYP2E*6*) restriction fragment length polymorphism was identified by minor modifications of methods described by Kato et al. (1995). Minor modifications of the *CYP2E1* methods included using AmpliTaq Gold Polymerase and Applied Biosystems PCR Buffer II in the reaction mixture and a 10 min preincubation of the reaction mixture at 95°C prior to beginning of the specified amplification program. PCR amplification and restriction products were examined on 2% agarose E-gels (Invitrogen Life Technologies, Carlsbad, CA) or 2% agarose gels prepared in the laboratory containing ethidium bromide and visualized on an ultraviolet transilluminator. *CYP2E1* has a polymorphic *DraI* restriction site in

intron six. The *DraI* restriction site is present in the *CYP2E1 DraI* Type D genotype, whereas the site is missing in the *CYP2E1 DraI* Type C genotype.

GSTM1 genotyping was based on the method of Huang *et al.* (1997). The PCR reaction mixture contained thermophilic DNA polymerase 10X buffer with 15 mM MgCl₂, 2.5 pmol of each dNTP primer, 20 µM of each *GSTM1* primer, 20 µM of each β-globin primer, 2.75 µl Taq polymerase, and 2 µl of DNA template (100 – 300 ng) in a total reaction volume of 50 µl. The reaction mixture was placed in a thermocycler (model PTC-100, MJ Research Inc., Waltham, MA) for 30 cycles at 95°C for 30 sec followed by 45 sec at 56°C, 72°C for 45 sec, and a final step at 72°C for 10 min. PCR products were separated by electrophoresis on 2.5% agarose gels containing ethidium bromide and visualized on an ultraviolet transilluminator. The β-globin gene served as an internal control and generated a 100 bp PCR product in all reaction tubes. Only genomic DNA from GST-positive individuals served as a template for amplification of an additional 273 bp product.

GSTT1 genotype was identified using primers described by Hirvonen *et al.* (1996). The PCR reaction mixture contained 1X Applied Biosystems PCR Buffer II, 2 mM MgCl₂, 2.5 pmol of each dNTP primer, 30 pmol of *GSTT1* primer, 10 pmol of each β-globin primer, 1 unit AmpliTaq Gold Polymerase, and 2 µl of DNA extract in a total reaction mixture of 30 µl. The reaction mixture was placed in a thermocycler (model PTC-100, MJ Research Inc., Waltham, MA) for 10 min at 95°C followed by 25 cycles of 94°C for 1 min, 59°C for 1 min, 72°C for 1.5 min, and a final step of 72°C for 5 min. PCR products were examined as described for *CYP2E1* above. The β-globin gene served as an internal control and generated a 100 bp PCR product in all reaction tubes. *GSTT1-null* individuals were identified by their DNA templates that produced no amplification with *GSTT1* specific primer.

The method described by Wiemels *et al.* (1999), with minor modifications, was used to characterize the single nucleotide polymorphism rs1800566 in *NQO1*. Minor modifications in the *NQO1* methods and examination of PCR amplification and restriction products were carried out as described for *CYP2E1* above. This genetic variant has a C to T transition in nucleotide 609 in exon 6 of *NQO1* (Guha *et al.*, 2008).

Duplicate reactions for all genotypes were conducted on a minimum of 20% of the participants and all duplicate results were in complete concordance.

Statistical Analyses

All statistical analyses were conducted using the SAS software (V.9.1.3, SAS Institute, Cary, NC, USA) at a significance level of 0.10. All exposure and biomarker data (NKA levels, dermal, breathing-zone air, and breath naphthalene levels, as well as urine naphthalene and naphthol levels) were natural log-transformed to satisfy normality assumptions prior to statistical analyses. All statistical models were constructed and analyses conducted based on subjects with available data points; therefore, the sample sizes in these analyses differed due to missing values of independent variables.

The total NKA level for each worker was calculated by summing the four keratin-normalized adduct levels measured in all sampling sites. Pearson correlation was examined between the total NKA level and JP-8 exposure measures (*i.e.*, dermal, breathing-zone, pre- and post-exposure breath naphthalene levels) and urine biomarkers [*i.e.*, naphthalene, 1NAP, 2NAP, and total naphthol (TNAP = 1NAP + 2NAP) levels]. Analysis of variance (ANOVA) with the significance level of 0.1 was performed to test the effects of genetic variants in four metabolic genes (*CYP2E1*, *GSTM1*, *GSTT1*, *NQO1*), which have been indicated in naphthalene metabolism

(Tingle et al., 1993; Wilson et al., 1996; Yang et al., 1999; Lee et al., 2001), on the levels of total NKAs and urine biomarkers observed in this worker population.

Multiple linear-regression analyses were conducted to examine workplace and personal factors contributing to total NKA levels in all workers, and in *a priori* high-exposure group only, upon jet fuel exposure. Stepwise variable selection was used to determine final regression models, with $\alpha = 0.10$ as the inclusion level for predictors. The basic multiple linear regression model was as follows:

$$Y_i = \alpha + \sum_{j=1}^J \beta_j X_{ij} + \sum_{k=1}^K \gamma_k C_{ik} + \varepsilon_i \quad (1)$$

Where, Y_i is the natural log-transformed total NKA level measured in the i th worker's tape-strip samples, α is the intercept, X_{ij} represents the i th worker's j th dermal naphthalene level, C_{ik} represents the k th covariate value for the i th worker based on work diary and questionnaire, including exposure duration, job task, PPE, age, gender, and smoking status, as well as metabolic genotypes *CYP2E1*, *GSTM1*, *GSTT1*, and *NQO1*.

A second multiple linear regression analysis was used to investigate the relationship between the urine biomarkers and the total NKA levels as well as the contribution of the covariates also analyzed in model 1. Again, the models were constructed separately for all workers, and for the *a priori* high-exposure group only. The basic multiple linear regression model was as follows:

$$Y_i = \alpha + \sum_{j=1}^J \beta_j X_{ij} + \sum_{k=1}^K \gamma_k C_{ik} + \varepsilon_i \quad (2)$$

where, Y_i is the natural log-transformed urine biomarker level, measured in the i th worker's urine samples; α is the intercept; X_{ij} represents the i th worker's j th total NKA level; C_{ik} represents the k th covariate value for the i th worker, which included exposure data, PPE, metabolic genotypes *CYP2E1*, *GSTM1*, *GSTT1*, and *NQO1*, age, ethnicity, gender, and smoking status. Collinearity

was examined for all models using variance inflation, condition indices, and eigenvalues. Possible outliers were examined by studentized residuals.

Results

No significant correlation was observed between the total NKA levels and various exposure measures (i.e., dermal, breath, and breathing-zone naphthalene levels) or with the urine 1NAP, 2NAP, or TNAP levels with the exception of urine naphthalene level (Pearson correlation $r = 0.184$, $p = 0.061$) (Table 1).

The association between biomarker levels (i.e., total NKA level and urine 1NAP, 2NAP, TNAP, or naphthalene level) and the genotype of each of the four genes (*CYP2E1*, *GSTM1*, *GSTT1*, and *NQO1*) are presented in Table 2. A significant difference in total NKA level was observed between individuals with *GSTT1*-null genotype and those with *GSTT1*-plus (+/+-) genotype ($p = 0.080$). Individuals carrying *NQO1* homozygous variant (TT) also had significantly lower keratin adduct levels than those with homozygous wildtype (CC) ($p = 0.085$).

The regression analysis showed that total NKA level was influenced by dermal naphthalene level (Indermal), duration of exposure on the sampling day (exposure duration), and age (Table 3). The dermal naphthalene level and the age of the worker were inversely associated with total NKA level while exposure duration increased total NKA levels. When we investigated the factors related to workers assigned into the *a priori* high-exposure group only ($n = 68$), these same three predictors as for all workers were observed to be significant (Table 3). None of the four metabolic genotypes were observed to be significantly associated with total NKA level in either multiple linear-regression analysis when other significant covariates were included in the models.

Based on the Pearson correlation results (Table 1), a second regression model was constructed using urine naphthalene as the outcome variable. The significant predictors for urine naphthalene levels were total NKA level, post-exposure breath naphthalene level (Inpost-breath), *CYP2E1*6* DD (wild type), *GSTT1*-plus (+/+-) genotypes, and gender (Table 4). The total

NKA level, post-exposure breath naphthalene level, and male gender were associated with an increase in the urine naphthalene level while the *CYP2E1**6 DD and *GSTT1*-plus (++) genotypes were associated with a decrease in urine naphthalene level. When this analysis was limited to the workers in the *a priori* high-exposure group (n = 64), 24.5% of the total variance in the urine naphthalene level was explained by the total NKA and post-exposure breath naphthalene levels as well as presence of *GSTT1* genotype.

Discussion

Previously, we demonstrated the human skin's capacity to express enzymes for xenobiotic metabolism and to form naphthyl-keratin adducts in response to naphthalene exposure (Kang-Sickel et al., 2008; Kang-Sickel et al., 2010). We also demonstrated that these adducts can be quantitatively measured in the exposed skin of workers, facilitating naphthalene exposure assessment, particularly in regard to the dermal route (Kang-Sickel et al., 2008; Kang-Sickel et al., 2010). In addition, inhalation and dermal exposure were identified as significant factors contributing to the total systemic dose of naphthalene, measured as urine naphthol levels, in workers exposed to jet fuel (Serdar et al., 2004; Chao et al., 2006; Kim et al., 2007). Similarly, urine naphthalene was identified as a biomarker for both dermal and inhalation exposure to PAHs (Campo et al., 2009; Sobus et al., 2009a; Sobus et al., 2009b).

Here, we examined the relationship between the skin total NKA levels and urine biomarkers of naphthalene exposure, as a surrogate for JP-8 exposure. We observed a significant correlation between total NKA and urine naphthalene levels ($r = 0.184$, $p = 0.061$) but not between total NKA and breath naphthalene or urine naphthol levels. Jet fuel workers who entered and worked in the fuel tanks were equipped with in-line respirators and cotton coveralls. In this study, 75% of the fuel-cell maintenance workers (79/105) wore full-face supplied-air respirators. Thus, the principal route of contact and exposure to naphthalene is expected to be dermal. The NKA in the skin are formed via metabolism in the suprabasal layer of the skin (Kang-Sickel et al., 2010) and manifested after the processes of keratinocyte maturation, proliferation, differentiation, and migration to the surface of stratum corneum, which take approximately 28 days (Junqueira et al., 2005; Furukawa et al., 2008). Since each tape strip removes approximately 1 layer of keratinocytes, three sequential tape-strips removes approximately 3 layers of keratinocytes. Therefore, based on the average keratinocyte maturation and differentiation processes for a

healthy individual, the measured total NKA levels reflect dermal exposure that occurred approximately 3-4 weeks prior to sampling. Once naphthalene is metabolized into reactive naphthalene-1,2-oxide and the keratin adduction occurred in the skin, the intermediate reactive metabolites do not go through the nonenzymatic rearrangement and enter the system circulation as 1NAP or 2NAP to be further metabolized or excreted. If the individual's metabolic capacity in the skin became saturated, excess naphthalene would appear in the blood and enter the systemic circulation (Kim et al., 2006a; Kim et al., 2006b). Distribution would favor the kidneys due to the rates of perfusion. Metabolism of naphthalene could occur in the kidney cortex and outer medulla. Urine 1NAP and 2NAP levels reflect metabolism in both the epidermis and the kidney and, to a lesser extent, the liver. Exhaled or urine naphthalene levels observed at the end of the workday reflect the total excess naphthalene in the systemic circulation. Therefore, that the associations were stronger for the parent compound (naphthalene) than for naphthol metabolites in urine suggest that the levels of dermal exposure exceeded the skin's capacity to metabolize naphthalene.

The lack of association between the skin NKA levels and the breath naphthalene or urine naphthol levels is likely due to the fact that the inhalation exposure to jet fuel was overestimated (75% of the workers uses in-line respirators) and, thus, the relative contribution of dermal exposure to the systemic exposure was increased. This lack of association may be partially due to the differences in the biological half-lives of parent compound and its metabolites in different tissues/organs, and their representations of different exposure windows (Ruchirawat et al., 2002). For example, the half-life of naphthalene in breath is estimated to be 22 min (Egeghy et al., 2003), while half-lives for urine naphthols are 1.2 – 4.6 h in a rapid phase, and 14 – 46 h in a slow phase (Bieniek, 1994; Heikkila et al., 1995).

Our results indicating the lack of correlation between breath and urine biomarkers and skin protein adducts is supported by previously published studies on various urine biomarkers and protein adduct levels in regard to PAH exposure. Omland *et al.* (1994) observed no correlation between the levels of benzo(a)pyrene-albumin adducts and 1-hydroxypyrene (1-HP), a urinary biomarker for PAH exposure, in iron foundry workers. No correlation was observed between 1-HP and DNA or serum protein adducts in traffic police in Bangkok and Thailand, in Danish foundry workers, in Hungarian aluminum plant workers, or in Danish bus and postal workers, and garage workers (Sherson *et al.*, 1992; Nielsen *et al.*, 1996; Autrup *et al.*, 1999; Schoket *et al.*, 1999; Ruchirawa *et al.*, 2002). In addition, in our study the exposure intensity from one-day measurements may not have represented the exposure that occurred during the past 20-28 days, as measured by the keratin adduct levels, that the worker experienced. Therefore, this lack of correlation was expected.

Using multiple linear regression models in order to consider both environmental and genetic factors, we observed that the total NKA levels measured in the skin of fuel-cell maintenance workers were positively associated with exposure duration but inversely associated with the measured dermal naphthalene level and age. Interestingly, we observed previously that exposure duration was associated with lower dermal naphthalene level (Chao *et al.*, 2005). According to our study protocol, the subjects were to work for 4 h and then return to the examination station at the end of the work shift. Thus, the exposure duration was defined as the time between starting work and dermal sampling. Due to the lag-time between exposure and dermal sampling as well as the relatively fast dermal penetration rate of naphthalene, as duration of exposure increased, more naphthalene was absorbed into the stratum corneum, and metabolized by keratinocytes to form keratin adducts. Thus, less naphthalene remained on the surface of the stratum corneum for sampling by tape-stripping.

Based on the coefficient of age on the total NKA level (-0.019 for all workers and -0.022 for high-exposed group; Table 3), the data suggest an approximate 2% reduction of naphthalene metabolism in skin with each year of age increase in this subject population. This finding is consistent with a 3% reduction in naphthoquinone-albumin adduct level, and 2% reduction in 1,4-benzoquinone-albumin adduct level per year of increase in workers exposed to PAHs (Rappaport et al., 2002; Waidyanatha et al., 2004). Experimental evidence also suggests that percutaneous absorption of some chemicals decrease with increasing age (Christophers et al., 1965; Tagami, 1972; Roskos et al., 1989). This change may be due to the lower water and lipid contents in aged skin compared to young skin, which may account for decreased skin permeability (Blank et al., 1985) and partitioning of exogenous chemicals into the stratum corneum.

Because naphthalene is metabolized by phase I and II enzymes (Buckpitt et al., 2002; Preuss et al., 2003; Waidyanatha et al., 2004), we examined the effects of four metabolic genotypes (*CYP2E1*, *GSTM1*, *GSTT1*, and *NQO1*) on the measured total NKA levels in the skin and on the urine biomarker levels (Table 2). We also examined the effects of these genotypes on the total NKA (Table 3) and the urine naphthalene levels (Table 4) using linear regression modeling by considering both personal and environmental factors.

Higher levels of DNA adducts and urine naphthols have been observed in *GSTM1*-null in comparison to the *GSTM1*-plus individuals who were exposed to PAHs (Yang et al., 1999; Lee et al., 2001; Pavanello et al., 2008). However, our results indicated that *GSTM1* was not a significant contributing factor for keratin adduct or urine biomarkers levels (Table 2). In addition, *GSTM1* genotype was not observed to have an effect on dermal exposure, as measured by keratin adduct levels (Table 3), or systemic dose, as measured by urine naphthalene levels

(Table 4), when other significant factors were accounted for in the multiple linear regression analyses.

Our results showed that individuals with *GSTT1*-plus (++) genotype had significantly higher total NKA levels than the *GSTT1*-null individuals ($p = 0.080$) (Table 2). However, the effects of *GSTT1* became insignificant in regard to total NKA levels once other personal and environmental factors were considered in the linear regression model (Table 3). Interestingly, *GSTT1*-plus (++) genotype was negatively associated with the urine naphthalene levels in the linear regression model (Table 4). No significant association between *GSTT1* genotype and naphthalene metabolism has been observed (Lee et al., 2001; Nan et al., 2001). In other studies, individuals with *GSTT1*-null genotype were observed to have higher risk for benzene toxicity (Wan et al., 2002) and bladder cancer (Hung et al., 2004). On the contrary, Garte *et al.* (2007) observed a protective effect due to *GSTT1*-null genotype in regard to DNA damage. Our results and the conflicting data in the scientific literature indicate the lack of understanding of the involvement of GST in naphthalene metabolism and the complexity of investigating individual susceptibility factors in the context of environmental/occupational exposure. The effect sizes of the personal and environmental factors, such as exposure duration and dermal naphthalene level, may outweigh the effect associated with these metabolic genotypes. A single polymorphism may have only a weak effect, while susceptibility may be derived from effects of multiple genes, and the interaction from genes and the environment (Hung et al., 2004).

The second multiple linear regression model showed that urine naphthalene level increased as the total NKA and post-exposure breath naphthalene levels increased (Table 4). This observation also suggests that jet fuel exposure was sufficient to saturate the body's metabolic capacity since more naphthalene is being excreted without being metabolized. The induction of *CYP2E1* is known to increase the formation of naphthols from naphthalene, and the

polymorphisms in this gene are associated with urine 2NAP levels in Korean coke oven workers, who were exposed to PAHs via both inhalation and dermal routes (Wilson et al., 1996; Nan et al., 2001). Therefore, the inverse association between *CYP2E1**6 wild-type (DD) and the urine naphthalene levels was expected. Interestingly, the same association was not observed in the model constructed with *a priori* high-exposure group. It is possible that in the high-exposure group, the metabolic capacity of CYP2E1 had been reached, which prevented observation of a significant effect of its genotypes on urinary naphthalene levels in this subject population.

In conclusion, this is the first study to investigate the contribution of both environmental and genetic factors in regard to the formation of skin keratin adducts and the relationships between total NKA levels and other JP-8 exposure biomarkers in occupationally exposed workers. Only naphthalene through dermal route of exposure can induce formation of NKAs in the skin (*i.e.*, these adducts are route specific indicators of exposure; inhalation exposure will not contribute to the formation of these adducts). Therefore, quantitation of naphthyl-keratin protein adducts in the skin of jet fuel exposed individuals allows us to investigate the importance of dermal exposure, penetration, metabolism, and adduction of naphthalene and to predict more accurately the contribution of chronic dermal exposure to total body burden for use in exposure assessment models. The total NKA levels were affected by both the work scenarios and extrinsic and intrinsic personal factors, and were associated with the urine naphthalene levels, indicating their potential as quantitative biomarkers of dermal exposure. In addition, this novel dermal exposure biomarker represents the past exposure that occurred 20 – 28 days prior to sampling, and can be utilized in exposure assessment for other common PAHs and environmental pollutants. Further studies are needed in regard to the relationship between skin NKAs, biological effect markers, and JP-8 health effects to further explore the potential application of these adducts as biomarkers of naphthalene exposure and related health effects.

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Table 1. Pearson correlation coefficients (r) between the total skin naphthyl-keratin adduct levels [$\ln(\text{ng adduct}/\mu\text{g keratin})$] and the exposure and biomarker levels observed in the fuel-cell maintenance workers exposed to jet fuel.

Exposure or Biomarker	n	Pearson Correlation (r)	p-value
Dermal naphthalene (ng/m ²)	105	-0.038	0.704
Pre-exposure breath naphthalene [$\ln(\text{ng}/\text{m}^3)$]	103	0.149	0.133
Post-exposure breath naphthalene (ng/m ³)	101	0.038	0.707
Breathing-zone naphthalene [$\ln(\text{ng}/\text{m}^3)$]	100	0.043	0.668
Urine 1-naphthol [$\ln(\mu\text{g}/\text{l})$]	104	0.080	0.418
Urine 2-naphthol [$\ln(\mu\text{g}/\text{l})$]	104	0.012	0.907
Total urine naphthols [$\ln(\mu\text{g}/\text{l})$]	104	0.033	0.740
Urine naphthalene [$\ln(\mu\text{g}/\text{l})$]	104	0.184	0.061

n = number of fuel-cell maintenance workers

Table 2. The log-transformed means, standard deviations, and ranges of total skin naphthyl-keratin adduct (NKA) level [$\ln(\text{ng}/\mu\text{g}$ keratin)] and urine biomarker levels [$\ln(\mu\text{g}/\text{l})$] as well as the associations between these biomarker levels and the specific genotypes in the fuel-cell maintenance workers exposed to jet fuel.

	Prevalence (%)	Total NKA	1-Naphthol	2-Naphthol	Total Naphthols	Naphthalene	Total Urine Biomarkers*
Average		1.03	9.58	9.96	10.54	5.4	10.56
STD		0.38	1.19	1.17	1.13	2.21	1.14
Range		0.24 – 2.04	6.18 – 11.31	6.18 – 12.49	6.88 – 12.53	-0.84 – 10.59	6.89 – 12.53
Association:							
<i>CYP2E1*6</i>							
DD	88/105 (84)	1.04	9.55	9.94	10.52	5.29	10.55
CD	14/105 (13)	0.91	9.78	10.07	10.65	6.13	10.67
CC	3/105 (3)	1.18	9.63	10.03	10.54	5.26	10.56
<i>GSTM1</i> -null	63/105 (60)	1.03	9.52	9.94	10.5	5.13	10.52
<i>GSTM1</i> -plus (++/+-)	42/105 (40)	1.03	9.68	10	10.6	5.82	10.64
<i>GSTT1</i> -null	24/105 (23)	0.91^a	9.81	9.99	10.64	5.88	10.65
<i>GSTT1</i> -plus (++/+-)	81/105 (77)	1.06	9.51	9.96	10.51	5.26	10.54
<i>NQO1</i>							
CC	66/105 (63)	1.06	9.75	10.08	10.69	5.6	10.71
CT	34/105 (32)	1	9.25	9.75	10.26	4.87	10.28
TT	5/105 (5)	0.69^b	9.68	9.97	10.56	6.43	10.578

* The log-transformed sum of urine naphthalene, 1-naphthol, and 2-naphthol levels.

^a Significant difference ($p = 0.080$) from the mean level in the *GSTT1*-plus group.

^b Significant difference ($p = 0.085$) from the mean level in the *NQO1* (CC) type.

Table 3. Regression analyses for the total skin naphthyl-keratin adduct levels [$\ln(\text{ng adduct}/\mu\text{g keratin})$] in the fuel-cell maintenance workers exposed to jet fuel.

Workers	n	R ²	Predictor	Parameter Estimate	Standard Error	p-value ^a
All workers	103	0.267	intercept	0.746	0.239	0.0024
			ln dermal ^b	-0.038	0.015	0.0161
			exposure duration ^c	0.004	0.001	<0.0001
			age	-0.019	0.006	0.0032
High-exposure group	68	0.326	intercept	0.873	0.298	0.0047
			ln dermal ^b	-0.047	0.019	0.0167
			exposure duration ^c	0.004	0.001	<0.0001
			age	-0.022	0.009	0.0214

n = number of workers

^a Stepwise regression variable inclusion and elimination decisions conducted at the α -level 0.10.

^b Log-transformed dermal naphthalene level [$\ln(\text{ng}/\text{m}^3)$].

^c Duration of exposure on sampling day (min).

Table 4. Regression analyses for the urine naphthalene levels [$\ln(\mu\text{g/l})$] in the U.S. Air Force personnel exposed to jet fuel.

Workers	n	R ²	Predictor	Parameter Estimate	Standard Error	p-value ^a
All	96	0.351	intercept	-1.606	1.310	0.2234
			naphthyl-keratin adducts ^b	0.826	0.418	0.0510
			$\ln\text{post-breath}^c$	0.870	0.149	<0.0001
			<i>CYP2E1*6 (DD)</i>	-1.102	0.417	0.0097
			<i>GSTT1</i> (++/+-)	-0.912	0.387	0.0207
			Gender	1.505	0.564	0.0091
High-exposure group	64	0.245	intercept	0.358	1.546	0.8178
			naphthyl-keratin adducts ^b	0.951	0.452	0.0396
			$\ln\text{post-breath}^c$	0.716	0.182	0.0002
			<i>GSTT1</i> (++/+-)	-0.940	0.433	0.0339

n = number of workers

^a Stepwise regression variable inclusion and elimination decisions conducted at the α -level 0.10.

^b Log-transformed total naphthyl-keratin adduct levels [$\ln(\text{ng adduct}/\mu\text{g keratin})$].

^c Log-transformed post-exposure breath naphthalene level [$\ln(\mu\text{g}/\text{m}^3)$].