Urinary Mutagenicity, CYP1A2 and NAT2 Activity in Textile Industry Workers

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Abstract: Urinary Mutagenicity, CYP1A2 and NAT2 Activity in Textile Industry Workers: Ana Fanlo, et al. Department of Pharmacology and Physiology, University of Zaragoza, Spain—The two major causes of bladder cancer have been recognised to be cigarette smoke and occupational exposure to arylamines. These compounds are present both in tobacco smoke and in the dyes used in textile production. Aromatic amines suffer oxidative metabolism via P450 cytochrome CYP1A2, and detoxification by the polymorphic NAT2. The aim of the present work was to assess the association between occupational-derived exposure to mutagens and CYP1A2 or NAT2 activity. This cross-sectional study included 117 textile workers exposed to dyes and 117 healthy controls. The urinary mutagenicity was determined in 24 h urine using TA98 Salmonella typhimurium strain with microsomal activation S9 (MIS9) or incubation with β-glucuronidase (MIβ). Urinary caffeine metabolite ratios: AFMU+1X+1U/17U, and AFMU/AFMU+1X+1U were calculated to assess CYP1A2 and NAT2 activities, respectively. The results show that workers present a strikingly higher urine mutagenicity than controls (p<0.0001), despite the implementation of the new restrictive norms forbidding the industrial use of the most carcinogenic arylamines. Neither NAT2 nor CYP1A2 activity had any effect on the markers of internal exposure to mutagens, since no significant differences were observed when the urinary mutagenicity of slow and fast acetylators (p>0.001) was compared, and the urinary mutagenicity was not significantly associated with the CYP1A2 activity marker (r=0.04 and r=–0.01 for MIS9 and MIβ, respectively). This study clearly indicates the need for further protective policies to minimise exposure to the lowest feasible limit in order to avoid unnecessary risks. (J Occup Health 2004; 46: 440–447)

Key words: NAT2, CYP1A2, Textile workers, Urine mutagenicity

Workers in textile production have for many years been exposed to dyes containing aromatic amine derivatives. Some of these compounds are able to induce tumours, particularly in the urinary bladder of humans and animals1, 2. Among them, benzidine, 2-naphthylamine, and 4-amino-biphenyl, are the most important carcinogenic substances which are also present in tobacco smoke. Between 1 and 19% of bladder cancer cases have been estimated to be occupation-related3. In our Spanish population an increased risk of bladder cancer has been found in individuals employed in textile factories4, 5. Tobacco smoking is one of the major causes of bladder cancer6, accounting for at least 50% of the bladder cancer cases in the population of western countries7.

The carcinogenic potential of arylamines depends on their metabolic activation. Aromatic amines are initially activated by N-hydroxylation, primarily in the liver by the cytochrome P450 (CYP1A2)8, 9. The N-hydroxyarylamine metabolites, once formed, may either be transported to the bladder or could be metabolised further in the liver by other Phase II enzymes, such as UDP-glucuronositransferase (UGT). The conjugates so formed may spontaneously hydrolyse to produce mutagenic hydroxylamines under the acidic conditions in the urine10.

The detoxification reaction, via polymorphic NAT211, competes with N-hydroxylation through CYP1A2 enzyme activity. Therefore, susceptibility to the carcinogenic effects of arylamines may depend on the balance between the individual activities of NAT2 and CYP1A2. A number of studies have indicated that slow
Acetylator individuals have an increased risk of bladder cancer, especially subgroups occupationally exposed to aromatic amines. In addition, 4-ABP-haemoglobin adducts have been found to be 2–3 times higher in slow acetylators than in individuals phenotyped as NAT2 rapid metabolisers. Among subjects occupationally exposed to arylamines, it has been observed that slow acetylators present significantly higher urine mutagenicity than rapid acetylators. With regard to CYP1A2, a relationship between high CYP1A2 activity and bladder cancer risk has been previously suggested.

In a previous work from our group, performed before implementation of the regulatory standards forbidding the industrial use of the most carcinogenic arylamines, such as 4-aminobiphenyl, benzidine and 2-naphthylamine, we found data suggesting that workers in textile factories were highly exposed to aromatic amines from dyes. Despite the exclusion of the abovementioned carcinogenic arylamines, other aromatic amines continue to be used as textile dyes. Among them are aniline-based compounds and o-toluidine derivatives, which have been found to be correlated with increased bladder cancer risk. In addition, environmental exposure to azo pigments, which are extensively used in textile factories, may result in internal exposure to aromatic amines which may be released by either reducing cleavage of azo bonds, or by hydrolysis of anilido bonds. Most of the aromatic amines are suspected to have carcinogenic potential.

The present work has been primarily undertaken to assess the association between both NAT2 and CYP1A2 activity and the internal exposure to mutagens with the potential to contact the urine bladder of workers. We also determine in this study whether the adoption of the regulatory standards mentioned has indeed produced a lower internal exposure to mutagens.

**Materials and Methods**

**Study subjects**

The participants, 117 control individuals and 117 textile workers gave their informed consent to participate in this study which was approved by the Ethics Committee for Clinical Research of the Hospital Clínico of the University of Zaragoza. Demographic data from cases and controls are shown in Table 1.

The volunteers responded to a questionnaire on smoking habits, diet, coffee and drug consumption. They were included in the study if they did not present idiosyncratic reactions to caffeine or histories of chronic diseases or recent illnesses and were not taking any medication. All of them were from the same area of Spain and followed a similar diet. They were asked to avoid the following during the study: diets or cooking procedures known to induce CYP1A2 activity or to increase urinary mutagenicity (e.g., cruciferous vegetables, watercress; charbroiled or grilled meat, or meat cooked at a high temperature; smoked foods; and toasted bread) or to inhibit CYP1A2 activity (e.g., grapefruit). Among the individuals, those who smoked were smokers of blonde tobacco. The occupationally exposed group consisted of 117 workers, involved in processing the dyes (dye weighers who also dissolved the dry dyes). They worked in 6 small textile factories from Mataró (province of Barcelona, Spain). In these factories, there were no systems to control environmental exposure. The most commonly used dyes were arylamine-related, such as o-toluidine based dyes, aniline derivatives and azo dyes. Interviews and sampling took place at the central office of the syndicate (CC.OO.) in Mataró. Control individuals were healthy volunteers, non occupationally exposed to dyes, paired for age (± 5 yr) with workers, and came from Zaragoza, Spain. All subjects of this study were men.

**CYP1A2 and NAT2 activity**

For 48 h before and during the five hours of the caffeine test, the volunteers refrained from consuming alcoholic drinks and foods or beverages containing methylxanthines. After 48 h each subject discarded the first morning urine and drank a glass of instant coffee containing 200 mg of caffeine in 250 ml of water. The urine 4–5 h after caffeine intake was collected in bottles preloaded with 1 mol/liter citric acid phosphate buffer.
pH=3. To analyse caffeine and its metabolites the HPLC method described by Grant et al. (1983)24 with some modifications was used. Caffeine and its metabolites were separated on a Waters Novapak C18 reverse-phase column (4 µm particle size, 25 cm × 4.6 mm internal diameter) (Millipore Ibérica S.A.), which was isocratically eluted with a mobile phase containing acetic acid/methanol/water (0.5:90:5.5 vol/vol/vol) at a flow rate of 1 ml/min and a pressure of 1,500 psi. The compounds were detected by UV absorbance at 280 nm. The chromatograph was an LC Module I Plus equipped with Millennium 2,010 software (Waters Corp., Madrid, Spain). Calibration curves were elaborated with known amounts of metabolites in a range from 5 to 80 µg/ml, added to blank urine samples, and then processed as described above. 1 U was dissolved under basic conditions (pH 9) by the addition of 10 N hydroxide and then neutralised to pH 7 with 12 N hydrochloric acid, according to Tang et al. (1991)25. To assess whether or not any urinary constituent could co-migrate with caffeine or caffeine metabolites used in the ratio, a Water 996 photodiode array detector was used to compare the peaks with the spectral libraries of standards. For data analysis, caffeine and its metabolites were expressed as mmol/l. The metabolite ratio (MR) used to assess NAT2 activity was MR1: AFMU/(AFMU+1 X+1 U)26, and that used to indicate CYP1A2 activity was MR2: (AFMU+1 X+1 U)/17 U27.

Urine Mutagenicity
The participants collected a 24 h urine sample 4 d prior the caffeine study and it was stored at ~80° C until analysis. Frozen urine was thawed and 75 ml of each sample was passed through a C18 resin column for recovering mutagens28. The concentrate was eluted with 400 µl of DMSO and stored at ~80° C before measuring mutagenicity.

The microsuspension assay was performed according to Malevelle et al. (1982)29 and Kado et al. (1983)30 with some modifications. Each sample was tested in two assays evaluated in the presence of 100 µl of concentrated bacterial culture medium (3–4 × 10^9 cells) of Salmonella typhimurium TA 98 and 20 µl of urinary extract (equivalent to the mutagens contained in 3.75 ml of urine). In the first assay we incorporated 10 µl of β-glucuronidase (118 units/ml; Sigma) and in the second, we added 100 µl of S9-mix, metabolic activation system, obtained from aroclor-induced male Sprague Dawley rats31. After incubating for 90 min at 37°C in a shaking water bath, the mixtures were plated as described by Maron and Ames (1983)31. The revertant colonies were counted after 3 d of incubation at 37°C, and mutagenicity was expressed as the number of revertant colonies (after subtracting the spontaneous revertants) per mmole of urinary creatinine, which was determined in an aliquot of each 24 h urine sample32. Urine extracts were included in the study only if they produced at least a doubling or more revertant colonies in relation to the number of spontaneous revertants in the control plate. The results are expressed as mutagenic indexes: MIB/M or MIS9.

Statistical method
The variables MIB, MIS9 and MR2 were logarithmically transformed (x to logx) to stabilise the variance. The influence of the smoking exposure and occupational exposure on the 2 mutagenic indexes was determined by multivariate analysis, after checking that the data fitted a normal distribution (by using the Kolmogorov Smirnov’s test). When a relationship between independent and dependent variables could be demonstrated, ANOVA was used for comparisons. Single comparisons of means were performed by Student’s t-test for independent variables. Correlation analysis was carried out by linear regression. Differences in either smokers or slow acetylators between groups were analysed by X^2 test. The statistical evaluation was performed with an SPSS statistical program. The null hypothesis was rejected when p<0.05.

Results
Multivariate analysis showed the two mutagenic indexes to be different in their dependency profiles on the variables involved in the study, such as occupational exposure and smoking. With regard to occupational exposure, MIB was the most influenced index (p<0.001), followed by MIS9 (p<0.01). MIB was not dependent on tobacco smoking (p=0.32), but the mutagenicity in the presence of microsomal activation (MIS9) was highly dependent on the smoking habit (p<0.001). The interaction between tobacco smoking and occupational exposure was detected by MIB (p=0.01), but not by MIS9 (p>0.05). The smokers ratio was not statistically different in controls and workers (X^2=1.71; p>0.05)

Figures 1 and 2 show that workers, as a group, exhibited significantly higher urine mutagenicity than controls with both MIB (p<0.001) and MIS9 (p<0.001). Since both groups, controls and workers, were made up both by non smokers and smokers, the comparison between nonsmokers and smokers, among workers, gives better information about the influence of the occupational exposure. It can be seen in Figures 1 and 2 that increased levels of mutagenicity in workers in relation to controls, all of them non smokers, can be detected by the two mutagenic indexes (p<0.001 and p<0.001 for MIB and MIS9, respectively), which can be attributed to exposure at the workplace. Among smokers, workers had significantly higher levels of urine mutagenicity when measured by MIB (p<0.0001) or MIS9 (p<0.01) than controls.

In the total sample of individuals, there were 149 NAT2
slow acetylators and 85 NAT2 rapid acetylators. The ratio of slow to fast acetylators was similar in controls and workers ($X^2=3.12, p=0.07$). The antimode in the frequency distribution of the ratio AFMU/AFMU+1X+1U was 0.24. By analysing the differences in urine mutagenicity between the two phenotypes of N-acetylation, non statistically significant differences were observed, in either the control group or the group of workers, regardless of whether they were smokers or not (Table 2). With regard to CYP1A2 activity, MR2 values were significantly correlated to the urine mutagenicity only in smokers in the control group (Table 3). Urinary mutagenicity was not significantly higher in workers with the combination of phenotypes NAT2 slow/CYP1A2 EM
than in the other combinations of the two phenotypes (3.25 ± 0.58 versus 3.15 ± 0.45 revertants/mmol creatinine, p>0.05 for MI\( \beta \), and 2.91 ± 0.4 versus 2.96 ± 0.45 revertants/mmol creatinine, p>0.05 for MIS9). Similar results were obtained in both smokers and non smokers workers (p>0.05 in all cases).

### Discussion

In the present work, higher levels of urine mutagenicity have been observed in workers than in controls, thus indicating that the occupational environment contains mutagenic substances. N-glucuronides accumulation in workers, as indicated by the high levels of MI\( \beta \) mutagenicity in relation to controls, may represent a risk factor of higher exposure to mutagens in their bladders. Arylamine N-glucuronides are labile and may be easily hydrolysed to hydroxylamines in the weak acidic media of the urinary bladder\(^3\). These hydroxylamines can lose water to yield an electrophilic arylnitrenium ion which can, in turn, react with DNA and macromolecules and initiate tumorigenesis\(^3\). In addition, N-glucuronides of hydroxylamines may be directly responsible for the induction of bladder tumors\(^3, 3\). N-glucuronides in urine may also derive from the conjugation of PAHs metabolites, present in tobacco smoke, with glucuronic acid. This could be one reason to explain the ability of the MI\( \beta \) to detect higher urine mutagenicity in smokers.

The results found here indicate that MI\( \beta \) is more sensitive to detect exposure to arylamines and other compounds present at the workplace, and MIS9 is more able to indicate tobacco-derived mutagenicity. Studies performed in arylamine-exposed animals and humans have consistently demonstrated a relevant increase in the mutagenicity of MI\( \beta \) urines\(^1, 3, 7\).

By comparing the results of the present study with those of our previous work in textile workers\(^1\), some differences are apparent. Among them, the magnitude of the urine mutagenicity was strikingly greater (MI\( \beta \) values 30 times higher than controls) than that in the present study. Bearing this fact in mind and considering that the microsuspension method used here is more sensitive than that of plate incorporation\(^9\), which was used in the previous work, it seems to indicate that the arylamine exposure at the workplace has decreased, probably as a consequence of the implementation of regulatory norms forbidding the use of the most mutagenic/carcinogenic

### Table 2. Lack of significant differences in urine mutagenicity (revertants/mmol creatinine) between slow and fast acetylators

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>Slow</th>
<th>Fast</th>
<th>n</th>
<th>Slow</th>
<th>Fast</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Sample</td>
<td>149</td>
<td>2.94 ± 0.58</td>
<td>85</td>
<td>2.87 ± 0.58</td>
<td>85</td>
<td>2.78 ± 0.50</td>
</tr>
<tr>
<td>Controls</td>
<td>68</td>
<td>2.64 ± 0.52</td>
<td>49</td>
<td>2.65 ± 0.53</td>
<td>49</td>
<td>2.66 ± 0.50</td>
</tr>
<tr>
<td>Nonsmokers</td>
<td>28</td>
<td>2.49 ± 0.52</td>
<td>26</td>
<td>2.54 ± 0.51</td>
<td>26</td>
<td>2.51 ± 0.53</td>
</tr>
<tr>
<td>Smokers</td>
<td>40</td>
<td>2.74 ± 0.50</td>
<td>23</td>
<td>2.77 ± 0.54</td>
<td>23</td>
<td>2.83 ± 0.42</td>
</tr>
<tr>
<td>Workers</td>
<td>81</td>
<td>3.19 ± 0.51</td>
<td>36</td>
<td>3.18 ± 0.52</td>
<td>36</td>
<td>2.96 ± 0.46</td>
</tr>
<tr>
<td>Nonsmokers</td>
<td>44</td>
<td>3.25 ± 0.60</td>
<td>20</td>
<td>3.19 ± 0.44</td>
<td>20</td>
<td>2.79 ± 0.31</td>
</tr>
<tr>
<td>Smokers</td>
<td>37</td>
<td>3.12 ± 0.37</td>
<td>16</td>
<td>3.16 ± 0.62</td>
<td>16</td>
<td>3.17 ± 0.53</td>
</tr>
</tbody>
</table>

MI\( \beta \): Mutagenic index (revertants/mmol creatinine) in the presence of \( \beta \)-glucuronidase. MIS9: Mutagenic index (revertants/mmol creatinine) in the presence of S9 mix.

### Table 3. Correlation coefficients between urine mutagenicity (revertants/mmol creatinine) and CYP1A2 activity

<table>
<thead>
<tr>
<th></th>
<th>Log MI( \beta )</th>
<th>Log MIS9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>0.220(^a)</td>
<td>0.235(^a)</td>
</tr>
<tr>
<td>Non smokers</td>
<td>0.009</td>
<td>0.052</td>
</tr>
<tr>
<td>Smokers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Workers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Log MI( \beta )</td>
<td>0.015</td>
<td>-0.055</td>
</tr>
<tr>
<td>Log MIS9</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.048</td>
<td>-0.216</td>
</tr>
</tbody>
</table>

\( ^a p<0.05 \)

MI\( \beta \): Mutagenic index (revertants/mmol creatinine) in the presence of \( \beta \)-glucuronidase. MIS9: Mutagenic index (revertants/mmol creatinine) in the presence of S9 mix.
arylamines as industrial dyes. In fact, o-toluidine, aniline derivatives and azo dyes, which currently are the most commonly used dyes, have been reported to exhibit weaker mutagenic properties\(^{22, 40, 41}\).

Among the different bacterial mutagenicity assays, we selected the TA 98 strain of Salmonella typhimurium. De Marini et al. (1997)\(^{42}\) found that Salmonella strain YG1024 was more sensitive than TA98 to mark exposure to benzidine, since YG1024 strain overproduces acetyltransferase, so providing additional acetylation activity. Our study, however, was directed to indicate differences between individuals' ability for N-acetylation, and hence, any exogenous acetylation could introduce a confounding factor, and therefore, the use of YG1024 strain was rejected in this work. The absence of differences in urine mutagenicity between slow and rapid N-acetylators suggests a low level of exposure in the workplace. In fact, Weber and Hein (1985)\(^{34}\) noted that low levels of aromatic amines, such as those that accompany cigarette smoking, may be detoxified nearly as well by slow acetylators as by rapid acetylators. In contrast, at higher doses, the lower detoxication ability of slow acetylators may become apparent. In this context, it is important to consider that only in studies where workers were heavily exposed to aromatic amines, a strong association between the acetylator phenotype and bladder cancer risk could be found\(^{13, 15, 43–46}\).

Since CYP1A2 enzyme activity is involved in the initial bioactivation pathway of arylamines, a relationship between CYP1A2 activity and urine mutagenicity with \(\beta\)-glucuronidase (without external hepatic mixed oxidases) could be expected. The results indicate a lack of association between urine mutagenicity from occupationally exposed individuals and their CYP1A2 activities. Tobacco-derived mutagenicity, by contrast, is influenced by CYP1A2 activity, as seen by the correlation coefficient obtained between mutagenicity in smokers from the control group and their MRs values (Table 3). This result is consistent with a previous report\(^{39}\), and could probably be due to the fact that cigarette smoke is a known source of arylamine carcinogens which are bioactivated by CYP1A2\(^{48}\) but also contains PAHs which induce CYP1A2 activity\(^{49}\). The absence of a higher urinary mutagenicity in workers with the combination of phenotypes presumed at risk (NAT2 slow/CYP1A2 EM) in relation to the other combination of the two phenotypes reinforces the notion about the relatively low level of exposure to CYP1A2 and NAT2 promutagenic substrates in the workplace.

In summary, the data suggest a lack of influence of CYP1A2 activity and NAT2 status in the urine mutagenicity of textile workers exposed to industrial dyes. On the other hand, workers continue to exhibit higher urinary mutagenicity than the general population despite the implementation of the regulatory norms forbidding the use of the most carcinogenic arylamines. This fact indicates the need for further protective policies to minimise the exposure of workers to the lowest feasible limit, in order to avoid unnecessary risks.

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Abbreviations:

<table>
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<tr>
<th>Acronym</th>
<th>Definition</th>
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<tr>
<td>17U</td>
<td>1,7-dimethyurate</td>
</tr>
<tr>
<td>1U</td>
<td>1-methyluric-acid</td>
</tr>
<tr>
<td>1X</td>
<td>1-methylxanthine-acid</td>
</tr>
<tr>
<td>AFMU</td>
<td>5-acetylamino-6-formylamino-3-methyluracil</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>Cytochrome P450 1A2</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>MRS</td>
<td>Metabolite ratio</td>
</tr>
<tr>
<td>NAT1</td>
<td>N-acetyltransferase 1</td>
</tr>
<tr>
<td>NAT2</td>
<td>N-acetyltransferase 2</td>
</tr>
<tr>
<td>PAH</td>
<td>Polycyclic aromatic hydrocarbon</td>
</tr>
<tr>
<td>UGT</td>
<td>UDP-glucuronosyltransferase</td>
</tr>
<tr>
<td>PMs</td>
<td>Poor metabolisers</td>
</tr>
<tr>
<td>Ems</td>
<td>Extensive metabolisers</td>
</tr>
</tbody>
</table>

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