

# A Belgian case control study on bladder cancer: rationale and design

by

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## Abstract

*Previous analyses of the Limburg Cancer registry (LIKAR) indicated the existence of a geographical cluster of bladder cancer incidence, particularly transitional cell carcinomas, amongst males in the surrounding area of the Belgian cities Hasselt and Alken. In subsequent ecologic analyses no risk factors were identified which could explain the existence of this cluster. Therefore, a case-control study has been started in the province of Limburg to explore the determinants of the cluster. In the coming years, the following determinants will be investigated: socio-demographic characteristics (e.g. age, sex, socio-economic status), life style factors (smoking, alcohol and diet), occupational expo-*

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*sure and genetic predisposition. The research setup combines a traditional population based case-control design with a case-only design. 203 Cases, defined by a recent histological confirmed diagnosis of transitional cell carcinoma (after January 2000), have been recruited through the LIKAR registry, the pathologist and the referring urologist. Population based controls (n=380) have been selected, through simple random sampling, from the inhabitants of the province of Limburg above the age of 50. Individual data have been collected through personal (structured) interviews by two trained interviewers. Genetic polymorphisms have been determined using genomic DNA extracted from blood samples. Determinant distributions of cases have been compared with those from controls in logistic regression analyses with special emphasis on gene-environment and gene-gene interactions. This paper will introduce this study and will report on its methodology in more detail than is possible in subject specific results papers.*

**Keywords:** *Bladder neoplasm, case-control study, risk factors, epidemiology*

## **Introduction**

The province of Limburg is situated in the north-east of Belgium and covers 2422 square km. The population is estimated at 798,583 inhabitants (2002) and is relatively young: 25% is younger than 20 years, only 17% is older than 60 years. In north and middle Limburg the major economical activities are related to the petrochemical, electronic and automobile industry. In southern Limburg fruit farming remains one of the major economical activities. LIKAR (the Limburg Cancer registry) was founded in 1993. Its major objective is to follow the incidence and trends of histological or cytological confirmed cancers of all inhabitants of Limburg and to continuously analyse the collected data. These data are provided by all the pathological, cytological and haematological laboratories located in the province and some outside the province which more regularly examine samples from Limburg inhabitants. Sex-stratified and age-standardised incidence rates have been calculated for each municipality and for most cancers in order to identify possible clusters of increased cancer incidence. Analyses within the LIKAR-register learned that bladder cancer is the fifth most common cancer among males (1996-2000) in this province. The male/female ratio is approximately five to one. Very few cases occur under the age of 40 years with two thirds of the cases occurring over the age of 65 years (1). Recent analyses of the data indicated a geographical cluster of bladder cancer incidence amongst males in the surroundings of Hasselt

and Alken (2). Using a conditional autoregressive model, smoothed standardised incidence ratios (SIR) above 1.5 were identified in all municipalities of the cluster with a SIR of 2 in Alken, the municipality with the highest SIR ( $p < 0.01$ ).

In females too, similar or higher age-standardised SIRs were found in these municipalities. They disappeared, however, after smoothing (2). This was probably due to a lower power resulting from a lower number of bladder cancer cases in women. When focusing on transitional cell carcinomas (TCC), (82% of all bladder cancers), the results were even stronger. Five municipalities with a SIR above 2.0 within this cluster could be identified (2).

Following the discovery of the bladder cancer cluster a further ecological analysis of the data was performed. The standardized incidence ratio of male bladder cancer of each municipality was related to an index of the degree of urbanisation by linear regression and no relation was found. However, it was found that the incidence rate was related to a municipality-specific index of the socio-economic status (SES). A higher standardised incidence rate was found in municipalities with a higher SES. This explained 11% of the variance of the incidence rates. No relationship was found with the proportion of migrants per municipality or the proportion of 'ever-smokers' in the municipalities (1). Hence, an epidemiological study with the individual as the unit of analysis was warranted, in order to explore the reasons and determinants of the cluster. This paper will introduce this study and will report on its methodology in more detail than is possible in subject specific results papers.

### **Risk factors for bladder cancer**

Current literature divides the main known risk factors of bladder cancer in four broad categories: socio-demographic characteristics, life style (smoking habits and diet), occupation and genetics.

#### ***Socio-demographic characteristics: race, gender, age and socio-economic status***

The incidence and mortality rate of bladder cancer vary worldwide. The highest rates are found in North America and Europe. Race also has a significant influence on bladder cancer incidence. Bladder cancer is twice as common among American Caucasian men as among African American men and roughly 1.5 times more common among White American women than African American women (3). The LIKAR register mentions an incidence rate of invasive bladder cancer per 100,000

person-years of 290 among males and 63 among females. Very few patients under the age of 40 are diagnosed with bladder cancer, with two thirds of the cases occurring over the age of 65 (2). Socio-economic status is not extensively studied as risk-factor for bladder cancer. A Spanish case-control study found no pronounced differences by social class for bladder cancer (4). Although in most industrialised countries the prevalence of cigarette smoking, an important risk-factor for bladder cancer, is higher in low compared to high social classes (5).

### ***Lifestyle: smoking and diet***

#### *Smoking*

Cigarette smoking is one of the most documented risk factors for bladder cancer. Approximately 50% of the bladder cancers in Western countries have been found attributable to cigarette smoking (6). A pooled analysis of 11 European case-control studies of male cases published from 1985 to 1999, found a linear increasing risk of bladder cancer with increasing duration of smoking.

The odds ratio increased from 1.96 (95% CI 1.5-2.6) after 20 years of smoking to 5.6 (95% CI 4.2- 7.4) after 60 years of smoking. A dose relationship was observed between numbers of cigarettes smoked per day and bladder cancer up to a threshold limit of 15-20 cigarettes per day.

Smoking cessation was observed with a decrease of the risk of bladder cancer (7). Similar results were found by a meta-analysis on the effect of cigarette smoking on urinary tract cancer risk. The meta-analysis identified 43 follow-up and case-control studies published until December 2000. Smoking cessation and age at first exposure proved to be negatively associated with the risk of urinary tract cancer. The age and gender adjusted summary odds ratios for current and former cigarette smokers compared with those for non-smokers were 3.3 (95% CI 2.6-4.2) and 1.98 (95% CI 1.7-2.3) (8). A large case-control study of 1514 matched pairs in the US observed a gender differential in smoking-related risk. Smoking seems to have a stronger effect on the occurrence of bladder cancer in females compared to males. The authors concluded a higher risk of bladder cancer in female smokers when compared to male smokers with comparable duration and dose of smoking habits (9).

The mechanistic evidence related to tobacco smoking and bladder cancer has focused on arylamines, in particular the potent carcinogen 4-aminobiphenyl. This carcinogen forms DNA-adducts in exfoliated bladder cells and bladder biopsy specimens from smokers. Hemoglobin

adducts formed by 4-aminobiphenyl are markedly increased in smokers, particularly in smokers of black tobacco (10). The literature is less clear on the effect of cigar, pipe and passive smoking. A pooled analysis of 6 European case-control studies, published between 1980 and 1995, found an odds ratio of pure pipe smoking of 1.9 (95% CI 1.2-3.1) and of pure cigar smoking of 2.3 (95%CI 1.6-3.5) (11).

However, tobacco (including all forms of smoking) is considered as a cause of bladder cancer (10).

### *Diet*

Many compounds contained in food and their metabolites are excreted through the urinary tract and thus a role of dietary factors in bladder carcinogenesis is plausible (12).

#### Alcohol

The association between alcohol consumption and bladder cancer appears not to be significant. In 1986, in the Netherlands a large-scale cohort study on diet and cancer among 120,852 subjects aged 55-69 years at baseline started. The follow-up data included the incidence of bladder cancer. A slightly increased risk of bladder cancer has been associated with alcohol consumption among men, although not clinically relevant (relative risk: 1.4, 95%CI: 1.0-1.9) (13). However, a population based cohort study in the USA found no significant association between alcohol consumption and the risk of bladder cancer. Beer consumption was associated with a reduced risk of bladder cancer, whereas wine and spirit consumption were not (14).

#### Food groups

A meta-analysis (consisting of 38 articles) of six dietary factors and bladder cancer concluded that increased risks of bladder cancer were associated with low fruit intake (relative risk of 1.4, 95%CI: 1.1-1.8), with diets low in vegetable intakes (relative risk of 1.2; 95%CI: 1.0-1.3) and diets high in fat intake (relative risk of 1.4; 95%CI: 1.2-1.6). No association was found between diets high in meat intake, low in retinol and beta-carotene (15).

Another meta-analysis of six case-control studies and three cohort studies published between 1973 and 2001 confirmed the protective effect of fruit consumption, but found no significant association for vegetable consumption.

The summary odds ratio for the case-control studies was found to be 0.8 (95%CI: 0.7-0.9) for fruit consumption and 0.9 (95%CI: 0.8- 1.0) for vegetable consumption. The odds ratio for the cohort studies was 0.8 (95%CI 0.6-1.0) for fruit consumption and 0.9 for vegetable consumption (95%CI: 0.7-1.1) (16).

### Nutrients

One review of 7 clinical trials, 16 cohort and 36 case-control studies published between 1990 and 1996, addressed the association between supplemental nutrients and cancer risk. Specifically for bladder cancer, the authors concluded that only case-control studies have reported an inverse association with vitamin C (17).

The Netherlands cohort study mentioned above also studied the association between the toenail selenium status and bladder cancer risk. The authors found evidence for an inverse association between selenium and bladder cancer risk. The relative risk decreased by seven percent for each increment of one standard unit of toenail selenium (0.202 $\mu$ g/g) (18).

### Coffee and fluid consumption

The relationship between coffee consumption and bladder cancer risk appears to be weak. A pooled analysis of ten European case-control studies conducted between 1985 and 1999, found no excess risk in 'ever coffee drinkers' compared to 'never drinkers'. But a statically significant excess risk was seen for subjects (both men and women) having drunk more than ten cups per day (odds ratio of 1.8; 95% CI 1.0-3.3). There was no association of the risk with duration or type of coffee consumption (19).

A review of epidemiological studies published from 1990 up to 1999, concluded that the risk tends to be higher in coffee drinkers than in those who do not drink coffee, but the excess risk is generally moderate and is neither dose- nor duration-related (20).

The relationship between total fluid consumption and bladder cancer risk remains controversial. A review of epidemiological studies (14 case-control and 3 cohort studies) conducted between 1963 and 2001, described that some studies found a decreased risk, others found a direct trend in risk or no association at all (21).

### **Occupation**

The first described occupational risk factor of bladder cancer was exposure to arylamines, accounting for a high incidence of bladder

cancer among workers in the dye and rubber industry. Aromatic amines and polycyclic aromatic hydrocarbons have been identified as suspected bladder cancer carcinogens. Due to strict regulations, the use of arylamines in the industry is limited nowadays (6). A meta-analysis of 11 case-control studies conducted in Europe between 1976 and 1996, concluded that metal workers, machinists, transport equipment operators and miners are among the major occupations contributing to occupational bladder in men (22). A meta-analysis of epidemiological studies for textile workers, published up to 2002, found an increased risk for bladder cancer in dyers, attributable to textile dye exposure (23). A meta-analysis of 6 cohort, 10 surveillance and 8 case-control studies undertaken to investigate the association between foundry work and bladder cancer, and published between 1980 and 2001, found a weak association (24). A review of studies conducted from 1982 to 1996 on cancer risk among workers in the rubber industry found an excess risk of bladder cancer in most studies (25).

A meta-analysis of 35 studies concluded that exposure to diesel exhaust is suggested to increase the risk on bladder cancer (26). A meta-analysis of our group, including 87 case-control and cohort studies published up to November 2004, found increased sex-specific relative risk estimates exceeding 1.2 for artists, leather workers, rubber workers, machinists, metal processors, chimney sweeps, printers, hair-dressers, and dye production workers (27).

### **Genetics**

Genetic susceptibility to cancer may be due to highly-penetrant mutations in genes that are directly involved in carcinogenesis. Less penetrant genetic conditions in which the function of the protein (an enzyme involved in the metabolism of toxic chemicals or carcinogens) is impaired, result in a greater susceptibility to the effect of environmental toxicants as a consequence. Therefore the mode of action of these low-penetrance genes (of interest in bladder cancer) is gene-environments interaction (28). This explains why only a small number of individuals exposed to environmental carcinogens will actually develop bladder cancer. Many chemical substances require activation by enzyme systems in order to become active carcinogens that can provoke tumour initiation. Other enzymes are involved in the detoxification of chemical carcinogens and are therefore related to a protective role. N-acetyl-transferases (NATs) and glutathione-S-transferase (GSTs) are two enzyme families involved in the detoxification of arylamines and are both polymorphically expressed. These alleles and genotypes are frequent in the general population. Previous studies indicated that *GSTM1*, *GSTT1*

and *NAT-2* null genotypes are independently associated with the risk of bladder cancer.

A meta-analysis of 17 articles published up to 2000, obtained a summary odds ratio of 1.4 (95%CI 1.2-1.7) for the *GSTM1* null status (29). A meta-analysis of 16 studies published up to 1999, indicated that smokers who are slow acetylators have a higher risk of bladder cancer than smokers who are rapid acetylators and slow acetylators who do not smoke are at a similar risk compared to non-smoking rapid acetylators (30). Another meta-analysis of 22 studies published up to the end of 1998, suggested that the relationship of *NAT2* slow acetylation and bladder cancer might differ by geographical region (31). Another meta-analysis of 21 published case-control studies found that the pooled odds ratio of bladder cancer associated with slow acetylator status was 1.31 (95% CI: 1.1-1.5) (32). Subjects with the slow acetylator phenotype tend to diverge more from rapid acetylators at low levels of tobacco dose than at high levels or the difference in risk between slow and rapid acetylators is more evident at low doses (33). A large study of 1592 case-control pairs observed that the carotenoid effect on smoking-related bladder cancer is modified by the *NAT2* polymorphisms (and *CYP1A2* and *NAT1\*10*) (34). Interaction between occupational risk and the slow acetylator phenotype has been described in several studies (35-37).

Several studies indicated that the *GSTT1* genotype is associated with a modest increase in the risk of bladder cancer (37-39). Sulfotransferase 1A1 is also involved in the metabolism of aromatic amines. *SULT1A1 Arg213His* polymorphism showed a marginal protective effect (40-41).

Table 1 gives an overview of the odds ratios for the relation between the main genetic polymorphisms and bladder cancer risk and their frequencies in the general population. It has been calculated that the attributable risks for bladder cancer are 8.2% for *NAT2\*5* and 22% for *GSTM1* among Caucasians (28).



TABLE 1  
Odds ratios for the relation between the main genetic polymorphisms  
and bladder cancer risk

Genetic polymorphism	Odds Ratio *	95%CI	Frequencies in Caucasians	Reference
<u>NAT2 genotype</u>	2.1	1.2-3.8 (Europe)		(63)
	0.9	0.7-1.3 (USA)		(64)
NAT2*5			0.482 (heterozygous)	(65)
NAT2*6			0.430 (heterozygous)	(66)
NAT2*7			0.05 (heterozygous)	(67)
<u>GSTM1 genotype</u>	1.42	1.26-1.60		(68)
			0.531	(69)
<u>GSTT1 genotype</u>	1.74	1.02-2.95		(70)
			0.197	(70)
<u>SULT1A1</u>	0.72	0.54-0.97		(71)

\* Odds ratio of the risk on bladder cancer risk for people with the null genotype compared to people with the wild type of the genetic polymorphisms, as published before.

## Objectives

The main objective of the Belgian case-control study is to identify those risk factors that may explain the high incidence and the cluster of transitional cell carcinoma of the bladder around Hasselt-Alken. More specifically, the following risk factors will be investigated: socio-demographic characteristics (age, sex, socio-economic status), life style factors (smoking, alcohol consumption and diet), occupational exposures to potential carcinogens (polycyclic aromatic hydrocarbons, diesel exhaust and aromatic amines), genetic predisposition (*NAT2\*5-6-7-14*, *SULT1A1 (R213H)*, *GSTM*, *GSTT1*) and the interaction between these risk factors.

## Methods

### **Recruitment of the subjects**

For this research both cases and healthy population based controls are recruited and analysed in a case-control and a case-only design.

### *Recruitment of the cases*

A case is defined as a patient with histologically confirmed transitional cell carcinoma of the bladder, diagnosed in 1996 or later. Recruitment of the cases is performed by the participating urologists or

general practitioners. Persons belonging to the black African, Asian, Hispanic race are excluded (as cases and controls) because frequencies of genetic polymorphisms vary between different ethnic groups (42). All subjects had to master the Dutch language and may be excluded due to illness, mental sickness or incapacity to comprehend the questions as evaluated by the treating clinician.

### *Recruitment of the controls*

The controls are selected from the registry of the inhabitants of the province of Limburg through simple random sampling, stratified by municipality and socio-economic status, among citizens above 50 years of age without a diagnosis of transitional cell carcinoma of the bladder. Different socio-demographic characteristics amongst respondents and non-respondents might reveal selection bias. The possibility of selection bias, arising when a large part of the population refuses to cooperate, will be monitored by comparing the socio-demographic characteristics (age, sex and region) between respondents and non-respondents.

### *Sample size*

The calculated sample size is based solely on considerations with respect to the genetic polymorphisms. An optimal sample size cannot be determined. A sample size of 203 cases and 380 controls is able to detect with sufficient power a minimum odds ratio of 1.5 for common polymorphism, for example *GSTM1 null status* which has allele frequency of 0.53; and a minimum odds ratio of 1.6 for relatively rare polymorphisms, for example *GSTT1 null status*, which has allele frequency of 0.19.

Table 2 indicates the required sample size (per group) to detect different odds ratios.

TABLE 2

Calculations of the sample size (per group), required to detect different odds ratios in case-control analyses, assuming  $\alpha=0.05$ ; power=80% and ratio cases/controls=0.7

Exposure frequency in controls	Minimal detectable odds ratio			
	1.5	2.0	2.5	3.0
5%	1870/1309	570/399	294/206	189/133
10%	870/609	265/186	135/95	85/60
20%	377/264	110/77	54/38	33/24
30%	215/151	60/42	27/19	15/11

## **Data collection**

Participants are interviewed by three medically trained interviewers. The interviewers cannot be blinded for the case/control status due to practical reasons. However, since the questionnaire is standardised, this is not expected to substantially affect the validity of the data collection. Interview data are entered twice and processed in a standardised manner in order to minimise observed bias in coding and interpretation of the data.

The data collection covers socio-demographic characteristics, lifestyle factors, occupational history and genetic polymorphisms.

### *Socio-demographic characteristics*

The requested information includes date of birth, information on ethnic and racial background, gender, marital status, educational level, a categorised medical history, a history of bladder cancer in first degree family members (parents, brothers, sisters and children) and a residential history. The residential history starts from the current residence and goes back 20 years in time. Residential history includes the full street address and city and the beginning and ending year of the residency. In addition we obtain information about the type of primary source of drinking water of the residence (municipality water supply, house well or bottled). Furthermore we require whether or not the participants grow vegetables in their own garden and whether they use pesticides.

### *Lifestyle*

#### Smoking

Subjects will be asked questions concerning their smoking status, average daily consumption, age at first exposure, age at cessation and years of smoking. This information will be collected separately for cigarette smoking, cigar smoking and pipe smoking.

#### Diet

A standardised food frequency questionnaire, the IMMIDIET questionnaire, is used to register nutritional characteristics (43). The IMMIDIET project studies the dietary habits and genetic polymorphisms in an Italian, Belgian and British population sample in order to evaluate the myocardial risk profile of these populations.

The IMMIDIET questionnaire was developed in Limburg and therefore adapted to the cross-cultural context of the province. The food table is composed of 788 food items and was based on three existing food tables

(the NEVO table of the Netherlands, the Nubel table of Flanders and the IPL table of Francophone Belgians) (44-46). The questionnaire is sent by mail to the participants of the study. During the home visit, the interviewer reviews the answers, possibly correcting or completing some questions. After the questionnaire is electronically scanned, a computerised programme provides linkage to food composition tables. The generated data will include information on several food composition groups and nutrients. Special attention will be given to those identified in the literature as possibly being related to bladder cancer, such as consumption of coffee, tea, fruits and vegetables (raw and cooked) and the intake of vitamins A, C and E.

### *Occupation*

A life-time occupational history lists all jobs (including official jobs and jobs done outside working hours) lasting more than six months and consists of the job title, the industry or type of business, employment dates and duration, company name and location, tasks and materials used (47-48). Each occupational history is reviewed to decide on the exposure status of three potential carcinogenic exposures: polycyclic aromatic hydrocarbons, diesel exhaust and aromatic amines. This will be done without contacting the interviewer or the respondent and blinded for the case/control status of the subject. Three different exposure categories will be defined: no exposure, possible exposure, and nearly certain exposure.

### *Genetics*

Additionally to the personal interview, blood samples and/or buccal swabs will be collected.

Genomic DNA will be extracted from peripheral blood lymphocytes or buccal swabs using standard methods. DNA will be resuspended in Tris-EDTA (TE) buffer and stored until further use. Genotyping will initially be performed for the most common polymorphisms in the *GSTM1*, *GSTT1*, *SULTA1A* and *NAT2* genes. Genotyping for the *GSTM1* null genotype (=homozygously deficient for the *GSTM1* gene) will be performed according to the method of Comstock et al. (56). Briefly, a fragment of the 5'-region of exon 4 to the 3'-end of exon 5 will be co-amplified with a control fragment of the human  $\beta$ -actin gene. The PCR products will then be separated on 1.5% agarose gels.

Genotyping for the *NAT2* \*5, 7 and 14 alleles will be performed with PCR-RFLP assays as previously described (49). The *NAT2* \*6 will also

be genotyped by PCR-RFLP, with primers GCM355 and GCM356 (Table 3). PCR conditions are as follows: initial denaturation of 10 min at 95°C, followed by 35 cycles of melting 95°C for 45 sec, annealing 50°C for 45 sec, extension 72°C for 45 sec. A final extension 72°C for 10 min will terminate the procedure. PCR mix will consist of 1x PCR buffer, 0.2 mM dNTP's, 1.5 mM MgCl<sub>2</sub>, 250 nM of each primer, 10% DMSO and 0.75 U of *ampliTaq Gold*. The 327 bp PCR product will be digested for 3 hours at 65°C with restriction enzyme *Taq I*, after which the resulting fragments will be separated on a 3% agarose gel. The wild type allele will result in two fragments of 192 and 135 bps respectively, while the \*6 allele will remain uncut (327 bp). For a complete overview of the assays and primers used, see table 3. By screening for seven NAT SNPs (G191A, C282T, T341, C481T, G590A, A803G and G857A) the risk of genotype misclassification is substantially decreased and therefore the sample size required to detect gene-environment interaction (50). The SULT1A1 R213H polymorphism will be detected with a PCR-RFLP method as described by Coughtrie et al. (51).

## Design

The proposed case-control design combined with analysis of the data according to a case-only design is suitable to determine the association between the disease, transitional cell carcinoma, and several risk factors. Transitional cell carcinoma is a relatively rare disease with a long induction time. A cohort study would require a large number of patients and a long follow-up time, factors that would add to the cost price. Case-control studies offer considerable savings in time and resources and are therefore more efficient to study the influence of background factors, lifestyle factors (smoking, diet, alcohol) and occupational exposures but are unfortunately prone to recall bias. Recall bias does not influence the collected genetic data. The case-control design is the method of choice when the objective is to study associations between genotype and disease risk (52). Data will be processed through unconditional multiple logistic regression. Additionally, the data will be analysed according to a case-only analysis method to evaluate gene-environment interaction (53). In case-only studies, the association between an environmental exposure and a genotype is examined among cases only (subjects with transitional cell carcinoma) (54). This approach will allow assessment of the magnitude of the association between the exposure and the susceptibility of genotype but has some limitations. Independence between environmental exposure and genotype in the controls is assumed. If this assumption is accepted, analyses of case-only studies offer a higher precision for estimating gene-environment interaction than those based

TABLE 3  
An overview of the discussed genetic polymorphisms involved in bladder cancer

Gene	Polymorphism	Allele	Primers	Type of assay
GSTM1	Gene deletion	Null genotype	GCM349: 5'-CTGCCCTACTTGGATTGATGGG-3'	Multiplex PCR
			GCM350: 5'-CTGGATTGTAGCAGATCATGC-3'	
			GCM351: 5'-GGGCACGAAGGCTCATCATT-3' ( $\beta$ -actin)	
			GCM352: 5'-GGCCCCCTCCATCGTCCACCG-3' ( $\beta$ -actin)	
GSTT1	Gene deletion	Null genotype	GCM353: 5'-GGAACAAAATTGGACTTGG-3'	PCR-RFLP
			NAT2	
NAT2	481 T/C	NAT2*5	GCM354: 5'-TCTAGCATGAATCACTCTGC-3'	(Kpn I)
			NAT2	
NAT2	590 G/A (R197Q)	NAT2*6	GCM355: 5'-GCCCTAGAAATTAATTTCTGGG-3'	PCR-RFLP
			NAT2	
NAT2	857 G/A (G286E)	NAT2*7	GCM356: 5'CTATAGGTGAGGATGAAGCC-3'	(Tag I)
			NAT2	
NAT2	191 G/A (R64Q)	NAT2*14	GCM353: 5'-GGAACAAAATTGGACTTGG-3'	PCR-RFLP
			GCM354: 5'-TCTAGCATGAATCACTCTGC-3'	
				(Msp I/Alu I)

on cases and controls (smaller standard errors due to the elimination of control group variability) (55). It is not possible to evaluate the independent effects of the exposure or the genotype alone, merely departure from multiplicative effects. The independence assumption will be verified by analyzing amongst the controls.

### **Ethical considerations**

Prior to the study an informed consent is obtained from each participant. The informed consent clearly describes the purpose of the study, the requested information and emphasises voluntary participation. The privacy and confidentiality of the participants is guaranteed. The study protocol was approved by the ethical review board of the medical faculty of the University of Leuven. According to Belgian law, the commission for the protection of privacy has also been notified.

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