NAT2 slow acetylation and *GSTM1* null genotypes may increase postmenopausal breast cancer risk in long-term smoking women

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***N*-acetyltransferase (NAT) 1 and 2 and glutathione *S*-transferase (GST) M1 and T1 are phase II enzymes that are important for activation and detoxification of carcinogenic heterocyclic and aromatic amines, as present in cigarette smoke. We studied whether genetic polymorphisms in these genes modifies the relationship between smoking and breast cancer. A nested case–control study was conducted among participants in a Dutch prospective cohort. Breast cancer cases ($n = 229$) and controls ($n = 264$) were frequency-matched on age, menopausal status and residence. Compared to never smoking, smoking 20 cigarettes or more per day increased breast cancer risk statistically significant only in postmenopausal women [odds ratio (OR) = 2.17; 95% confidence interval (CI) 1.04–4.51]. Neither *NAT1* slow genotype, or *GSTT1* null genotype, alone or in combination with smoking, affected breast cancer risk. However, compared to individuals with rapid *NAT2* genotype, women with the very slow acetylator genotype (*NAT2*5*), who smoked for 20 years showed an increased breast cancer risk (OR = 2.29; 95% CI 1.06–4.95). Similarly, the presence of *GSTM1* null genotype combined with high levels of cigarette smoking (OR = 3.00; 95% CI 1.46–6.15) or long duration (OR = 2.53; 95% CI 1.24–5.16), increased rates of breast cancer. The combined effect of *GSTM1* null genotype and smoking high doses was most pronounced in postmenopausal women (OR = 6.78; 95% CI 2.31–19.89). In conclusion, our results provide support for the view that women who smoke and who have a genetically determined reduced inactivation of carcinogens (*GSTM1* null genotype or slow *NAT2* genotype (especially very slow *NAT2* genotype)) are at increased risk of breast cancer. *Pharmacogenetics* 13:399–407 © 2003 Lippincott Williams & Wilkins**

Pharmacogenetics 2003, **13**:399–407

Keywords: *N*-acetyltransferase, glutathione-*S*-transferase, smoking, breast cancer, nested case–control

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Sponsorship: The studies outlined in this manuscript were supported in part by United States Public Health Service grant CA34627 from the National Cancer Institute and from the Dutch Cancer Society (UU 98–1707) and Sineke ten Horn fund, the Netherlands.

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Received 16 September 2002
Accepted 4 April 2003

Introduction

Breast cancer is the most common cancer in women in Western society [1]. Approximately one-third of new cases of cancer in women are breast cancer. Genetic factors, acquired environmental factors or, most often, a combination of both probably causes breast cancer. A family history of breast cancer and several reproductive characteristics are acknowledged risk factors. For smoking, the results are less conclusive, although Khuder *et al.* [2] summarized 40 studies and showed a 10% higher risk for women who ever smoked [pooled relative risk = 1.10; 95% confidence interval (CI) 1.02–1.18] [2]. Another recent meta-analysis suggests no relation between smoking and breast cancer overall [3]. However, there may be women who are more susceptible for smoking compared to other individuals because of their genetic make-up. Cigarette smoke contains rodent mammary carcinogens, such as polycyclic aromatic hydrocarbons, nitrosamines, aromatic amines and heterocyclic amines. Individual cancer susceptibility following exposure to these tobacco carcinogens may be based on differences in the capacity of metabolic enzymes to activate or deactivate the carcinogens and form DNA adducts [4]. Perera *et al.* [5] showed that DNA adduct levels in human mammary tissue and smoking habits were related in breast cancer patients. Furthermore, Li *et al.* [6] demonstrated a significant elevation of DNA adducts levels in breast epithelial DNA in cancer patients compared to controls. (In)activation of aromatic amine carcinogens is catalysed by metabolic enzymes including *N*-acetyltransferase 1 (NAT1) and *N*-acetyltransferase 2 (NAT2) and glutathione-*S*-transferase M1 (GSTM1) and T1 (GSTT1). Each of these phase II enzymes exhibit genetic polymorphisms in human populations.

0960-314X © 2003 Lippincott Williams & Wilkins

DOI: 10.1097/01.fpc.0000054106.48725.87

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Studies examining the relation of *NAT1* or *NAT2*, smoking and breast cancer are inconsistent

One meta-analysis showed a higher postmenopausal breast cancer risk in women with *GSTM1* null genotype [7]. No association with breast cancer has been observed with deletion of the *GSTT1* gene, but this is based on two studies. The interaction between smoking and *GSTM1* or *GSTT1* and breast cancer has been studied but significant associations have not been reported [8–11].

The aim of the present study was to investigate the effects of genetic polymorphisms in the relevant metabolic genes (*NAT1*, *NAT2*, *GSTM1* and *GSTT1*) and smoking on the risk of breast cancer in the Netherlands.

Methods

Study population

We conducted a nested case–control study using subjects enrolled in the Monitoring Project on Cardiovascular Disease Risk Factors conducted in three Dutch residences (Amsterdam, Maastricht and Doetinchem) between January 1987 and December 1991. More than 36 000 men and women were enrolled. A detailed description of this project is available elsewhere [12]. In brief, each year, a random sample of men and women, aged 20–59 years, was selected from the municipal registries of the three residences and invited to participate. The overall response rate was 50% for men and 57% for women. The study protocol was approved by the Medical Ethical Committee of the University of Leiden, the Netherlands.

In Doetinchem, some subjects participated more than once and duplicate observations from these participants ($n = 1097$; first record was used) were excluded. We further excluded subjects who could not be identified in the National Population Database ($n = 24$); whose vital status by 31 December 1997 was unknown ($n = 343$); who disagreed with the release of medical records from their general practitioner and were therefore not submitted for linkage to the cancer registry ($n = 597$); who did not provide a blood sample ($n = 705$), who were of presumed non-Caucasian ethnicity ($n = 1402$); or who had cancer previous to their inclusion into the cohort (except non-melanoma skin cancer and cervix cancer *in situ* or lobular breast cancer *in situ*) ($n = 542$). From the resulting cohort, we included all first incident breast cancer cases and a random sample from the controls as described below.

Follow-up for incident cancer from 1987–1997 was achieved via computerized record linkage with the Netherlands Cancer Registry (NCR) and with the three regional cancer registries (IKA, IKL and IKO) serving the areas of Amsterdam, Maastricht and Doetinchem, respectively. NCR is a national registry of all malignant tumors diagnosed from 1989 onwards in people living in the Netherlands. Completeness, data consistency and the possibility of duplicate records have extensively been checked [13]. Because data from NCR were complete only for the period 1989 to the end of 1996, additional information from the regional cancer registries was used. For 1987 and 1988, completeness of data from these registries varied between 60% and 100% depending on registry and year. For 1997, data from the three regional cancer registries were 100% complete and, for 1998, data were 100% complete for IKL only. Records from the cohort were linked using a method based on the two-stage process developed by Van den Brandt *et al.* [14]. In total, 251 breast cancer cases could be identified. A random sample of controls matched to the cases with the same age (5-year intervals), menopausal status and residence was drawn. We oversampled our control population by 20% because the success rate of DNA isolation was expected not to be 100%. Our study population consisted of 251 cases and 300 controls.

Smoking

Smoking habits were recorded at baseline by use of a self-administered questionnaire. Exposure to tobacco smoke was assessed for cigarettes, cigars and pipe separately. Because only two women reported smoking cigars (one, one cigar per day for 10 years and the other, seven small cigars per day for 5 years) and none were pipe-smokers, we only used cigarette smoking data.

The questionnaire contained questions about current smoking status, current number of cigarettes smoked, age at start of smoking cigarettes, total number of years smoked and, in the case of past smoking, the daily number of cigarettes smoked in the past. We then computed a variable for average number of cigarettes smoked (dose). For women who recorded numbers of cigarettes smoked both for current smoking as for past smoking, we averaged past and present numbers of cigarettes.

Genotype

All participants provided a blood sample that was separated into plasma, erythrocytes and buffy coat and was stored at −20°C. Mean storage time until DNA isolation was 11.5 years. For three cases and two controls, no sample could be retrieved.

DNA was isolated from buffy coats of 229 cases and 264 controls (success rate of 91.2% for cases and 88% for controls). DNA was diluted to 20 ng/μl and stored at 4°C in deep-well microtitre plates. All genotyping was determined blind to case–control status.

We determined *NAT1* genotype by sequencing two

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parts of the *NAT1* gene (nucleotides 150–650 and 750–1150). Nucleotide sequence was determined after purification of the amplified polymerase chain reaction (PCR) products with Qiaquick PCR Purification Kit (Qiagen, Valencia, California, USA) using the Big-Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA). Electrophoresis and analysis of DNA sequence reactions were performed with an ABI 310 Genetic Analyzer (Perkin-Elmer Inc., Foster City, California, USA). *NAT2* genotype was determined using single nucleotide polymorphism-specific PCR primers and fluorogenic probes as described by Doll and Hein [15]. Six polymorphic sites were investigated, C282T, T341C, C481T, G590A, A803G and G857A. Because our population was Caucasian, we did not include G191A [16].

Presence or absence of the *GSTM1* and *GSTT1* gene was determined by multiplex PCR as described by Chen *et al.* [17]. Briefly, segments of *GSTM1* and *GSTT1* were amplified along with a segment of human β-globin. The PCR products were analysed on agarose gels. A fragment of 215 bp indicated the presence of *GSTM1*, a fragment of 480 bp indicated the presence of *GSTT1* and a fragment of 268 bp indicated the positive internal control β-globin.

Data analysis

Smoke dose was categorized as never, < 10, 10–20 and $\geqslant 20$ cigarettes per day. Duration of smoking was classified as never, < 15, 15–30 and $\leqslant 30$ years. For analyses according to menopausal status, for the very slow *NAT2* genotype, and for gene–gene smoking interaction, numbers were small and therefore dose and duration were categorized into three categories (never, < 20 and $\geqslant 20$ cigarettes per day and never, < 20 and $\geqslant 20$ years, respectively). Women with artificial menopause were categorized as postmenopausal.

Although an initial report on increased activity associated with the *NAT1*10* allele [18] could not be supported in subsequent studies [19–22], we maintain the distinction between *NAT1*10* and *non NAT1*10* in this study. Women with at least one *NAT1*10* allele were classified as rapid acetylators whereas women with at least one *NAT1*14A*, *NAT1*14B*, *NAT1*15*, *NAT1*17* and *NAT1*22* were combined and classified as slow acetylators. This last group is small and therefore women with no *NAT1*10* allele were classified as one group. Rapid acetylators served as the reference group in all analyses.

For *NAT2* genotype, carriers of a *NAT2*4*, *NAT2*12* or *NAT2*13* allele were classified as rapid and the rest as slow acetylators [23]. Rapid acetylators were used as a reference group. According to some data, individuals homozygous for *NAT2*5* alleles are the slowest acetylators [24], and therefore we also analysed these individuals compared to the rapid acetylators.

Absence of *GSTM1* or *GSTT1* (null genotype) in women was compared to women with those genes present (reference group). Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated using logistic regression models. We first analysed the effect of smoking, with breast cancer as the dependent variable and smoking exposure as the independent variable. Subsequently, the effect of each of the putative at-risk genotypes was assessed. To evaluate the combined effect of smoking and metabolic genotype, a logistic regression model was used with breast cancer as the dependent variable and a combination of smoking and genotype as the independent variable. Combined gene and environment effects were analysed using two-by-eight tables. Tests for trend were calculated.

For all models, age, menopausal status, and residence were included as confounders (frequency matching). Other factors considered for confounding were body mass index (BMI) (continuous), age at first full-term pregnancy (not applicable, < 22, 22–26 and $\geqslant 26$), menarchal age (continuous), and education (primary, technical, secondary and academic). We decided to include confounders in the model if exclusion changed the estimate for the association with cancer risk by more than 10%. Because this was never the case, the final models contain breast cancer as dependent variable and the determinants of interest (smoking, genotype or a combination) and age, menopausal status, and residence as independent variables.

Tests for Hardy–Weinberg equilibrium were conducted by comparing observed and expected polymorphisms and genotype frequencies using a chi-square test. All analyses were conducted using SPSS version 9.0 (SPSS, Chicago, Illinois, USA).

Results

Effect of smoking

Characteristics of the study population are shown in Table 1. No significant difference was observed between the genotyped and non-genotyped persons with respect to these characteristics (data not shown).

Compared to never smoking, neither dose nor duration of smoking significantly increased breast cancer risk, although the data suggested a dose-response relation ($P = 0.06$ and $P = 0.07$ for trend; respectively) (Table 2). For postmenopausal women, this dose-response relation was significant ($P = 0.04$ for trend) with breast cancer risk more than doubled in women who smoked the highest dose (OR = 2.17; 95% CI 1.04–4.51).

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Table 1 Characteristics of the study population

	Cases ($n = 229$)	Controls ($n = 264$)
Mean age at recruitment, years (SD)	47.5 (8.0)	47.0 (9.1)
Mean age menopause (SD)	49.2 (3.5)	48.9 (3.9)
Menopausal state, No (%)		
Pre-	127 (55.5)	136 (51.5)
Natural menopause	68 (29.7)	85 (32.2)
Artificial menopause	34 (14.8)	43 (16.3)
Mean age at menarche (SD)	13.4 (1.6)	13.4 (1.5)
Parity, No (%)	51 (22.3)	60 (22.7)
Mean no. of children (SD)	2.0 (0.8)	2.4 (1.4)
Mean age first full-term pregnancy (SD)	25.6 (4.0)	24.6 (3.9)
Mean height, cm (SD)	164.9 (6.8)	165.0 (6.6)
Mean weight, kg (SD)	68.3 (11.3)	69.5 (11.9)
Mean body mass index, kg/m^2 (SD)	25.1 (4.1)	25.5 (4.2)
Highest level of education, No. (%)		
Primary school	60 (26.3)	69 (26.2)
Technical	106 (46.5)	122 (46.4)
Secondary	31 (13.6)	32 (12.2)
Academic	31(13.6)	40 (15.2)
Mean age start smoking (SD)	18.7 (4.3)	18.4 (4.7)
Mean years smoked (SD)	23.1 (11.2)	22.1 (11.1)
Smoking status, No. (%)		
Never	76 (33.2)	104 (39.4)
Former	55 (24.0)	62 (23.5)
Current	98 (42.8)	98 (37.1)

Values are means (SD) or *n* (%).

Effect of genotype

Table 3 shows allele frequencies in cases and controls. For *NAT1* and *NAT2*, all polymorphisms were in Hardy–Weinberg equilibrium with $P > 0.05$ by the chi-square test. For *GSTM1* and *GSTT1*, we did not determine the heterozygotes and Hardy–Weinberg equilibrium could not be determined. The distribution of *NAT1*, *NAT2* and *GSTT1* genotypes was similar among cases and controls (with and without stratification for menopausal status) and revealed no increased breast cancer risk. *NAT2*5B* was the most common *NAT2* allele, present in 43% of the breast cancer cases and in 41% of the controls. Analysing *NAT2*5* alleles separately, the slowest *NAT2* acetylators, revealed 55 cases (24% of the total cases) and 52 controls (20% of the total controls) who have two *NAT2*5* alleles. They were compared with women with a rapid acetylator genotype, and women with two *NAT2*5* alleles had no increased risk (OR = 1.32; 95% CI 0.82–2.14).

The *GSTM1* null genotype was present in 58% of cases and 49% of controls. Women with the *GSTM1* null genotype showed an increased breast cancer risk (OR = 1.46; 95% CI 1.02–2.09) (Table 3), with the risk more pronounced among postmenopausal women (OR = 1.83; 95% CI 1.07–3.13) than among premenopausal women (OR = 1.18; 95% CI 0.73–1.94).

Combined effect of gene and smoking

Neither *non NAT1*10* nor *GSTT1* null genotype significantly modified the effect of smoking. Compared to women with a rapid *NAT2* genotype and who had never smoked, women with a slow *NAT2* genotype and who smoked 20 cigarettes per day or smoked more than 30 years tended to have an increased breast cancer risk (OR = 1.82; 95% CI 0.91–3.64 and OR = 1.66; 95% CI 0.80–3.44, respectively) (Table 4). Individuals with the very slow acetylator genotype (*NAT2*5*), high levels of cigarette smoking or duration similarly showed increased risk of breast cancer (OR = 2.19; 95% CI 0.83–5.82 and OR = 2.29; 95% CI 1.06–4.95, respectively) (Table 4).

Compared to women with *GSTM1*, individuals possessing *GSTM1* null genotype, high levels of cigarette smoking and long duration were again associated with breast cancer (OR = 3.00; 95% CI, 1.46–6.15 and OR = 2.53; 95% CI 1.24–5.16) (Table 5).

After stratification for menopausal status, postmenopausal women with slow *NAT2* acetylator genotype and who smoked 20 cigarettes or more per day showed a four-fold higher risk of breast cancer compared to women who never smoked with the rapid *NAT2* genotype (OR = 4.20; 95% CI 1.34–13.19); premenopausal group (OR = 1.03; 95% CI 0.42–2.52) (Table 6). Furthermore, postmenopausal women with *GSTM1* null

Table 2 Smoking and breast cancer risk

	Cases, *n* (%) (total = 229)	Controls, *n* (%) (total = 263)	Odds ratio[a] (95% confidence interval)
Dose			
Never	76 (33.2)	104 (39.5)	1.00
< 10 cigarettes/day	40 (17.5)	48 (18.2)	1.22 (0.72–2.06)
10–20 cigarettes/day	60 (26.2)	63 (23.9)	1.37 (0.86–2.18)
⩾ 20 cigarettes/day	53 (23.1)	49 (18.6)	1.55 (0.94–2.54)
			P-value for trend 0.06
Duration			
Never	76 (33.2)	104 (39.5)	1.00
< 15 years	34 (14.8)	41 (15.6)	1.22 (0.68–2.18)
15–30 years	68 (29.7)	71 (27.0)	1.37 (0.86–2.16)
⩾ 30 years	51 (22.3)	47 (17.9)	1.55 (0.92–2.61)
			P-value for trend 0.07

[a]Adjusted for age, menopausal status and residence.

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Table 3 ***NAT1*, *NAT2*, *GSTM1*, *GSTT1* alleles and genotypes in breast cancer cases and controls, and breast cancer risk**

Alleles/genotypes	Cases, *n* (%)	Controls, *n* (%)	Odds ratio[a] (95% confidence interval)
NAT1 alleles			
*NAT1*3*	12 (2.6)	13 (2.5)	
*NAT1*4*	334 (73.2)	394 (74.6)	
*NAT1*10*	87 (19.1)	97 (18.4)	
*NAT1*11*	8 (1.8)	8 (1.5)	
*NAT1*14A*	9 (2.0)	8 (1.5)	
*NAT1*14B*	0	1 (0.2)	
*NAT1*15*	1 (0.2)	3 (0.6)	
*NAT1*17*	2 (0.4)	3 (0.6)	
*NAT1*22*	3 (0.7)	1 (0.2)	
Total	456	528	
NAT1 genotype			
*NAT1*10*	77 (32.8)	88 (33.3)	1.00
*Non NAT1*10*	151 (66.2)	176 (66.7)	0.97 (0.67–1.43)
Total	228§	264	
NAT2 alleles			
*NAT2*4*	98 (21.3)	112 (21.3)	
*NAT2*5A*	10 (2.2)	14 (2.7)	
*NAT2*5B*	196 (42.8)	216 (41.1)	
*NAT2*5C*	9 (2.0)	4 (0.8)	
*NAT2*6A*	131 (28.7)	154 (29.3)	
*NAT2*6B*	1 (0.2)	0	
*NAT2*6C*	1 (0.2)	1 (0.2)	
*NAT2*7B*	11 (2.4)	17 (3.2)	
*NAT2*12A*	1 (0.2)	5 (0.9)	
*NAT2*12B*	0	2 (0.4)	
*NAT2*13*	0	1 (0.2)	
Total	458	526	
NAT2 genotype			
Rapid	86 (37.5)	106 (40.2)	1.00
Slow	143 (62.4)	158 (59.8)	1.13 (0.78–1.63)
Very slow *(NAT2*5/*5)*	55 (24.0)	52 (20.0)	1.32 (0.78–2.09)
Total	229	264	
GSTM1			
Present	96 (42.0)	134 (51.0)	1.00
Absent (null genotype)	133 (58.0)	129 (49.0)	1.46 (1.02–2.09)
Total	229	263§	
GSTT1			
Present	193 (84.0)	213 (81.0)	1.00
Absent (null genotype)	36 (16.0)	50 (19.0)	0.78 (0.49–1.26)
Total	229	263[b]	

[a]Adjusted for age, residence, menopausal status. [b]For one sample, it was not possible to determine *NAT1* genotype and, for another, it was not possible to determine *GSTM1* and *GSTT1* presence or absence.

genotype who smoked 20 cigarettes or more per day had an almost seven-fold increased risk compared to *GSTM1* positive women who never smoked (OR = 6.78; 95% CI 2.31–19.89); premenopausal group (OR = 1.28; 95% CI 0.47–3.52).

Combined gene–gene effect

Women with a combination of *NAT2* slow genotype, *GSTM1* null genotype and 20 years of smoking had an almost three-fold increased breast cancer risk compared to rapid *NAT2* acetylator genotype, *GSTM1* presence and never smoking (OR = 2.80; 95% CI 1.08–7.26). Due to small numbers, analyses according to menopausal status were not possible.

Discussion

The results of this study suggest that women who are long-term smokers and with a *NAT2* slow genotype or *GSTM1* null genotype are at increased breast cancer risk. This is especially clear for postmenopausal breast cancer.

A major advantage of a nested case–control study over a conventional case–control study is that exposure data are collected before disease occurrence thus excluding recall bias. Further exclusion of cases occurring in the first year of follow-up did not change the results, indicating that smoking status was probably not biased by presence of latent disease. Genotyping was performed blinded to case or control status and misclassification is therefore random which, if anything, dilutes

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Table 4 ***NAT2*** **genotype and** ***NAT2*5*** **genotype, smoking and breast cancer risk**

NAT2	Smoking	Cases, *n* (%) (total = 229)	Controls, *n* (%) (total = 263)	Odds ratio[a] (95% confidence interval)
Dose				
Rapid	Never	30 (13.1)	39 (14.8)	1.00
Rapid	< 10 cigarettes/day	15 (6.6)	19 (7.2)	1.10 (0.47–2.54)
Rapid	10–20 cigarettes/day	25 (10.9)	27 (10.2)	1.33 (0.64–2.76)
Rapid	⩾ 20 cigarettes/day	16 (7.0)	21 (8.0)	1.10 (0.48–2.49)
				P-value for trend 0.55
Slow	Never	46 (20.1)	65 (24.6)	0.97 (0.53–1.80)
Slow	< 10 cigarettes/day	25 (10.9)	29 (11.0)	1.27 (0.61–2.64)
Slow	0–20 cigarettes/day	35 (15.3)	36 (13.6)	1.35 (0.69–2.65)
Slow	⩾ 20 cigarettes/day	37 (16.2)	28 (10.6)	1.82 (0.91–3.64)
				P-value for trend 0.14
*NAT2*5*	Never[b]	13 (9.2)	24 (15.2)	0.75 (0.33–1.75)
*NAT2*5*	< 20 cigarettes/day	28 (19.9)	19 (12.0)	2.06 (0.96–4.42)
*NAT2*5*	⩾ 20 cigarettes/day	14 (9.9)	9 (5.7)	2.19 (0.83–5.82)
				P-value for trend 0.05
Duration				
Rapid	Never	30 (13.1)	39 (14.8)	1.00
Rapid	< 15 years	13 (5.7)	12 (4.6)	1.59 (0.61–4.14)
Rapid	15–30 years	24 (10.5)	33 (12.5)	1.01 (0.49–2.08)
Rapid	⩾ 30 years	19 (8.3)	21 (8.0)	1.30 (0.58–2.90)
				P-value for trend 0.71
Slow	Never	46 (20.1)	65 (24.7)	0.97 (0.53–1.80)
Slow	< 15 years	21 (9.2)	29 (11.0)	1.01 (0.47–2.19)
Slow	15–30 years	44 (19.2)	38 (14.4)	1.61 (0.84–3.12)
Slow	⩾ 30 years	32 (14.0)	26 (9.9)	1.66 (0.80–3.44)
				P-value for trend 0.12
*NAT2*5*	Never[b]	13 (9.2)	24 (15.3)	0.75 (0.33–1.75)
*NAT2*5*	< 20 years	13 (9.2)	10 (6.4)	1.78 (0.66–4.78)
*NAT2*5*	⩾ 20 years	29 (20.6)	18 (11.5)	2.29 (1.06–4.95)
				P-value for trend 0.05

[a]Adjusted for age, menopausal status and residence. [b]Due to small numbers, dose, duration and pack-years were divided into three categories instead of four.

Table 5 ***GSTM1*** **genotype, smoking, and breast cancer risk**

GSTM1	Smoking	Cases, *n* (%) (total = 229)	Controls, *n* (%) (total = 262)	Odds ratio[a] (95% confidence interval)
Dose				
Present	Never	27 (11.8)	50 (19.1)	1.00
Present	< 10 cigarettes/day	21 (9.2)	24 (9.1)	1.67 (0.78–3.56)
Present	10–20 cigarettes/day	29 (12.7)	33 (12.5)	1.67 (0.84–3.33)
Present	⩾ 20 cigarettes/day	19 (8.3)	27 (10.3)	1.33 (0.62–2.85)
				P-value for trend 0.26
Null	Never	49 (21.4)	54 (20.5)	1.66 (0.90–3.05)
Null	< 10 cigarettes/day	19 (8.3)	24 (9.1)	1.59 (0.73–3.47)
Null	10–20 cigarettes/day	31 (13.5)	29 (11.0)	2.10 (1.05–4.22)
Null	⩾ 20 cigarettes/day	34 (14.8)	22 (8.4)	3.00 (1.46–6.15)
				P-value for trend 0.003
Duration				
Present	Never	27 (11.8)	50 (19.1)	1.00
Present	< 15 years	15 (6.6)	18 (6.9)	1.58 (0.67–3.72)
Present	15–30 years	37 (16.2)	45 (17.2)	1.57 (0.82–3.01)
Present	⩾ 30 years	17 (7.4)	21 (8.0)	1.51 (0.67–3.40)
				P-value for trend 0.11
Null	Never	49 (21.4)	54 (20.6)	1.65 (0.90–3.05)
Null	< 15 years	19 (8.3)	23 (8.8)	1.70 (0.76–3.82)
Null	15–30 years	31 (13.5)	25 (9.5)	2.40 (1.17–4.92)
Null	⩾ 30 years	34 (14.8)	26 (9.9)	2.53 (1.24–5.16)
				P-value for trend 0.004

[a]Adjusted for age, menopausal status and residence.

the results. The cohort study was designed to estimate and monitor exposure to risk factors of cardiovascular diseases. Therefore, we lack information on family history of breast cancer, making evaluation of confounding impossible. However, we question whether 'family history of breast cancer' meets the criteria for confounding because it is assumed to be the result of a clustering of genetic predisposition (i.e. low penetrance or susceptibility genes) and shared environment, which is exactly the determinant under study. In this view, at

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Table 6 *NAT2*, *GSTM1*, smoking and breast cancer stratified by menopausal status

	Smoking	Cases, *n* (%)	Controls, *n* (%)	Odds ratio[a] (95% confidence interval)
NAT2		*n* = 127	*n* = 136	
Premenopausal				
Rapid	Never	20 (15.7)	21 (15.4)	1.00
Rapid	< 20 cigarettes/day	23 (18.1)	26 (19.1)	1.02 (0.44–2.36)
Rapid	⩾ 20 cigarettes/day	8 (6.3)	8 (5.9)	1.08 (0.33–3.50)
Slow	Never	23 (18.1)	28 (20.6)	0.91 (0.40–2.11)
Slow	< 20 cigarettes/day	35 (27.6)	33 (24.3)	1.31 (0.59–2.92)
Slow	⩾ 20 cigarettes/day	18 (14.2)	20 (14.7)	1.03 (0.42–2.52)
NAT2		*n* = 102	*n* = 128	
Postmenopausal				
Rapid	Never	10 (9.8)	18 (14.1)	1.00
Rapid	< 20 cigarettes/day	17 (16.7)	20 (15.6)	1.58 (0.57–4.40)
Rapid	⩾ 20 cigarettes/day	8 (7.8)	13 (10.2)	1.12 (0.34–3.72)
Slow	Never	23 (22.5)	37 (28.9)	1.12 (0.44–2.85)
Slow	< 20 cigarettes/day	25 (24.5)	32 (25.0)	1.39 (0.54–3.55)
Slow	⩾ 20 cigarettes/day	19 (18.6)	8 (6.3)	4.21 (1.34–13.19)
GSTM1		*n* = 127	*n* = 135	
Premenopausal				
Present	Never	17 (13.4)	20 (14.7)	1.00
Present	< 20 cigarettes/day	28 (22.0)	32 (23.5)	1.12 (0.49–2.58)
Present	⩾ 20 cigarettes/day	12 (9.4)	15 (11.0)	0.96 (0.35–2.64)
Null	Never	26 (20.5)	29 (21.3)	1.02 (0.44–2.38)
Null	< 20 cigarettes/day	30 (23.6)	26 (19.1)	1.48 (0.63–3.45)
Null	⩾ 20 cigarettes/day	14 (11.0)	13 (9.6)	1.28 (0.47–3.52)
GSTM1		*n* = 102	*n* = 128	
Postmenopausal				
Present	Never	10 (9.8)	30 (23.4)	1.00
Present	< 20 cigarettes/day	22 (21.6)	25 (19.5)	2.61 (1.03–6.57)
Present	⩾ 20 cigarettes/day	7 (6.9)	12 (9.4)	1.76 (0.53–5.81)
Null	Never	23 (22.5)	25 (19.5)	2.76 (1.11–6.87)
Null	< 20 cigarettes/day	20 (19.6)	27 (21.1)	2.30 (0.90–5.86)
Null	⩾ 20 cigarettes/day	20 (19.6)	9 (7.0)	6.78 (2.31–19.89)

[a]Adjusted for age and residence.

least some of the familial clustering is in the biological pathway and adjustment is not correct. Moreover, the prevalence of 'family history of breast cancer' in a group of postmenopausal women in the Netherlands was 8% [25], and this percentage will be lower in younger women. Therefore, if it is a confounder, adjustment would only marginally influence our estimates. This was also shown in two large-scale studies, where familial breast cancer showed not to confound the relations [26,27].

Other known breast cancer risk factors showed risks as expected (i.e. each year the age at first full-term pregnancy was delayed, the risk was 7% higher) (OR = 1.07; 95% CI 1.02–1.13).

Although the number of breast cancer cases that occurred during follow-up of the cohort is rather high, for analyses of interaction, the number is still limited. For example, the power to detect an increased risk of 2 (or higher) was 70% for the combined effect of *NAT2*5* and smoking compared to the reference group. However, for *GSTM1* and smoking (combined), the power was 50% to detect an OR of 2.0. Despite the limited numbers, we were able to show significant results for some gene-smoking relations, which are in accordance with previous studies. For the non-significant gene-smoking relations, the interpretation should be that there still might be significant relations, but these are likely to be lower than 2.

We did not observe significantly elevated breast cancer risks for dose or duration of smoking. However, overall tests for trend were of borderline statistical significance, but reached statistical significance in postmenopausal women. We did not observe independently elevated breast cancer risk for polymorphisms in *NAT1*, *NAT2* and *GSTT1* genes. This was expected because these enzymes have a specific role in activation and detoxification of carcinogens, and exposure to cigarette smoke is necessary for these polymorphisms to influence cancer risk. However, a significant association was observed for *GSTM1* null genotype (OR = 1.46; 95% CI 1.02–2.09) which is in concordance with a previous

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meta-analysis [7] and a recent study [28]. This may suggest that the enzyme is also important in metabolizing carcinogens other than from cigarette smoke, such as from well-cooked meat.

We did not observe an interaction between *NAT1* or *GSTT1* polymorphisms and smoking in the risk of breast cancer, in agreement with four previous studies [10,26,29]. By contrast, Zheng *et al.* [30] found an increased breast cancer risk for postmenopausal women with a *NAT1*11* allele that was more evident among smokers.

Moreover, two other studies found an interaction of smoking and *NAT1*10* in breast cancer risk (OR = 9.0; 95% CI 1.9–41.8 and OR = 13.2; 95% CI 1.5–116.0, respectively), although the group numbers were very small [30,31].

The tendency of elevated breast cancer risks in women who are slow *NAT2* acetylators and smoke is in concordance with the results of Ambrosone *et al.* [32], Huang *et al.* [33] and Chang-Claude *et al.* [34]. Pfau *et al.* [35] showed a significantly elevated DNA-adduct level in the mammary DNA from women with slow *NAT2* acetylator genotype [35]. However, five other studies did not report this association or the reverse was observed (i.e. higher risk for rapid acetylators) [4,26,27, 31,36]. The inconsistencies could partly be due to small numbers (i.e. number of cases varying from 113 to 492) or a low percentage of smokers in the studies (9% to 49%). Furthermore, differences in *NAT2* polymorphism determinations may sometimes lead to misclassification of slow or rapid acetylator genotype [16]. The inconsistent results cannot be explained by mixed ethnicity because all studies included Caucasian subjects, except one [31], although the authors did adjust for this.

Risk for breast cancer was increased in very slow acetylators (i.e. with two *NAT2*5* alleles) who smoked. This supports a detoxifying role rather than an activating role for NAT2. Cigarette smoke contains aromatic amine carcinogens, such as 4-aminobiphenyl, that readily undergo *N*-acetylation by NAT2 [37]. To complicate matters, rapid *NAT2* acetylators may be at increased risk for breast cancer following exposure to heterocyclic amines present in well-cooked meat [38] because these carcinogens do not undergo inactivation by *N*-acetylation [37], but rather are activated by *O*-acetylation [39].

Because all the enzymes examined in our study are involved in carcinogen metabolism, and they are not very substrate specific, it is possible that activation or detoxification of carcinogens of one enzyme is compensated for by another. The present study is not large enough to detect an interaction between all the enzymes in combination with environmental factors.

In conclusion, our results provide support for the view that women who smoke and have a genetically determined reduced inactivation (*GSTM1* null genotype or slow *NAT2* genotype, especially very slow *NAT2* genotype) are at increased breast cancer risk.

Acknowledgements

The data for this study originated from The Monitoring Project on Cardiovascular Disease Factors. G. L. Obermann-de Boer is kindly acknowledged for coordinating the study, and the epidemiologists and fieldworkers of the Municipal Health Services in Amsterdam, Doetinchem, and Maastricht are thanked for their contribution to data collection. We are thankful to L. J. Schouten (NCR/IKL), O. Visser (IKA) and J. van Dijck (IKO), for their support in the linkage with cancer registries. We further wish to thank the following individuals affiliated to the Netherlands Institute of Public Health and the Environment in Bilthoven, The Netherlands: A. Blokstra, E. den Hoedt, A. van Kessel and P. Steinberger for data management and retrieval; A. J. M. van Loon and M. C. J. F. Jansen for their help with retrieval of data from the cancer registries and data management; B. Hoebee, E. M. van Schothorst and P. van Impelen for help with retrieval of samples and DNA isolation. We thank B. Slothouber from Julius Center for Health Sciences and Primary Care for help with genotyping the samples.

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