

## Research Article

N-acetyltransferase 2 Phenotype, Occupation, and Bladder Cancer Risk: Results from the EPIC Cohort 

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## Abstract

**Background:** An association between N-acetyltransferase 2 (NAT2) slow acetylation and bladder cancer has been consistently observed in epidemiologic studies. However, evidence has been mainly derived from case-control studies and was sparse from cohort studies. We evaluated the association between NAT2 slow acetylation and bladder cancer in a case-control study nested in the European Prospective Investigation into Cancer and Nutrition.

**Methods:** Exposure to aromatic amines and polycyclic aromatic hydrocarbons (PAH) could be assessed for 754 cases and 833 controls for whom occupational information was documented. A semiquantitative job-exposure matrix was applied to at-risk occupations to estimate the exposure as low, medium, or high based on tertiles of the distribution of the exposure score in controls. Using a comprehensive genotyping, NAT2 acetylation status could be categorized from 6 single-nucleotide polymorphism genotypes as slow or fast in 607 cases and 695 controls with DNA from archived blood samples.

**Results:** Occupational exposure to aromatic amines and PAH was associated with an increased bladder cancer risk [upper tertile of the distribution of the exposure score: OR = 1.37; 95% confidence interval (CI), 1.02–1.84, and OR = 1.50; 95% CI, 1.09–2.05, respectively]. NAT2 slow acetylation did not modify these risk estimates and was not itself associated with bladder cancer risk (OR = 1.02; 95% CI, 0.81–1.29).

**Conclusions:** These findings confirm established or suspected occupational risk factors but not the anticipated role of NAT2 slow acetylation in bladder cancer. No interaction was detected between NAT2 and any exposure of interest, including smoking.

**Impact:** Genetic testing for NAT2 would be inappropriate in occupational settings. *Cancer Epidemiol Biomarkers Prev*; 22(11); 2055–65. ©2013 AACR.

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## Introduction

Exposure to aromatic amines (also known as arylamines) has been strongly linked to bladder cancer (1). The recognition of an occupational risk dates back to the observation of an excess of bladder cancer among aniline workers and was attributed to 4-aminobiphenyl and 2-naphthylamine (2). Both arylamines, as well as benzidine and ortho-toluidine, have been classified as Group 1 carcinogens by Working Groups of the International Agency for Research on Cancer (IARC) Monographs (<http://monographs.iarc.fr/>). Also polycyclic aromatic hydrocarbons (PAH) have been associated with bladder cancer (3). These groups of compounds are constituents of tobacco smoke, emissions from coke ovens and hot tar applications, and are present in a number of other occupational settings.

A reduced capacity for *N*-acetylation was the first enzyme polymorphism that was widely accepted as being associated with an increased cancer risk (4). Genetic testing of the encoding gene *N*-acetyltransferase 2 (*NAT2*) was introduced for purposes of compensation or preventive activities of workers with exposure to aromatic amines, but has been criticized because of the ethical questions and insufficient scientific evidence (5).

Several meta-analyses including mostly case-control studies showed higher bladder cancer risks for slow acetylators (6–10). So far, only two case-control studies had sufficient statistical power to detect an association between *NAT2* slow acetylation and bladder cancer (7, 11). Cohort studies in benzidine-exposed workers revealed a decreased risk (12, 13). This opposite effect has been attributed to differences in the metabolism of arylmonoamines and arylidiamines (12, 14). The arylidiamine benzidine was widely used in the production of azo dyes and can be cleaved by reductive degradation. Other cohort studies are inconclusive (15).

Using data from a case-control study nested in the European Prospective Investigation into Cancer and Nutrition (EPIC), we explore the impact of *NAT2* slow acetylation, as a main effect in bladder cancer etiology and as an effect modifier of the association between exposure to aromatic amines, PAHs, and bladder cancer.

## Materials and Methods

### Study groups

The study design and follow-up of 521,468 participants of EPIC for the investigation of bladder cancer risk have been reported elsewhere (16). A total of 976 subjects with bladder tumors were identified during 10 years of follow-up. A control group was identified consisting of 976 subjects randomly selected from all cohort members alive and free of cancer at diagnosis of the index case (except nonmelanoma skin cancer). They were 1:1 matched to the cases by gender, age at the time of enrolment ( $\pm 3$  years), study center, and other factors. A control could become a case at a later time (incidence density sampling). The pathology reports of cases were reassessed by a reference

pathologist according to the World Health Organization (WHO) criteria (17), resulting in 887 subjects with transitional cell bladder cancer. After exclusion of subjects with missing smoking information, 879 cases and 966 controls formed the final study population. The estimation of agent-specific ORs was based on 754 cases and 833 controls with occupational information. The analysis of *NAT2* slow acetylation was based on 607 cases and 695 controls with archived blood or DNA samples. Occupational exposure in combination with *NAT2* acetylation status could be assessed for 492 cases and 569 controls. The study was approved by the Institutional Review Board of IARC and the ethics committees in the participating countries.

### *NAT2* slow acetylation

DNA was available for 1,302 subjects (EPIC biobanks at IARC  $N = 929$ , Umea/Sweden  $N = 101$ , Malmö/Sweden  $N = 272$ ) and shipped to Institute for Prevention and Occupational Medicine of the German Social Accident Insurance (IPA, Bochum Germany). *NAT2* variants (rs1041983/282C>T, rs1801280/341T>C, rs1799929/481C>T, rs1799930/590G>A, rs1208/803A>G, rs1799931/857G>A) were genotyped using the MassARRAY system (Sequenom) as previously described (18, 19). A modified Bruker Autoflex matrix-assisted laser desorption ionization-time-of-flight mass spectrometer was used for data acquisition from the SpectroCHIP. Genotypes were called with MASSARRAY RT software v.4.0.20 (Sequenom).

Duplicates of plates allowed the genotyping of 1,030 subjects at a second Sequenom platform (concordance  $\geq 99.97\%$ ). LightCycler technology and sequencing were applied as additional quality control measures with previously described methods (20).

Minor allele frequencies and deviations from Hardy-Weinberg equilibrium (HWE) are shown in Supplementary Table S1. The best haplotype pairs were predicted from 6-single-nucleotide polymorphism (SNP) genotypes using PHASE v2.0.2. Three runs with different seeds revealed the same acetylation status for all subjects. Carriers of two slow alleles according to the *NAT* database (<http://louisville.edu/medschool/pharmacology/consensus-human-arylamine-n-acetyltransferase-gene-nomenclature/>) were classified as slow acetylators.

### Occupational exposure to aromatic amines and PAH

Occupational information was available for 1,587 subjects. Data on ever working in 52 at-risk occupations were assessed from a checklist or converted from textual job-title information. A semiquantitative job-exposure matrix (JEM) was developed by expert raters (T. Weiss, J. Angerer1, and B. Pesch) to assess the probability and intensity (0 = none, 1 = low, 2 = medium, 3 = high) of exposure to aromatic amines and PAH for these occupations. A score was generated for each agent and occupation by multiplying probability and intensity. Subjects who scored more than 0 in any at-risk occupation were considered as "potentially" exposed to aromatic amines or PAH, respectively. We categorized the subject's sum of

scores (further referred to as exposure score) as low, medium, or high using the tertiles of the distribution in controls according to an approach applied for the estimation of lung cancer risks (21).

### Statistical analyses

ORs were estimated using logistic regression models adjusting for gender, log-transformed age at interview, region (Northern, Western, and Southern Europe), smoking cigarettes (never, <20 cigarettes/day, ≥20 cigarettes/day, unknown), and smoking of other tobacco types. Because matching was broken by various exclusions, unconditional models were run. Conditional models gave identical results. To allow for multiple testing when analyzing 52 occupations, we calculated the Bayesian false discovery probability with *a priori* probabilities for occupations with excess risk (5/52, 15/52, 29/52),  $\epsilon = 0.05$  for each risk estimate, and two-sided *P* value <0.05 (22). A likelihood ratio test was conducted to evaluate deviations from a multiplicative interaction. A random effect model was applied for the meta-analysis; heterogeneity was assessed with  $I^2$  statistics. Analyses were done in SAS version 9.2.

### Results

#### Occupational exposure and bladder cancer

The study population comprised Europeans (99% of Caucasian descent; Table 1). We observed increased ORs for bladder cancer in transportation and welding in men that became nonsignificant after controlling for smoking and multiple testing (Supplementary Table S1). Notably high were the smoking-adjusted ORs for hairdressers, but based on small numbers [men: OR = 2.24; 95% confidence interval (CI), 0.62–8.15, women: OR = 5.29; 95% CI, 0.57–49.32]. An OR of 1.53 (95% CI, 1.03–2.28) was estimated for subjects ever working in occupations with potential exposure to dyestuffs. No increased ORs were observed in the chemical and rubber industry, in the manufacture of shoes or leather, and in asphalt workers and roofers.

High occupational exposure to aromatic amines, with the upper tertile of the distribution of the exposure scores among controls as cutoff, was associated with an OR for bladder cancer of 1.37 (95% CI, 1.02–1.84) and high exposure to PAH with an OR of 1.50 (95% CI = 1.09–2.05; Table 2). The exposure scores and, hence, tertiles reflect a combination of probability and intensity of exposure as well as the number of at-risk jobs during a subject's lifetime. For example, the majority of subjects (70%) categorized as "highly exposed to aromatic amines" performed at least one occupation with a high probability of exposure. About 1% of the subjects in the high-exposure group performed various occupations with a low-exposure probability that cumulatively led to high-exposure scores.

The estimates of the relative risks were lower when additionally adjusted for mutual coexposure (aromatic amines adjusted for PAH: OR = 1.14; 95% CI, 0.78–1.67; PAH adjusted for aromatic amines: OR = 1.38; 95% CI, 0.92–2.08), suggesting that these exposures jointly occurred in several jobs. The OR for exposure to either aromatic amines or PAH (OR = 1.29; 95% CI, 0.95–1.73) was similar to that in workers exposed to both the agents (OR = 1.29; 95% CI, 1.02–1.64).

The relative bladder cancer risk of PAH exposure was stronger among smokers (OR = 3.48; 95% CI, 2.51–4.84) than among non-smokers (OR = 2.43; 95% CI, 1.78–3.32; Table 3). When additionally adjusting for exposure to aromatic amines, the risk estimates decreased. Smokers who were highly exposed to PAH had an OR of 3.19 (95% CI, 1.86–5.48) compared with an OR of 2.23 (95% CI, 1.50–3.30) in smokers who were never exposed to PAH (Supplementary Table S2). The corresponding joint effects for aromatic amines and ever-smoking were 3.41 (95% CI, 2.42–4.81) for potential exposure and 2.98 (95% CI, 1.73–5.15) for high occupational exposure. Smokers who were never occupationally exposed to aromatic amines showed an OR of 2.68 (95% CI, 1.91–3.76; Table 3).

**Table 1.** Characteristics of bladder cancer cases and controls from EPIC

	Cases (N = 879)	Controls (N = 966)	OR <sup>a</sup> (95% CI)
Age at recruitment (y), median (range)	59 (23–76)	59 (23–76)	
Age at diagnosis (y), median (range)	64 (30–82)		
Gender, n (%)			
Men	645 (73.4)	703 (72.8)	
Women	234 (26.6)	263 (27.2)	
European region, n (%)			
Northern	498 (56.7)	539 (55.8)	
Southern	212 (24.1)	234 (24.2)	
Western	169 (19.2)	193 (20.0)	
Smoking, n (%)			
Never	173 (19.7)	385 (39.9)	1
Ever	706 (80.3)	581 (60.1)	2.89 (2.32–3.60)
<20 cigarettes/d	363 (41.3)	317 (32.8)	2.76 (2.16–3.54)
≥20 cigarettes/d	152 (17.3)	103 (10.7)	3.70 (2.67–5.12)
Unknown intensity	191 (21.7)	161 (16.7)	2.71 (2.02–3.63)

<sup>a</sup>OR (95% CI) adjusted for gender, age, and region.

**Table 2.** Association of occupational exposure to aromatic amines and PAHs with bladder cancer (879 cases and 966 controls from EPIC)

Occupational exposure	Cases n (%)	Controls n (%)	OR <sup>a</sup> (95% CI)	OR <sup>b</sup> (95% CI)
Aromatic amines (AA) <sup>c</sup>				
Never	329 (37.4)	407 (42.1)	1	1
Potential	425 (48.4)	426 (44.1)	1.20 (0.97–1.48)	1.05 (0.79–1.38)
Low	150 (17.1)	169 (17.5)	1.07 (0.81–1.41)	0.99 (0.72–1.36)
Medium	124 (14.1)	126 (13.0)	1.20 (0.89–1.62)	1.03 (0.72–1.48)
High	151 (17.2)	131 (13.6)	1.37 (1.02–1.84)	1.14 (0.78–1.67)
Unknown	125 (14.2)	133 (13.8)	-	-
PAH <sup>c</sup>				
Never	378 (43.0)	476 (49.3)	1	1
Potential	376 (42.8)	357 (37.0)	1.27 (1.03–1.58)	1.24 (0.93–1.65)
Low	166 (18.9)	164 (17.0)	1.24 (0.94–1.63)	1.20 (0.87–1.67)
Medium	83 (9.4)	91 (9.4)	1.09 (0.77–1.54)	1.06 (0.72–1.57)
High	127 (14.4)	102 (10.6)	1.50 (1.09–2.05)	1.38 (0.92–2.08)
Unknown	125 (14.2)	133 (13.8)	-	-
Combined exposure				
Never AA or PAH	289 (32.9)	373 (38.6)	1	
Either AA or PAH	129 (14.7)	137 (14.2)	1.29 (0.95–1.73)	
AA and PAH	336 (38.2)	323 (33.4)	1.29 (1.02–1.64)	
Complex compounds				
Never	329 (37.4)	407 (42.1)	1	
Dyestuffs	69 (7.9)	54 (5.6)	1.53 (1.03–2.28)	
Combustion	43 (4.9)	40 (4.2)	1.22 (0.76–1.96)	
Others	313 (35.6)	332 (34.4)	1.13 (0.90–1.42)	

<sup>a</sup>OR (95% CI) adjusted for gender, age, region, and smoking.

<sup>b</sup>OR (95% CI) additionally adjusted for mutual exposure to PAHs or aromatic amines, respectively.

<sup>c</sup>Low, medium, and high exposure based on the tertiles of the distribution of exposure scores among controls.

### NAT2 slow acetylation and bladder cancer

Single *NAT2* variants showed no gene-dose effects, but there were marginally more heterozygous cases at nts 341 and 481 (Supplementary Table S3). The "wild-type" allele 4 was observed in 19.9% of the controls and in 20.2% of the cases. About 80% of the subjects carried at least two SNPs, where haplotypes can only be deduced. The haplotypes 4, 5B, and 6A comprised altogether more than 90% of the *NAT2* variation. Overall, 64.1% of the cases and 64.0% of the controls were classified as slow acetylators (Table 4).

We observed no main effect of slow acetylation on the risk of bladder cancer (all cases OR = 1.02; 95% CI, 0.81–1.29; high-grade bladder cancer OR = 0.98; 95% CI, 0.73–1.31; Table 4). The risk estimates did not vary when stratified by smoking (ever-smokers OR = 1.04; 95% CI, 0.79–1.37) or occupational exposure (high exposure to aromatic amines: OR = 0.89; 95% CI, 0.47–1.71; high exposure to PAH: OR = 0.97; 95% CI, 0.48–1.98). Restricting attention to high-grade bladder cancer revealed slightly lower ORs of slow acetylation in subjects exposed to dyestuffs (OR = 0.68; 95% CI, 0.27–1.71) and in women (OR = 0.60; 95% CI, 0.34–1.08).

### Effect modification by NAT2 slow acetylation

Logistic regression revealed no deviation from multiplicative interaction between *NAT2* slow acetylation and smoking or occupational exposure to aromatic amines and PAH (Table 5 for potential exposure and Supplementary Table S4 for low, medium, and high occupational exposure). Ever-smoking was associated with ORs of 2.60 (95% CI, 1.76–3.83) in slow acetylators and 2.52 (95% CI, 1.67–3.82) in fast acetylators. The joint effect of slow acetylation and high exposure to aromatic amines was 1.20 (95% CI, 0.67–2.16) if additionally adjusted for PAH exposure. The corresponding OR for slow acetylation and PAH exposure was 1.22 (95% CI, 0.66–2.25). The relative risk estimates of high occupational exposure were similar in slow versus fast acetylators (aromatic amines: OR = 1.50; 95% CI, 0.95–2.37 vs. OR = 1.46; 95% CI, 0.80–2.68; PAH: OR = 1.52; 95% CI, 0.93–2.49 vs. OR = 1.51; 95% CI, 0.80–2.85; Supplementary Table S5).

### Meta-analysis of slow acetylation and bladder cancer risk

We restricted the meta-analysis to recent studies in predominantly Caucasians that applied high-throughput genotyping methods. We estimated a summary OR of 1.11

**Table 3.** Association of smoking and occupational exposure with bladder cancer (754 cases and 833 controls from EPIC)

Smoking	Occupational exposure to aromatic amines						P
	Never		Potential		Never	Potential	
	Cases	Controls	Cases	Controls	Joint effect OR <sup>a</sup> (95% CI)		
Never	72	166	54	139	1	0.98 (0.64–1.51)	
Ever	257	241	371	287	2.68 (1.91–3.76)	3.41 (2.42–4.81)	0.05 <sup>b</sup>
						Interaction	0.28 <sup>c</sup>
Intensity							
Never	72	166	54	139	1	1.01 (0.65–1.55)	
<20 cig/d	137	144	203	153	2.39 (1.64–3.47)	3.62 (2.49–5.27)	0.01 <sup>b</sup>
≥20 cig/d	51	32	90	66	4.43 (2.57–7.64)	3.83 (2.44–6.03)	0.60 <sup>b</sup>
Unknown	69	65	78	68	2.63 (1.67–4.15)	2.99 (1.90–4.71)	0.59 <sup>b</sup>
Smoking	Occupational exposure to PAH						P
	Never		Potential		Never	Potential	
	Cases	Controls	Cases	Controls	Joint effect OR <sup>a</sup> (95% CI)		
Never	89	194	37	111	1	0.81 (0.51–1.29)	
Ever	289	282	339	246	2.43 (1.78–3.32)	3.48 (2.51–4.84)	0.003 <sup>b</sup>
						Interaction	0.03 <sup>c</sup>
Intensity							
Never	89	194	37	111	1	0.83 (0.52–1.31)	
<20 cig/d	171	167	169	130	2.42 (1.72–3.41)	3.34 (2.31–4.83)	0.05 <sup>b</sup>
≥20 cig/d	52	46	89	52	2.91 (1.78–4.77)	4.48 (2.84–7.05)	0.11 <sup>b</sup>
Unknown	66	69	81	64	2.19 (1.41–3.39)	3.16 (2.02–4.96)	0.13 <sup>b</sup>
Smoking	Occupational exposure to aromatic amines or PAH						P
	Never		Potential		Never	Potential	
	Cases	Controls	Cases	Controls	Joint effect OR <sup>a</sup> (95% CI)		
Never	67	152	59	153	1	0.98 (0.64–1.50)	
Ever	222	221	406	307	2.51 (1.76–3.57)	3.52 (2.47–5.01)	0.01 <sup>b</sup>
						Interaction	0.14 <sup>c</sup>
Intensity							
Never	67	152	59	153	1	1.00 (0.65–1.54)	
<20 cig/d	126	133	214	164	2.36 (1.60–3.48)	3.57 (2.43–5.25)	0.01 <sup>b</sup>
≥20 cig/d	43	31	98	67	3.85 (2.18–6.80)	4.13 (2.61–6.54)	0.80 <sup>b</sup>
Unknown	53	57	94	76	2.27 (1.39–3.69)	3.25 (2.07–5.09)	0.15 <sup>b</sup>

<sup>a</sup>OR (95% CI) adjusted for gender, age, and region.

<sup>b</sup>Wald test for the comparison of ORs for potential versus never occupationally exposed smokers.

<sup>c</sup>Likelihood ratio test for the deviation from multiplicative interaction between smoking and occupational exposure (comparing logistic models with and without interaction terms).

(95% CI, 0.89–1.38) for slow acetylation and bladder cancer based on two powerful case-control studies and on studies nested in large cohorts (Fig. 1; refs. 7, 11, 15). There was a significant heterogeneity between these studies ( $P = 0.02$ ). Exclusion of the Spanish hospital-based study yielded much greater homogeneity ( $P = 0.43$ ) and a meta-OR of 1.01 (95% CI, 0.87–1.17).

## Discussion

When reassessing the role of metabolic genes as susceptibility factors, it has been concluded that *NAT2* might be one of the few examples of a consistent association with cancer (23). The study of *NAT2* polymorphisms has been supported by the mechanistic assumption that slow acetylation could be associated with increased bladder cancer

**Table 4.** Association of *NAT2* slow acetylation with bladder cancer (607 cases and 695 controls from EPIC)

		Controls		All cases			High-grade bladder cancer		
		Fast	Slow	Fast	Slow	OR <sup>a</sup> (95% CI)	Fast	Slow	OR <sup>a</sup> (95% CI)
All		250	445	218	389	1.02 (0.81–1.29)	110	191	0.98 (0.73–1.31)
Gender	Men	173	316	157	277	1.03 (0.78–1.36)	79	154	1.15 (0.82–1.62)
	Women	77	129	61	112	1.04 (0.68–1.60)	31	37	0.60 (0.34–1.08)
Smoking	Never	102	190	49	88	1.01 (0.66–1.56)	22	37	0.88 (0.48–1.58)
	Ever	148	255	169	301	1.04 (0.79–1.37)	88	154	1.00 (0.71–1.39)
	<20 cigarettes/d	71	119	81	125	0.92 (0.61–1.38)	44	66	0.86 (0.53–1.40)
	≥20 cigarettes/d	29	52	33	70	1.17 (0.63–2.17)	18	35	1.09 (0.53–2.28)
Occupational exposure	Never aromatic amines (AA)	103	176	84	131	0.88 (0.60–1.29)	40	55	0.71 (0.43–1.17)
	Potential exposure to AA	106	184	98	179	1.12 (0.78–1.60)	53	99	1.16 (0.75–1.79)
	High exposure to AA	29	53	39	63	0.89 (0.47–1.71)	22	36	0.93 (0.43–2.02)
	Never PAH	116	206	91	151	0.91 (0.64–1.30)	48	65	0.70 (0.45–1.10)
	Potential exposure to PAH	93	154	91	159	1.12 (0.76–1.64)	45	89	1.21 (0.76–1.93)
	High exposure to PAH	25	42	35	50	0.97 (0.48–1.98)	19	29	1.06 (0.46–2.40)
	Dyestuffs	24	46	23	40	0.93 (0.43–1.99)	15	19	0.68 (0.27–1.71)
	Combustion	21	34	20	34	1.10 (0.47–2.54)	11	21	1.17 (0.41–3.32)
Region	Northern Europe	103	204	102	173	0.87 (0.62–1.24)	50	88	0.90 (0.59–1.39)
	Western Europe	79	102	55	101	1.47 (0.93–2.33)	23	39	1.33 (0.71–2.49)
	Southern Europe	68	139	61	115	0.91 (0.58–1.41)	37	64	0.83 (0.49–1.40)

<sup>a</sup>OR (95% CI) adjusted for gender, age, region, and for smoking where appropriate.

risk, given the role of *NAT2* in the metabolism of aromatic amines (24). Here, we took advantage of the large database and biorepositories of the EPIC to explore *NAT2* slow acetylation. However, we found no main effect and could not observe support for an effect modification of the association of bladder cancer with exposure to tobacco smoke, aromatic amines, or PAH. An analysis of high-grade bladder cancers indicated a lower risk in dyestuff-exposed subjects, but the statistical power was limited for exploring subgroups.

*NAT2* slow acetylation was proposed for genetic testing for purposes of compensation or preventive activities among workers with exposure to aromatic amines. Few studies have explored the interaction of the *NAT2* acetylation status with occupational exposure to aromatic amines (24). Our results do not provide evidence for a strong interaction, but EPIC is a population-based cohort with a low prevalence of rare occupations like coke-oven works that are known to entail a bladder cancer risk. The statistical power of this analysis as well as of other population-based studies conducted so far is not sufficient to exclude the presence of interactions in rare at-risk occupations.

Although many studies reported on the main effect of slow acetylation, only two case-control studies had enough statistical power to assess the role of *NAT2* in bladder cancer. This is the first report on *NAT2* slow

acetylation from a large prospective study that had adequate statistical power to evaluate a 1.4-fold main effect for the association with bladder cancer (7). On the basis of fewer cases, an OR of 1.33 (95% CI, 0.77–2.31) was estimated in women from the Nurses' Health Cohort and of 0.78 (95% CI, 0.53–1.15) in men from the Health Professional's Follow-up Study (15). Previous meta-analyses included small historical case-control studies that used a variety of methods to deduce *NAT2* acetylation (6–10). Concerns can be raised about the phenotyping of cases with bladder cancer because both the disease and the treatment may influence urinary metabolite concentrations. Only two recent case-control studies were powerful enough to evaluate *NAT2* slow acetylation (7, 11). The Spanish study was hospital-based and reported an OR of 1.4 (95% CI, 1.2–1.7) and the population-based New England study estimated an OR of 1.04 (95% CI, 0.82–1.28) for the association of *NAT2* slow acetylation with bladder cancer. The summary OR of 1.11 (95% CI, 0.89–1.38) from the two large case-control studies and three prospective studies is lower than previously estimated with historical studies (6–10). There is still heterogeneity between these recent studies due to a higher OR in the Spanish study. The difference is at least partially explained by a 10% lower proportion of slow acetylators in Spanish hospital controls than in Spanish population controls from EPIC.

**Table 5.** Joint effects of the *NAT2* acetylation status and smoking (607 cases and 695 controls) or occupational exposure to aromatic amines and PAH (492 cases and 569 controls) on the risk of bladder cancer

	<i>NAT2</i> acetylation status						<i>P</i>
	Fast		Slow		Fast	Slow	
	Cases	Controls	Cases	Controls	Joint effect OR <sup>a</sup> (95% CI)		
<b>Smoking</b>							
Never	49	102	88	190	1	0.98 (0.64–1.49)	
Ever	169	148	301	255	2.52 (1.67–3.82)	2.60 (1.76–3.83)	0.83 <sup>b</sup>
						Interaction	0.83 <sup>c</sup>
<b>Aromatic amines</b>							
Never	84	103	131	176	1	0.92 (0.63–1.34)	
Potential	98	106	179	184	1.03 (0.68–1.57)	1.14 (0.78–1.65)	0.59 <sup>b</sup>
						Interaction	0.49 <sup>c</sup>
<b>PAH</b>							
Never	91	116	151	206	1	0.95 (0.66–1.35)	
Potential	91	93	159	154	1.09 (0.72–1.66)	1.20 (0.82–1.75)	0.62 <sup>b</sup>
						Interaction	0.57 <sup>c</sup>

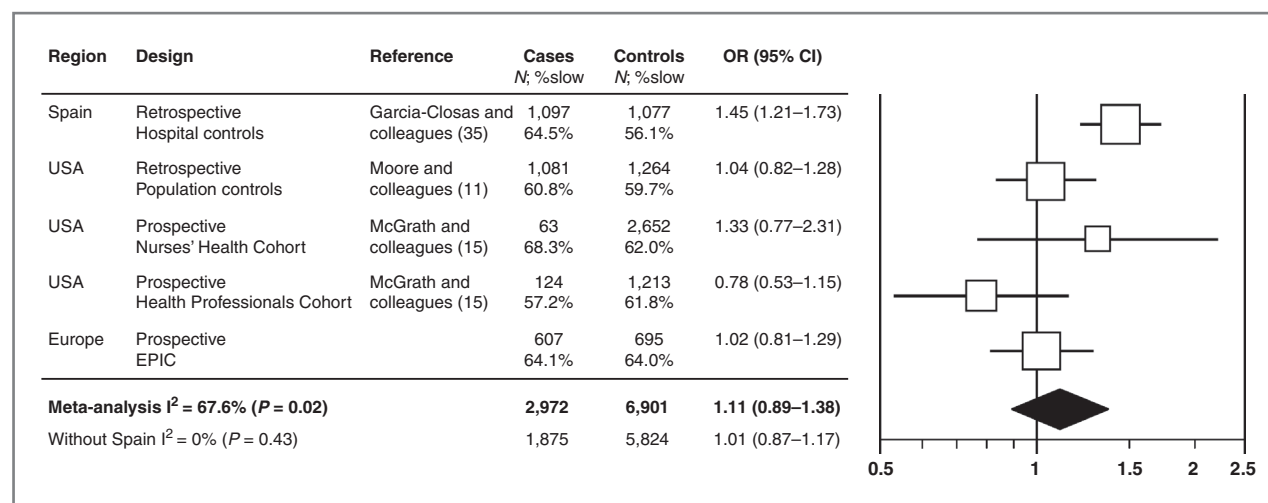
<sup>a</sup>OR (95% CI) adjusted for gender, age, region, and for smoking where appropriate.

<sup>b</sup>Wald test for the comparison of ORs for slow versus fast acetylation within the exposure groups.

<sup>c</sup>Likelihood ratio test for the deviation from multiplicative interaction (comparing logistic models with and without interaction terms).

Several methodologic reasons can be hypothesized that might explain the observed differences in the proportion of *NAT2* slow acetylators, which in turn can contribute to the heterogeneity between studies. *NATs* are phylogenetically

unstable genes with a large genetic diversity of *NAT2* alleles in humans. The slow variants of the *NAT2* gene may reflect a selective advantage in populations practicing farming (25). Although genomic features



**Figure 1.** Meta-analysis of *NAT2* slow acetylation and bladder cancer including two recent powerful case-control studies (11, 35) and risk estimates from three prospective designs (15). The horizontal axis shows ORs with 95% CI on a logarithmic scale. The size of the rectangles is proportional to the weight of the study in the risk estimation.

suggest a relatively homogenous European population, migration and isolation may result in genetic diversity (26). For example, Spanish Basques carry markers that indicate an isolated subpopulation (27). Population stratification may lead to false-positive associations of genetic variants with the disease if cases and controls include subjects who differ in geographic origin and may have a discordant ancestry level. Case-control studies are likely more prone to this specific bias than are prospective studies where both study groups are originating from the same target population.

Besides population stratification, minor uncertainties along the line from SNP selection to haplotype reconstruction might occur or even accumulate. Genotyping problems cannot be fully ruled out for this prominent locus (28). Part of the presumed evidence for slow acetylation has been derived from historical studies where quality control measures have been rarely reported. A visual inspection of the putative genotype can be prone to observer bias, whereas MALDI TOF MS calls genotypes from mass differences in the amplicons (29). It was frequently not possible to compare our genotype distributions with published data, because most *NAT2* reports show only the distribution of the deduced phenotypes. HWE deviations are supportive for genotype misclassification and can bias the risk estimates in meta-analyses (30). Our genotype distributions were in HWE and in good agreement with other large control populations in Europeans (31). Although PHASE can deduce alleles with incomplete data, missing calls can be biased toward a certain genotype (32). We obtained a full 6-SNP genotype for 91% of the subjects. Notably, we observed a slight difference in the genotype distribution at nt 341 compared with the New England study. This SNP is located in a CG-rich region and can pose genotyping problems. We found relatively more missing calls than for other SNPs, and most discordant pairs were related to the heterozygous genotype. CC and TT calls were determined as CT at the other Sequenom platform and *vice versa*, but not as TT or CC, respectively.

Another source of some uncertainty is the deduction of *NAT2* alleles from few SNPs because most Caucasians carry mutations. An allele is presented by a diploid DNA sequence of the gene. If short amplicons are analyzed, alleles can only be deduced for subjects carrying more than one heterozygous SNP. Computational algorithms like PHASE have been applied only recently to infer *NAT2* haplotypes (33). Despite an overall good performance, some subjects can be misclassified (34). Furthermore, the SNPs selected for genotyping should capture the genetic variation at this locus according to the underlying linkage disequilibrium structure. A good performance has also been shown for a tagging SNP (35, 36). The fraction of subjects with slow variants usually increases with an increasing number of SNPs. Many studies in Caucasians genotyped a 6-SNP set, whereas the studies nested in other large cohorts used a 3-SNP genotype (15), which is assumed to capture the acetylation status with high sen-

sitivity and specificity (28). Cascorbi and Roots considered 282C>T and 341T>C to be sufficient for predicting the acetylation phenotype in non-Africans (37). We achieved a coverage of 97% for 481C>T, 590G>A, and 857G>A and 92% for 282C>T and 341T>C.

Variation in risk estimates of *NAT2* slow acetylation may also arise from the crude categorization of subjects as slow and fast, or intermediate and rapid acetylators. Because of the small fraction of about 5% rapid acetylators (7, 11, 38), we refrained from a more extensive stratification of fast acetylators. Functional characterization of *NAT2* SNPs revealed multiple mechanisms conferring acetylation capacity, for example, by changing protein activity, thermostability, or substrate specificity (39). The *NAT2* polymorphism can be shown with a bimodal distribution of the ratio of urinary concentrations of two metabolites after administering caffeine (38). The variation of this ratio within groups of slow or fast acetylators is very large in common allele combinations, and phenotype data for rare allele combinations are yet inconclusive (40).

The established bladder cancer risk associated with aromatic amines and the observed differences in metabolic activity associated with *NAT2* variants have lent theoretical plausibility to the hypothesis that *NAT2* slow acetylation may modify the bladder cancer risk (41). The minor or even null main effect of *NAT2* slow acetylation on bladder cancer risk in our study also needs to be discussed with regard to the complexity of the metabolism. It is important to note that canids even lack *NAT* expression (42), and *NAT* knockout mice do not exhibit a different phenotype (43). Human *NATs* form a cluster of three genes, comprising a pseudogene and the functional genes *NAT1* and *NAT2* that share 81% of their sequence (44). Both isoforms catalyze *N*- and *O*-acetylation reactions but vary in substrate specificity (45). Caffeine, which is not an aromatic amine, has been administered to deduce the acetylation status based on a ratio of metabolites that are excreted into urine following *N*-acetylation as detoxifying mechanism of arylamines. However, less is known about the influence of *NAT2* variants on the *O*-acetylation due to a limited access to the target tissue in healthy humans. In addition, other highly polymorphic enzymes like P4501A2 contribute to the metabolism of arylamines (46). A pathway analysis will be subject of another report.

An accurate exposure assessment is important for exploring gene-environment interactions (47). The assessment of occupational exposure poses various challenges in population-based studies. Job-exposure matrices are a general tool of population-based studies to estimate exposure probability and intensity associated with job titles (48). Combining different dimensions into an exposure score may partially misclassify some subjects, in addition to differing evaluations of various experts when generating JEMs. Aromatic amines have been rarely assessed with a JEM. On the basis of a semiquantitative assessment



of occupational exposure, we could confirm the bladder cancer risk associated with aromatic amines and dyestuffs (49). However, the risk estimates are lower than in older studies, in particular, for occupations in the European chemical and rubber industry or in asphalt workers (50, 51). This is likely due to the ban of carcinogenic aromatic amines from many industrial processes and the substitution of tar by bitumen. Concern has been raised about the use of hair dyes, as hairdressers were among the occupations with an elevated bladder cancer incidence, for example in Northern Europeans (51). We also found an excess risk of bladder cancer, but it was not significant likely due to small numbers.

We could further provide support for an increased bladder cancer risk associated with PAH exposure as previously shown in a pooled analysis of European case-control studies (50). Combustion of organic matter is a common source of PAH and occurs at many workplaces. This has been frequently addressed before, also in combination with aromatic amines during the hot application of tar or in coke production and coal gasification (52). The cancer risk for agents that occur jointly at the same workplaces can hardly be disentangled by statistical means. Mutual adjustment attenuates the risk estimates and may underestimate the effect of the driving factor with a higher carcinogenic potency. We found higher risks in smokers with substantial occupational exposure as compared with smokers who were never exposed to aromatic amines or PAH.

However, we found no support for an effect modification by *NAT2* slow acetylation. EPIC was not aimed at investigating occupational exposures, and due to its population-based design, the prevalence of certain high-exposed occupations was low. Risk estimates for single occupations are additionally impaired by inflation of type I error. Also lacking information on the occupational history and duration of exposure weakens the detection of work-related bladder cancer risks. Additional analyses provided no evidence for a residual confounding by smoking.

We confirmed tobacco consumption as a major risk factor for bladder cancer, where arylamines are suspected to be the primary causative agents (53). Certain PAH compounds are another group of carcinogens in tobacco smoke. A comprehensive analysis of smoking and bladder cancer in EPIC has been reported elsewhere (54). Reviews and meta-analyses supported a joint effect of *NAT2* slow acetylation with tobacco smoking (7, 10, 11). We found no interaction with smoking or a difference of the relative risk estimates of slow acetylation in smokers compared with never-smokers. However, the statistical power was not sufficient to rule out a weak effect modification in heavy smokers.

A striking feature of bladder cancer is its heterogeneous clinical behaviour, likely due to different molecular pathways (55). We therefore explored *NAT2* slow acetylation also in high-grade bladder cancer. We estimated similar

risks of slow acetylation except a slightly lower risk in dyestuff-exposed subjects that is in line with a lower risk in Chinese benzidine-exposed workers (12). We further found a lower risk in women, though not significant. Gender differences are known in bladder cancer risk, recurrence, and survival (56). A survival analysis will be subject of another report.

In conclusion, the findings from this prospective investigation are compatible with previous evidence showing excess risks of bladder cancer associated with occupational exposure to aromatic amines and are supportive for a role of PAHs in the development of bladder cancer. However, neither found an excess risk of bladder cancer associated with *NAT2* slow acetylation, nor did we find that *NAT2* slow acetylation modified the relative risk associated with exposure to aromatic amines, PAH, or tobacco smoke. *NAT2* acetylation status is unlikely to be a useful marker for predicting bladder cancer in the population at large, or among workers.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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# Cancer Epidemiology, Biomarkers & Prevention

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