Author's response to reviews

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Authors:

Vibeke Andersen (va9791@gmail.com)
Mette Østergaard (mette.oestergaard@viborg.rm.dk)
Jane Christensen (jane@cancer.dk)
Kim Overvad (ko@dce.au.dk)
Anne Tjønneland (annet@cancer.dk)
Ulla Vogel (ulbvo@food.dtu.dk)

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Polymorphisms in the xenobiotic transporter Multidrug Resistance 1 (MDR1) gene and interaction with meat intake in relation to risk of colorectal cancer in a Danish prospective case-cohort study.

Vibeke Andersen[1], Mette Østergaard[1,2], Jane Christensen[3], Kim Overvad[4], Anne Tjønneland[3], Ulla Vogel[5,6]

[1]Medical Department, and [2]Clinical Biochemical Department, Viborg Regional Hospital, DK-8800 Viborg,

[3]Danish Cancer Society, Institute of Cancer Epidemiology, DK-2100 Copenhagen,

[4]Department of Clinical Epidemiology, Aarhus University Hospital, DK-9100 Aalborg,

[5]National Research Centre for the Working Environment, DK-2100 Copenhagen,

[6]National Food Institute, Technical University of Denmark, DK-2860 Søborg.

e-mails addresses:

Mette.Oestergaard@viborg.rm.dk

ulbvo@food.dtu.dk

annet@cancer.dk

jane@cancer.dk

vibeke.andersen@viborg.rm.dk

ko@dce.au.dk

Abbreviations: *BCRP*, Breast Cancer Resistance Protein; BMI, body mass index; CI, confidence interval; *COX-2*, Cyclooxygenase-2; CRC, Colorectal Cancer; HRT, hormone replacement therapy; *MDR1*; Multidrug Resistance 1; IRR, incidence rate ratio; NSAID, non-steroidal anti-inflammatory drug; QPCR, real-time quantitative PCR; SNP, single nucleotide polymorphism.

Correspondence:

Vibeke Andersen

Medical Department

Viborg Regional Hospital

Heibergs Allé 4,

DK-8800 Viborg

Denmark

Tel +45 8927 2641, Fax +45 8927 3484, Email: vibeke.andersen@viborg.rm.dk

Abstract

Background: Genetic variation that modifies the gut barrier function or the inflammatory response may modify the risk of colorectal cancer (CRC). The intestinal xenobiotic transporters, Multidrug Resistance 1 *(MDR1/ABCB1)* and Breast Cancer Resistance Protein *(BCRP/ABCG2)* contribute to the intestinal homeostasis by exporting a broad spectrum of substrates including potential carcinogens from the intestinal lining into the lumen. Cyclooxygenase-2 (*COX-2/PTGS2/PGHS2*) plays a key role in the intestinal immune response to luminal antigens. The aim of this study was to investigate if polymorphisms in these genes were associated with CRC risk, and to investigate possible interactions with lifestyle factors such as alcohol consumption, smoking, meat consumption and use of non-steroidal anti-inflammatory drugs (NSAID).

Methods: *MDR1* C3435T (rs1045642) and intron3 G-rs3789243-A, *BCRP* C421A (rs2231142), and *COX-2* A-1195G (rs689466), G-765C (rs20417), and T8473C (rs5275) polymorphisms were assessed together with lifestyle factors in a nested case-cohort study of 364 cases and a random cohort sample of 772 participants from the Danish prospective Diet, Cancer and Health study.

Results: Carriers of the variant allele of *MDR1* intron 3 were at 1.52-fold higher risk of CRC compared with homozygous wild type allele carriers (Incidence rate ratio (IRR) =1.52. 95 % Confidence Interval (CI): 1.12-2.06). Homozygous carriers of the T-allele of *MDR1* C3435T had a lower risk of CRC compared with homozygous C-allele carriers (IRR= 0.66. 95% CI: 0.45-0.98). There was interaction between the studied *MDR1* polymorphisms and intake of red and processed meat in relation to CRC risk. Homozygous *MDR1* C3435T C-allele carriers were at 8% increased risk pr 25 g meat/day (CI: 1.00-1.16) whereas variant allele carriers were not at increased risk (p for

interaction = 0.02). There was interaction between NSAID use and *MDR1* C3435T and *COX-2* T8473C (p-values for interaction 0.001 and 0.04, respectively). Use of NSAID was associated with an increased risk among homozygous wild type allele carriers (*MDR1* C3435T: IRR=2.34 (1.22-4.48) and *COX-2* T8473C: IRR=1.38 (0.89-2.14), but not among variant allele carriers.

Conclusions: Two polymorphisms in *MDR1* were associated with higher risk of CRC and there was interaction between the polymorphisms and meat intake in relation to CRC risk.

Keywords: Breast Cancer Resistance Protein, Cyclo-oxygenase-2, gene-environmental interaction, intestinal barrier function, meat exposure, non-steroidal anti-inflammatory drugs, population based prospective study

Background

Colorectal cancer (CRC) is one of the leading causes of cancer-related mortality in the Western World, with great impact on the life quality for the affected persons. Both genetic and environmental factors contribute to the pathogenesis, and gene-environmental interactions may modulate cancer risk. Multiple low-penetrance genes have been shown to confer susceptibility to CRC**.**

Diet components, alcohol consumption and cigarette smoking are risk factors for CRC [1-3]. Red and processed meat and tobacco are sources of carcinogens, among others the polycyclic aromatic hydrocarbons (PAH). Smoking and alcohol intake have been shown to induce inflammation [4,5], a well-known risk factor for gastrointestinal cancer development.

The transport proteins P-glycoprotein (encoded by the Multidrug Resistance 1 (*MDR1/ABCB1*) gene) and Breast Cancer Resistance Protein (BCRP, encoded by the *BCRP/ABCG2* gene) are abundant in the intestine [6,7]. They transport a broad spectrum of compounds [8], including PAH [9-11], to the intestinal lumen, thereby protecting the intestinal cells from exposure to potential carcinogens. Also various cytokines, such as interleukin-1β, and chemokines involved in inflammation, seem to be P-glycoprotein substrates [12], thus making a potential link between P-glycoprotein function and inflammation-induced carcinogenesis.

Significant *MDR1* gene heterogeneity has been demonstrated and several *MDR1* polymorphisms have been described [13,14]. The *MDR1* C3435T polymorphism has been most extensively investigated. The variant allele has been associated with lower *in vitro* activity due to a lower mRNA stability [15-18]. Nevertheless, *in vivo* studies have been inconsistent [19-21]. The variant allele has been associated with higher risk of colorectal cancer [22,23], although the numbers of study participants in these studies were relatively small. More recently, using a haplotype tagging approach, a SNP in intron 3 of *MDR1*, G-rs3789243A, has been associated with risk of inflammatory bowel

disease [24,25], a known risk factor for CRC. Also intestinal adenomas increase the risk of CRC. We have previously made a case-cohort study of the intron 3 polymorphism in relation to the development of adenomas and carcinomas in a Norwegian population but found no associations [26]. So far, the functional effect of this SNP is unknown [24].

The variant allele of the non-synonymous *BCRP* C421A (Q141K) polymorphism has been associated with lower protein levels and lowered transport activity *in vitro* [27-29]. However, no association was found with the intestinal expression of the protein [30] or the risk of CRC [31] .

Cyclooxygenase-2 (COX-2) plays a key role in gastrointestinal carcinogenesis, affecting angiogenesis, apoptosis, and invasiveness [32]. In line with this, inhibition of COX-2 activity by non-steroidal anti-inflammatory drugs (NSAID) is associated with reduced risk of CRC [33,34]. Moreover, COX-2 plays a key role in the regulation of the intestinal immune response to luminal antigens [35] and may thus modulate the interaction between carcinogen exposure and intestinal barrier function by regulation of the intestinal immune homeostasis. Two functional polymorphisms, A-1195G and G-765C in the promoter region of *COX-2* have been described [36]. Carriers of the variant G-allele of -1195 lack a c-Myb binding site resulting in lowered COX-2 mRNA levels [36]. The G to C substitution at nucleotide -765 eliminates an Sp1 binding site, but meanwhile creates an E2F binding site [36]. Even though *in vitro* studies have revealed a lower COX-2 expression from the C-allele of G-765C allele [36], *in vivo* studies have shown the opposite effect [36,37]. A *COX-2* haplotype containing the variant allele in position -765 together with the variant allele of the T8473C SNP in the 3´ untranslated region (UTR) has been associated with high COX-2 activity [38], indicating that the 3'UTR variant allele may stabilize the mRNA level considerably.

COX-2 polymorphisms were associated with increased risk of CRC in a large Chinese study [39] but not in a large French study [40], thus suggesting a possible population heterogeneity for COX-2 [41]. On the other hand, an association was found between a polymorphism in *COX-2* exon 10 and CRC risk in Caucasians [42]. *COX-2* polymorphisms have not been associated with risk of intestinal adenomas *per se* [43,44], but interactions with NSAID use have been found [43], thereby possibly elucidating the role of *COX-2* in CRC carcinogenesis. The role of genetic variation in *MDR1, BCRP* and *COX-2* and the interaction with alcohol intake, smoking, consumption of meat, and use of NSAID, were assessed in relation to the risk of developing CRC in a case-cohort study nested in the prospective population-based Danish Diet, Cancer and Health cohort study.

Methods

Studied Subjects

The subjects were selected from the Danish Diet, Cancer and Health study, an ongoing prospective cohort study [45]. Between December 1993 and May 1997, 160,725 individuals aged 50 to 64 years, born in Denmark, living in the Copenhagen and Aarhus areas and having no previous cancers at the time of invitation, were invited to participate in the study. A total of 57,053 persons accepted the invitation.
In total, 405 cases (184 women and 221 men) of colorectal cancer were diagnosed among the cohort members between 1994 and 2003 and registered in the files of the nationwide Danish Cancer Registry. Within the cohort we defined a sub-cohort sample including 368 women and 442 men who were randomly selected. Cases and the sub-cohort sample were frequency-matched on gender. Blood samples were available for 397 cases and 800 sub-cohort members. All information on genotypes and lifestyle factors was available for 372 cases and 765 sub-cohort members. 13 colorectal cancer cases diagnosed with carcinoid tumor or various other histological subtypes were excluded from the analysis, leaving 359 adenocarcinoma cases.

Meat, alcohol, NSAID, and other lifestyle variables

At enrolment, detailed information on diet, lifestyle, weight, height, medical treatment, environmental exposures, and other socio-economic factors were collected [45]. In the food-frequency questionnaire, meat consumption was assessed in 12 categories of predefined responses, ranking from ’never’ to ’eight times or more per day’. The daily intake was then calculated by using FoodCalc [46], this program uses population specific standardized recipes and portion sizes. Intake of red meat in grams per day was calculated by adding up intake of beef,

veal, pork, lamb and offal. Intake of processed meat in grams per day was calculated by adding up intake of processed red meat, including bacon, smoked ham, salami, frankfurter, Cumberland sausage, cold cuts and liver pâté and processed fish that is fish prepared by pickling, salting or smoking. Alcohol intake was recorded as the average frequency of intake of six types of alcoholic beverage over the preceding year: the frequency of consumption of three types of beer was recorded in bottles (330 ml), wine in glasses (125 ml), and fortified wine in drinks (60 ml) and spirits in drinks (30 ml). The predefined responses were in 12 categories, ranging from "never" to "8 or more times a day". The alcohol content was calculated as follows: one bottle of light beer, 8.9 g ethanol; one bottle of regular beer, 12.2 g ethanol; one bottle of strong beer, 17.5 g ethanol; one glass of wine, 12.2 g ethanol; one drink of fortified wine, 9.3 g ethanol; and one drink of spirits, 9.9 g ethanol. We did not differentiate between red and white wine.

The lifestyle questionnaire included this question regarding use of NSAID: "Have you taken more than one pain relieving pill per month during the last year?" If the answer was yes, the participant was asked to record how frequently they took each of the following medications: "Aspirin", "Paracetamol", "Ibuprofen", or "Other pain relievers". The latter category included NSAID preparations other than aspirin and ibuprofen. Based on all records, we classified study subjects according to use of "any NSAID" (≥ 2 pills per month during one year) at baseline.

Data on hormone replacement therapy (HRT), intake of dietary fibre and red meat, and anthropometric measurements was obtained as previously described [45]. The body mass index (BMI) was calculated as weight (kg) per height (m) squared.

Blood sampling and storage

Blood was collected at enrolment and prepared as previously described [47]. In short, a total of 30 ml blood was collected in citrated (2x10 ml) and plain (1x10 ml) Venojects from each non-fasting participant. Plasma, serum, lymphocytes, and erythrocytes were isolated and frozen at -20 ^{o}C within 2 hours. At the end of the day of collection, all samples were stored in liquid nitrogen, at -150 ˚C.

Genotyping

All analyses were run blinded to the case-control status. DNA was isolated from frozen lymphocytes as described by Miller et al [48]. Generally, 100 µg DNA were obtained from 10^7 lymphocytes. Genotyping was performed by TaqMan real-time quantitative PCR (QPCR). *MDR1* C3435T and G-rs3789243-A, *BCRP* C421A, and *COX-2* G-765C were genotyped on an Mx3000 machine (Stratagene, La Jolla, CA, USA), using the Allelic Discrimination feature of the MxPro software (Stratagene). Reactions were carried out essentially as previously described [25], except the reaction volume was 15 µl. In brief, each reaction contained 1x TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA, USA), approximately 20 ng DNA, and the relevant sets of primers and locked nucleic acid (LNA)-containing probes. All reactions were run for 50 cycles with two PCR steps, denaturation and combined annealing and elongation, respectively, except for *MDR1* G-rs3789243-A, where annealing and elongation were split. Verified genotype controls were included in each run. To confirm reproducibility, 20 samples for each SNP were randomly selected within each of the three genotype groups and repeated. The genotypes showed 100 % identity.

COX-2 A-1195G and T8473C were genotyped on an ABI7500 machine by endpoint readings as previously described [47]. Twenty ng of DNA were genotyped in 5 µl containing 1x Mastermix (Applied Biosystems, Nærum, Denmark), 100 nM probes, and 900 nM primers. Controls were included in each run, and repeated genotyping of a random 10 % subset yielded 100 % identical genotypes.

Statistical Analysis

The analyses were performed according to the principles for the analysis of case-cohort studies as described by Barlow [49]. The analyses were performed unweighted. Age was used as the time scale in the Cox regression model. Tests and confidence intervals were based on Wald's test using the robust estimate of the variance-covariance matrix for the regression parameters in the Cox regression model [50]. All models were adjusted for baseline values of established risk factors for colorectal cancer such as BMI (kg/m^2, continuous), NSAID (yes/no), use of HRT (never/past/current, among women), smoking status (never/past/current), and intake of dietary fibers (g/day, continuous) and red meat (g/day, continuous).

We investigated possible interactions between the genes and selected environmental factors using the likelihood ratio test. The procedure PHREG in SAS (release 9.1; SAS Institute Inc., Cary, NC, USA) was used for the statistical analyses.

Ethics

All participants gave verbal and written informed consent. Diet, Cancer and Health and the present sub-study were approved by the regional Ethics Committees on Human Studies in Copenhagen and Aarhus (Jr.nr. (KF)11-037/01 and jr.nr. (KF)01-045/93), and by the Danish Data Protection Agency.

Results

Associations between genotype and CRC risk

Characteristics of the study population and risk factors for CRC are shown in Table 1.

The genotype distributions among the participants in the sub-cohort sample did not deviate from Hardy-Weinberg equilibrium (results not shown). Carriers of the variant allele of *MDR1* G-rs3789243-A were at 1.52-fold (95% confidence interval (CI): 1.12-2.06) higher risk of CRC compared with homozygous carriers of the wild type allele (Table 2). Homozygous carriers of the T-allele of *MDR1* C3435T hada lowered risk of CRC compared with homozygous C-allele carriers (IRR 0.66; CI: 0.45-0.98). *COX-2* and *BRCP* polymorphisms were not associated with CRC risk (Table 2).

Haplotype analyses

Analysis of the combination of the *MDR1* G-rs3789243-A and C3435T polymorphisms (supplemental Table 5) showed that the genotype combinations encompassing the A-allele of *MDR1* the G-rs378943-A were associated with increased risk of CRC, whereas the effect of C3435T was less clear (supplemental Table 5).

Haplotype analyses combining the polymorphisms at position -1195, -765, and 8473 of *COX-2* revealed that four haplotypes included 97% of the observed genotype combinations in the cohort sample. *COX-2* haplotypes were not associated with CRC risk (supplemental Table 6).

Gene-gene and gene-environment analyses

Since we observed no gene-dose effects, variant genotypes were combined in subsequent analyses to obtain power to interaction analyses.

No gene-gene interaction between the *MDR1* G-rs3789243-A and *COX-2* T8473C polymorphisms was found (data not shown).

There was interaction between the studied *MDR1* polymorphisms and intake of red and processed meat in relation to CRC risk (Table 3). Variant allele carriers of *MDR1* G-rs3789243-A were at 3% increased risk pr 25 g meat/day (CI: 0.98-1.09) whereas homozygous carriers of the wild type allele were not at increased risk (IRR pr 25g/day 0.95, CI: 0.89-1.02) (p for interaction= 0.01). Homozygous *MDR1* C3435T C-allele carriers were at 8% increased risk pr 25 g meat/day (CI: 1.00-1.16) whereas variant allele carriers were not at increased risk (IRR pr 25g/day 1.00, CI: 0.95-1.06) (p for interaction= 0.02). No interaction was found for the *BRCP* polymorphism.

We found interaction between *MDR1* C3435T and NSAID use in relation to risk of CRC (Table 4). Among homozygous carriers of the C-allele, NSAID use was associated with 2.34-fold (CI:1.22-4.48) higher risk of CRC compared to non-users, whereas NSAID use had no effect among variant allele carriers (p for interaction 0.001). Likewise, there was a marginally statistically significant interaction between *COX-2* T8473C and NSAID use (p for interaction 0.04). A non-significant increased risk of CRC by NSAID use was found among homozygous wild type allele carriers (IRR 1.38, CI:0.89-2.14) compared to non-NSAID users, whereas NSAID use was not associated with increased CRC risk among variant allele carriers.

There was no interaction between the polymorphisms and alcohol intake, BMI, or smoking status in relation to CRC risk (data not shown).

Discussion

We found that *MDR1* polymorphisms were associated with risk of CRC in the Danish population and, interaction between the same polymorphisms and meat intake in relation to CRC risk. Furthermore, there was interaction between NSAID use and the *MDR1* and *COX-2* polymorphisms in relation to risk of CRC.

The present study had a strong epidemiological design. Cases and cohort sample were selected from the same cohort, which together with complete follow up of the participants, minimised the risk of selection bias. Information on lifestyle factors were collected at enrolment for all participants which minimised the risk of differential misclassification of cases and comparison group. However, lifestyle factors were only collected once, and may thus not be representative for the lifestyle during follow-up. Heterozygous and homozygous variant genotype carriers were combined for the analyses of interactions due power-considerations. Therefore, in the light of the obtained P-values and the number of statistical testes performed, we cannot exclude that our positive findings are due to chance. On the other hand, the fact that we find interaction between both of the MDR1 polymorphisms and meat intake in relation to CRC risk makes a chance finding less likely.

The biological function of *MDR1* G-rs3789243-A is unknown. The polymorphism was associated with risk of ulcerative colitis in the Scottish population [24] and weakly with inflammatory bowel diseases in the Danish population [51]. *MDR1* polymorphisms have also been studied in relation to CRC susceptibility [22,23,52,53]. The majority of the findings are in agreement with the present results. Furthermore, associations have been found between other *MDR1* polymorphisms and CRC [22,54]. In a Slovenian case-control study of

400 cases and 395 controls, *MDR1* C1236T and C2677C variant allele carriers were at increased risk of CRC, whereas there was no association with C3435T [52]. The *MDR1* G-rs3789243-A polymorphism was not associated to CRC or colorectal adenomas in a Norwegian population-based study [26]. Other, smaller case-control studies found no primary association with *MDR1* genotypes, including the C3435T polymorphism, among Caucasians (184 cases and 188 controls), in the European part of Russia (285 cases and 275 controls) and in a Korean population (111 cases and 93 controls) [23,53]. However, in subgroup analyses associations between *MDR1* C3435T variant allele and CRC were found in of patients being diagnosed before the age of 50 years [22] and in life-long nonsmokers of more than 63 years of age [23]. These studies have, relative to our study, a weaker design, being small case-control studies.

Interestingly, we found interaction between the CRC-associated *MDR1* genotypes and meat intake. Red meat and processed meat have been associated with increased risk of CRC [3], which may be due to carcinogenic heterocyclic amines and polycyclic aromatic hydrocarbons formed by cooking meat at a high temperature. In our cohort, intake of the two kinds of meat was not associated with increased risk of CRC (Table 1). *MDR1* is involved in the mucosal defence against xenobiotics and a higher risk of CRC may be caused by lower P-glycoprotein mediated xenobiotic transport and thereby increased carcinogen exposure [55]. Differences in exposure profiles may theoretically explain the different findings in the Norwegian [56], however, the consumption of meat seems not to differ in the Danish and Norwegian cohort [56]. The absence of interaction with the transporter *BCRP* may suggest that the different substrate specificities of P-glycoprotein and BCRP may be relevant in relation to CRC development, and elucidation hereof will help to the identification of which processes may contribute to malignancy.

We observed an interaction between *MDR1* C3435T and NSAID use such that NSAID use was associated with increased CRC risk among homozygous carriers of the wild type allele only. In cell lines, COX-2 has been shown to induce P-glycoprotein [57] whereas COX-2 inhibition prevented the expression and function of P-glycoprotein [58], thereby affecting apoptosis [59]. A similar relationship has been found *in vivo* [60]. Thus, NSAID use seems to affect P-glycoprotein tumorigenesis in an incompletely understood manner.
Taken together, while *MDR1* seems to play a role in CRC carcinogenesis, much is to be learned about the function of P-glycoprotein in relation to carcinogenesis.

We found no association between *COX-2* polymorphisms or *COX-2* haplotypes and risk of CRC. The haplotype pattern was similar to what has previously been found for Danes [47]. Variant allele carriers of *COX-2* G-765C have been reported to be at higher CRC risk among Han Chinese and in a Spanish population [39,42]. In accordance with our results, no association between *COX*-2 polymorphisms and CRC was demonstrated in a large French case control study [40]. Other studies suggest that the effects of polymorphisms on *COX-2* expression levels are large enough to have biological impact provided that *COX-2* expression is important in CRC [47]. On this basis, our results suggest that COX-2 plays a limited role in colon carcinogenesis in the present study population.
Interaction between NSAID and *COX-2* polymorphisms in relation to risk of CRC and colorectal adenomas has been investigated previously [42,43,61]. In contrast to the present results, Ulrich et al. found that *COX-2* G-765C homozygous wild type allele carriers were at lower risk of adenomas among NSAID users [43]. The effect of NSAID use has been reported for the present study population [34] and

long-term consistent use appears to be necessary to achieve a protective effect against CRC. However, using a higher intake of NSAID as cut-off value (weekly use) did not change our results regarding NSAID use (results not shown).

Conclusion

In conclusion, we found associations between *MDR1* polymorphisms and risk of CRC in the studied Danish population. Moreover, there was interaction between the polymorphisms and meat intake in relation to CRC risk. Therefore, the results from this study are consistent with the assumption that a higher risk of CRC may be caused by increased carcinogen exposure in genetic susceptible subjects. We found interactions between NSAID use and both *COX-2* and *MDR1* polymorphisms in relation to CRC risk. However, the very small effects of the *COX-2* polymorphisms suggest that *COX-2* plays a limited role for CRC in the present study population.

Competing interests

None declared.

Authors' contributions

MØ and UV carried out the molecular genetic studies. UV, KO, AT participated in the design of the study and JC performed the statistical analysis. VA conceived the study, and participated in its design and coordination and VA, UV and JC drafted the manuscript. All authors read and approved the final manuscript.

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Reference List

1. Buc E, Kwiatkowski F, Alves A, Panis Y, Mantion G, Slim K: **Tobacco smoking: a factor of early onset of colorectal cancer.** *Dis Colon Rectum* 2006, **49:** 1893-1896.

2. Boffetta P, Hashibe M: **Alcohol and cancer.** *Lancet Oncol* 2006, **7:** 149-156.

3. Cross AJ, Leitzmann MF, Gail MH, Hollenbeck AR, Schatzkin A, Sinha R: **A prospective study of red and processed meat intake in relation to cancer risk.** *PLoS Med* 2007, **4:** e325.

4. Imhof A, Froehlich M, Brenner H, Boeing H, Pepys MB, Koenig W: **Effect of alcohol consumption on systemic markers of inflammation.** *Lancet* 2001, **357:** 763-767.

5. Wang JJ, Tung TH, Yin WH, Huang CM, Jen HL, Wei J *et al.*: **Effects of moderate alcohol consumption on inflammatory biomarkers.** *Acta Cardiol* 2008, **63:** 65-72.

6. Hilgendorf C, Ahlin G, Seithel A, Artursson P, Ungell AL, Karlsson J: **Expression of thirty-six drug transporter genes in human intestine, liver, kidney, and organotypic cell lines.** *Drug Metab Dispos* 2007, **35:** 1333-1340.

7. Albermann N, Schmitz-Winnenthal FH, Z'graggen K, Volk C, Hoffmann MM, Haefeli WE *et al.*: **Expression of the drug transporters MDR1/ABCB1, MRP1/ABCC1, MRP2/ABCC2, BCRP/ABCG2, and PXR in peripheral blood mononuclear cells and their relationship with the expression in intestine and liver.** *Biochem Pharmacol* 2005, **70:** 949-958.

8. Schinkel AH: **The physiological function of drug-transporting P-glycoproteins.** *Semin Cancer Biol* 1997, **8:** 161-170.

9. Ebert B, Seidel A, Lampen A: **Identification of BCRP as transporter of benzo[a]pyrene conjugates metabolically formed in Caco-2 cells and its induction by Ah-receptor agonists.** *Carcinogenesis* 2005, **26:** 1754-1763.

10. Vache C, Camares O, De GF, Dastugue B, Meiniel A, Vaury C *et al.*: **Drosophila melanogaster p-glycoprotein: a membrane detoxification system toward polycyclic aromatic hydrocarbon pollutants.** *Environ Toxicol Chem* 2006, **25:** 572-580.

11. van Herwaarden AE, Jonker JW, Wagenaar E, Brinkhuis RF, Schellens JH, Beijnen JH *et al.*: **The breast cancer resistance protein (Bcrp1/Abcg2) restricts exposure to the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine.** *Cancer Res* 2003, **63:** 6447-6452.

12. Johnstone RW, Ruefli AA, Smyth MJ: **Multiple physiological functions for multidrug transporter P-glycoprotein?** *Trends Biochem Sci* 2000, **25:** 1-6.

13. Kroetz DL, Pauli-Magnus C, Hodges LM, Huang CC, Kawamoto M, Johns SJ *et al.*: **Sequence diversity and haplotype structure in the human ABCB1 (MDR1, multidrug resistance transporter) gene.** *Pharmacogenetics* 2003, **13:** 481-494.

14. Goldstein DB, Hirschhorn JN: **In genetic control of disease, does 'race' matter?** *Nat Genet* 2004, **36:** 1243-1244.

15. Jamroziak K, Balcerczak E, Smolewski P, Robey RW, Cebula B, Panczyk M *et al.*: **MDR1 (ABCB1) gene polymorphism C3435T is associated with P-glycoprotein activity in B-cell chronic lymphocytic leukemia.** *Pharmacol Rep* 2006, **58:** 720-728.

16. Morita N, Yasumori T, Nakayama K: **Human MDR1 polymorphism: G2677T/A and C3435T have no effect on MDR1 transport activities.** *Biochem Pharmacol* 2003, **65:** 1843-1852.

17. Wang D, Johnson AD, Papp AC, Kroetz DL, Sadee W: **Multidrug resistance polypeptide 1 (MDR1, ABCB1) variant 3435C>T affects mRNA stability.** *Pharmacogenet Genomics* 2005, **15:** 693-704.

18. Takane H, Kobayashi D, Hirota T, Kigawa J, Terakawa N, Otsubo K *et al.*: **Haplotype-oriented genetic analysis and functional assessment of promoter variants in the MDR1 (ABCB1) gene.** *J Pharmacol Exp Ther* 2004, **311:** 1179-1187.

19. Larsen UL, Hyldahl OL, Guldborg NC, Eriksen J, Jakobsen P, Ostergaard M *et al.*: **Human intestinal P-glycoprotein activity estimated by the model substrate digoxin.** *Scand J Clin Lab Invest* 2007, **67:** 123-134.

20. Hoffmeyer S, Burk O, von RO, Arnold HP, Brockmoller J, Johne A *et al.*: **Functional polymorphisms of the human multidrug-resistance gene: multiple sequence variations and correlation of one allele with P-glycoprotein expression and activity in vivo.** *Proc Natl Acad Sci U S A* 2000, **97:** 3473-3478.

21. Moriya Y, Nakamura T, Horinouchi M, Sakaeda T, Tamura T, Aoyama N *et al.*: **Effects of polymorphisms of MDR1, MRP1, and MRP2 genes on their mRNA expression levels in duodenal enterocytes of healthy Japanese subjects.** *Biol Pharm Bull* 2002, **25:** 1356-1359.

22. Kurzawski M, Drozdzik M, Suchy J, Kurzawski G, Bialecka M, Gornik W *et al.*: **Polymorphism in the P-glycoprotein drug transporter MDR1 gene in colon cancer patients.** *Eur J Clin Pharmacol* 2005, **61:** 389-394.

23. Osswald E, Johne A, Laschinski G, rjomand-Nahad F, Malzahn U, Kirchheiner J *et al.*: **Association of MDR1 genotypes with susceptibility to colorectal cancer in older non-smokers.** *Eur J Clin Pharmacol* 2007, **63:** 9-16.

24. Ho GT, Soranzo N, Nimmo ER, Tenesa A, Goldstein DB, Satsangi J: **ABCB1/MDR1 gene determines susceptibility and phenotype in ulcerative colitis: discrimination of critical variants using a gene-wide haplotype tagging approach.** *Hum Mol Genet* 2006, **15:** 797-805.

25. Ostergaard M, Ernst A, Labouriau R, Dagiliene E, Krarup HB, Christensen M *et al.*: **Cyclooxygenase-2, multidrug resistance 1, and breast cancer resistance protein gene polymorphisms and inflammatory bowel disease in the Danish population.** *Scand J Gastroenterol* 2009, **44:** 65-73.

26. Andersen V, Agerstjerne L, Jensen D, Ostergaard M, Saebo M, Hamfjord J *et al.*: **The multidrug resistance 1 (MDR1) gene polymorphism G-rs3789243-A is not associated with disease susceptibility in Norwegian patients with colorectal adenoma and colorectal cancer; a case control study.** *BMC Med Genet* 2009, **10:** 18.

27. Mizuarai S, Aozasa N, Kotani H: **Single nucleotide polymorphisms result in impaired membrane localization and reduced atpase activity in multidrug transporter ABCG2.** *Int J Cancer* 2004, **20;109:** 238-246.

28. Imai Y, Nakane M, Kage K, Tsukahara S, Ishikawa E, Tsuruo T *et al.*: **C421A polymorphism in the human breast cancer resistance protein gene is associated with low expression of Q141K protein and low-level drug resistance.** *Mol Cancer Ther* 2002, **1:** 611-616.

29. Kondo C, Suzuki H, Itoda M, Ozawa S, Sawada J, Kobayashi D *et al.*: **Functional analysis of SNPs variants of BCRP/ABCG2.** *Pharm Res* 2004, **21:** 1895-1903.

30. Zamber CP, Lamba JK, Yasuda K, Farnum J, Thummel K, Schuetz JD *et al.*: **Natural allelic variants of breast cancer resistance protein (BCRP) and their relationship to BCRP expression in human intestine.** *Pharmacogenetics* 2003, **13:** 19-28.

31. Campa D, Pardini B, Naccarati A, Vodickova L, Novotny J, Forsti A *et al.*: **A gene-wide investigation on polymorphisms in the ABCG2/BRCP transporter and susceptibility to colorectal cancer.** *Mutat Res* 2008, **645:** 56-60.

32. Chan AT, Ogino S, Fuchs CS: **Aspirin and the risk of colorectal cancer in relation to the expression of COX-2.** *N Engl J Med* 2007, **356:** 2131-2142.

33. Flossmann E, Rothwell PM: **Effect of aspirin on long-term risk of colorectal cancer: consistent evidence from randomised and observational studies.** *Lancet* 2007, **369:** 1603-1613.

34. Friis S, Poulsen AH, Sorensen HT, Tjonneland A, Overvad K, Vogel U *et al.*: **Aspirin and other non-steroidal anti-inflammatory drugs and risk of colorectal cancer: A Danish cohort study.** *Cancer Causes Control* 2009 in press DOI: 10.1007/s10552-008-9286-7..

35. Fukata M, Chen A, Klepper A, Krishnareddy S, Vamadevan AS, Thomas LS *et al.*: **Cox-2 is regulated by Toll-like receptor-4 (TLR4) signaling: Role in proliferation and apoptosis in the intestine.** *Gastroenterology* 2006, **131:** 862-877.

36. Zhang X, Miao X, Tan W, Ning B, Liu Z, Hong Y *et al.*: **Identification of functional genetic variants in cyclooxygenase-2 and their association with risk of esophageal cancer.** *Gastroenterology* 2005, **129:** 565-576.

37. Szczeklik W, Sanak M, Szczeklik A: **Functional effects and gender association of COX-2 gene polymorphism G-765C in bronchial asthma.** *J Allergy Clin Immunol* 2004, **114:** 248-253.

38. Sanak M, Szczeklik W, Szczeklik A: **Association of COX-2 gene haplotypes with prostaglandins production in bronchial asthma.** *J Allergy Clin Immunol* 2005, **116:** 221-223.

39. Tan W, Wu J, Zhang X, Guo Y, Liu J, Sun T *et al.*: **Associations of functional polymorphisms in cyclooxygenase-2 and platelet 12-lipoxygenase with risk of occurrence and advanced disease status of colorectal cancer.** *Carcinogenesis* 2007, **28:** 1197-1201.

40. Kury S, Buecher B, Robiou-du-Pont S, Scoul C, Colman H, Le NT *et al.*: **Low-penetrance alleles predisposing to sporadic colorectal cancers: a French case-controlled genetic association study.** *BMC Cancer* 2008, **8:326.:** 326.

41. Goodman JE, Bowman ED, Chanock SJ, Alberg AJ, Harris CC: **Arachidonate lipoxygenase (ALOX) and cyclooxygenase (COX) polymorphisms and colon cancer risk.** *Carcinogenesis* 2004, **25:** 2467-2472.

42. Cox DG, Pontes C, Guino E, Navarro M, Osorio A, Canzian F *et al.*: **Polymorphisms in prostaglandin synthase 2/cyclooxygenase 2 (PTGS2/COX2) and risk of colorectal cancer.** *Br J Cancer* 2004, **91:** 339-343.

43. Ulrich CM, Whitton J, Yu JH, Sibert J, Sparks R, Potter JD *et al.*: **PTGS2 (COX-2) -765G > C promoter variant reduces risk of colorectal adenoma among nonusers of nonsteroidal anti-inflammatory drugs.** *Cancer Epidemiol Biomarkers Prev* 2005, **14:** 616-619.

44. Gunter MJ, Canzian F, Landi S, Chanock SJ, Sinha R, Rothman N: **Inflammation-related gene polymorphisms and colorectal adenoma.** *Cancer Epidemiol Biomarkers Prev* 2006, **15:** 1126-1131.

45. Tjonneland A, Olsen A, Boll K, Stripp C, Christensen J, Engholm G *et al.*: **Study design, exposure variables, and socioeconomic determinants of participation in Diet, Cancer and Health: a population-based prospective cohort study of 57,053 men and women in Denmark.** *Scand J Public Health* 2007, **35:** 432-441.

46. Foodcalc 1.3 Computer program 1998. 2009.
Ref Type: Internet Communication

47. Vogel U, Christensen J, Dybdahl M, Friis S, Hansen RD, Wallin H *et al.*: **Prospective study of interaction between alcohol, NSAID use and polymorphisms in genes involved in the inflammatory response in relation to risk of colorectal cancer.** *Mutat Res* 2007, **624:** 88-100.

48. Miller SA, Dykes DD, Polesky HF: **A simple salting out procedure for extracting DNA from human nucleated cells.** *Nucleic Acids Res* 1988, **16:** 1215.

49. Barlow WE, Ichikawa L, Rosner D, Izumi S: **Analysis of case-cohort designs.** *J Clin Epidemiol* 1999, **52:** 1165-1172.

50. Barlow WE: **Robust variance estimation for the case-cohort design.** *Biometrics* 1994, **50:** 1064-1072.

51. Ostergaard M, Ernst A, Labouriau R, Dagiliene E, Krarup HB, Christensen M *et al.*: **Cyclooxygenase-2, multidrug resistance 1, and breast cancer resistance protein gene polymorphisms and inflammatory bowel disease in the Danish population.** *Scand J Gastroenterol* 2008, 1-9.

52. Potocnik U, Glavac D, Dean M: **Common germline MDR1/ABCB1 functional polymorphisms and haplotypes modify susceptibility to colorectal cancers with high microsatellite instability.** *Cancer Genet Cytogenet* 2008, **183:** 28-34.

53. Bae SY, Choi SK, Kim KR, Park CS, Lee SK, Roh HK *et al.*: **Effects of genetic polymorphisms of MDR1, FMO3 and CYP1A2 on susceptibility to colorectal cancer in Koreans.** *Cancer Sci* 2006, **97:** 774-779.

54. Potocnik U, Ferkolj I, Glavac D, Dean M: **Polymorphisms in multidrug resistance 1 (MDR1) gene are associated with refractory Crohn disease and ulcerative colitis.** *Genes Immun* 2004, **5:** 530-539.

55. Yamada T, Mori Y, Hayashi R, Takada M, Ino Y, Naishiro Y *et al.*: **Suppression of intestinal polyposis in Mdr1-deficient ApcMin/+ mice.** *Cancer Res* 2003, **63:** 895-901.

56. Skjelbred CF, Saebo M, Hjartaker A, Grotmol T, Hansteen IL, Tveit KM *et al.*: **Meat, vegetables and genetic polymorphisms and the risk of colorectal carcinomas and adenomas.** *BMC Cancer* 2007, **%19;7:228.:** 228.

57. Ziemann C, Schafer D, Rudell G, Kahl GF, Hirsch-Ernst KI: **The cyclooxygenase system participates in functional mdr1b overexpression in primary rat hepatocyte cultures.** *Hepatology* 2002, **35:** 579-588.

58. Zatelli MC, Luchin A, Piccin D, Tagliati F, Bottoni A, Vignali C *et al.*: **Cyclooxygenase-2 inhibitors reverse chemoresistance phenotype in medullary thyroid carcinoma by a permeability glycoprotein-mediated mechanism.** *J Clin Endocrinol Metab* 2005, **90:** 5754-5760.

59. Fantappie O, Solazzo M, Lasagna N, Platini F, Tessitore L, Mazzanti R: **P-glycoprotein mediates celecoxib-induced apoptosis in multiple drug-resistant cell lines.** *Cancer Res* 2007, **67:** 4915-4923.

60. Patel VA, Dunn MJ, Sorokin A: **Regulation of MDR-1 (P-glycoprotein) by cyclooxygenase-2.** *J Biol Chem* 2002, **277:** 38915-38920.

61. Sansbury LB, Millikan RC, Schroeder JC, North KE, Moorman PG, Keku TO *et al.*: **COX-2 polymorphism, use of nonsteroidal anti-inflammatory drugs, and risk of colon cancer in African Americans (United States).** *Cancer Causes Control* 2006, **17:** 257-266.

Table 1. Baseline characteristics of study participants selected from the Danish Diet, Cancer and Health prospective cohort study.

	Cases		Sub-cohort		IRR[a] (95% CI)	
	No. (%)	Median (5-95%)	No. (%)	Median (5-95%)		
Total	359 (100)		765 (100)			
Gender						
Men	200 (56)		419 (55)			
Women	159 (44)		346 (45)			
Age at inclusion		59 (51-64)		56 (50-64)		
Topology						
Proximal segment of colon	42 (12)		-			
Distal segment of colon	142 (40)		-			
Rectal	129 (36)		-			
Not specified	46 (13)		-			
BMI, kg/m^2		26 (21-34)		26 (20-33)	1.02[b]	(0.96-1.09)
Food intake						
Alcohol, g/day		14 (1-69)		13 (1-64)	1.06[b]	(1.00-1.13)
Red meat, g/day		82 (36-170)		82 (32-175)	1.02[b]	(0.94-1.12)
Processed meat, g/day		26 (6-80)		26 (4-78)	1.00[b]	(0.85-1.19)
Dietary fibers g/day		20 (10-32)		21 (11-34)	0.62[b]	(0.37-1.02)
Smoking status at inclusion						
Never	111 (31)		258 (34)		1.00	-
Former	112 (31)		239 (31)		1.02	(0.73-1.42)
Present	136 (38)		268 (35)		1.15	(0.83-1.61)
NSAID use						
No	244 (68)		528 (69)		1.00	-
Yes	115 (32)		237 (31)		1.07	(0.80-1.42)

HRT use among women				
Never	93 (58)	181 (52)	1.00	-
Former	25 (16)	60 (17)	0.68	(0.39-1.18)
Present	41 (26)	105 (30)	0.70	(0.44-1.10)

[a] Mutually adjusted.

[b] BMI pr. 2 kg/m^2. Alcohol pr. 10 g/day. Red meat, processed meat and dietary fibers pr 25 g/day.

Observed median values (5-95 percentiles) or percents of the distribution of alcohol, NSAID, smoking and potential colorectal cancer confounders among cases and members of the comparison group.

Table 2. Incidence rate ratio for colorectal cancer for the studied gene polymorphisms.

	N_{Case}	$N_{Sub\text{-}cohort}$	IRR[a]	(95% CI)	IRR[b]	(95% CI)	P-value[c]
MDR1 G-rs3789243-A							
GG	81	224	1.00	-	1.00	-	0.03
GA	194	365	1.55	(1.13-2.12)	1.55	(1.12-2.14)	
AA	84	176	1.43	(0.98-2.08)	1.45	(0.99-2.13)	
GA and AA	278	541	1.51	(1.12-2.04)	1.52	(1.12-2.06)	
MDR1 C3435T							
CC	73	118	1.00	-	1.00	-	0.11
CT	174	385	0.69	(0.49-0.99)	0.74	(0.51-1.07)	
TT	112	262	0.66	(0.45-0.96)	0.66	(0.45-0.98)	
CT and TT	286	647	0.68	(0.48-0.95)	0.71	(0.50-1.00)	
COX-2 G-765C							
GG	267	566	1.00	-	1.00	-	0.91
CG	83	186	1.02	(0.75-1.39)	1.03	(0.75-1.41)	
CC	9	13	1.45	(0.61-3.48)	1.21	(0.49-2.98)	
CG and CC	92	199	1.05	(0.78-1.41)	1.04	(0.77-1.42)	
COX-2 A-1195G							
AA	230	482	1.00	-	1.00	-	0.88
AG	116	258	0.94	(0.71-1.24)	0.93	(0.70-1.23)	
GG	13	25	0.90	(0.44-1.85)	0.94	(0.45-1.95)	
AG and GG	129	283	0.93	(0.71-1.22)	0.93	(0.71-1.23)	
COX-2 T8473C							
TT	147	315	1.00	-	1.00	-	0.37
CT	178	355	1.12	(0.85-1.48)	1.11	(0.83-1.47)	
CC	34	95	0.82	(0.52-1.29)	0.81	(0.51-1.28)	
CT and CC	212	450	1.06	(0.81-1.38)	1.05	(0.80-1.37)	
BRCP C421A							

CC	296	592	1.00	-	1.00	-	0.16
CA	58	161	0.72	(0.51-1.01)	0.71	(0.50-1.01)	
AA	5	12	1.10	(0.38-3.21)	1.17	(0.39-3.57)	
CA and AA	63	173	0.74	(0.53-1.03)	0.73	(0.52-1.04)	

[a]Crude

[b]Adjusted for status of HRT, smoking status, alcohol, dietary fibre, red meat, BMI and NSAID.

[c]p-value for trend.

Table 3. Interaction between intake of red and processed meat and *MDR1* and *BCRP* polymorphisms in relation to CRC risk.

	N_{Case}	$N_{Sub\text{-}cohort}$	IRR[a]	(95% CI)	IRR[b]	(95% CI)	P-value[c]
MDR1 G-rs3789243-A							
GG	81	224	0.95	(0.89-1.02)	0.95	(0.89-1.02)	0.01
GA and AA	278	541	1.03	(0.98-1.08)	1.03	(0.98-1.09)	
MDR1 C3435T							
CC	73	118	1.07	(1.00-1.15)	1.08	(1.00-1.16)	0.02
CT and TT	286	647	1.00	(0.94-1.05)	1.00	(0.95-1.06)	
BRCP C421A							
CC	296	592	1.02	(0.97-1.07)	1.02	(0.97-1.08)	0.40
CA and AA	63	173	0.99	(0.91-1.07)	0.99	(0.91-1.08)	

[a]Crude

[b]Adjusted for status of HRT, smoking status, alcohol, dietary fibre, red meat, BMI and NSAID.

[c]p-value for trend.

Table 4. Interactions between nonsteroidal anti-inflammatory drug (NSAID) use and *MDR1, COX-2* and *BCRP* polymorphisms.

Polymorphism	N_{case} / $N_{sub\text{-}cohort}$		IRR[a] (95% CI)				IRR[b] (95% CI)				p-value[c]
	NSAID[d]		NSAID[d]				NSAID[d]				
MDR1 C3435T	NO	YES	NO		YES		NO		YES		
CC	41/79	32/39	1.00	-	2.21	(1.17-4.17)	1.00	-	2.34	(1.22-4.48)	0.001
CT and TT	204/449	82/198	0.92	(0.60-1.41)	0.83	(0.52-1.33)	0.99	(0.63-1.54)	0.86	(0.53-1.39)	
MDR1 G- rs 3789243-A											
GG	54/160	27/64	1.00	-	1.35	(0.77-2.35)	1.00	-	1.31	(0.74-2.32)	0.26
GA and AA	191/368	87/173	1.65	(1.15-2.38)	1.65	(1.09-2.49)	1.66	(1.15-2.41)	1.61	(1.06-2.46)	
COX-2 G-765C											
GG	180/397	87/169	1.00	-	1.22	(0.89-1.69)	1.00	-	1.20	(0.86-1.67)	0.06
CG and CC	65/131	27/68	1.22	(0.86-1.75)	0.93	(0.57-1.51)	1.23	(0.85-1.78)	0.90	(0.54-1.48)	
COX-2 A-1195G											
AA	156/329	74/153	1.00	-	1.05	(0.75-1.48)	1.00	-	0.99	(0.69-1.41)	0.46
AG and GG	89/199	40/84	0.91	(0.66-1.27)	1.04	(0.67-1.60)	0.88	(0.63-1.23)	1.04	(0.67-1.61)	
COX-2 T8473C											
TT	94/222	53/93	1.00	-	1.42	(0.92-2.18)	1.00	-	1.38	(0.89-2.14)	0.04
CT and CC	151/306	61/144	1.23	(0.89-1.70)	1.09	(0.73-1.62)	1.22	(0.87-1.69)	1.05	(0.70-1.58)	
BCRP C421A											
CC	199/414	97/178	1.00	-	1.20	(0.88-1.63)	1.00	-	1.18	(0.85-1.62)	0.09
CA and AA	46/114	17/59	0.86	(0.58-1.27)	0.62	(0.35-1.10)	0.86	(0.57-1.29)	0.60	(0.33-1.08)	

[a]Crude

[b]Adjusted for status of HRT, smoking status, alcohol, dietary fibre, red meat and BMI.

[c]p-value for interaction.

[d]Study subjects were classified according to use of "any NSAID" (≥ 2 pills per month during one year) at baseline