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Cumulative genetic defects in carcinogen metabolism may increase breast cancer risk (The Netherlands)

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Abstract

Variants in the metabolic genes *NAT1*, *NAT2*, *GSTM1* or *GSTT1*, may cause differences in individual detoxifying capacity of possible carcinogens. We examined the cumulative effect of putative at risk genotypes on breast cancer risk and we examined the extent to which these polymorphisms modify the association between smoking and breast cancer. A case cohort study was conducted in the DOM cohort with 676 breast cancer cases and a random sample of 669 individuals. No effect of the *NAT1*, *NAT2* or *GSTM1* genotypes on breast cancer risk was observed. However, women with *GSTT1* null genotype had a 30% increased breast cancer risk compared to women with *GSTT1* present (RR = 1.30 (95% confidence interval (CI) 1.04–1.64)). Smoking did not influence breast cancer risk nor did genetic variations in *NAT1*, *NAT2* or *GSTM1* in combination with smoking. Compared to women who never smoked with *GSTT1* present, women with *GSTT1* null genotype and who formerly smoked showed an increased breast cancer risk (RR = 2.55 (95% CI 1.10–5.90)), but current smokers who smoked 20 cigarettes or more per day did not (RR = 1.06 (95% CI 0.51–2.18)). Increasing numbers of putative at risk genotypes increased breast cancer risk in a dose dependent manner (p for trend 0.01). The risk was more than doubled in women with all four risk genotypes, RR = 2.45 (95% CI 1.24–4.86), compared to women with zero putative at risk genotypes. In conclusion, the results of this study suggest that presence of three or more putative at risk genotypes increases breast cancer risk.

Abbreviations: BMI – body mass index; CI – confidence interval; DOM – diagnostic study on breast cancer; GST – glutathione S-transferase; IRR – incidence rate ratio; NAT – N-acetyltransferase

Introduction

N-acetyltransferase 1 and 2 (NAT1, NAT2), glutathione S-transferase M1 (GSTM1) and T1 (GSTT1) are phase II enzymes, involved in carcinogen metabolism. The genes coding for the NAT enzymes contain polymorphic sites, which cause variable enzymatic activity [1]. *GSTM1* or *GSTT1* null genotype results in a complete lack of enzymatic activity [2]. Individual efficiency to metabolize carcinogens is determined by genetic factors. Polymorphisms in these genes have been associated with an increased risk of several cancers [1].

Several risk factors for breast cancer have been studied, and they may be genetic or environmental or a combination. Among those, smoking is one of the most widely studied environmental risk factor for breast cancer. Results for smoking are inconclusive, but a pooling of 40 studies showed an increased risk for ever smoking of 1.10 (95% confidence interval (CI)

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1.02–1.18) [3]. Another recent meta-analysis suggests no relation between smoking and breast cancer overall [4]. However, there may be women who are more susceptible for smoking than others because of their genetic composition. Groups of tobacco related carcinogens are converted into metabolites by cytochrome P450 related enzymes. Phase II enzymes are involved in subsequent detoxification of activated metabolites of carcinogens. Both NAT1 and NAT2 are involved in the activation and deactivation of aromatic and heterocyclic amines that can form DNA adducts [5]. Carcinogen-DNA adducts associated with tobacco smoke have been detected in human breast tissue and the mean adduct value was higher for breast cancer cases than for controls [6]. Several studies examined the relation between one genotype and breast cancer, but not all included smoking data or did combine different genotypes [7–9].

The aim of the present study was to investigate both the combined effects of smoking and genetic polymorphisms in relevant metabolic genes (*NAT1*, *NAT2*, *GSTM1* and *GSTT1*) and the cumulative effect of putative at risk genotypes on breast cancer risk.

Subjects and methods

Study subjects

A population-based screening program for early detection of breast cancer was started in 1974, in Utrecht, The Netherlands, the 'DOM' project (Diagnostisch Onderzoek Mammacarcinoom). The project was carried out in four cohorts. Between 1974 and 1979, all women born between 1911 and 1925 (DOM 1 cohort) were invited for screening, in 1981 and 1983 all women born between 1926 and 1931 (DOM 2 cohort), in 1982 until 1985, all women born between 1932 and 1941 (DOM 3 cohort) and in 1985 and 1986, all women born between 1942 and 1945 (DOM 4 cohort). In DOM 1, DOM 3 and DOM 4 participants were asked to bring an overnight urine sample. A total of 25,769 women (DOM 1: 12,242), (DOM 3: 10,229), (DOM 4: 3298) attended the screening and provided a urine sample, that was stored at −20 °C. Women of the DOM 2 cohort did not provide a urine sample. The participation rate was 70% [10].

In cooperation with all general practitioners in the region, a breast cancer incidence registry was set up, starting in 1974. From 1987 onwards, the regional cancer registry covered the identification of new cancers, as part of the Netherlands Cancer Registry. Follow-up from 1974 until January 1, 1996 revealed 942 incident breast cancer cases. For the present study 1000 controls were randomly selected from the DOM 1 (800), DOM 3 (160) and DOM 4 (40) cohorts based on the baseline numbers of each DOM cohort with a relatively oversampling of DOM 1 cohort because most cases were expected in this subcohort. During the follow-up 31.1% was censored due to moving out of the study area or death (DOM 1, 32.7%; DOM 3, 42.0%; DOM 4, 12.5%). The mean follow-up time for the random cohort sample was 15.8 years (standard deviation 5.20). The institutional review board for human studies of the University Medical Center Utrecht approved the study.

DNA isolation

DNA was isolated from urine by alcohol precipitation as described earlier [11]. Urine samples of the study population were thawed overnight at room temperature, mixed vigorously and 50 ml was used for DNA isolation. In brief, after centrifugation, DNA was isolated from the urine pellet by protein precipitation and DNA was precipitated by alcohol. DNA was finally resuspended in 40 μl (10 mM Tris, 1 mM EDTA, pH 7.6 (TE)).

Genotype

NAT1 polymorphisms were detected by radioactive dot blot as described previously (11), preceded by PCR using *NAT1* specific primers [12]. Briefly, distinction between the *NAT1*3*, **4*, **10* and **11* alleles was carried out by hybridization at 42 °C overnight of allele specific oligonucleotides to PCR products spotted on a membrane. The *NAT1*3*, **4*, **10* and **11* alleles were detected by hybridizing an antisense oligonucleotide for each allele to a separate membrane: *NAT1*3* (5′γ ^{32}P-ATP-agg cca tct tta aaa tac att tat tat ta), *NAT1*4* (5′γ ^{32}P-ATP-gcc atc ttt aaa aga cat tta tta tta tt), *NAT1*10* (5′γ ^{32}P-ggc cat ctt taa aat aca ttt ttt att at) and *NAT1*11* (5′γ ^{32}P-aaa ata cat tta tta tta tta tta ttt gaa aag g). Non-specific binding was removed by 30 min washing in 2× SSC, 0.1% SDS at 60 °C for *NAT1*4*, *NAT*10*, NAT*11 and at 59 °C for *NAT1*3*. In each experiment three blood samples of known genotype were used as controls for PCR as well as for hybridization. Their sequence was confirmed by DNA sequencing.

For genotype determination four X-ray films were read at the same time. The samples were classified in duplicate as *NAT1*3*, *NAT1*4*, *NAT1*10* or *NAT1*11* homozygote or heterozygote, by two independent observers.

NAT2 polymorphisms at positions 341, 590, 803 and 857 were detected by restriction fragment length polymorphism (RFLP) as described earlier [13]. In each experiment three known blood samples were used as

controls for PCR as well as for RFLP analyses and the sequence was confirmed by DNA sequencing. The alleles were determined according to the known nomenclature [14]. $G^{191}A$ is not determined because it is not seen in Caucasian people [1] and we did not determine the silent mutations at nt 282 and 481.

Presence or absence of the *GSTM1* and *GSTT1* gene was determined by multiplex PCR as described by Chen *et al*. [15]. Briefly, segments of *GSTM1* and *GSTT1* were amplified along with a segment of human β-globin. The PCR products were analyzed on 2% 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. All laboratory analyses were performed blinded for case or control status and two observers independently classified all sample results.

Smoking

Smoking status was assessed at baseline by self-administered questionnaire. In this analysis three smoking classes were considered. Women, who reported smoking in the past, were classified as former smokers. Women who smoked at baseline were classified as current smokers and women who have never smoked were classified as never smokers. For women who smoked currently, information on dose was available and categorized as <10, 10–19, $\geqslant 20$ cigarettes/day.

Data analysis

For ten women the smoking status was not known. DNA was insufficient or the genotyping method failed for *NAT1* in 368 participants (19% cases and 19% subcohort), for *NAT2* in 260 women (10% cases and 12% subcohort), and *GSTM1* or *GSTT1* genotyping was not possible in 458 women (24% cases and 22% subcohort). The percentage successful genotyping is slightly higher for *NAT1* and *NAT2* than for the *GSTM1* and *GSTT1*. *GSTM1* and *GSTT1* were determined by a multiplex PCR. An internal control is necessary as control for the PCR success in case of a null genotype, which results in two sets of primers instead of one. This could influence the percentage visible PCR product. Furthermore, we did a pilot study for evaluating such a multiplex PCR and decided that it was more efficient to perform one multiplex PCR with three primers than two multiplex PCR with two primer sets.

For *NAT1* the corresponding phenotype is not clear. An initial report on increased activity associated with the *NAT1*10* allele [16] could not be supported in subsequent studies [17–20]. However, we maintain the distinction of *NAT1*10* and non *NAT1*10* in our studies. Women with at least one *NAT1*10* allele were classified as rapid acetylators and the rest as slow acetylators. Rapid acetylators served as the reference group in all analyses.

For *NAT2* genotype carriers of a *NAT2*4* or *NAT2*12* allele were classified as rapid and the rest as slow acetylators [14]. Rapid acetylators were used as reference group. According to some data individuals homozygous for *NAT2*5* alleles are the slowest acetylators [21], and therefore we also analyzed these individuals compared to the rapid acetylators.

Homozygous *GSTM1* null carriers were compared to the reference group of women with at least one *GSTM1* present, analogous to *GSTT1*.

Since the 1000 women constitute a random sample of the total cohort ($n = 25{,}769$), the case cohort analysis was adopted. Multiplication of the person years in the reference group by 25.8 (the inverse of the subcohort non-cases sampling fraction) enabled us to analyze the case cohort as a full cohort, in which person years are unbiased estimates of true person years. Incidence rate ratios (IRR) and corresponding 95% CIs for breast cancer were estimated using Poisson regression models. Robust 95% CIs were calculated to account for additional variance introduced by sampling for the cohort [22].

We included women for whom the genotyping was successful, for *NAT1* genotyping this resulted in 764 cases and 810 controls, for *NAT2* genotyping this were 845 cases and 875 controls, and for *GSTM1* and *GSTT1* this resulted in 717 cases and 767 controls. For 676 cases and 669 controls all genotyping was successful. We first analyzed the main effects of smoking and of the different genotypes separately. The joint effect of each at risk genotype and smoking was analyzed by combining genotype and smoking status. p Values for tests for trend were calculated.

We also evaluated the effect of a combination of several putative at risk genotypes. According to the number of putative at risk genotypes they possess, women were categorized in five subgroups: none (reference group), women with only one putative at risk genotype (defined as 'non *NAT1*10*' or '*NAT2* slow genotype' or '*GSTM1* null genotype or '*GSTT1* null genotype'), two, three and four putative at risk genotypes.

Factors considered for confounding were age, DOM cohort (1,3,4), body mass index (BMI) (continuous), nulliparity (yes/no), age at first full term pregnancy (not applicable, <24, 24–27, $\geqslant 28$), menopause (yes/no), menopausal age (continuous), family history of breast cancer (yes/no). We decided to include possible

confounders in the model if exclusion changed the estimate by more than 10%. Since this was the case for age and DOM cohort, the final models contain the determinants of interest (smoking, genotype or a combination) and the variables age and DOM cohort. Analyses were performed using SPSS 9.0 and Stata 6.0.

Results

General characteristics, allele frequencies for *NAT1* and *NAT2* and genotype frequencies for *GSTM1* and *GSTT1* of the breast cancer cases and the cohort sample are presented in Table 1. No significant difference was observed between the genotyped and non-genotyped persons with respect to these characteristics (data not shown). Breast cancer in first degree relatives was more frequent in cases than in the cohort sample. The distribution of *NAT1*, *NAT2* (and very slow *NAT2*5*) and *GSTM1* genotypes was similar among cases and the cohort sample and revealed no increased breast cancer risk. From the cases, 38% and from the cohort sample 29% had the *GSTT1* null genotype.

Women with *GSTT1* null genotype showed a statistically significant, increased breast cancer risk (RR = 1.30 (95% CI 1.04–1.64)), compared to women with *GSTT1* present (Table 2). When we analyzed the effects of each genotype separately in the subsample of cases and controls for which we had complete genotype information (676 cases; 669 controls), results were largely the same.

Table 1. Baseline characteristics of the study population

	Cases $N=676$	Cohort sample $N=704$
Age	52.2 (6.9)	54.0 (6.4)
Study recruitment (range)		40–65
Height (cm)	163.8 (6.0)	162.5 (6.2)
Weight (kg)	69.8 (11.8)	69.2 (10.5)
BMI (m^2/kg)	26.0 (4.3)	26.2 (3.7)
Age at first full term pregnancy	26.0 (4.1)	26.3 (3.7)
Number of children	2.8 (1.4)	3.1 (1.7)
Nulliparous	130 (19)	134 (20)
Menopausal status		
Premenopausal	286 (42)	213 (30)
Natural postmenopausal	287 (43)	372 (53)
Artificial postmenopausal	103 (15)	119 (17)
Age menopause	49.7 (4.1)	49.6 (4.4)
Family history of breast cancer (first relatives)		
Yes	105 (16)	54 (8)
No	571 (84)	650 (92)
Current use oral contraceptive		
Yes	37 (6)	26 (4)
No	639 (94)	678 (96)
Smoking		
Current	189 (28)	193 (28)
NAT1 alleles		
*NAT1*3*	31 (2)	47 (3)
*NAT1*4*	1101(72)	1143 (71)
*NAT1*10*	373 (24)	399 (25)
*NAT1*11*	23 (2)	31 (2)
NAT2 alleles		
*NAT2*4*	384 (23)	387 (22)
NAT2*5A	68 (4)	87 (5)
*NAT2*5B*	661 (39)	700 (40)
*NAT2*5C*	17 (1)	41 (2)
*NAT2*6A*	467 (28)	445 (25)
*NAT2*6C*	7 (0)	4 (0)
*NAT2*7B*	48 (3)	33 (2)
*NAT2*12A*	38 (2)	53 (3)
GSTM1		
Present	355 (50)	396 (52)
Null genotype	362 (50)	371 (48)
GSTT1		
Present	443 (62)	542 (71)
Null genotype	274 (38)	225 (29)

Values are mean (SD) and numbers (percentage).

Table 2. *NAT1*, *NAT2*, *GSTM1*, *GSTT1* genotypes and breast cancer

	Cases	Person years	IRR*
NAT1 genotype			
*NAT1*10*	209	104,910	1.00
Non *NAT1*10*	555	222,400	1.18 (0.94–1.48)
Total	764		
NAT2 genotype			
Rapid	364	147,863	1.00
Slow	481	207,510	0.94 (0.78–1.14)
NAT2*5	172	85,178	0.81 (0.62–1.05)
Total	845		
GSTM1			
Present	355	160,560	1.00
Null genotype	362	146,890	1.14 (0.93–1.42)
Total	717		
GSTT1			
Present	443	220,427	1.00
Null genotype	274	87,021	1.30 (1.04–1.64)
Total	717		

* Adjusted for cohort (categorical) and age (continuous).

Table 3. Smoking and breast cancer risk

	Cases	Person years	IRR*
Never	584	280,744	1.00
Former	63	11,120	1.29 (0.81–2.03)
Current			
< 10 cig/day	106	44,747	1.01 (0.74–1.36)
10–20 cig/day	112	54,036	1.03 (0.77–1.38)
> 20 cig/day	63	17,010	1.28 (0.83–1.96)
Total	928		

* Adjusted for age (continuous), cohort (categorical).

Table 4. *NAT1*, *NAT2*, *GSTM1* genotype, smoking and breast cancer risk

Genotype		Cases	Person years	IRR*
NAT1				
*NAT1*10*	Never	133	68,485	1.00
*NAT1*10*	Former	13	2552	1.48 (0.58–3.75)
	Current			
*NAT1*10*	< 10 cig/day	23	14,963	0.73 (0.41–1.33)
*NAT1*10*	10–20 cig/day	25	14,976	0.94 (0.53–1.69)
*NAT1*10*	> 20 cig/day	13	3,910	1.24 (0.50–3.06)
Non *NAT1*10*	Never	346	155,159	1.12 (0.85–1.48)
Non *NAT1*10*	Former	42	6762	1.40 (0.76–2.56)
	Current			
Non *NAT1*10*	< 10 cig/day	61	24,727	1.14 (0.73–1.77)
Non *NAT1*10*	10–20 cig/day	61	27,746	1.10 (0.72–1.70)
Non *NAT1*10*	> 20 cig/day	36	7562	1.54 (0.83–2.86)
Total		753		
NAT2				
Rapid	Never	243	102,387	1.00
Rapid	Former	25	4430	1.25 (0.61–2.56)
	Current			
Rapid	< 10 cig/day	33	18,250	0.66 (0.40–1.09)
Rapid	10–20 cig/day	35	17,034	0.92 (0.55–1.52)
Rapid	> 20 cig/day	22	5690	1.31 (0.63–2.70)
Slow	Never	286	143,277	0.85 (0.66–1.08)
Slow	Former	35	5583	1.24 (0.66–2.33)
	Current			
Slow	< 10 cig/day	61	20,743	1.19 (0.78–1.83)
Slow	10–20 cig/day	61	28,765	0.89 (0.60–1.33)
Slow	> 20 cig/day	32	8740	1.08 (0.59–1.97)
Total		833		
GSTM1				
Present	Never	220	108,957	1.00
Present	Former	17	4113	0.92 (0.42–2.01)
	Current			
Present	< 10 cig/day	47	19,013	0.94 (0.43–2.05)
Present	10–20 cig/day	40	22,203	1.08 (0.68–1.72)
Present	> 20 cig/day	26	6222	0.96 (0.60–1.54)
Null genotype	Never	235	103,808	1.17 (0.90–1.52)
Null genotype	Former	34	3774	1.99 (0.99–3.97)
	Current			
Null genotype	< 10 cig/day	31	17,919	0.87 (0.52–1.46)
Null genotype	10–20 cig/day	38	16,267	1.13 (0.68–1.86)
Null genotype	> 20 cig/day	19	4714	1.30 (0.60–2.80)
Total		707		

* Adjusted for age (continuous) and cohort (categorical).

Compared to women who never smoked, women smoking a high dose of cigarettes (20 or more per day) showed a modest increase in breast cancer risk, although not statistically significant (RR = 1.28 (95% CI 0.83–1.96)) (Table 3). No joint effect of smoking and genotype was observed for *NAT1*, *NAT2* nor *GSTM1* genotype (Table 4), nor for smoking in combination with the very slow *NAT2*5* (data not shown). Compared to women who never smoked with *GSTT1*, women without *GSTT1* (null genotype) who smoked in the past had an increased breast cancer risk, RR = 2.55 (95%CI 1.10–5.90). However, for current smokers no increased risk was observed (Table 5).

Table 5. *GSTT1* genotype, smoking and breast cancer risk

GSTT1		Cases	Person years	IRR*
Present	Never	289	156,652	1.00
Present	Former	26	5783	1.10 (0.57–2.11)
	Current			
Present	< 10 cig/day	52	26,015	1.03 (0.68–1.55)
Present	10–20 cig/day	43	25,844	0.95 (0.61–1.46)
Present	> 20 cig/day	25	6037	1.97 (0.99–3.94)
Null genotype	Never	166	56,115	1.43 (1.07–1.89)
Null genotype	Former	25	2105	2.55 (1.10–5.90)
	Current			
Null genotype	< 10 cig/day	26	10,917	1.02 (0.57–1.82)
Null genotype	10–20 cig/day	35	12,626	1.29 (0.76–2.20)
Null genotype	> 20 cig/day	20	4,909	1.06 (0.51–2.18)
Total		707		

* Adjusted for age (continuous) and cohort (categorical).

When women were classified according to their cumulative number of putative at risk genotypes (non *NAT1*10* or *NAT2* slow genotype, *GSTM1* or *GSTT1* null genotype), none, one, two, three or four, an increased breast cancer risk with increasing number of putative at risk genotype was apparent. The effect showed a dose-response relation (p for trend 0.01). The risk was more than doubled in women with all four risk genotypes, RR = 2.45 (95% CI 1.24–4.86) (Table 6). Numbers were too small to determine interaction with smoking.

Results did not change, when analyzing pre- and postmenopausal women separately.

Discussion

The present study shows that women with increasing number of putative at risk genotypes showed increased breast cancer risk in a dose dependent manner. An effect of *GSTT1* null genotype on breast cancer risk was also

Table 6. Breast cancer risk according to cumulative number of putative at risk genotypes (non *NAT1*10* or *NAT2* slow genotype or *GSTM1* or *GSTT1* null genotype)

	Cases	Person years	IRR*
No	26	20,250	1.00
One	97	46,001	1.47 (0.84–2.59)
Two	292	125,756	1.58 (0.94–2.64)
Three	214	77,951	1.81 (1.07–3.06)
Four	47	11,767	2.45 (1.24–4.86)
Total	676		

* Adjusted for age (continuous) and cohort (categorical).

observed, especially for women who have smoked in the past.

A major advantage of case cohort approach over a nested case control study is that it is analyzed as a full cohort and breast cancer incidence rates can be estimated. The incidence rate observed in the first DOM cohort (50 to 70 years in 1975, follow-up to 1996), IR = 2.64/1000 person years, is of the same order of magnitude as the incidence rate for The Netherlands for 60 to 64 years old women in 1990, IR = 2.52/1000 person years in 1990 [23]. This implies that follow-up of the cohort for breast cancer is virtually complete.

Moreover, in this study smoking data were collected prior to 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 reporting of smoking habits was not biased by presence of latent disease. Genotyping was done blinded to case or control status and misclassification is therefore random and will, if anything, dilute results.

We did not observe increased breast cancer risk for different *NAT1*, *NAT2* and *GSTM1* genotypes separately. However, a significant 30% increased breast cancer risk was observed for *GSTT1* null genotype (RR = 1.30 (95% CI 1.04–1.64), especially among former smokers (RR = 2.55 (95% CI 1.10–5.90). An effect of *GSTT1* null genotype on breast cancer risk is in agreement with three previous studies [24–26] but such risk was not observed in eight other studies [27–34]. Our observation may be a chance finding, also in view of the absence of an association for current smokers, even for those who smoke more than 20 cigarettes per day. Another explanation might be that former smokers reflect more accurately exposure to carcinogens and the necessary subsequent latency time. Duration may thus be important, but unfortunately this was not asked in the questionnaire. Also, information on exposure to environmental tobacco smoke is lacking.

Our result that *NAT2* did not modify smoking effect is in accordance with five other studies [5, 8, 35–37]. In contrast, three studies showed an interaction [38–40], and it was found that DNA-adduct level were significantly elevated in the mammary DNA from women with slow *NAT2* acetylator genotype [41, 42]. We therefore analyzed the very slow *NAT2* acetylators (*NAT2*5)* separately. This revealed no increased breast cancer risk, neither by itself, nor in combination with smoking status. Inconsistencies in results in studies may partly be explained by differences in *NAT2* polymorphism determinations, which may lead to misclassification of acetylator type imputed from the genotype [1].

We observed an increased breast cancer risk for women with increasing number of putative at risk genotypes, which was in a dose-response manner. Two other studies reported a positive association of two putative at risk genotypes (*GSTM1* and *GSTT1* null genotype) and breast cancer [24, 25] and one study reported that genetic polymorphisms of *NAT1* and *NAT2* modulated breast cancer risk in the presence of *GSTM1* and *GSTT1* [43]. This may indicate that other enzymes compensate detoxification or activation of carcinogens in case of a single putative at risk genotype.

In conclusion, the results of this study suggest that presence of three or more metabolic putative at risk genotypes increase breast cancer risk in women, but none of these genotypes alone modified the effect of smoking on breast cancer.

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