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Smokers and Urinary Genotoxins: Implications for Selection of Cohorts and Modulation of Endpoints in Chemoprevention Trials

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Abstract Urinary genotoxicity assays measure the internal dose of genotoxic carcinogens, thereby providing a particularly sensitive endpoint for selecting cohorts of individuals exposed to cigarette smoke or other mutagens excreted with urines, as well as for evaluating the modulation of this parameter after administration of chemopreventive agents. Mutagenicity of urines was investigated in smoking Italian volunteers, who received oral *N*-acetylcysteine (NAC) at the same doses which are usually prescribed for the long-term treatment of chronic bronchitis. The daily excretion of mutagens, concentrated on XAD-2 columns and tested in *Salmonella typhimurium* YG1024 with S9 mix, was significantly and remarkably decreased by NAC in the majority of the subjects examined so far. Time-course experiments showed that this effect starts since the first day of drug administration and reverses when treatment is withdrawn. In addition, NAC administration almost totally prevented urinary genotoxicity in one subject whose concentrated urines induced a differential lethality in *Escherichia coli* strains having distinctive DNA repair capacities. The decrease of urinary genotoxicity produced by NAC in the majority of smokers correlates with the ability of this thiol to prevent tumors and to affect a variety of intermediate biomarkers in animal models. Modulation of the urinary excretion of mutagens is one of the biomarkers evaluated in two ongoing Phase II chemoprevention trials. One study involves the oral administration of NAC in Dutch smokers. The pretreatment urine samples of all the subjects so far recruited are clearly mutagenic. The other study involves the oral administration of the dithiolethione oltipraz to individuals living in the Qidong County of the People's Republic of China, an area of high endemy for HBV infection and of high exposure to aflatoxins. Additionally, a large proportion of the recruited male subjects are smokers. A total of 500 urine specimens will be assayed from 240 subjects according to a complex protocol arranged in three consecutive phases. J. Cell. Biochem. 25S: 92–98.

Key words: biomarkers; chemoprevention; DNA repair; mutagenicity; *N*-acetylcysteine; oltipraz; urinary mutagens

URINE GENOTOXICITY ASSAYS

Genotoxicity assays of urines provide internal dose biomarkers evaluating the amounts of mutagenic and DNA-damaging agents excreted with this biological fluid. Tobacco smoking is the major source of urinary genotoxins, although under certain conditions a positive response has been also recorded following either dietary, occupational, pathological or therapeutic exposures [see, e.g., references 1 and 2 for reviews]. Although xenobiotics circulating in the blood are already concentrated by the kidney, their dilution in urines requires a prelimi-

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nary concentration step prior to any genotoxicity assay. The most common method involves filtration of urines through resin columns, e.g., XAD-2 nonpolar resin [3], but other concentration methods, such as the blue rayon technique [4], have been used as well. The concentrated material is assayed in any short-term test system for genotoxicity, and typically in the Ames' reversion assay using *Salmonella typhimurium his*$^-$ strains [5]. Here we also present the results obtained in a DNA repair assay in *Escherichia coli*, evaluating the differential lethality in the DNA repair-proficient strain WP2 and in its DNA repair-deficient counterpart CM871 (*uvrA*$^-$ *lexA*$^-$ *recA*$^-$). As confirmed in the present experiments, this assay is less sensitive than the reversion assay in detecting promutagens but is more sensitive in detecting direct-acting, toxic genotoxins [6]. Since genotoxins may be eliminated under conjugated form, not only a metabolic system (S9 mix) but also deconjugating enzymes, such as β-glucuronidase or arylsulfatase, can be used in genotoxicity test systems.

The urinary genotoxicity assay has several advantages, including the circumstances that collection of samples is noninvasive, genotoxicity assays yield quantitative data, and the costs are reasonable. Moreover, since the renal elimination of genotoxins is rapid and continuous, their detection in urines reflects acute exposures. On the other hand, the assay is not suitable for detecting cumulative exposures. Other disadvantages are that concentration methods may not be effective for all classes of mutagens, and that the biological significance and interpretation of results are sometimes uncertain.

The sensitivity of mutagen detection in smokers' urines, which is mainly due to the contribution of aromatic amines in this complex mixture, has been considerably improved by using nitroreductase (NR)- and *O*-acetyltransferase (OAT)-overproducing bacteria, which were derived from the classical *S. typhimurium* strains TA98 and TA100 [7]. With YG1024, the most sensitive strain, we observed a doubling of spontaneous revertants, after metabolic activation with as little as 1/110 of the mainstream smoke condensate recovered from one cigarette [8]. The average YG1024:TA98 sensitivity ratio was 6.6:1 when testing urine specimens from Italian smokers, and even moderate smokers were unequivocally positive by eliminating tens of

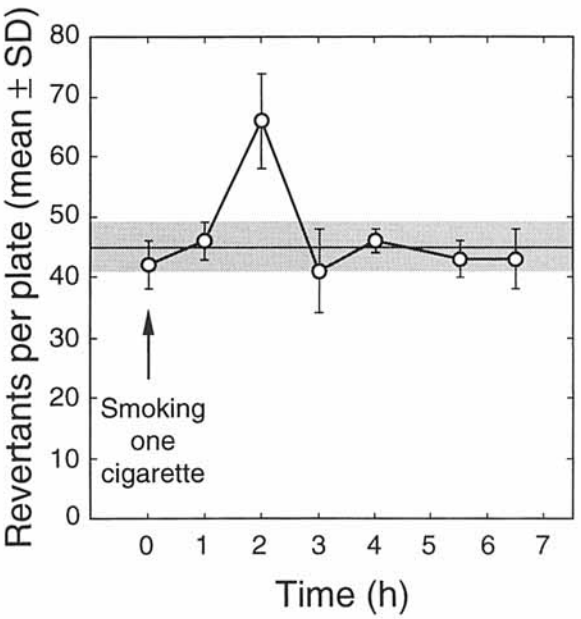

Fig. 1. Time-course of mutagenicity in strain YG1024 of *S. typhimurium*, in the presence of S9 mix, of urines concentrated 100-fold on XAD-2 resin (20 µl/plate). Urine samples were obtained, at varying time intervals, from a volunteer smoking a single filter cigarette having a declared content of 14 mg tar and 0.9 mg nicotine. The horizontal lines and dashed area denote the mean spontaneous revertants and their SD values.

thousands revertants in 24 hours [8]. Recent experiments showed that appearance of a mutagenicity peak can be detected in the urines of a volunteer, usually nonsmoker, two hours after smoking a single cigarette (Fig. 1). In another experiment in the same volunteer, the overnight urines became toxic to *E. coli* strains after smoking at bedtime two cigarettes only. Both in the presence and in the absence of S9 mix, toxicity was significantly higher ($P < 0.05$) in CM871 than in WP2, thereby indicating the occurrence of genotoxic effects. Drinking a superalcoholic drink (50 ml whisky) during smoking increased both toxicity and genotoxicity, but not to a significant extent (data not shown). Therefore, urinary genotoxicity appears to be a particularly sensitive endpoint for selecting cohorts of individuals exposed to cigarette smoke or other mutagens excreted with urines, as well as for evaluating the modulation of this parameter after administration of chemopreventive agents. In principle, no other biomarker is expected to react so rapidly to variations either in exposure to mutagens or in their bioavailability and excretion.

There is an interindividual variability in the urinary elimination of smoke-related muta-

gens, which in part can be ascribed to polymorphisms in the metabolism of carcinogens. For instance, smokers with no glutathione *S*-transferase-encoding *GSTM1* gene in peripheral blood cells were found to eliminate significantly higher amounts of mutagens with urines than smokers with the gene, whereas no difference was observed in the levels of urinary mutagens between slow-acetylator and fast-acetylator *NAT2* genotype [9]. The urinary excretion of mutagens can be also modulated exogenously. For instance, some decrease of urinary mutagenicity could be detected following treatment with *Lactobacillus acidophilus*-fermented milk in individuals eating cooked beef [10], with *Lactobacillus casei* in individuals eating fried ground beef [4], or with turmeric in individuals smoking beedies or cigarettes [11].

We report here the preliminary results of three studies aimed at assessing the modulation of urinary genotoxicity in smokers treated with the thiol *N*-acetylcysteine (NAC) [12] and the dithiolethione oltipraz [13], which are among the most promising cancer chemopreventive agents [14]. The evaluation of urinary mutagenicity is one of the endpoints investigated in two ongoing Phase II chemoprevention trials with these drugs.

FOLLOW-UP OF SMOKERS TREATED WITH NAC

This study is being carried out in Genoa (Northern Italy) and in the surrounding area. Smoking volunteers were asked to supply 24-h urine samples for three consecutive days, after which they received for seven days tablets of NAC according to the schedules indicated in Table I. During the last three days of treatment with NAC, the smokers supplied additional 24-h urine samples. Throughout the experiments, lasting 10 days, the smokers tried to smoke a constant number of cigarettes of the same brand, and maintained their standard diet and alcohol consumption habits. The urine samples were concentrated 100-fold on XAD-2 columns. Mutagenicity of concentrates was evaluated in *S. typhimurium* strain YG1024, in the presence of S9 mix incorporating 10% liver S12 fractions from Aroclor-pretreated rats, at three dose levels (100, 50, and 25 µl/plate) in triplicate plates, with and without addition of β-glucuronidase (1,000 units/plate). The mutagenic response was then related to the original volume of urine excreted in 24 h.

Table I summarizes the results so far obtained by analyzing 54 samples from nine volunteers, smoking 9–37 cigarettes per day. All pretreatment samples were clearly mutagenic, with 14,800 to 92,400 revertants eliminated per day. There was a significant correlation between number of smoked cigarettes and mutagenicity ($r = 0.786$, $P < 0.05$). Addition of β-glucuronidase did not significantly affect the results (data not shown). After treatment of smokers with NAC the urinary excretion of mutagens was decreased to a significant and considerable extent in five subjects. In three subjects there was no significant change of mutagenicity, whereas in one subject a significant increase was observed (Table I).

The same urine samples were assayed in the selective lethality test using the two *E. coli* strains differing in their DNA repair ability, according to a spot test procedure [6]. As shown in Table I, in three subjects the urines were more toxic in the repair-deficient strain CM871 than in the wildtype WP2, and treatment with NAC decreased in all cases genotoxicity. The most interesting case was subject G.R., in whom the reversion assay had failed to detect any decrease of genotoxicity following NAC treatment.

Thus, on the whole, a significant decrease of genotoxicity was recorded in six out of the nine smokers so far examined (66.7%). It is noteworthy that this effect was achieved by administering NAC at pharmacological doses (600–800 mg/day), which are usually prescribed on a large scale for the long-term treatment of chronic bronchitis. It is also remarkable how rapid is the effect of NAC on the urinary excretion of mutagens. As shown in the time-course experiment reported in Figure 2, the decrease of urinary mutagenicity started since the first day of drug administration, and reversed when treatment was withdrawn.

The interpretation of these results is not easy, but it is generally assumed that a decreased excretion of mutagens with urines reflects a decrease in the systemic exposure to the same mutagens [15]. In fact, inhibition of this internal dose biomarker in humans, as shown by the present experiments, is consistent with the protective effects produced by NAC in a large variety of studies in vitro as well as in animal models evaluating the biologically effective dose, early biological damage, and preneoplastic or neoplastic lesions [12,14].

TABLE I. Modulation of the Urinary Excretion of Genotoxic Agents in Smokers Receiving Oral *N*-Acetylcysteine (NAC)

Subject	Sex	Age (years)	Weight (kg)	NAC (mg/day)[a]	Cigarettes smoked per day (no.)[b]	Mutagenicity in *S. typhimurium* YG1024 (S9+)[c]	Selective lethality in *E. coli* strains[d]			
							WP2 (wildtype)		CM871 ($uvrA^-$ $recA^-$ $lexA^-$)	
							S9−	S9+	S9−	S9+
R.M.	F	41	45	0	9.3 ± 2.1	33.3 ± 2.6	UD	UD	6.2 ± 0.8	UD
				800	9.3 ± 1.5	18.7 ± 1.9**	UD	UD	UD	UD
S.P.	F	59	58	0	11.7 ± 2.1	21.6 ± 5.0	UD	UD	UD	UD
				800	12.0 ± 2.0	10.8 ± 2.8*	UD	UD	UD	UD
G.P.C.	M	40	72	0	26.7 ± 1.5	42.2 ± 6.5	UD	UD	UD	UD
				800	27.7 ± 1.5	24.2 ± 3.9*	UD	UD	UD	UD
Y.F.	F	46	54	0	11.7 ± 2.1	22.9 ± 4.4	UD	UD	UD	UD
				800	14.3 ± 0.3	31.9 ± 7.6	UD	UD	UD	UD
A.S.	F	53	55	0	13.7 ± 4.6	14.8 ± 3.2	UD	UD	UD	UD
				800	16.3 ± 2.5	23.8 ± 4.3*	UD	UD	UD	UD
M.O.	M	27	87	0	19.0 ± 1.0	42.2 ± 7.5	UD	UD	11.5 ± 5.7	12.6 ± 4.2
				800	18.7 ± 1.5	32.4 ± 4.5*	UD	UD	5.5 ± 0.0	UD*
A.S.	M	45	71	0	36.7 ± 0.6	92.4 ± 10.3	UD	UD	UD	UD
				600	36.3 ± 0.6	63.4 ± 8.7*	UD	UD	UD	UD
G.R.	M	39	78	0	18.0 ± 0.0	26.5 ± 5.6	8.0 ± 0.9	8.0 ± 1.6	11.9 ± 2.6	12.0 ± 2.4
				600	19.0 ± 1.0	25.1 ± 10.9	6.6 ± 1.1	5.7 ± 0.3*	6.8 ± 1.1**	5.6 ± 0.2***
A.D.B.	M	47	78	0	29.7 ± 4.0	32.9 ± 7.4	UD	UD	UD	UD
				600	27.6 ± 9.3	40.8 ± 10.3	UD	UD	UD	UD

[a]800 mg NAC schedule: two 200 mg tablets 30 minutes before breakfast and lunch; 600 mg NAC schedule: single administration at wake-up time.
[b]Mean ± SD of the cigarettes smoked during the three days preceding NAC administration or during the last three days of treatment with NAC for one week.
[c]The results are expressed as induced revertants excreted in 24-h × 10^3 (mean ± SD of three 24-h urine samples).
[d]The results are expressed as diameter (mm) of the zone of bacterial growth inhibition (mean ± SD) produced by urine concentrates (spot test). UD = undetectable lethality.
Statistical analysis: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (Student's t test), as compared to pre-NAC samples.

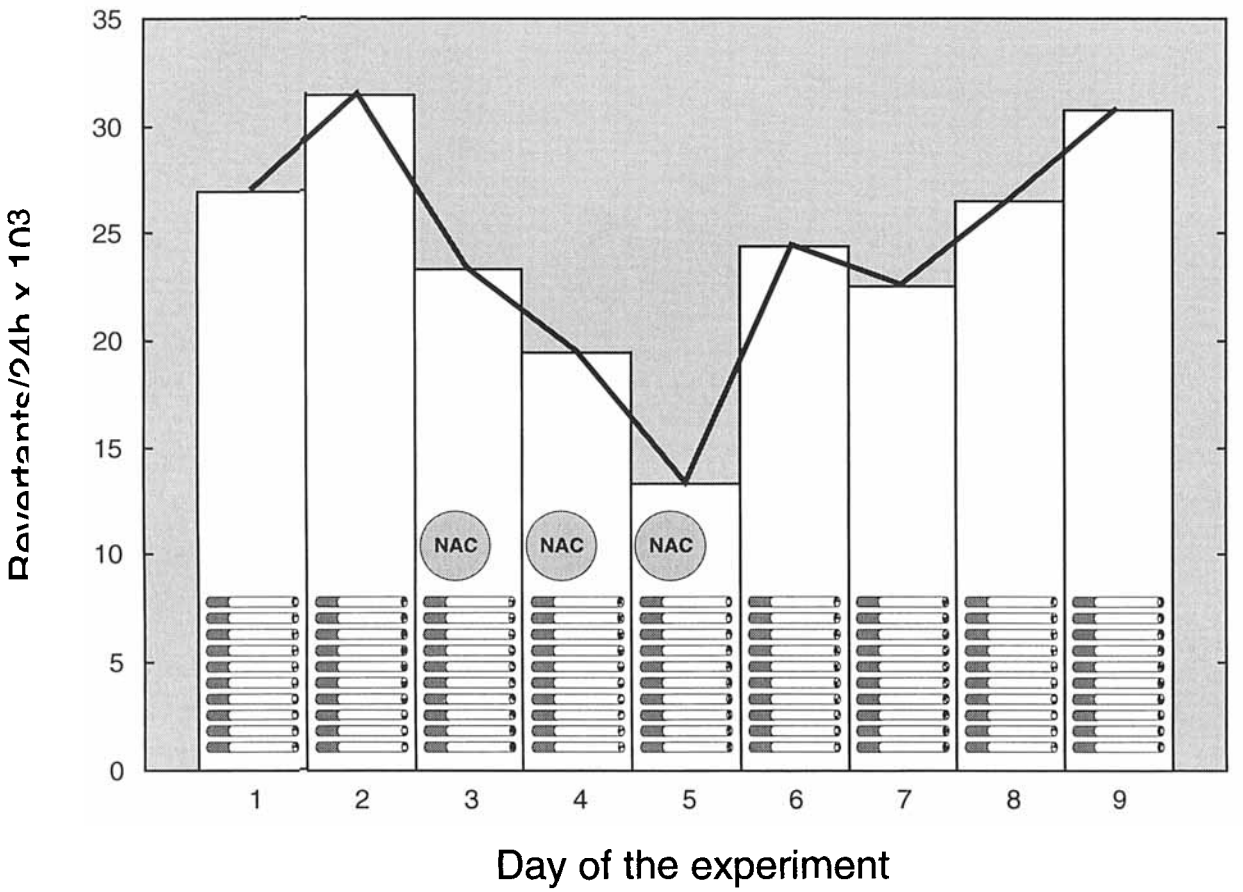

Fig. 2. Time-course of the urinary excretion of mutagens reverting strain YG1024 of *S. typhimurium*, in the presence of S9 mix, in a volunteer (subject R.M. in Table I) smoking 10 cigarettes for 10 consecutive days and receiving oral tablets of NAC (800 mg/day) on days 3–5 of the experiment.

PHASE II CHEMOPREVENTION TRIAL WITH NAC IN THE NETHERLANDS

Besides a Phase III cancer chemoprevention trial with NAC and/or retinol palmitate, denominated Euroscan [16], a Phase II trial in smokers has been implemented at the Netherlands Cancer Institute, under the coordination of Nico van Zandwijk. So far, 41 subjects have been recruited for this study and randomized in two groups (placebo and NAC). NAC will be given in two daily 600 mg oral tablets, one in the morning and one in the evening, for six months. The monitored biomarkers include the measurement of carcinogen-DNA adducts in pulmonary alveolar macrophages and in leucocytes, as assessed by both ^{32}P postlabeling and synchronous fluorescence spectrophotometry, the involvement of *p53*, detection of a proliferation marker (Ki-67) and frequency of micronuclei in the bronchial epithelium, the count and cytological formula of bronchoalveolar lavage cells, and urinary mutagenicity. Molecular dosimetry biomarkers will be evaluated at The University of Limburg in Maastricht (The Netherlands) and in part at The University of Genoa (Italy), where urinary mutagenicity is assessed.

So far, 24-h pretreatment samples were obtained from 17 subjects, 5 males and 12 females, aged 25–60 years, currently smoking 5–55 cigarettes per day. All urine concentrates were clearly positive in *S. typhimurium* YG1024 strain, in the presence of S9 mix. A weaker positivity was observed in strain YG1029. The results obtained in YG1024, as related to the number of cigarettes smoked per day, are shown in Figure 3. Both by relating the results to the 24-h volume of urines and to their creatinine content, a positive correlation was pointed out but it did not reach the threshold of statistical significance. This can be ascribed to important deviations from the regression line, e.g., in a subject smoking 55 cigarettes per day

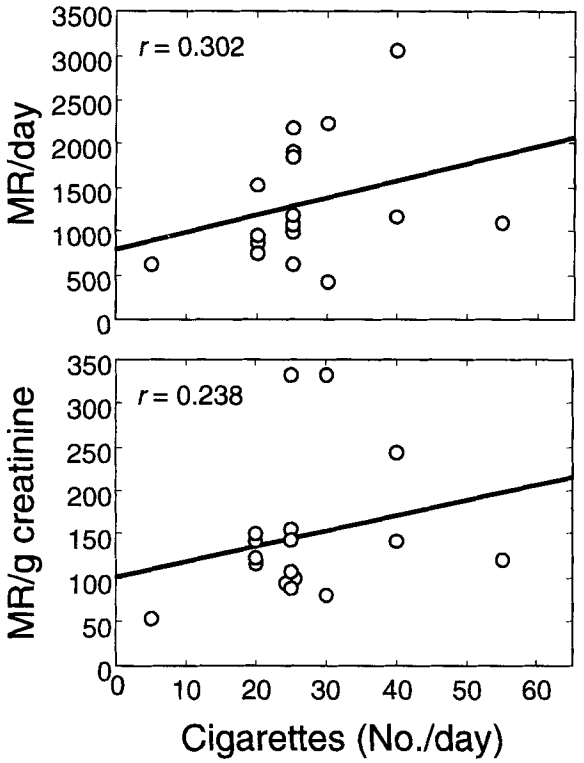

Fig. 3. Relationships between number of cigarettes smoked per day and mutagenicity of urines concentrated 100-fold on XAD-2 resin in *S. typhimurium* YG1024, in the presence of S9 mix, in 17 volunteers recruited in The Netherlands for a Phase II chemoprevention trial with NAC. Mutagenicity is expressed as MR and related either to the daily volume of urines or to their creatinine content. MR was calculated according to the formula [induced revertants—spontaneous revertants]/spontaneous revertants. Both *r* values are below the threshold of statistical significance.

and having a relatively poor excretion of urinary mutagens. As already discussed, these variations mainly reflect metabolic polymorphisms and underlie the convenience of measuring the internal dose for predicting the risk. Interindividual variations do also occur when measuring the biologically effective dose in smokers, e.g., by relating the levels of carcinogen-DNA adducts in pulmonary alveolar macrophages to the number of currently smoked cigarettes [17].

PHASE II CHEMOPREVENTION TRIAL WITH OLTIPRAZ IN CHINA

This clinical trial, which started in June 1995, has been organized by the Johns Hopkins University School of Hygiene and Public Health (Baltimore, MD), with the collaboration of the Qidong Liver Cancer Institute, the Shanghai Cancer Institute (People's Republic of China) and the University of Genoa (Italy). The study is being carried out in Qidong, Jiangsu Province, where hepatocellular carcinoma is the leading cause of cancer death. Approximately 10% of the population is carrier of hepatitis B virus (HBV), and the prevalence of residents positive for aflatoxin biomarkers in their blood and/or urine exceeds 50% [13]. A highly significant association was found between the presence of urinary aflatoxins, serum HBsAg positivity and risk of primary hepatocellular carcinoma [18]. The observed synergistic interaction between viral and chemical risk factors is consistent with studies demonstrating that infection with *hepadnaviridae* in both humans (HBV) and woodchucks (WHV) results in a decrease of protective mechanisms, e.g., a depletion of reduced glutathione (GSH), and a concomitant stimulation of a number of activating mechanisms [reviewed in reference 19], accompanied by an enhanced formation of carcinogen-DNA adducts in the liver [20]. In addition to the risk factors for hepatocellular carcinoma, a majority of male residents in Qidong are tobacco smokers.

The chemoprevention trial will involve 240 subjects divided into 3 groups, i.e., a group receiving a placebo, and two groups receiving oltipraz [5-(2-pyrazinyl)-4-methyl-1,2-dithiole-3-thione] p.o., according to two different treatment schedules. Two specific aflatoxin biomarkers, i.e., aflatoxin-albumin adducts in blood and aflatoxin-N^7-guanine adducts in urines, will be measured in the 240 subjects twice monthly throughout the 2-month intervention and 2-month follow-up period. Taking into account the mechanisms of oltipraz, including enhancement of GSH stores and induction of Phase II enzymes [13], we expect that this drug may affect the aflatoxin biomarkers as well as the excretion of urinary mutagens.

The assessment of urine mutagenicity will follow two stages, i.e., a pilot study with 20 untreated individuals not recruited into the trial, and the definitive study with 240 recruited subjects, examined at four and six weeks on the intervention trial. Thus, a total of 500 urine specimens, each pooled from overnight urines collected on three consecutive days, will be concentrated 100-fold on XAD-2 columns at the Shangai Cancer Institute, and assayed for mutagenicity at the University of Genoa. Urine concentrates will be analysed in *S. typhimurium* strains TA100 and YG1024 (TA98 OAT^+)

at 4 dose levels (12.5, 25, 50, and 100 μl/plate), each in triplicate plates, under three metabolic conditions (without S9, with S9, and with S9 plus 1,000 units β-glucuronidase). A single dose (100 μl/plate) will be additionally tested in other *S. typhimurium* strains having distinctive genetic characteristics, such as TA97a, TA98, TA102, YG1021 (TA98 NR^+), YG1026 (TA100 NR^+) and YG1029 (TA100 OAT^+). Positive controls will include aflatoxin B_1 and a cigarette smoke condensate. The pilot study is now in progress.

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