**Carcinogenesis** vol.23 no.6 pp.907–922, 2002

**REVIEW**

# Human urinary carcinogen metabolites: biomarkers for investigating tobacco and cancer

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**Measurement of human urinary carcinogen metabolites is a practical approach for obtaining important information about tobacco and cancer. This review presents currently available methods and evaluates their utility. Carcinogens and their metabolites and related compounds that have been quantified in the urine of smokers or non-smokers exposed to environmental tobacco smoke (ETS) include *trans,trans*-muconic acid (tt-MA) and *S*-phenylmercapturic acid (metabolites of benzene), 1- and 2-naphthol, hydroxyphenanthrenes and phenanthrene dihydrodiols, 1-hydroxypyrene (1-HOP), metabolites of benzo[*a*]pyrene, aromatic amines and heterocyclic aromatic amines, *N*-nitrosoproline, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol and its glucuronides (NNAL and NNAL-Gluc), 8-oxodeoxyguanosine, thioethers, mercapturic acids, and alkyladenines. Nitrosamines and their metabolites have also been quantified in the urine of smokeless tobacco users. The utility of these assays to provide information about carcinogen dose, delineation of exposed vs. non-exposed individuals, and carcinogen metabolism in humans is discussed. NNAL and NNAL-Gluc are exceptionally useful biomarkers because they are derived from a carcinogen- 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)- that is specific to tobacco products. The NNAL assay has high sensitivity and specificity, which are particularly important for studies on ETS exposure. Other useful assays that have been widely applied involve quantitation of 1-HOP and tt-MA. Urinary carcinogen metabolite biomarkers will be critical components of future studies on tobacco and human cancer, particularly with respect to new tobacco products and strategies for harm reduction, the role of metabolic polymorphisms in cancer, and further evaluation of human carcinogen exposure from ETS.**

## Introduction

This review will discuss human urinary carcinogen metabolites as biomarkers in tobacco carcinogenesis. Tobacco products cause ~30% of all cancer death in developed countries (1,2). In spite of this, there are over one billion smokers in the world and millions of people who use smokeless tobacco products (1,3,4). Exposure to environmental tobacco smoke (ETS) is also a recognized cause of cancer (5). Mortality from cancers caused by tobacco products – lung, larynx, oral cavity, esophagus, pancreas, kidney, liver, bladder, stomach, and colon – will continue to be significant in the foreseeable future (6–8). Biomarkers can increase our understanding of tobacco-related cancer mechanisms. This knowledge can be applied to develop preventive strategies which may decrease the toll of these cancers.

Carcinogens link nicotine addiction and cancer (9). Nicotine addiction is the reason that people continue to use tobacco products. Each cigarette or smokeless tobacco product contains multiple carcinogens. It is these agents which are responsible for cancer caused by tobacco products. The carcinogens in most cases require metabolic activation to reactive electrophiles that bind to DNA. There are competing detoxification processes which are protective. The DNA adducts can be removed by cellular repair mechanisms. But if they persist, miscoding can occur leading to permanent mutations. If the mutations occur in critical regions of genes involved in regulation of growth such as oncogenes or tumor suppressor genes, normal cellular growth control mechanisms can be lost and ultimately cancer can develop. Carcinogen dose depends on the amount of a tobacco product which an individual may use, and the way in which he or she uses it. Individuals differ in the extents to which they metabolically activate tobacco carcinogens and in the ways that they process DNA adducts. These and other factors may determine cancer susceptibility. Biomarkers can capture some of the quantitative aspects of the cancer development process and provide information pertinent to cancer risk (10).

Several types of carcinogen-related biomarkers have been used. DNA adduct measurements give direct information on the extent of carcinogen reactions with DNA (11–14). If measured in target tissues or cells, DNA adduct levels could relate directly to risk because DNA adducts are central to the carcinogenic process. Protein adducts have frequently been used as surrogates for DNA adducts because the two parameters tend to be correlated and protein, such as hemoglobin or albumin, is relatively easy to obtain in large quantities (15,16). A third type of carcinogen biomarker measurement – urinary compounds – is the subject of this review. Measurement of urinary compounds has certain advantages. Important among these is their quantity, which is generally great enough that, with the use of modern analytical methods, reliable data can almost always be obtained. This is frequently not the case when one is measuring DNA adducts or protein adducts. One will obtain many ‘not detected’ values. A large number of these severely limits a study’s ultimate value, frustrates graduate students and other laboratory personnel, and can drive the research group leader to distraction, early retirement, or worse. Urine is simple to obtain in large quantities and

**Abbreviations:** BaP, benzo[*a*]pyrene; ETS, environmental tobacco smoke; GC–TEA, gas chromatography with nitrosamine selective detection; 1-HOP, 1-hydroxypyrene; *iso*-NNAC, 4-(methylnitrosamino)-4-(3-pyridyl)butyric acid; NMTCA, *N*-nitroso-2-methylthiazolidine 4-carboxylic acid; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NNAL-Gluc, a mixture of 4-(methylnitrosamino)-1-(3-pyridyl)-1-(*O*-β-D-glucopyranuronosyl)butane (NNAL-*O*-Gluc) and 4-(methylnitrosamino)-1-(3-pyridyl-*N*-β-D-glucopyranuronosyl)-1-butanolonium inner salt (NNAL-*N*-Gluc); NPRO, *N*-nitrosoproline; NSAR, *N*-nitrososarcosine; NTCA, *N*-nitrosothiazolidine 4-carboxylic acid; 8-oxo-dG, 8-oxodeoxyguanosine; PAH, polycyclic aromatic hydrocarbons; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; PREPS, potential reduced-exposure products; *S*-PMA, S-phenylmercapturic acid; *trans-anti*-BaP-tetraol, *r*-7,*t*-8,9,*c*-10-tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; tt-MA, *trans,trans*-muconic acid.

compliance is almost never a problem. Although urinary compounds in most cases are considered to be quite distant from the DNA target, metabolite profiling could give an indication of relative extents of carcinogen activation and detoxification in an individual, analogous to caffeine phenotyping as an indicator of cytochrome P450 1A2 activity (17). A potential disadvantage of urinary biomarkers is their transitory nature. However, this is mitigated by the chronic use of tobacco products which provides a consistent level of urinary biomarkers.

The major use of urinary compound measurements to date has been to estimate carcinogen dose. This is presently a particularly relevant question in the area of tobacco and cancer. As I write, new products are being introduced by the tobacco industry. Advertisements for these new products claim substantially reduced levels of well-known tobacco carcinogens such as polycyclic aromatic hydrocarbons (PAH) and tobacco-specific nitrosamines. These products have been termed 'potential reduced-exposure products' (PREPS) by the Institute of Medicine in their 2001 report 'Clearing the Smoke' which assesses the science base for tobacco harm reduction (18). Constituents of PREPS have been determined by the industry using standardized machine methods. However, such methods have clearly been shown not to reflect actual human use patterns for tobacco products, because smokers may change the way they use the new products (smokers' compensation) (19,20). Therefore, we do not know whether carcinogen uptake by users of PREPS will in fact be reduced. Urinary biomarkers can determine, in a straightforward way, whether carcinogen exposure has actually been altered in people who use PREPS. This would be the essential first step in evaluating the potential reduced risk of these products. The evaluation of such products is mandatory in view of the findings of the recent report 'Risks Associated With Smoking Cigarettes with Low Machine-Measured Yields of Tar and Nicotine' which demonstrates that smokers of 'light' cigarettes marketed over the past several decades are not protected and that the popularity of these brands resulted in a sustained increase in lung cancer among older smokers (21).

Cotinine and its further transformation products, major metabolites of nicotine, have been extensively used as biomarkers of nicotine uptake from tobacco products (22,23). However, cotinine and nicotine are not carcinogenic and, although cotinine measurements do provide a measure of nicotine exposure, they say little about potential carcinogenicity. In addition, cotinine measurements cannot be used to assess tobacco constituent uptake in people using nicotine replacement therapy during smoking reduction programs. Minor alkaloids such as anatabine and anabasine have been used for this purpose, since they are found in all tobacco products but not in significant quantities in nicotine replacement products (24). Anatabine and anabasine like nicotine and cotinine are non-carcinogenic.

Hoffmann *et al.* listed over 60 carcinogens in cigarette smoke for which there is sufficient evidence for carcinogenicity in either laboratory animals or humans, according to the International Agency for Research on Cancer (25). These include benzene, various PAH, aromatic amines, heterocyclic aromatic amines, *N*-nitrosamines, a variety of low molecular weight organic compounds such as formaldehyde, acetaldehyde, and butadiene, and several metals. In this paper, I will review urinary compounds related to organic carcinogens on this list.

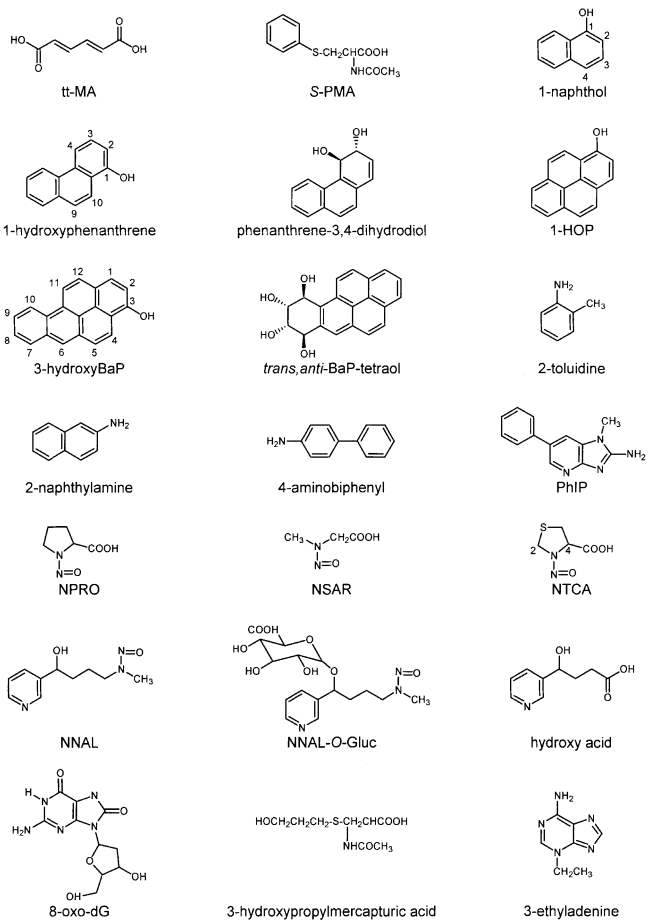


**Fig. 1.** Structures of compounds discussed in this review.

## Urinary compounds as biomarkers in smokers

### *trans,trans-muconic acid (tt-MA), S-phenylmercapturic acid (S-PMA) and other benzene metabolites*

Benzene causes leukemia in humans (26). Its concentration in mainstream smoke is relatively high, 6–70 μg/cigarette (25). One pathway of the complex metabolism of benzene proceeds via ring oxidation and ultimately ring cleavage to *trans,trans*-muconaldehyde and finally to tt-MA (see Figure 1 for structures of compounds discussed here). This metabolite has been widely used as a biomarker of benzene uptake. A recent review summarizes the pertinent literature (27). Significantly elevated levels of tt-MA were found in the urine of smokers in 12 of 14 studies (27). Levels of tt-MA were 1.4–4.8 times greater in smokers than in non-smokers and the additional amount of tt-MA excreted by smokers ranged from 0.022 to 0.20 mg per g creatinine (27). However, sorbic acid, a food constituent that can be transformed metabolically to tt-MA, can contribute to background levels of tt-MA, thereby decreasing its specificity (27,28).

*S*-PMA is formed by normal degradation of the glutathione conjugate of benzene oxide and has the potential to be quite specific (29–33). In one study, levels of *S*-PMA were significantly higher in smokers (1.71 μmol/mol creatinine) compared with non-smokers (0.94 μmol/mol creatinine) whereas levels of tt-MA were not significantly different (29). Another recent report also found significant differences in *S*-PMA levels between smokers and non-smokers (34). *S*-PMA

and tt-MA are believed to be the most sensitive biomarkers for low level benzene exposure (33).

Phenol, hydroquinone, catechol, and 1,2,4-trihydroxybenzene are also urinary metabolites of benzene. Mixed results have been obtained in studies relating urinary levels of these metabolites to occupational exposure to benzene, because background levels are high (33,35–38). We found no difference in urinary catechol levels between smokers and non-smokers (39). Diet was a major source of urinary catechol (39).

*1- and 2-Naphthol*

Inhalation studies of naphthalene provide clear evidence of carcinogenicity in rats (40). Naphthalene caused increased incidences of respiratory epithelial adenoma and olfactory epithelial neuroblastoma of the nose. Naphthalene is a component of all PAH mixtures. Its concentration in mainstream cigarette smoke is 2–4 μg/cigarette (25). Naphthalene is metabolized to 1- and 2-naphthol which are excreted in urine as glucuronide and sulfate conjugates. These metabolites are elevated in smokers compared with non-smokers (41–47). For example, Nan *et al.* found levels of 3.94 ± 1.89 μmol/mol creatinine 2-naphthol in smokers compared with 1.55 ± 2.19 μmol/mol creatinine in non-smokers ($P < 0.01$) (46). There is some indication that urinary naphthols may be particularly appropriate as biomarkers of inhalation exposure to PAH, possibly due to the high volatility of naphthalene (41,46,47). It has been proposed that urinary 2-naphthol is a better biomarker than urinary 1-naphthol for inhalation exposure (46). Urinary 2-naphthol correlated with urinary cotinine more closely than did 1-naphthol (47). Levels of urinary 2-naphthol can be affected by genetic polymorphisms in carcinogen metabolizing genes such as *CYP2E1* and *GSTM1* (46).

*Polycyclic aromatic hydrocarbons (PAH)*

*Hydroxyphenanthrenes and phenanthrene dihydrodiols*

Phenanthrene is the simplest PAH with a bay region and is a reasonable model for metabolism studies of carcinogenic PAH molecules with bay regions. PAH are a well established group of environmental carcinogens which are likely to contribute substantially to the carcinogenic activity of cigarette smoke (6,9,48,49). Phenanthrene however is inactive as a carcinogen (50). Concentrations of phenanthrene in mainstream smoke are 85–620 ng/cigarette (6). Hydroxyphenanthrenes and phenanthrene dihydrodiols have been quantified in human urine. Heudorf and Angerer, using HPLC with fluorescence detection, reported highly significant differences between smokers and non-smokers and dose-response relationships to cigarettes smoked per day in levels of 2-, 3-, and 4-hydroxyphenanthrene, but not 1-hydroxyphenanthrene (51). For example, levels of 3-hydroxyphenanthrene were 473 ± 302 ng/g creatinine in smokers ($N = 100$) and 305 ± 299 ng/g creatinine in non-smokers ($N = 288$) ($P = 0.001$). Jacob *et al.* examined levels of phenanthrene metabolites by GC-MS (52). They found no significant differences in levels of hydroxyphenanthrenes or phenanthrene dihydrodiols between smokers and non-smokers, possibly due to the small size of the study. Reported values for 3-hydroxyphenanthrene were 598 ± 267 ng/24 h in smokers ($N = 9$) and 395 ± 187 in non-smokers ($N = 10$). Clearly, there are important sources of phenanthrene exposure other than smoking. This has been well documented in environmental and occupational settings with high PAH exposure (53–55). Jacob *et al.* found a lower ratio of phenanthrene-1,2-dihydrodiol to phenanthrene-3,4-dihydrodiol in smokers than in non-smokers suggesting that smoking induces formation of phenanthrene-3,4-dihydrodiol via induction of cytochrome P450 1A2. Similar results were obtained by Heudorf and Angerer who found that the ratio of 1- plus 2-hydroxyphenanthrene to 3- plus 4-hydroxyphenanthrene decreased with increased cigarette consumption. Both groups also observed a decreased ratio of phenanthrene metabolites to 1-hydroxypyrene (1-HOP) with increased smoking, reflecting greater intake of pyrene than phenanthrene in smokers (52,56). Phenanthrene metabolites appear to have considerable promise for probing human PAH metabolism.

*1-Hydroxypyrene (1-HOP)*

Pyrene is a non-carcinogenic component of all PAH mixtures. Its concentration in mainstream cigarette smoke is 50–270 ng/cigarette (6). The major urinary metabolite of pyrene is 1-HOP glucuronide. Jongeleelen pioneered the development of a method for measurement of 1-HOP in urine (57). After enzymatic hydrolysis, the released 1-HOP is enriched by reverse phase chromatography and quantified by HPLC with fluorescence detection. Variations of this method have been described. 1-HOP has been measured in hundreds of studies of occupational and environmental PAH exposure. Data on the effects of smoking have been reviewed by Jongeneelen (58,59), van Rooj *et al*. (60), Heudorf and Angerer (51), and Levin (61). Most studies find significantly higher levels of 1-HOP in smokers than in non-smokers. Some representative data from recent investigations of urinary 1-HOP are summarized in Table I. These data are from non-occupationally exposed individuals. Levels in the urine of non-smokers vary considerably and are likely to be influenced by environmental pollution and diet. In most studies, 1-HOP levels in smokers' urine are about twice as great as in non-smokers, although greater differences have been reported. Levels may be influenced by genetic polymorphisms in carcinogen metabolizing enzymes (46,70,71,74).

*Benzo[a]pyrene (BaP) metabolites*

The carcinogenic activity of BaP in laboratory animals has been conclusively established and its presence in cigarette smoke has been repeatedly demonstrated. Therefore, measurement of BaP metabolites in the urine of smokers could potentially provide a direct assessment of carcinogen dose. However, unlike the lower molecular weight PAH considered above, the concentrations of BaP in cigarette smoke are quite low, presently ~9 ng/cigarette (75), and in laboratory animals its metabolites are excreted mainly in the feces. Therefore, BaP metabolites are difficult to quantify in smokers' urine.

3-HydroxyBaP is a major metabolite of BaP *in vitro* and is excreted in urine as its glucuronide. Methods for quantitation of 3-hydroxyBaP in human urine have been described (54,76,77). These methods are based on HPLC with fluorescence detection or GC-MS. Reported levels are quite low ranging from ~1–14 ng/l in exposed workers. Limited data are available on smokers. One study reported 0.1–0.8 ng/l in smokers ($N = 3$) compared with not detected – 0.2 in non-smokers ($N = 3$) (76). High levels of 3-hydroxyBaP have been reported in Chinese exposed to smoky coal (78).

*r*-7,*t*-8,9,*c*-10-Tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (*trans-anti*-BaP-tetraol) is a hydrolysis product of *anti*-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene, the major established ultimate carcinogen of BaP. We have

**Table I.** I-Hydroxypyrene in the urine of smokers and non-smokers: representative recent studies

| 1-Hydroxypyrene level[a] | | | | |
|---|---|---|---|---|
| Non-smoker | Smoker | Fold increase | Significant difference? | Reference |
| 0.12 μmol/mol C[b] | 0.21 μmol/mol C | 1.8 | yes | (62) |
| 0.55 nmol/l | 1.04 nmol/l | 1.9 | yes | (63) |
| 0.089 μmol/mol C | 0.176 μmol/mol C (<15 cig/day) | 2.0 | yes | (64) |
| | 0.226 μmol/mol C (>15 cig/day) | 2.5 | | |
| 0.784 nmol/24 h | 1.59 nmol/24 h | 2.0 | yes | (65) |
| 1.10 nmol/l | 2.47 nmol/l | 2.2 | yes | (66) |
| 1.0 nmol/24 h | 2.77 nmol/24 h | 2.8 | yes | (52) |
| 0.04 μmol/mol C | 0.20 μmol/mol C : light | 5.0 | | |
| | 0.46 μmol/mol C : medium | 11.5 | yes | (67) |
| | 1.16 μmol/mol C : heavy | 29 | | |
| 0.27 nmol/12 h | 0.51 nmol/12 h | 1.9 | yes | (68) |
| 0.03 μmol/mol C[c] | 0.04 μmol/mol C[c] | 1.3 | yes | (69) |
| 0.10 μmol/mol C | 0.17 μmol/mol C | 1.7 | yes | (70) |
| 0.04 μmol/mol C[d] | 0.05 μmol/mol C[d] | 1.3 | no | (46) |
| 0.27 μmol/mol C | 0.70 μmol/mol C | 2.6 | yes | (71) |
| 0.03 μmol/mol C | 0.05 μmol/mol C | 1.7 | yes | (72) |
| 0.11 μmol/mol C | 0.57 μmol/mol C | 5.2 | yes | (73) |

[a]Arithmetic mean unless noted otherwise.
[b]C, creatinine.
[c]Median.
[d]Geometric mean.

developed a method for quantitation of this metabolite in human urine by GC-negative ion chemical ionization-MS (79). The method was applied to psoriasis patients treated with a coal tar ointment, coke oven workers, and smokers. Levels of *trans-anti*-BaP-tetraol in the urine of smokers were lower than in the other two groups, ranging from not detected to 0.2 fmol/μmol creatinine. It was not detectable in urine samples from 12 of 21 smokers (79). Immunoaffinity-synchronous fluorescence spectroscopy methods for quantitation of BaP-tetraols in human urine have also been reported, but they have not been applied to smokers' urine (80–82).

BaP metabolites can be converted to BaP by treatment with HI. This reaction has been employed as the basis for a technique to determine urinary metabolites of BaP and several other PAH (83–87). In occupationally non-exposed individuals, levels of BaP determined by this method ranged from 4–19 ng/mmol creatinine in non-smokers ($N = 5$) and 18–102 ng/mmol creatinine in smokers ($N = 4$) (84). Problems with this method include different conversions for various metabolites and low analytical recoveries (87).

An unstable BaP-DNA adduct, 7-(benzo[*a*]pyrene-6-yl) adenine, has been reported in the urine of 3 of 7 smokers and its quantity was estimated in one as 0.6 fmol/mg creatinine (88).

*Other PAH metabolites*

Several studies have reported the presence of other PAH metabolites in urine, including hydroxychrysenes and chrysene dihydrodiols (54), 3-hydroxybenz[*a*]anthracene (77), and – under high exposure conditions – 3-hydroxyfluoranthene, 6-hydroxychrysene, and 6-hydroxyindeno[1,2,3-*cd*]pyrene (78). None of these studies investigated the relationship of these metabolites to smoking. In addition, several PAH have been reported in urine treated with HI, as described above (83–85). These include fluorene, phenanthrene, anthracene, fluoranthene, benz[*a*]anthracene, chrysene, and benzo[*e*]pyrene, but there are insufficient data to evaluate the utility of this methodology as a biomarker of PAH uptake in smokers.

*Aromatic amines and heterocyclic aromatic amines*

Aromatic amines were first identified as carcinogens due to industrial exposures in the dye industry. Among these, 2-naphthylamine and 4-aminobiphenyl are well established human bladder carcinogens (89,90). Aromatic amines cause tumors at a variety of sites in laboratory animals. One member of this class, 2-toluidine, is only weakly carcinogenic (91), but has been proposed as a cause of human bladder cancer (92). Levels of 2-naphthylamine, 4-aminobiphenyl, and 2-toluidine in mainstream cigarette smoke are 1–334, 2–5.6, and 30–337 ng/cigarette, respectively (25).

Aromatic amines but not their metabolites have been quantified in human urine. In one study, smokers excreted 6.3 ± 3.7 μg/ 24 h of 2-toluidine while levels in non-smokers were 4.1 ± 3.2 μg/24 h, not significantly different from smokers (93). Another investigation reported higher levels of 2-toluidine in smokers than in non-smokers (94). There appear to be significant sources of human uptake of 2-toluidine in addition to cigarette smoke. Smokers excreted similar amounts of 4-aminobiphenyl (78.6 ± 85.2 ng/24 h) as non-smokers (68.1 ± 91.5 ng/24 h), and similar amounts of 2-naphthylamine (84.5 ± 102.7 ng/24 h) as non-smokers (120.8 ± 279.2) (95). Hemoglobin adducts appear to be better biomarkers of aromatic amine exposure from tobacco-smoke than urinary metabolites (16).

DNA adducts have been detected by $^{32}$P-postlabelling in exfoliated urothelial cells isolated from urine of smokers and non-smokers. At least four adducts may have been related to smoking and one was qualitatively similar to *N*-(deoxyguanosin-8-yl)-4-aminobiphenyl (96). This approach requires further investigation.

Heterocyclic aromatic amines are well-known mutagens and carcinogens in cooked foods (97). Several of these compounds are listed as carcinogens in cigarette smoke (25). Among these, analyses of urinary metabolites have been carried out only in the case of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) (reviewed in ref. 98). Most studies have focused on the

effects of diet, but one found no effect of smoking on levels of PhIP in urine, after adjustment for ethnicity (99).

### *N-Nitrosamines*

#### *N-Nitrosoproline (NPRO) and other nitrosamino acids*

In 1981, Ohshima and Bartsch demonstrated that NPRO could be formed endogenously in humans after ingestion of proline and nitrate (100). This finding evolved into a test for endogenous nitrosation by measurement of NPRO in urine of people who ingested proline and nitrate, or proline alone with or without ascorbic acid, an inhibitor of nitrosation (101). The test is safe because NPRO is not metabolized and is not carcinogenic. This test has been applied in several studies designed to compare endogenous nitrosation in smokers and non-smokers. The results indicate that endogenous formation of NPRO occurs in smokers, and that thiocyanate catalysis may be important (101–103). However, mixed results have been obtained in population-based studies (103).

Several other nitrosamino acids are present in human urine. The major ones are *N*-nitrososarcosine (NSAR), *N*-nitrosothiazolidine 4-carboxylic acid (NTCA) and *trans*- and *cis*-isomers of *N*-nitroso-2-methylthiazolidine 4-carboxylic acid (NMTCA) (101,102). NTCA and NMTCA are formed by reactions of formaldehyde or acetaldehyde with cysteine, followed by nitrosation. Some studies demonstrate increased levels of urinary NTCA and NMTCA in smokers (102). Total nitrosamino acids correlated with urinary nicotine plus cotinine in smokers (104). Mixed results have been obtained in other studies (103). Collectively, the available data support the concept that nitrosamines can be formed endogenously in smokers under some conditions.

4-(Methylnitrosamino)-4-(3-pyridyl)butyric acid (iso-NNAC) was suggested as a potential monitor of endogenous nitrosation of nicotine (105). However, no evidence for its formation after oral administration of nicotine or cotinine to abstinent smokers could be found (106).

#### *Volatile nitrosamines*

Low molecular weight nitrosamines such as *N*-nitrosodimethylamine (NDMA) and *N*-nitrosopyrrolidine (NPYR) are extensively metabolized, but small amounts of the unchanged compounds have been quantified in urine (103). One investigation found that smokers excreted higher levels of NDMA than non-smokers (107), but in two other studies there were no effects of smoking on levels of volatile nitrosamines in urine (108,109). Most research on volatile nitrosamines in urine has not focused on smoking.

#### *4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), its glucuronides (NNAL-Gluc), and other metabolites of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)*

NNK, a tobacco-specific nitrosamine, is a systemic lung carcinogen. NNK induces pulmonary tumors in rats, mice, and hamsters independent of the route of administration (110,111). Its lung tumorigenicity is particularly strong in rats, in which total doses as low as 6 mg/kg, administered by s.c. injection, or 35 mg/kg administered in the drinking water, produced statistically significant incidences of lung tumors (112,113). Even lower doses induced lung tumors when considered in dose–response trend analyses. NNK is the most prevalent systemic lung carcinogen in tobacco products (9,25). Its presence has been repeatedly demonstrated and confirmed in multiple international studies (114). Levels of NNK in cigarette smoke are typically 100–200 ng/cigarette as measured by the standard machine smoking (FTC) method, but higher under actual smoking conditions (20). Since NNK is formed by nitrosation of nicotine or the related minor alkaloid 'pseudo-oxynicotine' [(4-methylamino)-1-(3-pyridyl)-1-butanone], it is found only in tobacco products, and possibly in other nicotine containing products but never in the diet or the general environment unless there is pollution by tobacco smoke. This is important with respect to the use of NNK metabolites as biomarkers.

NNAL and its glucuronides are quantitatively significant metabolites of NNK in rodents and humans (110). NNAL is also a pulmonary carcinogen with particularly strong activity in the rat (110). Glucuronidation of NNAL at the pyridine nitrogen gives NNAL-*N*-Gluc while conjugation at the carbinol oxygen yields NNAL-*O*-Gluc. NNAL-*N*-Gluc and NNAL-*O*-Gluc each exist as a mixture of 2 diastereomers and each diastereomer is a mixture of *E*- and *Z*-rotamers (115). The NNAL glucuronides are collectively referred to as NNAL-Gluc. (*R*)-NNAL-*O*-Gluc is inactive as a tumorigen in mice (116).

NNAL and NNAL-Gluc can readily be determined in urine by gas chromatography with nitrosamine selective detection (GC–TEA) (117–119). The presence of these metabolites in human urine has also been established by MS methods, but these are less convenient and sensitive than GC–TEA at present (117,120–123). Typical levels are ~1 nmol/24 h NNAL and 2.2 nmol/24 h NNAL-Gluc; unchanged NNK is not detected. Investigations of NNAL and NNAL-Gluc in human urine are summarized in Table II. Several points are noteworthy. In studies to date, this biomarker is absolutely specific to tobacco exposure. It has not been detected in the urine of non-tobacco users unless they were exposed to ETS. Since NNAL is not present in cigarette smoke, the origin of NNAL and NNAL-Gluc in urine is metabolism of NNK. Most investigations to date demonstrate a correlation between NNAL plus NNAL-Gluc and cotinine (Figure 2A) indicating that NNAL plus NNAL-Gluc is an effective biomarker of lung carcinogen (NNK) uptake whereas cotinine is an effective biomarker of nicotine uptake. NNAL-Gluc:NNAL ratio varies at least 10-fold in smokers and, since NNAL-Gluc is a detoxification product whereas NNAL is carcinogenic, this ratio could be a potential indicator of cancer risk (118,125). In human urine, (*S*)-NNAL-*O*-Gluc is the predominant diastereomer of NNAL-*O*-Gluc while (*S*)-NNAL is slightly in excess over (*R*)-NNAL (120). (*S*)-NNAL is the more tumorigenic enantiomer of NNAL in A/J mouse lung (116). NNAL and NNAL-Gluc are only slowly released from the human body after smoking cessation and this has been linked to particularly strong retention of (*S*)-NNAL, possibly at a receptor site (119).

In rodents, NNK and NNAL undergo metabolic oxidation at the pyridine nitrogen giving NNK-*N*-oxide and NNAL-*N*-oxide, respectively (110). Both metabolites are less tumorigenic than NNK and NNAL (110). Analysis of human urine for NNAL-*N*-oxide demonstrated that its levels were less than those of NNAL; NNK-*N*-oxide was not detected (138). Thus, pyridine-*N*-oxidation is a relatively minor detoxification pathway of NNK and NNAL in humans.

4-Hydroxy-4-(3-pyridyl)butanoic acid (hydroxy acid) and 4-oxo-4-(3-pyridyl)butanoic acid (keto acid) are metabolites of NNK resulting from the α-hydroxylation metabolic activation pathway. Hydroxy acid and keto acid are potential biomarkers

**Table II.** NNAL and its glucuronides (NNAL-Gluc) in urine: biomarkers of NNK uptake

**A. Smokers**

| Study group | Main conclusions | Reference |
|---|---|---|
| 1. 11 smokers (9F)[a] 7 non-smokers | NNAL and 2 diastereomers of NNAL-*O*-Gluc identified in smokers' urine, but not in non-smokers' urine. NNK not detected | (117) |
| 2. 74 smokers (41F) | NNAL + NNAL-Gluc: levels stable day to day in smokers' urine; NNAL-Gluc:NNAL ratios fairly stable; range of NNAL-Gluc:NNAL ratios 0.7–10.8 | (118) |
| 3. 11 smokers (6F) | NNAL + NNAL-Gluc increased by 33.5% ($P < 0.01$) on days when watercress was consumed compared with baseline and follow-up periods | (124) |
| 4. 61 smokers (31F) | NNAL-Gluc:NNAL ratio higher in Caucasians than African–Americans | (125) |
| 5. 19 smokers | NNAL-Gluc: NNAL ratio and NNAL plus NNAL-Gluc fairly stable over a 2-year period in one individual | (126) |
| 6. 13 smokers (F) | Indole-3-carbinol caused significant decreases in levels of NNAL and NNAL plus NNAL-Gluc and increased NNAL-Gluc:NNAL ratio | (127) |
| 7. 27 smokers (13F) | NNAL and NNAL-Gluc highly persistent after smoking cessation; 34.5% of baseline amount remained after 1 week, 15.3% after 3 weeks. No effect of nicotine patch use on levels or persistence of NNAL or NNAL-Gluc | (119) |
| 8. 30 smokers (18F) | Enantiomeric distribution of NNAL, 54%(*S*); diastereomeric distribution of NNAL-Gluc, 68%(*S*) | (120) |
| 9. 23 smokers (13F) | Reduction in smoking caused a significant decrease in NNAL-Gluc but not NNAL | (128) |
| 10. 20 smokers (M) | Levels of NNAL and NNAL-Gluc were (mean ± SD) 1494 ± 1090 and 1724 ± 946 pmol/day, respectively | (129) |
| 11. 10 smokers | NNAL-*N*-Gluc identified in urine, comprises 50 ± 25% of total NNAL-Gluc | (130) |

**B. Smokeless tobacco users**

| Study group | Main conclusions | Reference |
|---|---|---|
| 1. 7 toombak users (M) | Exceptionally high levels of NNAL and NNAL-Gluc (0.12–0.44 mg) excreted daily. (*S*)-NNAL-*O*-Gluc:(*R*)-NNAL-*O*-Gluc ratio, 1.9 | (131) |
| 2. 47 smokeless tobacco users (chewers and snuff-dippers) (M) | NNAL and NNAL-Gluc levels similar to those in smokers. Significant association between total NNAL + NNAL-Gluc and oral leukoplakia | (132) |
| 3. 13 smokeless tobacco users (M) | Distribution half-lives of NNAL and NNAL-Gluc significantly shorter in smokeless tobacco users than smokers. Ratios of (*S*)-NNAL:(*R*)-NNAL and (*S*)-NNAL-Gluc:(*R*)-NNAL-Gluc significantly higher 7 days after cessation than at baseline, suggesting receptor site for (*S*)-NNAL | (133) |
| 4. 10 smokeless tobacco users and 4 toombak users | NNAL-*N*-Gluc identified in urine, comprises 24 ± 12% of total NNAL-Gluc | (130) |

of NNK metabolic activation in humans. However, these metabolites are also formed from nicotine, which is 1400–13 000 times more abundant in cigarette smoke than is NNK. Since hydroxy acid is chiral, it was possible that one enantiomer would be formed preferentially from NNK, while the other would be produced from nicotine. Studies in rats demonstrated that this was plausible. However, hydroxy acid is a more abundant nicotine metabolite in humans than in rats and consequently even the minor enantiomer, as formed from nicotine, was far greater in concentration than that which would be produced from NNK. Because of the abundant metabolism of nicotine to hydroxy acid and keto acid, these metabolites cannot be used as biomarkers of tobacco-specific nitrosamine metabolism in humans (139,140).

**Table II.** *Continued*

**C. ETS and transplacental exposure**

| Study group | Main conclusions | Reference |
|---|---|---|
| 1. 5 men exposed to ETS | Significantly increased levels of NNAL plus NNAL-Gluc after exposure to ETS in a chamber: mean ± SD after exposure, ~0.16 ± 0.10 pmol/ml | (134) |
| 2. 5M, 4F exposed to ETS 5 controls unexposed | Significantly increased levels of NNAL-Gluc in workers exposed to ETS compared with negative controls: mean ± SD in exposed workers, 0.059 ± 0.028 pmol/ml | (121) |
| 3. 30 non-smokers (13F) | NNAL + NNAL- Gluc levels correlated with nicotine levels on personal samplers. NNAL, 20.3 ± 21.8 pmol/day, NNAL-Gluc, 22.9 ± 28.6 pmol/day in exposed non-smokers | (129) |
| 4. 45 non-smoking women, 23 exposed to ETS in the home, 22 non-exposed | NNAL and NNAL-Gluc significantly higher in exposed than in non-exposed women. NNAL + NNAL-Gluc in exposed women, 0.050 ± 0.068 pmol/ml | (135) |
| 5. 204 non-smoking elementary school-aged children | 34% with total cotinine ≥5 ng/ml; 52/54 of these samples had detectable NNAL or NNAL-Gluc, 93-fold range. Mean ± SD, NNAL + NNAL-Gluc 0.056 ± 0.076 pmol/ml | (123) |
| 6. 31 newborns of mothers who smoked; 17 newborns of mothers who did not smoke | NNAL-Gluc detected in 71% of urines of newborns of smokers, NNAL in 13%; neither detected in urines of newborns of non-smokers, a significant difference; NNAL + NNAL-Gluc in urine of newborns of smoking mothers, 0.13 ± 0.15 pmol/ml | (122) |
| 7. 21 smokers and 30 non-smokers | NNAL detected in amniotic fluid of 52.4% of smokers and 6.7% of non-smokers, a significant difference. NNAL levels in amniotic fluid of smokers, 0.025 ± 0.029 pmol/ml | (136) |
| 8. 12 smokers and 10 non-smokers | NNAL and NNAL-Gluc not detected in follicular fluid | (137) |

[a]Number and letter in parentheses represent number and gender of subjects.

## Other compounds

### *Products of oxidative DNA damage*

Cigarette smoke contains free radicals and induces oxidative damage (141,142). The gas phase of freshly generated cigarette smoke contains large amounts of nitric oxide and other unstable oxidants (9). The particulate phase is postulated to contain long-lived radicals that may undergo quinone-hydroquinone redox cycling (141). The presence of such free radicals and oxidants can lead to oxidative DNA damage resulting in the formation of products such as 8-oxodeoxyguanosine (8-oxo-dG), thymine glycol, thymidine glycol, and 5-hydroxymethyl-uracil. Repair of these modified DNA constituents ultimately leads to their excretion in urine. 8-oxo-dG has been quantified frequently in the urine of smokers and non-smokers. This work has been reviewed (143–145). Cigarette smoking usually results in modestly increased levels of 8-oxo-dG in urine, in amounts 16–50% greater than in non-smokers, although negative results have also been reported (146). Smoking cessation caused a decrease in the excretion of 8-oxo-dG by 21% (145). A recent longitudinal study showed that intra-individual variation in urinary 8-oxo-dG was greater than the increase due to smoking, suggesting that there may be a complex pattern of factors determining levels of this biomarker in urine (147,148). No effect of smoking on urinary levels of 5-hydroxymethyluracil was observed (149).

### *Thioethers and mercapturic acids*

Conjugation of electrophiles with glutathione ultimately results in the excretion of mercapturic acids (*N*-acetylcysteine conjugates) in the urine (150,151). A method for determination of total thioethers in urine has been applied in a substantial number of studies comparing smokers and non-smokers. This work has been reviewed (6,151,152). Cigarette smokers excrete significantly higher levels of thioethers than non-smokers. There is considerable interindividual variation when diet, a major source of sulfur containing compounds, is not controlled (152,153). This assay provides no information about the structure of the electrophiles which ultimately are detected in urine as conjugates.

More specific methods have been applied to investigate the presence of mercapturic acids in human urine. Levels of 3-hydroxypropylmercapturic acid, a likely detoxification product of acrolein, are significantly elevated in the urine of smokers (154,155). Excretion of methylmercapturic acid was significantly correlated to smoking dose parameters, but no relationship

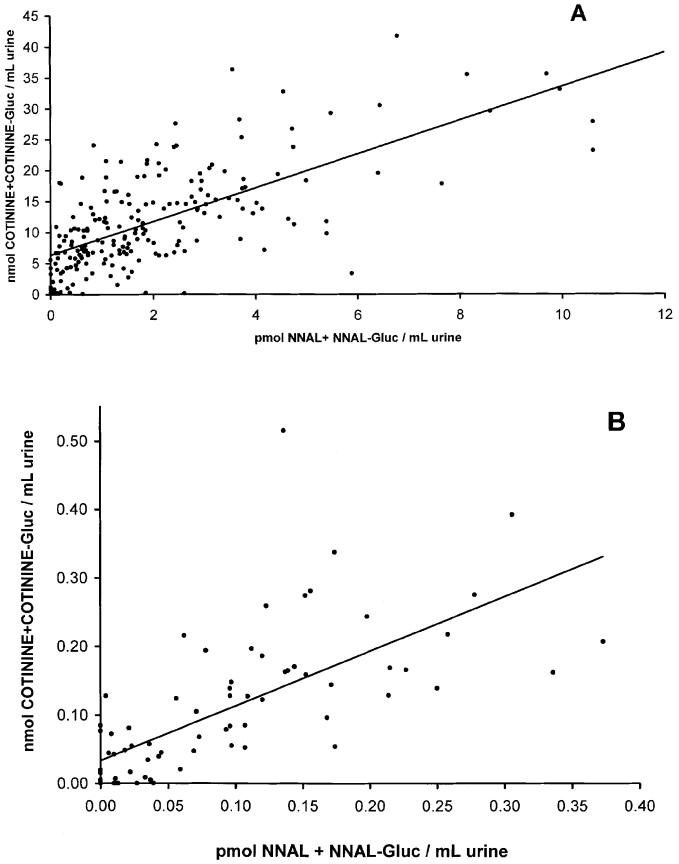


**Fig. 2.** Relationship between the NNK metabolites NNAL plus NNAL-Gluc and the nicotine metabolites cotinine plus cotinine-Gluc in human urine. (**A**) smokers, r =0.68, $P < 0.01$. (**B**) children exposed to ETS, r = 0.71, $P < 0.01$.

of smoking to levels to 2-cyanoethylmercapturic acid and 2-hydroxyethylmercapturic acid was found (155).

*Alkyladenines and alkylguanines*

Reaction of alkylating agents with DNA results in the formation of alkyladenines and alkylguanines among other products (156). Alkylation at the 3-position of deoxyadenosine or at the 7-position of deoxyguanosine gives products that have an unstable glycosidic bond and are readily removed either spontaneously or by glycosylases resulting in the excretion of 3-alkyladenines and 7-alkylguanines in urine. 3-Alkyladenines have been more extensively investigated as biomarkers of exposure to alkylating agents because background levels in urine are expected to be lower than those of 7-alkylguanines. However, substantial amounts of 3-methyladenine occur in the diet (157,158). Nevertheless, two controlled studies demonstrated increased excretion of 3-methyladenine in the urine of smokers (159,160). Background levels of 3-ethyladenine are lower than those of 3-methyladenine (157). Two studies demonstrated convincing increases in urinary levels of 3-ethyladenine in smokers, indicating the presence in cigarette smoke of an unidentified ethylating agent (159,160). There was no effect of smoking on levels of 3-(2-hydroxyethyl)adenine in urine (160). A population-based study found higher levels of both 3-methyladenine and 7-methylguanine in smokers than in non-smokers while a second found no difference in 3-methyladenine levels (161,162).

## Urinary compounds as biomarkers in smokeless tobacco users

*N-Nitrosamines*

*NPRO and other nitrosamino acids*

Betel quid chewing is a major cause of oral cavity cancer in India and other Asian countries (163). The quid frequently contains tobacco and lime. Among subjects dosed with proline, NPRO was significantly elevated in the urine of individuals who chewed tobacco plus lime, and its formation was inhibited by ascorbic acid (164). Levels of some other nitrosamino acids were not significantly affected by betel quid chewing (164,165). NPRO levels were unaffected by tobacco chewing in a group of Nebraskans (166).

*NNAL and NNAL-Gluc*

Levels of NNK in smokeless tobacco products marketed in the US are typically ~1–2 μg/g tobacco (167). NNK and *N′*-nitrosonornicotine are the most abundant strong carcinogens in unburned tobacco products and likely play a role in the induction of oral cavity tumors in snuff-dippers (110). NNAL and NNAL-Gluc are readily determined in the urine of snuff-dippers and tobacco chewers. Levels are similar to those found in smokers (132). After cessation of smokeless tobacco use, NNAL and NNAL-Gluc disappear slowly from the body, as in smokers (133). NNAL and NNAL-Gluc levels in the urine of Sudanese men who use toombak, an oral tobacco product, are exceptionally high, in keeping with the high levels of NNK in this product (131).

## Urinary compounds as biomarkers in non-smokers

Human uptake of carcinogens from ETS has been reviewed (168). Mixed results have been obtained in studies on the relationship of tt-MA to ETS exposure, with some studies showing marginally higher amounts in people exposed to ETS while others found no effect (169–174). Levels of 1-HOP and hydroxyphenanthrenes in urine are not consistently increased by exposure to ETS, although small effects have been seen under some high exposure conditions (60,65,175–177). Levels of aromatic amines and 8-oxo-dG in urine were unaffected by exposure to ETS (95,147).

All studies reported to date show significantly higher amounts of NNAL plus NNAL-Gluc, or NNAL-Gluc, in the urine of ETS-exposed humans than in unexposed controls (Table IIC, entries 1–5). In one study, uptake of NNK was over six times higher in women who lived with smokers compared with women who lived with non-smokers (135). In another investigation, widespread uptake of NNK was demonstrated in a group of economically disadvantaged school-children (123). As in smokers, a correlation between levels of cotinine and NNAL plus NNAL-Gluc in urine has been consistently observed in ETS-exposed non-smokers (Figure 2B). The assay for NNAL and NNAL-Gluc in urine is ideally suited to investigations of ETS exposure for two reasons. First, it has the required sensitivity to measure relatively low levels (typically ~0.05 pmol/ml urine). Second, since NNK is a tobacco-specific compound, detection of NNAL and NNAL-Gluc in urine specifically signals ETS exposure. The uptake of NNK by non-smokers exposed to ETS provides a biochemical link between ETS exposure and lung cancer.

NNAL-Gluc has been detected in the urine of newborns of women who smoked, indicating that NNK, a transplacental carcinogen, crosses the placental barrier and is taken up by

**Table III.** Representative concentrations of biomarkers in smokers' urine

| Biomarker | Amount (nmol/24 h) | Precursor (ng/cigarette) | Reference |
|---|---|---|---|
| tt-MA | 1100 | 6000–70 000 | (34) |
| 2-Naphthol | 73[a] | 2000–4000 | (44) |
| 3-Hydroxyphenanthrene | 3.1 | 85–620 | (52) |
| 1-Hydroxypyrene | 1.6 | 50–270 | (65) |
| *trans-anti*-BaP-tetraol | 0.0008 | 9 | (79) |
| NNAL + NNAL-Gluc | 3.2 | 100–200 | (119) |
| 8-Oxo-dG | 25 | ? | (147) |
| 3-Ethyladenine | 0.85 | ? | (159) |

[a]Estimate based on 1.5 l urine excreted per day.

the fetus (122). Consistent with these results, NNAL was detected in amniotic fluid of pregnant smokers (136). However, neither NNAL nor NNAL-Gluc could be detected in follicular fluid (137).

Urinary 3-ethyladenine was unaffected by exposure to ETS (159). No evidence of significantly increased thioethers was found in studies of ETS exposure (152,178,179). 3-Hydroxypropyl mercapturic acid, possibly from acrolein exposure, was identified in the urine of ETS-exposed non-smokers (175).

## Discussion

Urinary biomarkers potentially can provide three types of information. The first is carcinogen dose. This is important for assessing overall carcinogen exposure in people who use tobacco products. It has particular relevance with respect to the use of PREPS. A second type of information emanating from measurements of urinary compounds is the distinction between individuals exposed or not exposed to tobacco products. Issues of sensitivity and specificity are important here. Analytically, sensitivity refers to the limit of detection while specificity guarantees that the method is really measuring the substance of interest (180). In an epidemiologic context, sensitivity is the probability that a person with the condition (such as ETS exposure) will be classified correctly as having the condition on the basis of the biomarker, while specificity is defined as the probability that a person without the condition will be classified correctly as not having the condition (e.g. not exposed to ETS) (181). Such information is particularly important with respect to ETS exposure. The third type of information that can be obtained from urinary biomarkers relates to mechanisms of carcinogen metabolism in humans. Smokers and other tobacco users are the world's largest group of carcinogen-exposed humans. Much can be learned about mechanisms of carcinogen metabolism from biomarker studies on this group of people and the conclusions may be applicable to carcinogen-exposed non-smokers as well. These three aspects of urinary biomarkers will be discussed with respect to smokers, smokeless tobacco users, and people exposed to ETS.

### *Smokers*

Representative levels (nmol/24 h) of eight urinary biomarkers discussed in this paper are summarized in Table III. The amounts of these compounds in urine are generally proportional to the levels of their parent compounds in cigarette smoke, with the possible exception of 8-oxo-dG and 3-ethyladenine for which the precursors are not known. The compounds shown in Table III are representative of the biomarkers discussed in this review, which in turn covers essentially all organic urinary compounds that are related to documented carcinogens in tobacco products. However, it should be noted that there are a number of established tobacco smoke carcinogens for which no urinary biomarkers have been validated in smokers and non-smokers. Examples include formaldehyde, acetaldehyde, butadiene, ethylene oxide, and vinyl chloride (25).

Most of the urinary biomarkers discussed here provide only a rough estimate of carcinogen dose because the extent of metabolism of the parent compound to the biomarker in question is usually unknown and may vary from individual to individual. This problem can be avoided by using a biomarker that is not metabolized, such as NPRO, or by measuring all major metabolites, an approach that has been taken in some studies of nicotine uptake (182). Diet and pharmaceuticals may influence levels of a urinary biomarker. For example, we found that, in subjects who smoked a constant number of cigarettes per day, watercress consumption increased the level of NNAL plus NNAL-Gluc, presumably due to inhibition of NNK α-hydroxylation by cytochrome P450 1A2 (124). In contrast, smokers who took 400 mg of indole-3-carbinol per day had decreased urinary levels of NNAL plus NNAL-Gluc presumably due to induction of cytochrome P450 1A2 (127). Metabolic polymorphisms can also influence levels of urinary metabolite biomarkers, as has been documented for 2-naphthol and 1-HOP (46,70,71,74).

Among the urinary biomarkers discussed here, several consistently distinguish between smokers and non-smokers. These include tt-MA, *S*-PMA, 1- and 2-naphthol, 1-HOP, NNAL and NNAL-Gluc, 8-oxo-dG, and 3-ethyladenine. In all cases, analytical methods with requisite sensitivity and specificity are available. But none of these biomarkers, with the exception of NNAL and NNAL-Gluc, has high specificity with regard to smoking because sources other than tobacco smoke, including environmental, dietary, and occupational exposures, can contribute to urinary tt-MA, *S*-PMA, 1- and 2-naphthol, 1-HOP, 8-oxo-dG, and 3-ethyladenine. Since NNAL and NNAL-Gluc are metabolites of the tobacco-specific nitrosamine NNK, they are not found in the urine of non-smokers unless there has been exposure to ETS. It is possible that other sources such as nicotine replacement products, under certain special conditions, could contribute to urinary NNAL and NNAL-Gluc but this has not been demonstrated to date (183). We have seldom observed levels of NNAL plus NNAL-Gluc $<1$ pmol/ml in smokers whereas the highest levels in non-smokers exposed to ETS seldom exceed 0.4 pmol/ml (119,123).

With these considerations in mind, it is essential to determine

**Table IV.** Evaluation of urinary biomarkers of tobacco exposure[a]

| Biomarker | Well-established assay | Chemically specific | Distinguishes exposed and non-exposed | | | Specific to tobacco-exposure | Overall score (%) | |
|---|---|---|---|---|---|---|---|---|
| | | | Smokers | Smokeless tobacco users | ETS-exposed | | | |
| tt-MA | 3 | 2 | 3 | NA | 2 | 1 | 11/15 | (73) |
| *S*-PMA | 2 | 3 | 3 | NA | 1 | 1 | 10/15 | (67) |
| Naphthols | 2 | 3 | 3 | NA | ND | 1 | 9/15 | (60) |
| Phenanthrene metabolites | 2 | 3 | 2 | NA | 1 | 1 | 9/15 | (60) |
| 1-HOP | 3 | 3 | 3 | NA | 1 | 1 | 11/15 | (73) |
| BaP metabolites | 1 | 3 | ND | NA | ND | 1 | 5/15 | (33) |
| Aromatic amines | 2 | 3 | 1 | NA | 1 | 1 | 8/15 | (53) |
| Heterocyclic aromatic amines | 2 | 3 | 1 | NA | ND | 1 | 7/15 | (47) |
| NPRO | 3 | 3 | 2 | 2 | ND | 1 | 11/18 | (61) |
| Volatile nitrosamines | 3 | 3 | 1 | ND | ND | 1 | 8/18 | (44) |
| NNAL, NNAL-Gluc | 3 | 3 | 3 | 3 | 3 | 3 | 18/18 | (100) |
| 8-oxo-dG | 3 | 1 | 2 | ND | 1 | 1 | 8/18 | (44) |
| Thioethers | 3 | 1 | 3 | ND | 1 | 1 | 9/18 | (50) |
| Mercapturic acids | 2 | 2 | 2 | ND | 2 | 1 | 9/18 | (50) |
| 3-ethyladenine | 2 | 1 | 3 | ND | 1 | 1 | 8/18 | (44) |
| 3-hydroxypropylmercapturic acid | 2 | 1 | 2 | NA | 2 | 2 | 9/15 | (60) |

[a]3, high ranking; 2, moderate; 1, poor; ND, no data; NA, not applicable

whether the use of PREPS actually lowers carcinogen dose. Urinary biomarkers should be extremely useful in this evaluation. Some of the potential problems discussed above can be circumvented by well-designed studies in which smokers switch brands and serve as their own controls. It will also be important to determine whether reducing the number of cigarettes smoked actually results in a reduction of carcinogen uptake. This has been evaluated in limited studies to date and the results suggest that reductions in exposure are more modest than would have been expected based on reduction in cigarette numbers (128,184).

Urinary biomarkers can potentially provide mechanistic information on carcinogen metabolism in smokers. A large body of literature has explored the relationship between polymorphisms in PAH metabolizing genes such as *CYP1A1* and *GSTM1* and lung cancer, with inconsistent results (9,185–188). These studies attempt to test the hypothesis that variants in these genes will lead to differences in the amounts of activated and detoxified metabolites, and ultimately to differing cancer risk. Considering the complexity of PAH metabolism, it is unlikely that a variant in a single, or even multiple genes, will be predictive of the types of metabolites which are formed. Carcinogen metabolite phenotyping, potentially achieved by analysis of urinary metabolites, is likely to be a more fruitful way to test this hypothesis. Phenanthrene appears to be particularly attractive in this respect because it is the simplest PAH with a bay region and its metabolites are plentiful in urine. Results obtained to date indicate that smoking does affect the profile of phenanthrene metabolites in human urine (51,52). Further studies are required to obtain a more complete profile of urinary PAH metabolites. Another example of carcinogen metabolite phenotyping is the ratio NNAL-Gluc:NNAL. We have suggested that this ratio may relate to lung cancer risk, since NNAL-Gluc is non-carcinogenic but NNAL is a lung carcinogen (118). Therefore, individuals with higher NNAL-Gluc:NNAL ratios should be at lower risk. In most smokers, the ratio NNAL-Gluc:NNAL varies from 0.5–5. The possible relationship of this ratio to lung cancer risk is currently being investigated.

In one study, urinary NNAL and NNAL-Gluc were quantified in people who had stopped smoking (119). The results demonstrated that these two NNK metabolites were remarkably persistent in the body. One week after smoking cessation, 34.5% of the baseline amount of NNAL plus NNAL-Gluc was detected in urine, whereas the corresponding value for cotinine was 1.1%. These results suggested the presence of a high affinity compartment where NNAL was sequestered. Further studies demonstrated that the ratios (*S*)-NNAL:(*R*)-NNAL and (*S*)-NNAL-Gluc:(*R*)NNAL-Gluc in urine were significantly higher 7 days after cessation than at baseline, indicating stereoselective retention of (*S*)-NNAL (133). Similar results were obtained in a study of NNAL and NNAL-Gluc persistence after cessation of snuff-dipping (133). These results suggest that there may be a pulmonary receptor for (*S*)-NNAL, consistent with previous suggestions that NNK is a β-adrenergic agonist (189).

Another example of mechanistic information emanates from two studies which clearly demonstrate that urinary 3-ethyladenine is elevated in smokers (159,160). Its origin is unclear. It could result from metabolic activation of *N*-nitrosodiethylamine or *N*-nitrosoethylmethylamine, but the levels of these carcinogens in cigarette smoke are very low (25). A second possibility is from endogenous formation of an ethylating agent, perhaps by reaction of ethylamine and nitrogen oxides. This requires further investigation, but could indicate the existence of substantial amounts of a previously unrecognized alkylating agent in cigarette smoke.

*Smokeless tobacco users*

In contrast to cigarette smoke, tobacco-specific nitrosamines are clearly the most abundant strong carcinogens in smokeless tobacco products (111). Combustion products such as benzene, naphthalene, and PAH are not found in significant quantities in these products. The dominance of nitrosamines as carcinogens in unburned tobacco is reflected in studies on urinary biomarkers, which have focused only on nitrosamines among the compounds considered here.

Urinary NNAL plus NNAL-Gluc provide a good approxi-

mation of carcinogen dose in snuff-dippers. A correlation between number of tins or pouches of smokeless tobacco consumed per week and NNAL plus NNAL-Gluc in urine has been observed, as well as a correlation between salivary cotinine and NNAL plus NNAL-Gluc in urine of smokeless tobacco users (133,190). These correlations are analogous to those seen in smokers and people exposed to ETS (Figure 2). These results support the concept that NNAL plus NNAL-Gluc are excellent biomarkers of tobacco carcinogen uptake. We also observed a significant association between levels of NNAL plus NNAL-Gluc in the urine of smokeless tobacco users and the presence of oral leukoplakia, supporting the potential role of NNK as a causative factor for this lesion (132).

Toombak is an oral tobacco product used in Sudan. Extraordinarily high levels of NNAL plus NNAL-Gluc were detected in the urine of men who used this product (131). It was estimated that the minimum daily doses of NNK to which these users were exposed were 0.12–0.44 mg. This is the highest documented uptake of a non-occupational carcinogen. Tobacco-specific nitrosamines are considered to play a significant role in the induction of oral cavity tumors in people who use this product (191). The high exposure to NNK suggests that these individuals may also be at risk for lung cancer.

The presence of NNK in both smokeless tobacco products and cigarette smoke allowed us to compare pharmacokinetic parameters in people who use these products (133). The distribution half-lives $t_{1/2\alpha}$ (days) of NNAL (1.32 ± 0.85 versus 3.35 ± 1.86) and NNAL-Gluc (1.53 ± 1.22 versus 3.89 ± 2.43) were significantly shorter in smokeless tobacco users than in smokers. There were no significant differences in the terminal half-lives $t_{1/2\beta}$ (days) of NNAL (26.3 ± 16.7 versus 45.2 ± 26.9) and NNAL-Gluc (26.1 ± 15.1 versus 39.6 ± 26.0) in smokeless tobacco users and smokers. Together with the enantiomeric ratio data discussed above, these results support the presence in the human body of receptors for (*S*)-NNAL, the more carcinogenic NNAL enantiomer.

*Non-smokers*

NNAL and NNAL-Gluc are the only urinary carcinogen biomarkers that were consistently elevated in studies of exposure of non-smokers to ETS. This assay has the required specificity and sensitivity, both analytical and epidemiologic, to be useful in ETS studies. The contribution of non-tobacco sources to all other biomarkers discussed here confounds their validity in ETS studies, where carcinogen exposure is relatively low.

Epidemiologic studies of ETS exposure and lung cancer have focused in part on non-smoking women who lived with men who smoked (5). The excess risk for lung cancer in non-smoking women exposed to ETS is ~1–2% of that in smokers (5,192). The level of NNAL plus NNAL-Gluc in the urine of ETS-exposed women was ~5.6% as great as that of their smoking partners, indicating consistency between dose and excess risk for lung cancer (135). Other studies show a mean of ~0.05 pmol/ml NNAL plus NNAL-Gluc in non-smokers exposed to ETS (Table II). This is ~1.6% of the typical levels found in smokers (Table III), consistent with the results cited above. These data provide further biochemical support for the role of ETS as a lung carcinogen in non-smokers.

In a recent study in children, we found substantial and widespread uptake of NNK, as determined by urinary NNAL and NNAL-Gluc (123). A potentially important finding in this study was a 93-fold range of NNAL plus NNAL-Gluc levels. Epidemiologic studies on childhood exposure to ETS and cancer later in life have not produced consistent results (193). The large range of values may have important implications for interpretation of such studies, which have relied solely on questionnaire data. Clearly, it is necessary to incorporate biomarkers into epidemiologic studies on childhood exposure to ETS.

NNK is a potent transplacental carcinogen in the Syrian golden hamster (194). The offspring of NNK-treated hamsters have tumors in a variety of tissues including the respiratory tract, pancreas, and adrenal glands. The detection of NNAL and NNAL-Gluc in the urine of newborns of mothers who smoke demonstrates fetal uptake of NNK (122). Most urine samples had detectable NNAL-Gluc but few had free NNAL. In contrast, analysis of amniotic fluid taken at weeks 15–20 of gestation from mothers who smoked showed the presence of NNAL but rarely NNAL-Gluc (136). These results are consistent with transplacental passage of NNK or NNAL, followed by metabolism of NNAL to NNAL-Gluc only late in fetal development.

## Conclusions

An evaluation of the biomarkers discussed in this paper is presented in Table IV. Each category is given a subjective score with 3 being the highest ranking, 2 moderate, and 1 poor. The evaluation category 'well-established assay' refers to the amount of literature data demonstrating that a given assay is robust. 'Chemically specific' refers to knowledge of the precursor of the particular metabolite. The other categories are self-explanatory. The NNAL assay receives the highest score because it is specific to tobacco exposure and has been applied in multiple exposure situations. The next best assays are 1-HOP and tt-MA. These are well-established assays. The lack of specificity to tobacco exposure however detracts from their overall utility. *S*-PMA also receives a relatively high score which is to some extent diminished by low specificity to tobacco exposure. Among the assays with lower scores, 3-ethyladenine, 3-hydroxypropylmercapturic acid, and 2-naphthol may have considerable promise because of their clear elevation in smokers' urine. Phenanthrene metabolites may be useful for carcinogen metabolite phenotyping.

Quantitation of carcinogen-related organic compounds in urine has provided a wealth of data pertinent to our understanding of the relationship between exposure to tobacco products and cancer. In future studies, some of these assays should be applied to current areas of great topical interest such as the evaluation of PREPS, the relationship between reduced smoking and carcinogen uptake, the relationship of metabolic polymorphisms to cancer susceptibility, and the role of ETS as a human carcinogen.

## Acknowledgements

Research on tobacco and cancer in the author's laboratory is supported by grants CA-46535, CA-81301, CA-85702, CA-92025, and DA-13333 from the National Institutes of Health and by grant RP-138 from the American Cancer Society.

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*Received December 5, 2001; revised December 27, 2001;*
*accepted January 14, 2002*