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No modification of the beneficial effect of NSAIDs on colorectal cancer by *CYP2C9* genotype

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ABSTRACT

Background: CYP2C9 enzymes are involved in non-steroidal anti-inflammatory drug (NSAID) metabolism. Therefore, we investigated whether *CYP2C92 and *3 variant alleles, encoding for enzymes with lower activity, increased the protective effect of NSAIDs on colorectal cancer.**

Methods: Individual and combined associations of NSAIDs and *CYP2C92 and *3 variant alleles with colorectal cancer were studied in 7757 Caucasian individuals of The Rotterdam Study, a population-based prospective cohort since 1990. Additive and multiplicative effect modification models were used to examine drug-gene interactions.**

Results: There were 212 incident cases of colorectal cancer during follow-up. A reduced risk of colorectal cancer was observed in individuals who used NSAIDs for more than a year (HR 0.45; 95% CI 0.28 to 0.71), and in carriers of an *CYP2C9* variant allele associated with lower enzymatic activity (HR 0.67; 95% CI 0.47 to 0.96). The combination of both determinants was associated with a further risk reduction but without synergy.

Conclusion: Both NSAID use and *CYP2C92 and/or *3 carriage are associated with a reduced risk of colorectal cancer. However, no interaction between the determinants was found, which might indicate independent pathophysiological mechanisms.**

KEYWORDS

Anti-inflammatory agents, cohort studies, colorectal neoplasms, cytochrome p-450 enzyme system, interaction, non-steroid, polymorphism, single nucleotide

INTRODUCTION

Colorectal cancer is the second leading cause of cancer-related death in the Western world[1] and is considered to be the final stage of the sequence from adenoma to carcinoma by accumulation of genetic mutations in epithelial cells.[2] This may result from exposure to carcinogens and mutagens, which can be activated by xenobiotic-metabolising enzymes.[3] Furthermore, tumour development is dependent on vascularisation, cell proliferation and apoptosis.

Cytochrome P450 (CYP) enzymes, which metabolise most endogenous and exogenous substrates, are mainly expressed in the human liver, but also in normal intestinal epithelium and in colon adenocarcinoma.[4-7] The CYP2C subfamily accounts for 20% of all CYP in the liver, CYP2C9 being the main isoform.[8] The CYP2C9 isoform is capable of activating specific carcinogens and is related to the formation of DNA adducts.[9] Besides their role in (de)toxification, there is a physiological role for CYP2C9 in the metabolism of arachidonic acid, forming epoxyeicosatrienoic acids through epoxygenase.[10,11] An increase in arachidonic acid metabolism, resulting in decreased arachidonic acid levels, induces neo-vascularisation and cell growth and inhibits apoptosis,[12-15] conditions that might lead to tumour development. Additionally, CYP2C9 metabolises non-steroidal anti-inflammatory drugs (NSAIDs), which have been associated with a decreased risk of colorectal cancer (mortality).[16,17] In individuals of Caucasian descent, two allelic variants (*2 and *3) of the *CYP2C9* gene are relatively common. These *2 and *3 variants have been shown to result in a lower enzyme activity for several substrates, both *in vitro* and *in vivo*, compared with the activity of the wild-type allele.[5,18-20]

The objective of this cohort study was to investigate whether *CYP2C9*2* and *3 variant alleles are associated with an increase of the protective effect of NSAIDs on colorectal cancer risk in a Caucasian population.

METHODS

Setting

Data were obtained from The Rotterdam Study, a population-based prospective cohort study among inhabitants of the Rotterdam suburb of Ommoord. Between July 1989 and July 1993, all persons aged 55 years and older were invited (n=10,275). In total 7983 subjects (78%), including people living in one of the homes for the elderly, participated (4878 women and 3105 men). The design, ethical approval and rationale behind this study have been described earlier.[21]

At baseline, a home interview was performed followed by two visits to the research centre for clinical examinations. Blood samples were collected and DNA isolated. Baseline data collection was performed from October 1990 to July 1993. Since then, participants have been re-examined periodically. In addition, participants are continuously monitored for major events, including cancers, which occur during follow-up, through automated linkage with files from general practitioners. Information on medication use is available for all participants since January 1991. The seven computerised pharmacies that cover the research area are linked to one network. In this way, the date of prescription, the total amount of drug units per prescription and the prescribed defined daily dosage (DDD) are available per drug defined by an Anatomical Therapeutic Chemical (ATC) code.[22] Information on vital status is obtained regularly from municipal health authorities in Rotterdam and from general practitioners in the study district.

Cohort definition

Pharmacy data were available for 7857 (98%) subjects. Persons with a diagnosed colorectal cancer before 1 January 1991 (n=48) or who died or were lost to follow-up before this date (n=52) were excluded from the analyses. This resulted in a study cohort of 7757 (97%) individuals. Mainly due to a lack of blood samples, genotypes were determined in 6378 persons for whom genetic material was isolated and the assay was successfully performed. Follow-up time was defined as the period between 1 January 1991 and a diagnosis of colorectal cancer, death, or the end of the study period on 1 October 2004 whichever came first.

Exposure definition

The exposure of interest included both non-aspirin and aspirin NSAIDs. The following drugs were used by study subjects: acetylsalicylic acid, carbasalate calcium, diflunisal, sulindac, nabumetone, naproxen, ibuprofen, diclofenac, diclofenac/misoprostol, tolmetin, indomethacin, piroxicam, ketoprofen, dexketoprofen, flurbiprofen, azapropazone, meloxicam, celecoxib, etoricoxib, and rofecoxib. As previous studies showed that duration of use seemed more important than dose in reducing colorectal cancer risk, only cumulative time of use was considered in this study. Time-dependent exposure variables were defined by reference to the date of diagnosis of a colorectal cancer (index date) and to calculate cumulative duration for each case and the remainder of participants in the cohort until that date. Given this approach, subjects were eligible as controls as long as they were not a case or censored. Consequently, participants were used in several case-sets. Cases were censored at the date of diagnosis. The methodology of time-varying exposure has been described by Clayton and Hill.[23]

Genotyping

DNA was extracted using standard procedures and stored at -20°C until used for DNA amplification. *CYP2C9*2* (rs1799853) and *CYP2C9*3* (rs1057910) were determined using 2-ng genomic DNA with the Taqman Prism 7900HT 384 wells format allelic discrimination assay (Applied Biosystems, Foster City, California). Primer and probe sequences were optimised by using the SNP assay-by-design service of Applied Biosystems (for details, see http://store.appliedbiosystems.com). These allelic variants occur at appreciable frequency in the Caucasian population. The ALlele FREquency Database (ALFRED) reports allele frequencies of 13% for *CYP2C9*2* and 7% for *CYP2C9*3*.[24] The variants are the result of amino acid substitutions at position 144 (*CYP2C9*2*; $Arg_{(CGC)} \rightarrow Cys_{(TGC)}$) and position 359 (*CYP2C9*3*; $Ile_{(ATT)} \rightarrow Leu_{(CTT)}$). Since other variants are extremely rare among Caucasians, persons without *2 or *3 were considered as having the wild-type genotype (*1).

Case identification and validation

Three different databases were used for case identification. First, cases diagnosed by general practitioners in the research area were collected (International Classification of Primary Care (D75)). Second, the national registry of all hospital admissions was consulted to detect all malignancy-related hospital admissions for study participants. Third, regional pathology databases were linked to The Rotterdam Study to identify cases. Subsequently, colorectal cancer cases were validated by a physician (CS) on the basis of the general practitioners' medical records discharge letters, and pathology reports. The tenth edition of the International Classification of Diseases (ICD-10) was used to distinguish between the anatomical locations non-sigmoid colon (C18), sigmoid colon (C19) and rectal (C20) cancer. Because of their

low incidence and proposed different pathophysiology, anal cancers (n=3) were not included as cases. They were censored in the analyses at their date of diagnosis. Only pathologically confirmed cases were considered in the analyses. The index date was defined as the earliest date found in the pathology reports. Participants were not involved in any gastrointestinal screening programme.

Co-variables

On the basis of medical literature the following co-variables, assessed at baseline, were considered as potential confounders: age, gender, body mass index (BMI) (kg/m^2), total energy intake (kcal/day), alcohol (grams/day), vegetable (grams/day), fruit (grams/day), meat (grams/day), fibre (grams/day), and selenium intake (grams/day), hypercholesterolaemia (total cholesterol >6.5 mmol/l), physical activity (without difficulty, with some difficulty, with much difficulty, unable to do) and smoking (total pack-years). The method and validation of dietary assessment in The Rotterdam Study has been described elsewhere.[25]

Statistical analyses

Genotype proportions and allele frequencies were tested for deviations from the Hardy-Weinberg equilibrium using a χ^2 goodness-of-fit test. Cox proportional hazard models were used to study associations between NSAID use or *CYP2C9* variant alleles and colorectal cancer risk. A first model adjusted for age and gender. A second model was made with those co-variables that changed the point estimate by more than 10% or which were independent risk factors for the outcome, according to the literature.

NSAID use was studied both as a dichotomised variable (never / ever use) and in categories of cumulative duration (never use / 1-365 days use / >365 days use). The cut-off point of 365 days was chosen according to some previous studies that report a protective effect of NSAIDs after one year of cumulative use. The analyses were performed for total NSAID use and non-aspirin NSAIDs and aspirin NSAIDs separately. We defined five categories of medication exposure: never use of NSAIDs, 1 to 365 days of non-aspirin NSAID, >365 days of non-aspirin NSAID, 1 to 365 days of aspirin use and >365 days of aspirin use in which the 'never use' category served as a reference while the other categories could partly overlap when an individual used both aspirin- and non-aspirin NSAIDs during the study period. Trend analyses were performed to quantify a duration-effect response.

The association between genotype and colorectal cancer was studied in the total cohort, and in a subgroup of non-NSAID users to investigate the drug-independent effect of *CYP2C9* variant alleles. Carriers were defined as having at least one variant allele. The homozygous wild-type genotype (**1/*1*) served as the reference category. In addition to the association with the total group of colorectal cancers, the effect on anatomical subtypes (non-sigmoid colon, sigmoid colon, rectum) was investigated.

The combined effect of NSAIDs and *CYP2C9* genotype was studied by using the following groups: non-carriers without NSAID use (reference), variant carriers without NSAID use, non-carriers with NSAID use, variant carriers with NSAID use. Analyses were performed for the total colorectal cancer group as well as for anatomical subtypes. Drug-gene interactions were studied for the separate exposure subgroups (non-aspirin and aspirin NSAIDs) as well. When the interaction with non-aspirin NSAIDs was studied, the reference group was composed of only those without non-aspirin use and the analyses were adjusted for aspirin use. In a similar way, interaction of aspirin NSAIDs and genotype was studied. Trend analyses were performed on all these groups. The sequence for which the trends hold is based on the results of the separate analyses of NSAID use and *CYP2C9* variant allele carriage on colorectal cancer risk. Effect modification was studied with both additive (biological) and multiplicative interaction models. The relative excess risk reduction due to interaction (RERI) was used to evaluate departures from an additive scale. SAS software (Statistical Analysis Software version 8.2, Cary, NC) was used to derive regression coefficients (3) and covariance matrices (9). The numbers obtained were used to calculate RERIs ($RR_{combination} - RR_{exposure\ A} - RR_{exposure\ B} + 1$) and their corresponding 95% confidence limits.[26,27] If there is no biological interaction RERI is equal to 0. Interaction terms were added to the model to identify multiplicative effect modification. All analyses were performed with SPSS software (version 11.0.1; SPSS Inc., Chicago, USA). P values below the conventional level of significance ($p<0.05$) were considered statistically significant.

RESULTS

Individuals of whom the genotype was unknown (n=1379) were on average older, relatively more frequently female and smoker, and had a shorter follow-up time than those for whom genotype data were available. Baseline characteristics of the study group are presented in *table 1*. During a mean follow-up time of 9.8 years, 212 colorectal cancers (3 anal cancers not included) occurred. This was 3% of our cohort and corresponds with the incidence of colorectal cancer in the general Dutch population in persons aged ≥55 years.[28] The mean age was 68 years and 38% were males. Ninety-six percent of the population used an NSAID at any time during the study period. Non-aspirin NSAIDs were taken by 60% and aspirin NSAIDs by 30% of the population while 21% used both types during the study

Table 1. *Baseline characteristics of the total study population*

Total participants (genotyped)	7757 (6378)
Colorectal cancer cases (% of total participants) (genotyped)	212 (3%) (184)
Age, mean (SD)	68.1 (8.47)
Male gender, N (%)	2963 (38.2%)
Body mass index, mean (SD), N (%):	26.4 (3.7)
• Underweight (<18.5)	54 (0.7%)
• Normal weight (18.5-24.9)	2645 (34.1%)
• Overweight (25.0-29.9)	3367 (43.4%)
• Obesity (30-39.9)	1047 (13.5%)
• Extreme obesity (≥40)	23 (0.3%)
Smoking status, total pack years (SD), N (%):	26.7 (23.1)
• Never	2723 (35.1%)
• Former	3149 (40.6%)
• Current	1699 (21.9%)
Physical activity, N (%):	
• Without difficulty	4608 (59.4%)
• With some difficulty	1598 (20.6%)
• With much difficulty	496 (6.4%)
Unable to do	931 (12.0%)
Hypercholesterolaemia (>6.5 mmol/l), N (%)	3731 (48.1%)
Total energy intake (kcal/day), mean (SD)	1967 (501)
Vegetable intake (grams/day), mean (SD)	350 (137)
Fruit intake (grams/day), mean (SD)	230 (132)
Meat intake (grams/day), mean (SD)	108 (47)
Fibre intake (grams/day), mean (SD)	17 (5)
Selenium intake (grams/day), mean (SD)	33 (10)
Fat intake (grams/day), mean (SD)	40 (19)
Alcohol consumption (grams/day), mean (SD)	10 (15)
Genotypes, N (%):*	
• CYP2C9 *1/*1	4229 (66.3%)
• CYP2C9 *1/*2	1339 (21.0%)
• CYP2C9 *1/*3	593 (9.3%)
• CYP2C9 *2/*2	102 (1.6%)
• CYP2C9 *2/*3	89 (1.4%)
• CYP2C9 *3/*3	26 (0.4%)

***Hardy Weinberg χ^2 = 1.55 (p=0.34). Percentages do not sum up to 100% due to missing values. N = number; SD = standard deviation; CYP2C9 = cytochrome P450 2C9.**

period. Mean duration of non-aspirin and aspirin NSAIDs use was 90 and 280 days, respectively. Genotype data were in Hardy Weinberg equilibrium (χ^2 = 1.55; p=0.35). 33.7% of the study population carried at least one variant allele. Allele based frequencies of *CYP2C9*2* and *CYP2C9*3* were 12.8 and 5.8%, respectively.

Ever use of NSAIDs was associated with a 37% risk reduction of colorectal cancer (HR 0.63; 95% CI 0.47 to 0.85). Duration of use was inversely related to colorectal cancer incidence (p=0.001) (*table 2*). Both aspirin and non-aspirin NSAIDs were associated with a significant risk reduction for colorectal cancer, especially after more than one year of cumulative use. Total energy intake was the only potential confounder that changed the point estimate of NSAID use on the age and gender-adjusted colorectal cancer risk by more than 10%. The specified dietary factors did not change the risk when adjusted for the total intake. Other potential confounders were put into the model because they were considered as potential risk factors in the medical literature. Dietary data were not available for 23.1% of the population. Missing status of these and other factors had no effect on the association between NSAID use and colorectal cancer risk (p=0.11 to 0.51). Therefore, complete case analyses with a second model that consisted of age, gender, total energy intake, physical activity, body mass index, hypercholesterolaemia and the specified exposure were performed.

Carriage of a *CYP2C9* variant allele was also associated with a risk reduction (60%), primarily in the proximal parts of the colorectal tract (*table 3*). This risk reduction subsists for colon carcinoma in the analyses among non-NSAID users, although it is no longer significant. The number of cases was too small to investigate the individual effects of *CYP2C9*2* and **3* on cancer risk. Overall, the

Table 2. *Association between cumulative NSAID use and colorectal cancer*

Cumulative days of NSAID use	N	Model 1 HR (95% CI)	N	Model 2§ HR (95% CI)
Any NSAID use†#				
No use*	82	1.00 (reference)	63	1.00 (reference)
1-365 days use	90	0.72 (0.53-0.98)	69	0.65 (0.45-0.92)
>365 days use	40	0.48 (0.32-0.72)	32	0.45 (0.28-0.71)
Non-aspirin NSAID use‡				
No use*	82	1.00 (reference)	63	1.00 (reference)
1-365 days use	73	0.77 (0.55-1.06)	59	0.72 (0.50-1.05)
>365 days use	4	0.33 (0.12-0.91)	3	0.29 (0.09-0.95)
Aspirin NSAID use¥				
No use*	82	1.00 (reference)	63	1.00 (reference)
1-365 days use	17	0.48 (0.28-0.82)	10	0.34 (0.17-0.67)
>365 days use	36	0.55 (0.36-0.83)	29	0.51 (0.32-0.81)

***No use is defined as no use of non-aspirin or aspirin NSAIDs during the study period. §Complete case analyses. †Model 1: adjusted for age and gender, model 2: adjusted for age, gender, smoking, energy intake, physical activity, body mass index and hypercholesterolaemia. ‡Model 1: adjusted for age, gender and aspirin use, model 2: adjusted for age, gender, smoking, energy intake, physical activity, body mass index, hypercholesterolaemia and aspirin use. ¥Model 1: adjusted for age, gender and non-aspirin use, model 2: adjusted for age, gender, smoking, energy intake, physical activity, body mass index, hypercholesterolaemia and non-aspirin use. #Trend significant at 0.001 level.**
NSAID = non-steroidal anti-inflammatory drug; N = number; HR = hazard ratio; CI = confidence interval.

Table 3. *Association between CYP2C9 genotype and colorectal cancer*

	All participants				Non-NSAID users			
	N	Model 1 HR (95% CI)	N	Model 2 HR (95% CI)	N	Model 1 HR (95% CI)	N	Model 2 HR (95% CI)
Colorectal cancer	184		155		71		60	
CYP2C9	132	1.00 (reference)	115	1.00 (reference)	50	1.00 (reference)	44	1.00 (reference)
CYP2C9 variant	52	0.79 (0.57-1.09)	40	0.67 (0.47-0.96)	21	0.94(0.56-1.56)	16	0.78 (0.44-1.38)
Non-sigmoid colon	78		66		34	1.00 (reference)	30	
CYP2C9	61	1.00 (reference)	55	1.00 (reference)	27	0.59 (0.26-1.35)	25	1.00 (reference)
CYP2C9 variant	17	0.56 (0.33-0.96)	11	0.38 (0.20-0.72)	7		5	0.43 (0.16-1.13)
Sigmoid colon	65		52		21		16	
CYP2C9	46	1.00 (reference)	37	1.00 (reference)	14	1.00 (reference)	11	1.00 (reference)
CYP2C9 variant	19	0.82 (0.48-1.40)	15	0.79 (0.44-1.45)	7	1.09 (0.44-2.69)	5	1.00 (0.35-2.88)
Rectum	41		37		16		14	
CYP2C9	25	1.00 (reference)	23	1.00 (reference)	9	1.00 (reference)	8	1.00 (reference)
CYP2C9 variant	16	1.28 (0.69-2.41)	14	1.21 (0.62-2.36)	7	1.73 (0.64-4.66)	6	1.57 (0.53-4.62)

Model 1: Adjusted for age and gender. Model 2: Adjusted for age, gender, smoking, energy intake, physical activity, body mass index and hypercholesterolaemia. CYP2C9 = cytochrome P450 2C9; N = number; HR = hazard ratio; CI = confidence interval.

reduced risk of colorectal cancer associated with NSAID use seemed to be stronger than that associated with variant allele carriage.

Combinations of both variant allele carriage and NSAID use resulted in more protection than either of the factors alone (*table 4*). This effect was primarily seen in proximal parts with a significant trend for non-sigmoid colon cancer. However, significant effect modification on an additive or multiplicative scale did not occur (p>0.05). The results were similar for aspirin and non-aspirin NSAIDs.

DISCUSSION

This prospective population-based cohort study demonstrates associations between NSAID use, *CYP2C9**2 and *3 variant allele carriage and colorectal cancer incidence. Duration of NSAID use was inversely related to the incidence of colorectal cancer. Since both non-aspirin and aspirin NSAIDs have been associated with a decreased risk of colorectal cancer in former studies, we combined both types of NSAIDs and additionally

Table 4. *Combined effect of NSAID use and CYP2C9 genotype on colorectal cancer risk*

	Total NSAID use*		Non-aspirin NSAID use†		Aspirin NSAID use‡	
	N	HR (95%CI)	N	HR (95%CI)	N	HR (95%CI)
Colorectal cancer						
CYP2C9 wild-type, no use	50	1.00 (reference)	57	1.00 (reference)	100	1.00 (reference)
CYP2C9 variant, no use	21	0.92 (0.56-1.54)	25	0.94 (0.59-1.51)	39	0.79 (0.55-1.15)
CYP2C9 wild-type, use	82	0.64 (0.45-0.93)	75	0.87 (0.61-1.24)	32	0.62 (0.41-0.93)
CYP2C9 variant, use	31	0.47 (0.30-0.74)	27	0.60 (0.38-0.96)	13	0.50 (0.28-0.89)
Trend§	184	p=0.001	184	p=0.06	184	p=0.003
Non-sigmoid colon						
CYP2C9 wild-type, no use	27	1.00 (reference)	28	1.00 (reference)	51	1.00 (reference)
CYP2C9 variant, no use	7	0.57 (0.25-1.31)	10	0.77 (0.37-1.58)	11	0.44 (0.23-0.85)
CYP2C9 wild-type, use	34	0.50 (0.30-0.85)	33	0.81 (0.48-1.37)	10	0.38 (0.19-0.77)
CYP2C9 variant, use	10	0.29 (0.14-0.60)	7	0.33 (0.14-0.77)	6	0.45 (0.19-1.07)
Trend§	78	p<0.001	78	p=0.02	78	p=0.002
Sigmoid colon						
CYP2C9 wild-type, no use	14	1.00 (reference)	18	1.00 (reference)	32	1.00 (reference)
CYP2C9 variant, no use	7	1.10 (0.44-2.62)	8	0.95 (0.42-2.20)	15	0.94 (0.51-1.74)
CYP2C9 wild-type, use	32	0.93 (0.49-1.77)	28	1.02 (0.55-1.87)	14	0.88 (0.46-1.68)
CYP2C9 variant, use	12	0.67 (0.30-1.47)	11	0.76 (0.35-1.64)	4	0.50 (0.17-1.41)
Trend§	65	p=0.32	65	p=0.61	65	p=0.25
Rectum						
CYP2C9 wild-type, no use	9	1.00 (reference)	11	1.00 (reference)	17	1.00 (reference)
CYP2C9 variant, no use	7	1.73 (0.65-4.66)	7	1.38 (0.53-3.56)	13	1.57 (0.76-3.23)
CYP2C9 wild-type, use	16	0.63 (0.27-1.46)	14	0.77 (0.34-1.75)	8	0.85 (0.36-2.01)
CYP2C9 variant, use	9	0.68 (0.27-1.76)	9	0.96 (0.39-2.37)	3	0.63 (0.18-2.17)
Trend§	41	p=0.20	41	p=0.67	41	p=0.50

No use is defined as no use of the NSAID type of interest. For the total NSAID group this means no use of any non-aspirin or aspirin NSAID, for the non-aspirin NSAIDs group this means no non-aspirin NSAID use and for the group of aspirin NSAIDs this means no aspirin NSAID use §Sequence for which the trend holds is based on the results of the separate analyses that NSAID use seems to be more protective than carriage of a variant allele. *Adjusted for age and gender. †Adjusted for age, gender and aspirin use. ‡Adjusted for age, gender and non-aspirin use. NSAID = non-steroidal anti-inflammatory drug; CYP2C9 = cytochrome P450 2C9; N = number; HR = hazard ratio; CI = confidence interval.

performed separate analyses. No obvious differences were observed in the protective effect of aspirin and non-aspirin NSAIDs. Carriage of a *CYP2C9* variant allele was associated with a lower risk of non-sigmoid colon cancers, even in non-NSAID users, although for this last group no significance was reached, probably because of insufficient power. A combination of variant allele carriage and use of NSAIDs resulted in a larger reduction in colorectal cancer risk than one of the determinants independently. This seems to be due to independent pathophysiological mechanisms, since both additive and multiplicative interaction terms were not significantly different from the combined risk reduction. The influence on different regulatory pathways in the arachidonic acid metabolism and the influence CYP2C9 seems to have on the formation of DNA adducts might explain these independent mechanisms.[9]

Regular and long-term use of NSAIDs[17,29-35] and the effect of *CYP2C9* genotype[36-40] on colorectal cancer risk have both been studied before. Currently, there is increasing evidence that regular or long-term use of NSAIDs protects against malignancies in the gastrointestinal tract but final proof from randomised clinical trials is hardly available. In most observational studies and in line with our results, cumulative NSAID use is associated with a reduced cancer risk already after one to two years of cumulative use.[29-32] Other studies and trials, however, report a minimum required period of use of more than ten years.[33,34] Most trials were designed for their effect on adenoma risk reduction. The primary prevention of colorectal cancer, with at least ten-year latency of effect, is consistent with the understanding of the adenoma-carcinoma sequence.[41] The relatively short effect period found in a number of observational studies might be related to other mechanisms involving reduction of active tumour growth, bias by other health factors, stronger associations in smaller studies[41] and potential different effects of dose on COX-1 and COX-2 enzymes.[42] Despite the uniformity in chemopreventive effect of non-aspirin and aspirin NSAIDs, the inconsistency in desired duration of use between trials and observational studies needs further investigation. Furthermore, one most consider the effect of aspirin use on a more rapid manifestation by causing early bleeding from polyps that would therefore be identified in a less advanced stage. Nevertheless, this will most probably be random over genotypes.

More conflicting are the results of studies that investigated the CYP2C9-cancer relation. Two previously published studies were in line with our finding that *CYP2C9*2/*3* gene variant allele carriers had a decreased colon cancer risk.[36,38] Nevertheless, some others found inconsistent results.[37,39,40] The different risk per anatomical subtype might be the result of a different pathophysiological process that depends on a variety of environmental and genetic factors for proximal and distal cancers,[43] which would argue for performing studies by anatomical site.

The objective of our study was to investigate whether *CYP2C9*2* and **3* variant alleles are associated with an increase of the protective effect of NSAIDs on colorectal cancer risk due to a longer effective period of these drugs. The question whether there is synergism between *CYP2C9* variant alleles and NSAID use has been studied before in association with colon adenoma[44,45] and colorectal cancer.[37,46] Besides differences in outcome definition, the exposure definitions and methodology of these studies also vary. One of the previously published studies only presents results on the association with aspirin use,[45] while others report separate results for aspirin and non-aspirin NSAIDs[37,44] or aspirins and ibuprofen.[46] Similar to the first three studies, we did not find effect modification on a multiplicative scale. Additive modification of the drug effects was found in the first published study in approximately 500 cases and a similar number of controls.[44] The protective effect of aspirin on colorectal cancer risk appeared to be absent in those who carried a variant allele. Our results indicate the opposite with the lowest risk in both aspirin and non-aspirin NSAID users who are variant carriers. The different study outcome (adenoma *vs* carcinoma) or techniques to study additive effect modification might explain this contradiction.

Unlike earlier studies, we studied potential effect modification on an additive and multiplicative scale in both non-aspirin and aspirin users in one cohort to investigate whether there was synergism between *CYP2C9* variant alleles and NSAID use on colorectal cancer. For additive interaction we provided information about significance by using RERIs. None of the interaction terms were significant and consequently, based on our population-based study, there is no strong evidence that there is synergism and that *CYP2C9* variant carriage enhances the potential protective effects of NSAIDs on colorectal cancer risk. This seems in contrast with results from previous studies that *CYP2C9* variant allele carriers may accumulate NSAIDs and enhance their activity by a decreased metabolism.[47-49] However, these studies focussed on serum levels of the drug and not the protective effect of this accumulation in the human body. Although we can not be entirely certain about it as we have seen before, it is more or less established that duration of use is more important than dose in the prevention of colorectal cancer.[31] It is therefore possible that accumulation of an NSAID, resulting in higher serum levels of the drug, does not add so much extra protection against colorectal cancer. The observed reduced risks in persons with both factors present might therefore be considered as the sum of two independent risk factors. Hence, these factors most probably act primarily through different pathophysiological pathways. Nevertheless, enhancement of the effects of

NSAIDs on colorectal cancer by *CYP2C9* variants can still be present for specific NSAIDs, as was observed in a recently published American case-control study.[46] Due to a lack of power, we were not able to study the interaction between *CYP2C9* variant alleles and NSAIDs for individual products.

Observational studies might have some limitations. Selection bias due to including only those for whom blood samples were available seems unlikely since genotype data were in Hardy Weinberg equilibrium. Even though this group was slightly younger and possibly healthier, selection bias would not explain the risk reduction that we found in NSAID users. Next, 12% of persons were not able to do any physical activity. Most of these persons lived in one of the homes for the elderly. Although physical activity and colorectal cancer incidence are known to be associated, it was adjusted for in the analyses and this will probably not have led to any spurious results in the analyses on interaction, as there are no suspicions that physical activity is associated with genotype. As data on both disease status and medication use were prospectively gathered without knowledge of the research hypothesis, information bias is unlikely as well. However, NSAIDs have been available over-the-counter in the Netherlands since 1996 but to a limited extent and in a relatively low daily recommended dose. Moreover, it is unlikely that persons with a prescribed NSAID also use them over-the-counter, since all NSAIDs on prescription – including long-term use – are fully reimbursed. One of the strengths of this study was the use of pharmacy data on a day-to-day basis with little room for misclassification of the drug exposure due to recall bias. Misclassification of the anatomical subtypes would, if present, be random and lead to underestimation of the true estimates as well. Confounding of specified factors was adjusted for in the analyses. Furthermore, one must always consider the impact of insufficient power. For gene-environmental interactions the numbers of cases and controls required might be much higher than used in our analyses. However, the insignificant results of our interaction analyses could suggest that there might not be a very strong interaction. Nevertheless, it does not exclude a weaker interaction.

CONCLUSION

NSAID use and *CYP2C9* variant alleles are both associated with a reduced risk of colorectal cancer, primarily of the non-sigmoid colon. Both aspirin and non-aspirin NSAIDs account for this effect. Variant allele carriers who used NSAIDs experienced the strongest reduction in risk. This seems to be due to independent mechanisms, and not as a consequence of interaction. Nevertheless, interaction might be present for specific NSAIDs and future studies must include more cases to be able to identify differences in multiple subgroups, include longer follow-up times and include information on tumour stage, differentiation, treatment and use of other potentially interfering medications.

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