# Gene-gene and gene-environment interactions in prostate, breast and colorectal cancer

Tine Iskov Kopp
PhD Thesis
2015

DTU Food
National Food Institute

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# Data sheet

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ISBN: 978-87-93109-37-7

Front cover: Sussanne Carlsson; foto: Colourbox.

## List of papers included in this PhD thesis

This PhD thesis consists of the following papers. They are referred to in the text by their roman numbers:

I. Kopp TI, Friis S, Christensen J, Tjonneland A, Vogel U. Polymorphisms in genes related to inflammation, NSAID use, and the risk of prostate cancer among Danish men. *Cancer Genet.* 2013;206(7-8):266-278.

II. Kopp TI, Andersen V, Tjonneland A, Vogel U. Polymorphisms in *NFKB1* and *TLR4* and interaction with dietary and life style factors in relation to colorectal cancer in a Danish prospective case-cohort study. *Accepted for publication in PLoS One.*

III. Kopp TI, Andersen V, Tjonneland A, Vogel U. Polymorphisms in ABC transporter genes and interaction with diet and life style factors in relation to colorectal cancer in a Danish prospective case-cohort study. *Submitted to Cancer Genetics.*

IV. Andersen V, Kopp TI, Tjonneland A, Vogel U. No association between HMOX1 and risk of colorectal cancer and no interaction with diet and lifestyle factors in a prospective Danish case-cohort study. *Accepted for publication in International Journal of Molecular Sciences.*

V. Kopp TI, Jensen DM, Ravn-Haren G, Cohen A, Sommer HM, Dragsted LO, Tjonneland A, Hougaard DM, Vogel U. Alcohol-related breast cancer in postmenopausal women – effect of *CYP19A1*, *PPARG* and *PPARGC1A* polymorphisms on female sex-hormone levels and interaction with alcohol consumption and NSAID usage. *Manuscript in preparation.*

VI. Kopp TI, Lundqvist J, Petersen RK, Oskarsson A, Kristiansen K, Nellemann C, Vogel U. In vitro screening of inhibition of PPARγ activity as a first step in identification of potential breast carcinogens. *Accepted for publication in Human & Experimental Toxicology.*

## Related publications not included in this thesis

Andersen V, Holst R, Kopp TI, Tjonneland A, Vogel U. Interactions between diet, lifestyle and *IL10*, *IL1B*, and *PTGS2/COX-2* gene polymorphisms in relation to risk of colorectal cancer in a prospective Danish case-cohort study. *PLoS One.* 2013;8(10):e78366.

Vogel LK, Saebo M, Hoyer H, Kopp TI, Vogel U, Godiksen S, Frenzel FB, Hamfjord J, Bowitz-Lothe IM, Johnson E, Kure EH, Andersen V. Intestinal PTGS2 mRNA levels, *PTGS2* gene polymorphisms, and colorectal carcinogenesis. *PLoS One*. 2014;9(8):e105254.

Andersen V, Vogel LK, Kopp TI, Saebo M, Nonboe AW, Hamfjord J, Kure EH, Vogel U. High *ABCC2* and low *ABCG2* gene expression are early events in the colorectal adenoma-carcinoma sequence. *In review with PLoS One.*

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# Preface and acknowledgements

The present PhD project was carried out at Technical University of Denmark (DTU), the National Food Institute, Dept. of Toxicology and Risk Assessment in corporation with the Danish Cancer Society Research Center (DCRC). The project was funded by a PhD mobility grant (09-06 7572) from the Danish Council for Independent Research in the program 'An integrated approach to risk-benefit assessment of human health effects of food and food contaminants' (Forskeruddannelse 2009-10). Originally, this PhD project was initiated to investigate the association between Vitamin D and prostate cancer risk using biological material from the Danish "Diet, Cancer and Health" (DCH) cohort from the DCRC. However, as a consequence of some very unfortunate incidents, including the devastating flood at the Danish Cancer Society, this project was preliminary terminated, and we decided to pursue a hypothesis linking alcohol consumption, increased circulating estrogens and incidence of breast cancer. Later on, I got involved with the project ''Beef versus pork consumption in the etiology of cancers in the colon and rectum: investigations performed within the Diet, Cancer and Health cohort'' supported by the Danish Council for Independent Research I Medical Sciences (grant no. 09-073597), where my part was to investigate possible mechanisms explaining the association between red and processed meat and risk of colorectal cancer by use of genetic epidemiology. Due to the flood at the Danish Cancer Society, we were not able to update the study groups, which was especially problematic for *Paper I* which only included 370 prostate cancer cases.

I would like to thank everyone who has contributed to the making of this PhD thesis, especially my supervisor Ulla Vogel – without you this thesis would never have made it all the way through! Gitte Ravn-Haren and Christine Nellemann are thanked for daily and encouraging support at DTU, and Anne Tjønneland for giving me the opportunity to use biological material from the DCH cohort and helping me out with questions regarding the cohort.

Furthermore, I would like to thank:

- Lars O. Dragsted for giving me the opportunity to conduct the clinical intervention trial at his section "Bioactive Foods and Health" at NEXS, University of Copenhagen (UC); and Ümmühan Celik, Hanne L. Petersen, Lene S. D. Svensson and Annette Landin for technical assistance in relation to the trial.
- Karsten Kristiansen for allowing me to carry out the *in vitro* study at the Dept. of Biology at UC; and Rasmus K. Petersen for his help and guidance with the HEK293 cell assay.
- David Overgaard, Arieh Cohen and Susan S. Laursen at Statens Serum Institute for hormone measurements to the clinical intervention trial.
- Helle M. Sommer from DTU and Jane Christensen from the DCRC for priceless statistical support.
- Katja Boll and Nick Martinussen from the DCRC for DCH data management support.
- All my colleagues at the Dept. of Toxicology and Risk Assessment, especially fellow PhD students.
- Vibeke Andersen and Lotte Vogel for integrating me in their projects.

Last, but not least, my loving husband, Carsten, and two wonderful daughters, Maja and Dicte, are thanked for supporting and encouraging me throughout the entire project.

# Abstract in English

The incidence of cancer in the western world has increased steeply during the last 50 years. For three of the most prevalent cancer types in Denmark, prostate, breast and colorectal cancer (PC, BC and CRC, respectively), only a small fraction (1-15%) of the incidences are caused by highly penetrant single-gene mutations due to their low frequency in the general population. Overall, the contribution from hereditary factors to the causation of BC is only 27%, whereas genetics contributes to 35% and 42% for CRC and PC, respectively. Additionally, immigrations studies point to environmental factors as having strong influence on carcinogenesis. Therefore, very frequent, low effect polymorphisms may have a greater contribution on a population level in combination with environmental factors. Indeed, several dietary and life style factors are now well-established risk factors for different cancer types, such as alcohol consumption, smoking, obesity, inflammation and high meat intake; whereas other factors protect against cancer, such as high intake of dietary fibre, fruits and vegetables, and physical activity. Investigating the interactions between genetic variations and environmental factors, such as dietary and lifestyle factors may provide information about the underlying mechanisms and reveal new biological pathways.

The aim of this PhD thesis was to investigate relevant risk factors in relation to the three major cancer types in Denmark: PC, BC and CRC, respectively. The two major risk factors examined in this thesis are inflammation and alcohol consumption. With regard to inflammation, biological pathways involved in inflammation and the interaction with different dietary and lifestyle factors modulating the risk of CRC (*Paper II-IV*) and PC (*Paper I*), respectively, was examined. Moreover, a possible mechanism in alcohol-related BC in postmenopausal women involving a specific polymorphism in *PPARG* (coding the peroxisome proliferator-activated receptor (PPARγ)) and its interaction with the aromatase (encoded by *CYP19A1*) was investigated (*Paper V-VI*).

The Danish prospective “Diet, Cancer and Health” cohort study was used to examine gene-gene and gene-environment interactions in relation to risk of cancer (*Paper I-V*). A human intervention trial (*Paper V*) was conducted in order to directly examine the effect on concurrent use of nonsteroidal anti-inflammatory drugs (NSAIDs) and alcohol consumption on circulating female sex-hormones. Finally, *in vitro* assays were performed to study the effect on PPARγ transactivation and sex-hormone concentrations following exposure to other commonly used organic solvents than alcohol (*Paper VI*).

Based on the results from *Paper I*, inflammation did not seem to be a major risk factor for aggressive PC, whereas the results on non-aggressive PC were equivocal. In contrast, *Paper II* and *III* indicated that the immune system is indeed involved in the carcinogenesis of CRC. Carriage of a pro-inflammatory allele of the *NFKB1* gene, was not associated with aggressive PC risk, but was associated to lowered risk of non-aggressive PC, and to increased risk of CRC. Even though none of the results were strong statistically, they demonstrated that cancer is a very heterogeneous disease; and indicated that inflammation may not be a risk factor for (aggressive) PC, but in relation to CRC, inflammation seems important. In *Paper III*, a new possible mechanism involving the ATP-binding cassette (ABC) transporters, the cytokine interleukin (IL) 10

and dietary fibre in relation to protection against inflammation-induced CRC was found. Furthermore, use of NSAIDs seemed to interact with the ABC transporters and IL-10 in relation to CRC.

*Paper V* illustrated that genetic variations in *CYP19A1* predicts circulating sex-hormone levels in post-menopausal women, and that alcohol intake affects female sex-hormone concentrations in the blood. However, it was not possible to put PPARγ and the aromatase in the same pathway as hypothesized *a priori* in alcohol-related BC; and the possible effect modification of concurrent use of NSAIDs and alcohol consumption was not confirmed. Nevertheless, results from *Paper VI*, indicated that exposure to commonly used organic solvents may act via PPARγ modulating sex-hormone levels. However, whether there is a common mechanism linking the aromatase and PPARγ, and also whether the differences in hormone levels increases risk of BC, still needs to be elucidated. Furthermore, these studies illustrated that acute and chronic alcohol consumption may have different effects on sex-hormone biosynthesis and metabolism, and that it is not straightforward to compare observational studies with experimental studies.

Overall, this PhD thesis has shown that genetic epidemiology can be used to study biological mechanisms in combination with other mechanistic studies, although there are several limitations involved such as missing knowledge of confounders and limited statistical power to study gene-environment interactions.

Future research could establish whether and how dietary fibre, IL-10 and ABC transporters are connected in reducing the risk of CRC; and whether red meat *per se*, specific preparation methods or the life style associated with high red meat intake is carcinogenic. The acquired knowledge would improve the current dietary recommendations.

There also seem to be several yet unknown effects of NSAID usage that need to be clarified. Information of these potential (side) effects would lead to better and safer medication regimens and, hence, improved public health. Also, further knowledge of the harmful health effects related to alcohol consumption, including the potential effect modification with concurrent use of NSAIDs, would lead to improved public preventive strategies.

# Dansk resumé

Cancer incidensen i Vesten er steget stødt de sidste 50 år. For tre af de mest prævalente cancer typer i Danmark, prostata-, bryst- og kolorektal cancer, er kun en lille del (1-15%) af incidensen forårsaget af høj-penetrans enkelt-genmutationer pga. deres lave frekvens i populationen. Generelt set bidrager nedarvede faktorer til årsagsforhold for bryst cancer kun med 27%, hvorimod genetik bidrager med 35% og 42% for henholdsvis kolorektal- og prostata cancer. Derudover indikerer immigrationsstudier at miljø-faktorer har en stærk indflydelse på karcinogenesen. Derfor kan hyppige, lav-effekt polymorfier have et større bidrag på populationsniveau i kombination med miljøfaktorer. Flere kost- og livstilsfaktorer er nu veletablerede risikofaktorer for forskellige cancer typer, såsom indtag af alkohol, rygning, fedme, inflammation og højt indtag af kød; hvorimod andre faktorer beskytter mod cancer, såsom et højt indtag af kostfibre, frugt og grønt, og fysisk aktivitet. Ved at undersøge samspillet mellem genetiske variationer og miljøfaktorer, som kost- og livsstilsfaktorer, kan der opnås viden om de bagvedliggende mekanismer og afdækkes nye biologiske veje.

Formålet med denne ph.d. afhandling var at undersøge relevante risikofaktorer i forhold til de tre store cancer typer i Danmark: prostata-, bryst- og kolorektal cancer. De to primære risikofaktorer som blev undersøgt i denne afhandling var inflammation og alkohol indtag. I forhold til inflammation, blev biologiske veje der er involveret i inflammation og disses samspil med forskellige kost- og livsstilsfaktorer som ændrer på risikoen for henholdsvis kolorektal- (*Paper II-IV*) og prostata (*Paper I*) cancer undersøgt. Ydermere blev en mulig mekanisme i alkoholrelateret bryst cancer hos postmenopausale kvinder undersøgt (*Paper V-VI*). Denne mekanisme involverer en specifik polymorfi i *PPARG* (som koder for peroxisome proliferator-activated receptor (PPARγ)) og interaktion med aromatasen (som er kodet af *CYP19A1*).

Det danske prospektive "Kost, Kræft og Helbred" kohorte studie blev brugt til at undersøge gen-gen og gen-miljø interaktioner i forhold til risikoen for cancer (*Paper I-V*). Et humant interventions studie (*Paper V*) blev udført for direkte at undersøge effekten af samtidig indtag af non-steroide anti-inflammatoriske lægemidler (NSAID) og alkohol på cirkulerende kvindelige kønshormoner. Slutteligt blev der udført nogle *in vitro* forsøg for at undersøge effekten på PPARγ trans-aktivering og kønshormon niveau efter eksponering af andre almindeligt brugte organiske opløsningsmidler end alkohol (*Paper VI*).

På baggrund af resultaterne fra *Paper I*, syntes inflammation ikke at være en betydelig risikofaktor for aggressiv prostata cancer, hvorimod resultaterne fra non-aggressiv prostata cancer var mere tvetydige. Derimod indikerede *Paper II* og *III* at immunforsvaret er involveret i kolorektal karcinogenesen. At være bærer af det pro-inflammatoriske allel fra *NFKB1* genet var ikke associeret med aggressiv prostata cancer risiko, men var derimod associeret med nedsat risiko for non-aggressiv prostata cancer, og med øget risiko for kolorektal cancer. Selvom ingen af resultaterne var statistisk stærke, viste de at cancer er en meget heterogen sygdom; og indikerede at inflammation højst sandsynlig ikke er en risikofaktor for (aggressiv) prostata cancer, men tyder på at være vigtig i forhold til kolorektal cancer. I *Paper III* blev en ny mekanisme der involverer de ATP-bindende cassette (ABC) transportere, cytokinet interleukin (IL) 10 og kostfibre i

forhold til beskyttelse mod inflammations-induceret kolorektal cancer fundet. Ydermere tydede resultaterne på at brug af NSAID interagerer med ABC transporterne og IL-10 i forhold til kolorektal cancer.

*Paper V* illustrerede at genetiske variationer i *CYP19A1* prædikterer cirkulerende kønshormon niveauer hos postmenopausale kvinder, og at indtag af alkohol påvirker koncentrationen af kvindelige kønshormoner i blodet. Dog var det ikke muligt at anbringe PPARγ og aromatasen i den samme biologiske vej som antaget *a priori* i forhold til alkoholrelateret bryst cancer; og den formodede effekt modifikation af samtidigt brug af NSAID og alkohol blev ikke bekræftet. Ikke desto mindre indikerede resultaterne fra *Paper VI* at eksponering til almindeligt brugte organiske opløsningsmidler muligvis ændrer på kønshormonniveauer via PPARγ. Men om der er en fælles mekanisme som linker aromatasen og PPARγ; og ligeledes om ændringerne i hormonniveau øger risikoen for bryst cancer, er stadig uopklaret. Disse studier viste desuden at akut og kronisk alkoholindtag muligvis har forskellig effekt på kønshormon syntese og metabolisme, og at det ikke er ligetil at sammenligne observationelle studier med eksperimentelle studier.

Overordnet har denne ph.d. afhandling vist at genetisk epidemiologi kan bruges til at studere biologiske mekanismer i kombination med andre mekanistiske studier på trods af flere begrænsninger såsom manglende viden om confounding og begrænset statistisk power til at undersøge gen-miljø interaktioner.

Fremtidige studier vil kunne påvise om og hvordan kostfibre, IL-10 og ABC transportere er forbundet i forhold til at nedsætte risikoen for kolorektal cancer; og afklare hvorvidt rødt kød *per se*, tilberedelses metoden eller livsstilen, som er associeret med højt kød indtag, er karcinogent. Denne viden vil kunne forbedre de nuværende kostanbefalinger.

Der synes også at være adskillige hidtil ukendte effekter af NSAID forbrug som er nødvendige at klarlægge. Viden om disse mulige (bi-)virkninger ville kunne medføre bedre og mere sikker medicinering og derfor også forbedret folkesundhed. Yderligere viden om de sundhedsskadelige effekter som er relateret til indtag af alkohol, inklusiv den mulige effekt modificering af samtidig brug af NSAID, ville også føre til forbedrede forebyggende folkesundhedsstrategier.

# Abbreviations

| | |
|---|---|
| ABC: | ATP-binding cassette |
| AICR: | American Institute for Cancer Research |
| ARE: | Adelylate- and uridylate-rich element |
| BC: | Breast cancer |
| BCRP: | Breast cancer resistance protein |
| BMI: | Body mass index |
| CD: | Crohns' disease |
| CO: | Carbon monoxide |
| COX: | Cyclooxygenase |
| CRC: | Colorectal cancer |
| DCH: | Diet, Health and Cancer |
| EGFR: | Epidermal growth factor receptor |
| EP1-4: | $PGE_2$ cognate receptors 1-4 |
| ER: | Estrogen receptor |
| FFQ: | Food frequency questionnaires |
| GWAS: | Genome-wide association study |
| HCA: | Heterocyclic amine |
| HO-1: | Heme-oxygenase 1 encoded by *HMOX1* |
| HRT: | Hormone replacement therapy |
| $HXA_3$: | Eicosanoid hepoxilin $A_3$ |
| IBD: | Inflammatory bowel disease |
| IFN: | Interferon |
| IL: | Interleukin |
| IL-1Ra: | IL-1 receptor antagonist |
| IL-1R1/2: | IL-1 type 1 and 2 receptors |
| iNOS: | Inducible nitric oxide synthase |
| LPS: | Lipopolysaccharide |
| LRH-1: | Liver receptor homolog-1 |
| MAPK: | Mitogen-activated protein kinase |
| MDR: | Multidrug-resistance |
| MMP: | Matrix metalloprotease |
| MRP2: | Multidrug-resistance-associated protein 2 (encoded by *ABCC2*) |
| n-3/6 PUFA: | Omega 3/6 polyunsaturated fatty acid |
| NF-κB: | Nuclear factor kappa B |
| NOC: | Nitrite and N-Nitroso compound |
| NO: | Nitric oxide |
| NSAID: | Nonsteroidal anti-inflammatory drug |
| PAH: | Polycyclic aromatic hydrocarbon |
| PC: | Prostate cancer |
| PG: | Prostaglandin, including $PGD_2$, $PGE_2$ and $PGF_2$ |
| PGC-1α: | Perosixome proliferator-activated receptor gamma coactivator 1-alpha |
| $PGI_2$: | Prostacyclin |
| $PGJ_2$: | $PGD_2$ metabolite 15-deoxy-12,14 |
| Pgp/MDR1: | P-glycoprotein/multidrug resistance 1 (encoded by *ABCB1*) |
| PHIP: | 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine |
| PI3K: | Phosphatidylinositol 3'-kinase |
| PPAR: | Peroxisome proliferator-activated receptor |
| PPRE: | Peroxisome proliferator response element |
| PUFA: | Polyunsaturated fatty acid |
| ROS: | Reactive oxygen specie |
| RXR: | Retinoic X-receptor |
| SCFA: | Short-chain fatty acid |
| TLR: | Toll-like receptor |
| TNF-α: | Tumor necrosis factor α |
| $TXA_2$: | Thromboxane $A_2$ |
| UC: | Ulcerative colitis |
| VCAM: | Vascular cell adhesion molecule |
| VEGF: | Vascular endothelial growth factor |
| WCRF: | World Cancer Research Fund |

# Chapter 1

## Background

## 1.1. General introduction

The incidence of cancer in the western world has increased steeply during the last 50 years. In Denmark, one out of three will get cancer during a lifetime [1]. For three of the most prevalent cancer types in Denmark, prostate, breast and colorectal cancer (PC, BC and CRC, respectively) (Figure 1), only a small fraction (1-15%) of the incidences are caused by highly penetrant single-gene mutations due to their low frequency in the general population [2,3]. In twin studies, it has been estimated that the overall contribution from hereditary factors to the causation of BC is only 27% [3]. Correspondingly, the genetic contribution to the causation of CRC and PC has been estimated to 35% and 42%, respectively [3]. Additionally, immigrations studies point to environmental factors as having strong influence on carcinogenesis [4-7]. Therefore, very frequent, low effect polymorphisms may have a greater contribution on a population level in combination with environmental factors [8]. Indeed, several dietary and life style factors are now well-established risk factors for different cancer types, such as alcohol consumption [9-14], smoking [12,13], obesity [12-14], inflammation [15] and high meat intake [12-14,16,17]; whereas other factors protect against cancer, such as high intake of dietary fibre [13,14,18], fruits and vegetables [14], and physical activity [13,14]. Investigating the interactions between genetic variations and environmental factors, such as dietary and lifestyle factors may provide information about the underlying mechanisms and reveal new biological pathways.

In order to examine molecular mechanisms that cause a specific disease, different approaches have been used – from molecular *in vitro* studies to large epidemiological studies or genome wide association studies (GWAS). *In vitro* studies have the possibilities of investigating biological pathways directly without having to take ethic aspects into consideration, but it is challenging to extrapolate results from *in vitro* work to the biology of the intact organism. Human intervention studies are very valuable within limitations due to ethical reasons. These limitations can to a certain point be overcome in animal studies, but animals are not humans. GWAS can bring information on new possible biological pathways which can be further investigated in candidate gene studies where the information of confounders is more detailed than in the very large GWAS. Results from observational studies, however, are only associations, which are not necessarily causal, and it is therefore important to include all available information on conducted studies regarding the mechanism of interest when inferring causality. However, observational studies are excellent in generating or possibly even confirming hypotheses. New hypotheses can establish the fundament to new research in so far unknown biological pathways.

The knowledge acquired from research on gene-environment interactions can be used to understand the underlying mechanism of cancer and thereby reduce cancer incidence in the population by implementing national preventive and treatment strategies.

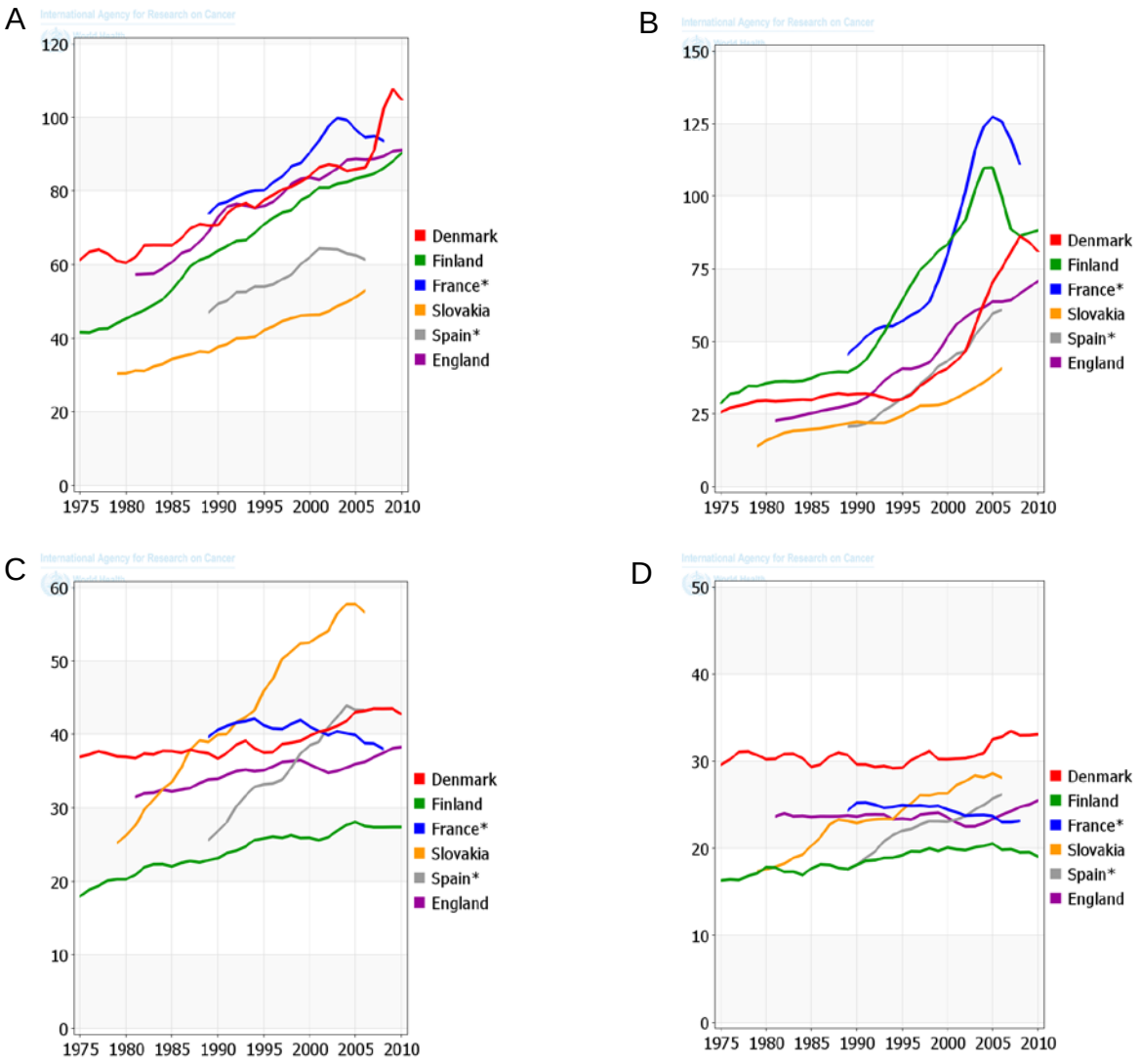


**Figure 1.** Age-standardized cancer rate per 100,000 in selected countries for breast, prostate and colorectal cancer, respectively. A. Breast cancer; B. Prostate cancer; C. Colorectal cancer among men; D. Colorectal cancer among women. Source: http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx.

## 1.2. General aims and objectives of this PhD thesis

The aim of this PhD thesis was to investigate relevant risk factors in relation to the three major cancer types in Denmark: PC, BC and CRC, respectively, primarily by use of genetic epidemiology, but also by *in vitro* studies and a human intervention trial to complement the observational findings. The two major risk factors examined in this thesis are inflammation and alcohol consumption. With regard to inflammation, biological pathways involved in inflammation and the interaction with different dietary and lifestyle factors modulating the risk of CRC and PC, respectively, will be described. Moreover, a description of a possible mechanism in alcohol-related BC in postmenopausal women involving a specific polymorphism in *PPARG* will be

presented. Hereafter, the Danish cohort and all polymorphisms investigated in *Paper I-V* are described. Finally, *Paper I-VI* are presented followed by a summarizing and supplementary discussion. Supplementary data (Tables and Figures termed "S" in front of the number) are provided in the Appendix section.

## 1.3. Inflammation-induced carcinogenesis

As early as 1863, Virchow speculated that there was an association between chronic inflammation and cancer. Virchow noticed "lymphoreticular infiltrate" at sites of cancer and hypothesized that these chronic inflammatory infiltrates reflected the origin of cancer [19]. Today, it is widely accepted that some cancers are caused by chronic inflammation, such as cancers of liver, colon, bladder, oesophagus and stomach. The molecular and cellular mechanisms mediating this association are very complex and can be caused by both infectious agents like *Helicobacter pyleri* and *Mycobacterium tuberculosis* and environmental factors like asbestos, UV irradiation, alcohol consumption, smoking and diet [15,20-22].

The term inflammation describes a local response to tissue injury. The cardinal signs of inflammation are rubor (redness), tumor (swelling), calor (heat), dolor (pain) and functio laesa (loss of function). It involves a vascular phase and a cellular phase. In the vascular phase, blood flow and capillary permeability increases leading to accumulation of blood proteins including components of the complement cascade. In the cellular phase, leukocytes (neutrophils, monocytes and eosinophils) and tissue mast cells migrate from the venous system to the site of inflammation. The neutrophils coordinate recruitment of the inflammatory cells to the site of tissue injury and the extracellular matrix where fibroblasts and endothelial cells proliferate and migrate in order to reconstruct the tissue: First, members of the selectin family of adhesion molecules are activated which facilitate rolling along the vascular epithelium. Secondly, leukocyte integrins are activated and up-regulated by cytokines and leukocyte-activating molecules. Third, the neutrophils are immobilized on the endothelium cells by integrins that binds to endothelial vascular cell-adhesion molecules. Fourth, the leukocytes migrate through the endothelium to the site of injury facilitated by extracellular proteases. At the site of inflammation, the chemokines – a family of chemotactic cytokines – recruits downstream effector cells and orchestrates the process of inflammation. At this point, it is extremely important that the cytokines/chemokines is tightly regulated resulting in fast resolution and wound healing. However, dysregulation of any of the inflammatory factors can lead to prolonged, chronic inflammation and ultimately neoplastic progression. After migration of the neutrophils to the site of injury, the monocytes, which differentiate into macrophages, are guided by chemotactic factors to the inflammation site. The activated macrophages are the main source of growth factors and cytokines which stimulate local endothelial, epithelial and mesenchymal cells to proliferate. Mast cells release histamine, cytokines, reactive oxygen species (ROS), nitric oxide (NO), different matrix metalloproteases (MMPs), and lipid mediators (see section 1.3.2.). Thus, chronic inflammatory states are characterized by an increased cell turnover, a large amount of growth factors, and activated phagocytic macrophages producing ROS and NO that are capable of damaging cells and DNA. Proliferation of epithelial cells in this environment may cause substantial potential for mutational and promoting effects. Therefore, chronic inflammatory states possess the potential of being both an initiator and a promoter of carcinogenesis [15,23-26].

One of the most convincing pieces of evidence linking inflammation and cancer, arises from several human observational and clinical studies illustrating that long-term regular use of Aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) reduces the incidence of cancer; particularly in CRC and other gastro-intestinal cancers [27-36]. Moreover, animal and *in vitro* studies have demonstrated that NSAIDs also are capable of inhibiting PC [37-40]. However, the observational data are less consistent although the majority of the studies point to a reduced risk of PC among long-time users of NSAIDs [29,41-56]. Additionally, numerous genetic epidemiological studies have found associations between polymorphisms in inflammatory genes and risk of cancer [57-62], and inflammatory bowel diseases (IBD) e.g., ulcerative colitis (UC) and Crohns' disease (CD), known to predispose to CRC [63-66].

### 1.3.1. Nonsteroidal anti-inflammatory drugs

NSAIDs function by inhibition of prostanoid synthesis when the drugs bind to cyclooxygenase (COX), an inducible pro-inflammatory enzyme that converts arachidonic acid to a range of prostaglandins (PGs) (see section 1.3.2.) [67]. Aspirin and ibuprofen are two of the classical NSAIDs. They are further divided in aspirin and non-aspirin (NA) NSAIDs due to differences in mechanisms of action. Only higher doses of aspirin inhibit the COX-2 isoform. The classical NSAIDs encompass anti-inflammatory, anti-pyretic and analgesic effects, and inhibit both COX-1 and COX-2 enzymes. Selective COX-2 inhibitors, so-called coxibs (e.g. celecoxib), are a newer class of NSAIDs that have little effect on COX-1, but potently inhibit COX-2 and therefore reduce the incidence of gastrointestinal ulcers and erosions associated with use of classical NSAIDs [68-70]. However, selective inhibition of COX-2 with coxibs blocks the formation of vasodilators (prostacyclins and PGs – see section 1.3.2.) and shifts the arachidonic acid metabolism to lipoxygenase pathway which may result in serious cardiovascular effects. Therefore, some of the selective COX-2 inhibitors have been withdrawn from the market and coxibs are now never used as routine therapy, but are reserved to patients in high risk of gastrointestinal bleeding [71]. In the present thesis, "NSAIDs" covers aspirin and NA-NSAID – not selective COX-2 inhibitors.

Due to the evidence of a protective effect of NSAIDs, it is reasonable to investigate functional polymorphisms in *COX-2* in relation to inflammation-induced cancer.

### 1.3.2. Cyclooxygenase-2 and its products, the prostanoids

COX or prostaglandin H2 synthase is the key enzyme in the synthesis of PGs from AAs and, as mentioned above, two isoforms of COX exist: COX-1 and COX-2 [72,73]. The two isoforms share significant sequence homology and catalytic activity, but their expression pattern is markedly different. COX-1 is often referred to as a "housekeeping" gene and is constitutively expressed in almost all tissues. The PGs produced by COX-1, are important for homeostatic functions. COX-2, in contrast, is an inducible enzyme with varying enzyme level due to different regulation in different tissues. COX-2 is significantly upregulated as a response to inflammation, and histological studies have found an upregulated COX-2 expression in several human cancers, including CRC [74-77] and PC [75,77-79].

Regulation of the *COX-2* gene expression is very complex since COX-2 is involved in numerous signalling pathways, depending on the specific stimulus and cell type (illustrated in Figure 2). The promoter region of the *COX-2* gene contains several potential transcriptional regulatory elements where transcription factors bind in many different ways [80-85]. Post-transcriptional regulation involves changes in mRNA stability [82,86]. The 3' untranslated region of *COX-2* contains multiple copies of adelylate- and uridylate-rich elements (AREs) composed of the sequence 5'-AUUUA-3'. The AUUUA motif, found in the 3' untranslated region, is characteristic of many unstable cytokine- and proto-oncogene-encoding mRNAs [87]. Specific ARE-binding factors bind to the AREs in response to interleukin (IL)-1β induced mitogen-activated protein kinase (MAPK) cascade signalling, and influence *COX-2* mRNA stability and also translational efficiency [88].

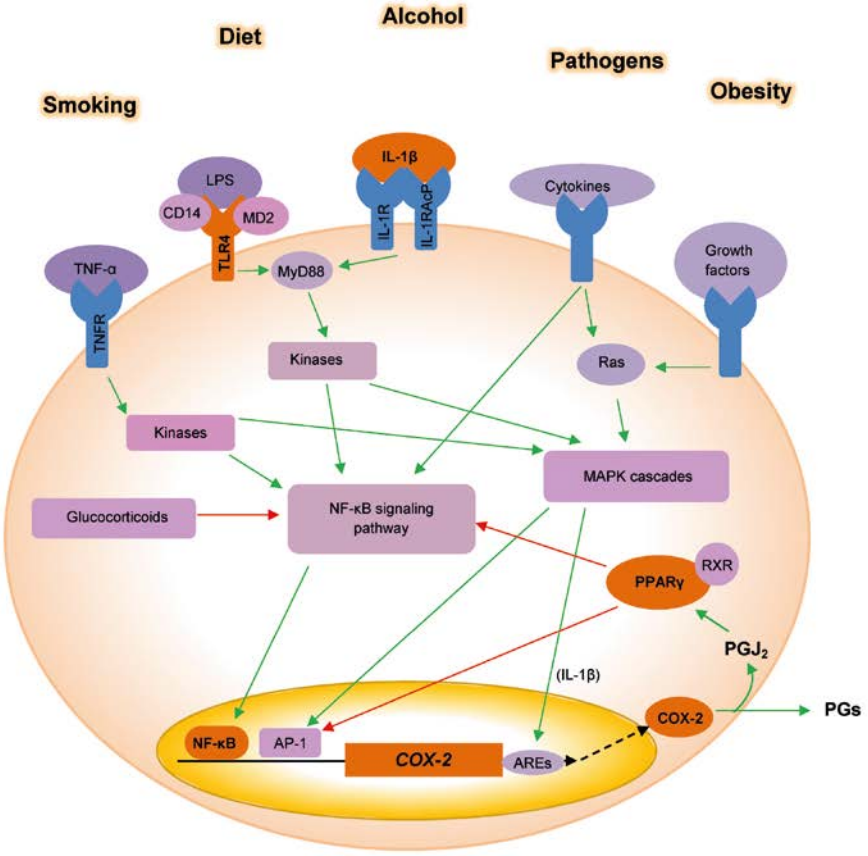


**Figure 2:** Simplified illustration of the main signalling pathways of *COX-2* gene induction and suppression including environmental triggers: *COX-2* gene expression is stimulated in response to several inducers such as pro-inflammatory factors, hormones, growth factors and oncogenes through receptor-mediated signalling and various signalling pathways including the mitogen-activated protein kinase cascade and the nuclear factor kappa B (NF-κB) signalling pathway [89]. The regulatory elements in the promoter region and in the 3' untranslated region regulate the *COX-2* gene expression in response to different signals. Peroxisome proliferator-activated receptor γ [90] and glucocorticoids [91] suppress *COX-2* gene expression by interfering with the NF-κB pathway and by inhibition of activator protein 1 [92]. Green arrows indicate induction and red arrows indicate suppression. Orange figures are the studied factors. AP: Activator protein; ARE: Adelylate- and uridylate-rich elements; CD14: Cluster of differentiation 14; COX: Cyclooxygenase; IL: Interleukin; LPS: Lipopolysaccharide; MAPK: Mitogen-activated protein kinase; PG: Prostaglandin; $PGJ_2$: $PGD_2$ metabolite 15-deoxy-12,14; ; PPARγ: Peroxisome proliferator-activated receptor γ; RXR: Retinoic X-receptor; TLR: Toll-like receptor; TNF: Tumor necrosis factor. (Based on [80,89-93]).

Prostanoids, which consist of prostacyclins ($PGI_2$), thromboxane $A_2$ ($TXA_2$) and PGs ($PGD_2$, $PGE_2$ and $PGF_2$), are paracrine and autocrine lipid mediators that coordinate intercellular events. They regulate the changes in blood flow required for leukocytes to exit the blood vessels in the initiation phase in inflammation, mediate a class switching of lipid mediators required for termination of the acute response, exert immunosuppressive effects by reducing the ability of leukocytes to phagocytosis and kill microorganisms, and are able to inhibit the production of downstream pro-inflammatory mediators. However, these PGs also contribute to fortification of the inflammatory response by enhancing and prolonging the signals of several pro-inflammatory mediators. Indeed, excess PGs has been shown to contribute to chronic inflammation [26,68,94]. Besides its role in inflammation, increasing evidence indicates that $PGE_2$ plays a dominant role in cancer progression [95-108], and increased $PGE_2$ production has been found in several types of neoplastic tissues compared to normal tissue [109]. The prostanoid $TXA_2$ has also been implicated in carcinogenesis by acting as a promoter of angiogenesis [110]. $PGE_2$ promotes tumour growth by binding to one of its four cognate receptors (EP1-4) which then activates downstream signalling pathways [27,111-113] as illustrated in Figure 3. In addition to COX-2, the epidermal growth factor receptor (EGFR) is activated in most human cancers [114]. $PGE_2$ can trans-activate EGFR, which results in stimulation of cell migration and invasion through increased phosphatidylinositol 3'-kinase/Akt signalling [95,100,115]. This enhances the cells ability to metastasise and is therefore often associated with a poor clinical outcome. The anti-apoptotic action of $PGE_2$ is mediated, among others, through activation of the apoptosis inhibitor, Bcl-2 [99] and peroxisome proliferator-activated receptor (PPAR) δ [98]. The ability to escape from apoptosis gives the cells a survival advantage, and may therefore increase resistance to chemotherapy. Activation of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor through EP1-4 leads to the formation of new blood vessels [102]. This property is important for the growth and survival of the tumour.

### 1.3.3. Nuclear factor kappa B

NF-κB is a transcription factor that, among others, regulates COX-2 expression [116]. NF-κBs are regulators of innate immune and inflammatory responses. In mammals, the NF-kB family consists of five members: p105/p50 (encoded by *NFKB1*), p52/p100 (encoded by *NFKB2*), p65/RelA (encoded by *RELA*), Rel/cRel (encoded by *REL*), and RelB (encoded by *RELB*) forming a variety of homodimers and heterodimers. As a p65/p50 heterodimer, the complex is pro-inflammatory, whereas the p50 homodimer has anti-inflammatory properties. In the inactive state, NF-κB is sequestered in the cytoplasm by inhibitory proteins called IκBs. In infectious or inflammatory states, signals such as pathogenic microorganisms, tumor necrosis factor α (TNF-α), Toll-like receptors (TLR) (see section 1.3.5.) or IL-1β (see section 1.3.4.) activate the dimers that lead to the phosphorylation, ubiquitylation, and consequent degradation of IκB. This allows for the NF-κB dimers to translocate into the nucleus and stimulate the transcription of specific genes via the NF-κB binding site in target genes [15,117-119].

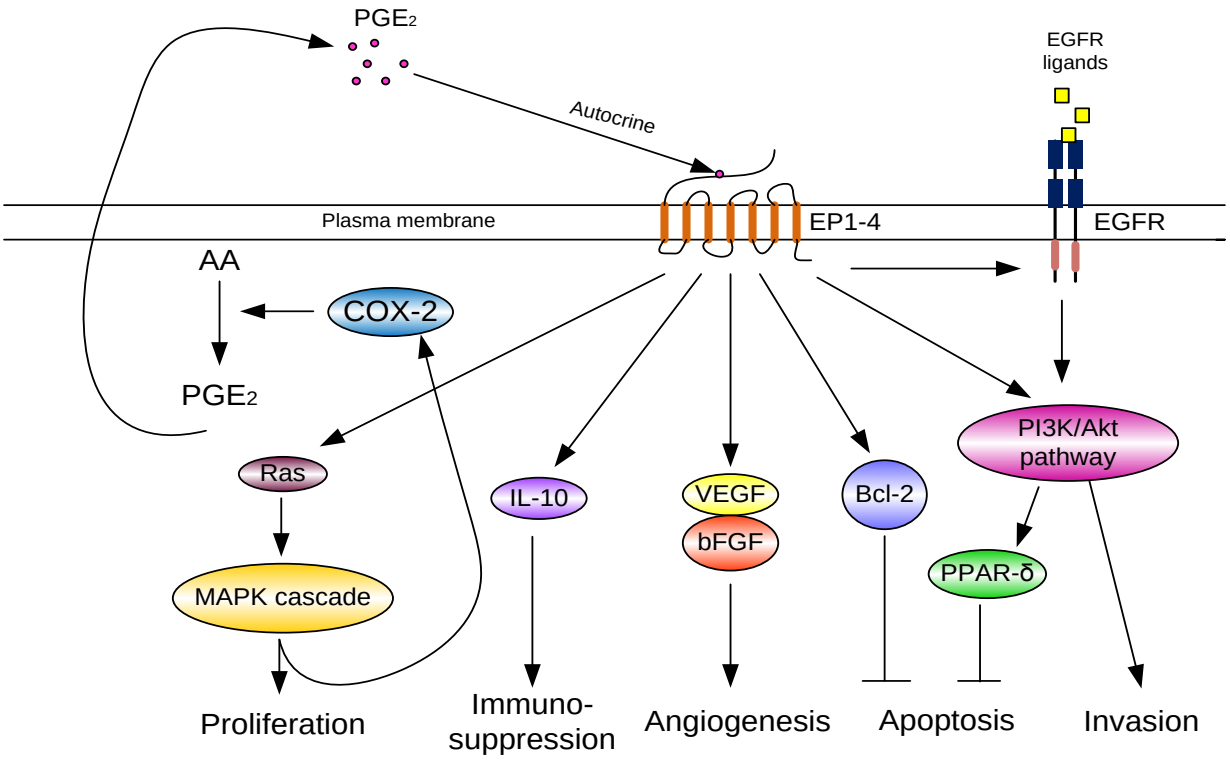


**Figure 3.** Cyclooxygenase 2-derived prostaglandin $E_2$ ($PGE_2$) in carcinogenesis. $PGE_2$ promotes tumour growth by stimulating $PGE_2$ receptor 1-4 downstream signalling and subsequent enhancement of cellular proliferation, suppression of immune responses, promotion of angiogenesis, and inhibition of apoptosis. Additionally, $PGE_2$ can activate epidermal growth factor receptor and thereby stimulate cell migration and invasion. AA: Arachidonic acid; Bcl-2: B-cell lymphoma 2; bFGF: basic fibroblast growth factor; COX-2: Cyclooxygenase 2; EGFR: Epidermal growth factor receptor; EP1-4: Prostaglandin cognate receptor 1-4; IL: Interleukin; MAPK: Mitogen-activated protein kinase; PI3K: Phosphatidylinositol 3'-kinase; PPAR: Peroxisome proliferator-activated receptor; VEGF: Vascular endothelial growth factor. (Based on [27]).

Dysregulation of NF-κB can lead to the constitutive overproduction of pro-inflammatory cytokines, which is associated with a number of chronic inflammatory disorders, including rheumatoid arthritis and CD and eventually leads to cancer [120-122]. Indeed, dysregulation of NF-κB has been found in several human cancers [119,123,124], and NF-κB encompasses the capacity of promoting cancer by several mechanisms. The molecules intercellular adhesion molecule 1, endothelial leukocyte-adhesion molecule 1 and vascular cell adhesion molecule 1 (VCAM-1) are all regulated by NF-κB [125-127] and mediate the ability to cross vessels which can lead to metastatic potential in tumor cells. Several angiogenic growth factors, e.g., VEGF and monocyte chemo-attractant protein 1 are also regulated by NF-κB [128-130]. These growth factors stimulate vascularization of tumor cells. NF-κB also regulates several tumor promoting and potential mutational genes apart from PGs via COX-2 [131], e.g., TNF-α [132], IL-1 [133], inducible nitric oxide synthase (iNOS) [134], MMPs [135], urokinase-type plasminogen activator [136] and many chemokines [137-139]. Finally, NF-κB possesses both pro-apoptotic (by regulating of e.g., Fas, c-myc, p53 and IκBα) and anti-apoptotic properties (by regulating of e.g., TNF receptor-associated factor 2, inhibitor of apoptosis proteins and Bcl-2 like proteins) [140-142]; and controls

regulation of cell cycle proteins (e.g., cyclin D1 and cyclin-dependent kinase 2) [143-145] which may increase cellular survival and proliferation.

Additionally, NSAIDs have been shown to inhibit NF-κB independent of COX inhibition [146]. Thus, inherent differences in NF-κB activity may modify the risk of inflammation-induced cancer and interact with NSAID intake.

### 1.3.4. Interleukin 1 beta

IL-1 is a pro-inflammatory cytokine that affects nearly all cell types and has an important regulatory role in inflammation and host defence; nearly all microbes and microbial products induce production of IL-1 proteins. The *IL-1* gene family consists of agonistic and antagonistic molecules, as well as receptors. IL-lα and IL-1β are the two major agonists, whereas IL-1 receptor antagonist (IL-1Ra) is a competitive antagonist of IL-1 that encompasses a signal peptide and requires transport to escape from the cell. IL-lα, IL-lβ and IL-1Ra are encoded by the three genes *IL1A*, *IL1B* and *IL1 RN,* respectively [147,148]. IL-lα and IL-lβ are processed into mature cytokines by specific proteases, and are agonists of cell membrane IL-1 type 1 and 2 receptors (IL-1R1 and 2). IL-1R1 is a signalling receptor, whereas IL-1R2 functions as a decoy target to reduce excessive amounts of IL-1 [149]. Upon binding to the IL-R1, the IL-1R acceptor protein is recruited. This heterodimeric complex triggers IL-1 signalling by activating an IL-1 receptor-associated kinase and MyD88 is recruited leading to the activation of NF-κB. IL-1β is induced by inflammatory mediators and is found in pathological fluids, whereas IL-1α is involved in homeostatic functions as an intracellular cytokine [147-149]. E.g., elevated levels of mucosal IL-1β have been detected in animal models of intestinal inflammation and in humans with IBD [150].

As mentioned in section 1.3.3., NF-κB regulates *COX-2* expression upon activation by IL-1. However, IL-1 is also able to stabilise the mRNA of *COX-2* [93]. As a result, COX-2 production is elevated for several hours and $PGE_2$ concentration increases steeply upon IL-1β stimulation [147]. Actually, many of the IL-1 induced changes are mediated by $PGE_2$ and NSAIDs are therefore potent inhibitors of IL-1-induced activity [147]. Moreover, IL-1β stimulates a cascade of other pro-inflammatory factors, e.g., iNOS, chemokines/cytokines and MMPs in an amplification loop where the molecules stimulate each other. This results in broad inflammation accompanied by tissue damage and immunosuppression [151]. Many of these properties alongside others make IL-1β capable of promoting carcinogenesis. Indeed, *IL-1β* knock out mice develop fewer tumors compared to IL-1α-deficient or wild type mice [152]. IL-1β also up-regulates expression of VEGF on endothelial cells providing a microenvironment for angiogenesis and tumor progression [153].

Thus, inherent variations in IL-1β activity may affect the risk of inflammation-induced cancer and interact with NSAID intake.

### 1.3.5. Toll-like receptor 4

The intestinal epithelium serves to protect the body from invading pathogens. However, a mutually beneficial relationship between the bacteria colonising the colon – the 'microbiota' - and the mucosal epithelium also exists, where the epithelial cells use short-chain fatty acids (SCFAs) produced by bacterial fermentation of

ingested dietary fibre, as a crucial energy source [154]. This complex interplay between the mucosal epithelium and the microbiota is orchestrated by the TLRs. At least 10 different TLRs exist in humans differing in their cellular locations and ligands [154]. The TLRs are type I transmembrane proteins with an extracellular domain consisting of a leucine-rich repeat region and an intracellular domain homologous to that of human IL1R [155]. The intracellular region mediates downstream signalling upon recognition of pathogen-associated molecular patterns which results in activation of one of two major signalling pathways: the MyD88-dependent pathway results in the activation of NF-κB and AP-1 and the TRIF-dependent pathway results in the activation of type I interferons (IFNs) [156]. Activation of TLR results in epithelial cell proliferation, IgA production, maintenance of tight junctions and antimicrobial peptide expression, which all contributes to maintaining a balance between microbiota and the mucosal immune system. However, they can also trigger pro-inflammatory responses by the underlying lamina propria immune cells [154]. Here, the TLR4 is central. TLR4 is the receptor for gram-negative bacterial lipopolysaccharide (LPS) together with its cofactors CD14 and MD2 which regulates cell proliferation in response to cell injury through induction of COX-2 expression in a cascade that involves activation of NF-κB resulting in the production of $PGE_2$ by the intestinal epithelial cells and epidermal growth factor [154,156-158]. This combination increases the potential for enhanced carcinogenesis in the setting of inflammation suggesting that TLR4 is an important element in the transition from inflammation to neoplasia. Certainly, increased expression of TLR4 has been linked to development of inflammation-associated neoplasia [159-162]. Thus, TLR4 is an important factor in inflammation-induced CRC and genetically determined alteration in TLR4 activity may modulate risk of CRC and also interact with intake of NSAIDs and dietary factors (see section 1.5.).

### 1.3.6. Peroxisome proliferator-activated receptor gamma

PPARγ is one of the three PPARs: PPARα, PPARδ and PPARγ [163,164]. The PPARs are members of the nuclear hormone receptor super family. They are ligand-dependent and form heterodimers with retinoic X-receptors (RXRs) and regulate transcription of various target genes after binding to their peroxisome proliferator response elements (PPREs). Four different isoforms of PPARγ exist generated from differential promoter usage and alternative splicing: PPARγ1, PPARγ2, PPARγ3 and PPARγ4. PPARs can be activated by fatty acids and PPARγ is involved in several mechanisms such as lipid metabolism, cell proliferation, adipocyte differentiation and insulin signalling [163,164].

However, PPARγ is also an essential anti-inflammatory modulator (see Figure 2). PPARγ inhibits COX-2 expression via NF-κB and AP-1, and inhibits mucosal production of inflammatory cytokines e.g., IL-1β and TNF-α, and chemokines, inhibits proliferation of inflammatory cells, represses generation of iNOS and MMPs and inhibits expression of adhesion molecules, mainly VCAM-1 [92,164-166]. The $PGD_2$ metabolite 15-deoxy-12,14 ($PGJ_2$) is a natural ligand for PPARγ; and COX-2 and PPARγ mRNA levels are inversely regulated through $PGJ_2$ in a negative feedback mechanism [90,165]. All of these properties provide PPARγ with important anti-cancer functions, which have been confirmed in several studies showing decreased expression of PPARγ in tumor tissue [167-171] and PPARγ ligand activation is a chemo-preventive target against cancer [172-174].

NSAIDs are known to activate PPARγ [175,176] and may therefore interact with genetically determined variations in PPARγ expression as well as different dietary and life style factors. Especially the interaction with alcohol has been investigated in this thesis. Therefore, in section 1.6., a further description of PPARγ in relation to alcohol-related BC in postmenopausal women will be discussed.

## 1.4. The ATP-binding cassette transporter family

The ATP-binding cassette (ABC) transporter superfamily is involved in both mucosal immune defence and interacts with dietary factors and drugs and will therefore be described separately in this section.

The ABC transporter superfamily consists of carrier proteins of major clinical significance due to their ability to transport drugs and to cause multidrug-resistance (MDR). They are also involved in homeostatic functions such as the excretion of lipids, bile salts, peptides for antigen presentation, toxic compounds from the liver, kidney and gastrointestinal tract and in limiting permeability of toxins to the brain, testes and placenta by forming a barrier. Genetic variations in these genes have various effects on their expression, mRNA stability, protein folding, intracellular localization, degradation, substrate binding, and/or transport kinetics [177-180], resulting in differences in the intestinal exposure of the transported compounds.

The transporter proteins bind ATP and use the energy to transport mostly hydrophobic components across the lipid bilayer of cell membranes. The functional transporter protein consists of four domains; two hydrophobic domains with 6-11 membrane-spanning α-helixes that determine the substrate specificity, and two ATP-binding catalytic domains in the inner side of the cell membrane. The ABC transporters are usually unidirectional and are formed either as full transporters containing two membrane-spanning domains and two ATP-binding domains. In other cases they are formed as homodimers or heterodimers that assemble into a full structure. The ABC genes are divided into seven ABC gene subfamilies based on gene-structure (half vs. full transporters), order of the different domains, and on sequence homology [181-183]. In this thesis, only three of the ABC transporter proteins will be described which are all involved in MDR: P-glycoprotein (Pgp)/MDR1 (encoded by *ABCB1*), multidrug-resistance-associated protein 2 (MRP2) (encoded by *ABCC2*) and breast cancer resistance protein (BCRP) (encoded by *ABCG2*). These transporter proteins are all efflux transporters and are expressed in various tissues including the apical surface of enterocytes where they mainly serve to restrict the body from exposure to potentially harmful substances (see Figure 4) [178,179,184,185]. Moreover, only their intestinal function will be described here. Apart from the ABC transporter superfamily, other efflux transporter proteins exists e.g., toxin extrusion proteins and transporter proteins that move substrates in to the cell – influx transporters – e.g., the organic anion transporters, organic cation transporters, oligopeptide transporters, etc., some of which are bidirectional [178].

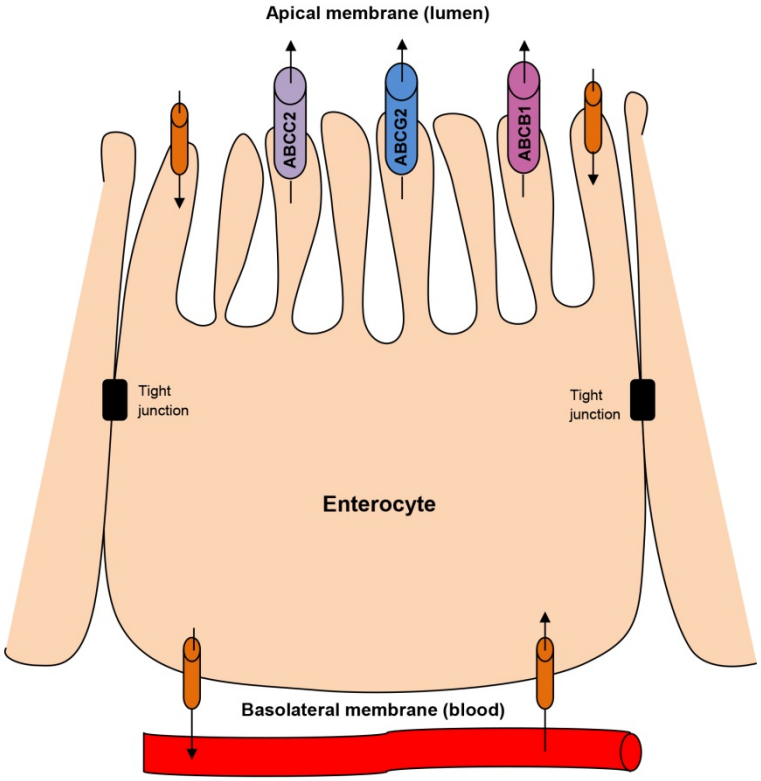


**Figure 4.** Illustration of the intestinal localization and transport direction of the three ATP-binding cassette transporters Pgp/MDR1, MRP2 and BCRP encoded by *ABCB1*, *ABCC2* and *ABCG2*, respectively. Other transporter proteins are shown in orange colour.

### 1.4.1. Pgp/MDR1 encoded by *ABCB1*

Pgp/MDR1 is the most thoroughly studied efflux transporter and was initially discovered via its ability to induce MDR in cancer cells after exposure to chemotherapy [186,187]. Pgp/MDR1 has a broad substrate specificity and today it is well-known that overexpression of the transporter protein during chemotherapy leads to MDR due to positive selection in those tumor cells that are capable of escaping death induced by the chemotherapeutic drugs [185,188]. However, Pgp/MDR1 is also involved in homeostatic functions, e.g., transport of steroids, regulation of chloride channels, transport of cytokines (particularly IL-1β, IL-2, IL-4 and IFN-γ), migration of antigen-presenting dendritic cells and T-lymphocytes, and inhibition of apoptosis [183,185]. The cytokines can either be transported actively by Pgp/MDR1 [183,188-190] or modulate expression or activity of Pgp/MDR1 [191]. However, it was shown that Pgp/MDR1 is pivotal for maintenance of healthy mucosal immune function in *mdr1a-/-* mice [192]. The *mdr1a-/-* mice spontaneously developed intestinal inflammation, which was reversed with the treatment of antibiotics suggesting that the colitis was developed as a result of a defect in the intestinal epithelial barrier. Moreover, Pgp/MDR1 expression is down-regulated in patients with UC [193-195] and in endotoxin-induced inflammation in rats [196]. Genetic variation in *ABCB1* is associated with disease distribution [197] and susceptibility to UC [197-199]. In line with this, low *ABCB1* gene and mRNA expression has been found in CRC tissue [124,200] indicating that low protein level of Pgp/MDR1 is an early

event in inflammation-related carcinogenesis of CRC which could be due to an impaired ability to protect the mucosal epithelial cells from toxic substances or imbalance in the interaction with inflammatory mediators.

Several NSAIDs have been shown to either inhibit Pgp/MDR1 expression or activity [201] or function as substrates (Pagliarulo et al. 2013). Interestingly, it has been proposed that NSAIDs also may regulate Pgp/MDR1 by a different mechanism [201]. As mentioned in section 1.3.3., NSAID can also inhibit NF-κB with subsequent down-regulation of COX-2. The *ABCB1* promoter contains putative binding sites for NF-κB, which is involved in *ABCB1* induction [202]. Consequently, inhibition of these factors by NSAIDs would result in down-regulation of *ABCB1* which could lead to increased exposure to toxic substances and/or impairment of the mucosal immune defence ultimately leading to cancer.

Dietary factors may also interact with Pgp/MDR1 modulating the intestinal immune response. In a review [203], the author described that numerous food components interact with Pgp/MDR1. Phytochemicals, aminopentol – a hydrolysis product of the mycotoxin Fumonisin $B_1$ - flavonoids and Chinese herbs modulate the activity of Pgp/MDR1 either by inhibiting the transporter activity or function as a substrate. Moreover, Pgp/MDR1 expression and activity is modulated by pesticides [204]. This is indeed especially relevant in terms of food-drug interactions, but may also affect the immunological functions and/or carcinogenic potential of Pgp/MDR.

### 1.4.2. MRP2 encoded by *ABCC2*

MRP2 is an organic anion transporter that participates in drug detoxification by transporting conjugated drugs and endogenous metabolites. The most critical function is the transport of bilirubin-glucuronides in the canalicular membrane in liver cells. Mutations in *ABCC2* lead to Dubin-Johnson syndrome, an autosomal recessive disorder characterized by conjugated hyperbilirubinemia [178,179,183]. Furthermore, it is involved in MDR, hence its name. In the intestine, MRP2 has both excretory and barrier functions since it actively pumps back conjugates formed in enterocytes, such as glutathione, glucuronide and sulphate derivatives of dietary factors into the intestinal lumen and form a barrier by transporting potentially toxic compounds out of the body [179].

MRP2 is also involved in mucosal immune defence. During an inflammatory attack, e.g., due to invading microorganisms, the eicosanoid hepoxilin $A_3$ ($HXA_3$) is formed in epithelial cells. In the apical membrane, $HXA_3$ is secreted in to the intestinal lumen to create a chemo-attractant gradient that recruits neutrophils from the submucosa to the luminal site of the inflammatory stimulus [205]. $HXA_3$ is transported by MRP2 and induction of intestinal inflammation up-regulates apical expression of MRP2 [206]. Additionally, inhibition of MRP2 has been shown to reduce inflammation [206] and high mRNA expression of *ABCC2* is associated with CRC (Andersen *et al.*, in prep.).

Apart from actively transporting several drugs, MRP2 is also inhibited or modulated by a range of drugs, including several NSAIDs [178,179,207,208]. Therefore, intake of NSAIDs could influence on the risk of inflammation-induced CRC.

As earlier mentioned, MRP2 transports conjugated derivates of dietary factors such as flavonoids and extracts of grape- and orange juices, but also mycotoxins found in cereal grains, e.g., deoxynivalenol and ochratoxin A. Variation in activity or expression of MRP2 could thus have influence on the intestinal exposure to these substances.

### 1.4.3. BCRP encoded by *ABCG2*

BCRP was initially discovered in relation to a multidrug resistance phenotype of a specific human BC cell line, MCF-7 [209]. It has broad substrate specificity, consistent with being a multidrug efflux pump. BCRP is also involved in physiological processes, such as uric acid excretion, protection of the fetus, export of nutrients into milk, regulation of uptake of substrates from the gastrointestinal tract, and transport of substrates across the endothelium of veins and capillaries [178,210,211].

Decreased mRNA expression of BCRP levels have been found in tissue from patients with UC compared to healthy controls [193]. Furthermore, the BCRP mRNA levels were inversely associated with the pro-inflammatory cytokine IL-6, indicating that the cytokine modulates BCRP expression. Indeed, several cytokines have been found to modulate BCRP expression and activity in different cell lines other than colon cells [210,212-214]. However, a down-regulation of BCRP has also been illustrated in CRC tissue [215,(Andersen *et al*., in prep.)] indicating a role for BCRP in inflammation-induced CRC.

Several dietary components and carcinogens are either transported by BCRP or modulate the activity or expression of the transporter protein. Numerous flavonoids are known to modulate BCRP or function as substrate [178,184,203]. BCRP also protects the body by actively transport dietary carcinogens such as mycotoxins [184] including the potent Aflatoxin $B_1$ [216], polycyclic aromatic hydrocarbons (PAHs) [217] and heterocyclic amines (HCAs) [216] including 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIPs) [218,219] which is one of the most abundant HCAs formed during cooking of meat at high temperatures (which will be further described in section 1.5.1.).

Thus, genetically determined differences in the expression and activity of the ABC transporters may have impact on the ability to restrict the body from exposure to dietary carcinogens and on the risk of developing inflammation-induced CRC. Dietary factors and NSAID use may interact with these transporters revealing important pathways in colorectal carcinogenesis.

## 1.5. Dietary and life style factors

In this section, the dietary and life style factors examined in this PhD thesis will be described, except for NSAID use and alcohol consumption, which are described separately in other sections. In Table 1, the modifiable dietary and life style factors that the World Cancer Research Fund (WCRF) in collaboration with the American Institute for Cancer Research (AICR), has found as having either convincing, probable or limited evidence as risk factors for CRC, BC or PC, respectively, are presented [220].

**Table 1. Modifiable dietary and lifestyle factors according to the WCRF/AICR.**

| Dietary and life style factors and CRC | | |
|---|---|---|
| | ***DECREASES RISK*** | ***INCREASES RISK*** |
| Convincing | Physical activity<br>Dietary fibre<br>NSAID use<br>HRT (women) | Red meat[a]<br>Processed meat[b]<br>Alcohol consumption (men)<br>Body/abdominal fatness<br>Smoking |
| Probable | Garlic<br>Milk<br>Calcium | Alcohol consumption (women) |
| Limited/suggestive | Non-starchy vegetables<br>Fruits<br>Vitamin D | Iron<br>Cheese<br>Animal fat<br>Sugar |
| Limited/no conclusion | Fish; glycaemic index; folate; vitamin C; vitamin E; selenium; low fat; dietary pattern | |
| **Dietary and life style factors and BC (premenopausal)** | | |
| | ***DECREASES RISK*** | ***INCREASES RISK*** |
| Convincing | Lactation | Alcohol consumption |
| Probable | Body fatness | |
| Limited/suggestive | Physical activity | |
| Limited/no conclusion | Dietary fibre; vegetables and fruits; soya and soya products; meat; fish; milk and dairy products; total fat; folate; vitamin D; calcium; glycaemic index; dietary patterns; abdominal fatness | |
| **Dietary and life style factors and BC (postmenopausal)** | | |
| | ***DECREASES RISK*** | ***INCREASES RISK*** |
| Convincing | Lactation | Alcohol consumption<br>Body fatness |
| Probable | Physical activity | Abdominal fatness<br>Adult weight gain |
| Limited/suggestive | | Total fat |
| Limited/no conclusion | Dietary fibre; vegetables and fruits; soya and soya products; meat; fish; milk and dairy products; folate; vitamin D; calcium; selenium; glycaemic index; dietary patterns; energy intake | |
| **Dietary and life style factors and PC** | | |
| | ***DECREASES RISK*** | ***INCREASES RISK*** |
| Convincing | No factor identified | No factor identified |
| Probable | Lycopene<br>Selenium | Calcium |
| Limited/suggestive | Leguminous fruits<br>Vitamin E<br>α-tocopherol | Processed meat<br>Milk and dairy products |
| Limited/no conclusion | Cereals (grains) and their products; dietary fibre; potatoes; non-starchy vegetables; fruits; meat; poultry; fish; eggs; total fat; plant oils; sugar (sucrose); sugary foods and drinks; coffee; tea; alcohol; carbohydrate; protein; vitamin A; retinol; thiamine; riboflavin; niacin; vitamin C; vitamin D; γ-tocopherol; vitamin supplements; iron; phosphorous; zinc; other carotenoids; physical activity; energy expendure; vegetarian diets; body fatness; abdominal fatness; energy intake | |

Since breast cancer mostly is hormone related, and the factors that modify risk of this cancer when diagnosed pre-menopausally and when diagnosed post-menopausally (much more common) are not the same, these risk factors are illustrated separately. Based on the continuous update of the WCRF/AICR reports on diet and cancer [220].

[a] The term 'red meat' refers to beef, pork, lamb, and goat .

[b] The term 'processed meat' refers to meats preserved by smoking, curing, or salting, or addition of chemical preservatives.

For CRC, dietary factors are considered as having particularly important roles in the carcinogenesis [12,13], whereas factors that are related to increased exposure to female sex-hormones and alcohol consumption are of more importance for BC [221,222]. However, for PC, no convincing risk factors have been established, except for age, ethnicity and family history of PC [4,5].

Not all dietary and life style factors that are associated with risk of the three studied cancer types will be examined in this PhD thesis. They will, though, be included as variables in the analyses whenever relevant e.g., body mass index (BMI), physical activity, hormone replacement therapy (HRT), educational level, number of births and age at first birth. Therefore, most of the risk factors described in this section are related to CRC and will also mostly be described in relation to colorectal carcinogenesis.

### 1.5.1. Red and processed meat

Even though the WCRF/AICR panel have determined that red and processed meat is convincingly a cause of CRC, the underlying mechanism is still not clear and several mechanism have been proposed which are either associated with specific components in the red meat or with toxic compounds generated during processing or cooking of the meat. 'Processing' is the methods used to preserve the meat e.g., smoking, curing (adding salt and other additives) and drying; whereas 'cooking' is the household preparation of meat, but cooking can also be part of the preservation process.

Heme is the iron-porphyrin pigment of red meat and the concentration of heme pigments is proportional to the red color [223]. Heme has been shown to induce hyperproliferation and hyperplasia of mucosal epithelium, induce lipid peroxidation of fecal water and to catalyze endogenous N-nitrosation, which increases nitrite and N-Nitroso compound (NOC) formation (see below) and the activation of HCAs [224,225]. Heme-oxygenase 1 (HO-1, encoded by *HMOX1*) is the inducible, rate-limiting enzyme converting heme to ferrous iron, carbon monoxide (CO) and biliverdin which is subsequently converted to bilirubin. HO-1 activity is induced during cellular oxidative stress, where the heme products, CO and bilirubin, exerts cytoprotective effects. Furthermore, CO inhibits pro-inflammatory cytokines including TNF-α, IL-1β, IL-2, IL-6, IFN-γ and COX-2, and stimulates the anti-inflammatory cytokine IL-10. However, HO-1 activity also produces free ferrous iron which produces ROS, including $H_2O_2$, which again induces genetic mutations and expression of many cytokines e.g., IL-6, IL-8, TNFα, NF-κB, leading to increased cytotoxicity and stimulation of an inflammatory response [12,226]. Thus, genetic variations in *HMOX1* may alter the risk of CRC among people with high intake of red meat.

When red meat is cooked at high temperatures e.g., frying, broiling or barbequing, HCAs are generated when amino acids react with creatinine. PAHs, on the other hand, are produced from incomplete combustion of organic compounds from cooked, smoked and barbequed meat. Many of the HCAs and PAHs are carcinogenic and/or mutagenic and may cause CRC [224]. As described in section 1.4.3., BCRP transports several HCAs and PAHs, and a high intake of red and processed meat in combination with inherent variations in *ABCG2* may have impact on the risk of CRC.

NOCs are alkylating agents that are capable of reacting with DNA leading to mutagenic and genotoxic effects [227]. Humans are exposed to NOCs from exogenous as well as endogenous sources. NOCs can be formed via nitrosation of organic compounds in meat via nitrite, which is added to meat during curing [223,228,229]. NOCs are also generated from fermentation processes of meat proteins by the microbiotic bacteria [230]. Endogenous NOC production has been shown to be increased in red, but not white meat (poultry), which could also explain why only intake of red, not white, meat is associated with CRC. Additionally, a diet high in red meat may alter the gut microbiota towards a more harmful composition with species generating carcinogenic metabolites [223]. Indeed, microbiota composition is different in people from the Western world compared to people from low-income countries, which have very low CRC rates [223]. A Western diet is typically composed of a high protein and fat content, and could thus explain the association between meat intake and CRC risk [231,232]. The modification in gut microbiota composition caused by red meat consumption may also modulate the mucosal immune response [233]. In fact, the mucosal microbiota has been shown to be necessary for development of colitis [223]. Therefore, intake of red meat may indirectly interact with modulators of the mucosal immune system via impact on gut bacteria, i.e., TLR4 and NF-κB in relation to inflammation-induced CRC.

The toxic compounds associated with red and processed meat intake mentioned above, have also been implicated in other cancer types, such as BC and PC [234-237]. However, in this PhD thesis, these mechanisms will not be examined.

### 1.5.2. Dietary fibre, cereals, fruits and vegetables

Dietary fibre is the indigestible part of food derived from plants and consists of polysaccharides, oligosaccharides, lignin, and associated plant substances. Insoluble fibre has bulky actions, which reduce fecal transit time and hence the exposure to food carcinogens and some of them are fermented by bacteria in the distal colon. Soluble fibre is fermented by bacteria in the proximal colon including the caecum [238]. Dietary fibre has rather consistently been associated with a reduced risk of CRC [18,239,240]. Fermentation of dietary fibre and starch provides the colonic epithelium with SCFAs e.g., acetate, propionate, and butyrate. SCFAs are used for energy metabolism by colonic epithelial cells and for maintenance of normal colonic function [238,241]. Butyrate is the major energy source for colonic mucosa and is important for colonic integrity [242], it inhibits growth of cancer cells in vitro [243,244] and has anti-inflammatory properties mainly by the inhibition of NF-κB [245]. In addition, a polymorphism in the anti-inflammatory gene *IL10* interacts with fibre intake so that only wild type carriers have decreased risk of CRC [246].

Whole grains, which are the natural form of cereals, are a major source of dietary fibre and contain germ, endosperm, and bran, in contrast with refined grains that only contain the endosperm, which mostly consists of starch. The germ and bran contain several nutrients e.g., vitamins, minerals, and phytochemicals, which are removed during the refining process. These nutrients have antioxidant properties and have been linked to CRC prevention [18,247]. However, as mentioned in section 1.4., many cereals also contain high amounts of toxic and carcinogenic mycotoxins [248], which could interact with the ABC transporters.

Fruits and vegetables are also sources of dietary fibre with potentially anticancer effects. However, when the dietary fibre intake is stratified by source, only those ingested from cereals are protective against CRC [18]. This may explain the lack of consistent evidence for a protective effect against CRC for fruits and vegetables. On the other hand, both fruits and vegetables contain several bioactive components with anticancer effects, such as flavonoids, β-carotene and lycopene [249,250], and it has been shown that genetic variations in *IL10* and *COX-2* interact with fruits and vegetables further decreasing the risk of CRC [246]. Flavonoids are phytochemicals that have been shown to possess antioxidant, antimutagenic, antibacterial, antiangiogenic and anti-inflammatory effects in both *in vitro* and *in vivo* studies, including the ability to inhibit COX-2 activity [249]. As described in section 1.4., they are substrates for and modulate ABC transporters and may therefore interact with genetic variations in these genes.

### 1.5.3. Fish

A diet high in fish intake is a source of Omega 3 (n-3) polyunsaturated fatty acids (PUFAs) which in experimental studies have been shown to reduce IBD activity through inhibition of COX-2 expression and activity, inhibition of cytokine production, improvement of epithelial barrier function and mucosal wound healing [251,252]. However, observational studies do not support the evidence of a chemo-protective effect of n-3 PUFAs on CRC risk [253]. Correspondingly, a protective effect of n-3 PUFA against PC has been suggested, but the evidence is also inconsistent [254]. Instead, there is evidence that the ratio between n-3 PUFA and the pro-inflammatory n-6 PUFA, which is abundant in meat, is of more importance, rather than the individual amount of each type of PUFA in the inflammation-induced cancer process. A Western diet, which is associated with high risk of CRC, typically consists of an n-6/n-3 PUFA ratio of 15:1, whereas the diet of our ancestors contains nearly equimolar ratios [251,252,255]. Alternative suggestions include the relatively high selenium or vitamin D content of fish as being the chemo-protective factors associated with fish intake [256,257]. Fish intake has been shown to interact with an *IL10* polymorphism so that only wild type carriers have decreased risk of CRC [246], and may also interact with other inflammatory mediators in relation to CRC.

### 1.5.4. Smoking

Tobacco contains several carcinogens e.g., HCAs, PAHs and NOCs [12] which are highly carcinogenic as described above. In addition, tobacco-specific nitrosamines bind to nicotinic acetylcholine receptors, which results in the production of ROS leading to oxidative stress. The oxidative stress activates the NF-κB and COX-2 inflammatory pathways [258]. Moreover, nicotine activates β-adrenoreceptors, which triggers inflammatory and metastatic signalling through the COX-2, $PGE_2$, and VEGF pathways [259]. Cigarette smoking is associated with increased risk of BC [8,260], PC [261,262] and CRC [12,263] and may interact with genetic variations in inflammatory mediators and/or ABC transporter genes.

## 1.6. Alcohol consumption

The term alcohol refers to ethanol in the present thesis, which is the type of alcohol used in alcoholic beverages. Chronic alcohol consumption is associated with systemic inflammation characterized by high circulating levels of pro-inflammatory cytokines [264] possibly mediated by gut microbiota-derived LPS [265] or through the production of ROS and NO during ethanol metabolism [266]. Intake of alcohol is associated with

increased risk of CRC [11,12,267] and BC [11,268], but not PC [269]. Overall, alcohol consumption is estimated to contribute to 3.6% of all cancer incidences; however, among women, 60% of cancers that are attributable to alcohol occur in the breast [270], indicating that the breast tissue is particularly sensitive to alcohol exposure. The underlying mechanism explaining the association between cancer and alcohol consumption is not fully understood and may differ by target organ. Apart from the evidence that intake of alcohol is associated with a systemic inflammatory state, which may induce cancer; other mechanisms have been proposed [10,271]. The primary ethanol metabolite, acetaldehyde, may cause DNA damage [272]. However, the evidence is weak [10] and genetic variation in the aldehyde dehydrogenase gene *ADH1C*, which causes phenotypic slow ethanol oxidation, is associated with high risk of BC, suggesting that ethanol rather than acetaldehyde is carcinogenic [273]. Alcohol may also acts as a solvent, enhancing penetration of tobacco or dietary carcinogens through mucosal cells [274]. Indeed, smoking is an important confounder for alcohol-related cancer risk [10,271]. Moreover, excessive alcohol consumption may lead to deficiency in essential nutrients such as folate, vitamin B12 and B6 [10,275]. The mechanisms discussed above are mostly involved in the carcinogenesis of oral cavity, pharynx, larynx, esophagus, colorectal and liver [10,271]. For BC, most evidence point to a mechanisms involving disruption in steroidogenesis since alcohol consumption is associated with increased level of circulating female sex-hormones [276-282]. Steroidogenesis is illustrated in Figure 5. Alcohol consumption is more strongly associated to hormone-sensitive BCs than hormone-insensitive subtypes [283,284] and the evidence is strongest among postmenopausal women [10,271]. In addition, alcohol is an inhibitor of PPARγ activity [285], which leads us to the hypothesis of PPARγ being an important modulator of alcohol-related BC among postmenopausal women, which will be discussed in the next section. The mechanisms explaining the link between alcohol intake and CRC will not be further examined in this PhD thesis.

### 1.6.1. Alcohol-related BC among postmenopausal women – a mechanism involving PPARγ

Estrogens are female steroid hormones controlling uterine and mammary gland development and function, external genitalia structure, secondary sex characteristics, sexual behaviour and menstrual cycle. The naturally occurring estrogens are estrone, estradiol and estriol, where estradiol is the predominant estrogen during reproductive years. During menopause, estrone replaces estradiol as the primary estrogen and throughout pregnancy estriol is the predominant circulating estrogen [286-288]. Estrogens play a central role in the cellular proliferation of both normal and neoplastic breast epithelium [268]. Approximately 60% of premenopausal and 75% of postmenopausal women with BC have estrogen-dependent carcinomas [289] and estradiol is considered the main determinant of proliferation of estrogen-dependent BC cells [290]. Moreover, estradiol is considered a carcinogen by the International Agency for Research on Cancer [291]. Estrogens exert their cellular actions by binding to the estrogen receptor (ER). ERs are present in malignant breast epithelial tissue at significant higher amounts than in normal breast epithelial cells [292]. This binding induces an activation of transcription of proliferative signalling and cell cycle progression genes [293], which may induce DNA damage [294]. Furthermore, BC is strongly associated with prolonged exposure of the breast epithelium to estrogens, i.e., early age at menarche, late age at menopause, nulliparity and old age at first birth [222,295], which is supported by several observational studies showing a positive correlation between female sex-hormone levels and risk of BC in postmenopausal women [296-299]. Conversely, high level of sex hormone-

binding globulin (SHBG) is associated with decreased risk due to reduced circulating free estrogen levels [297,299]. Use of HRT is also considered a risk factor for BC [300-302]; and has an additive effect when combined with intake of alcohol. Thus, women who drink alcohol and use HRT have higher estradiol concentrations than expected from use of HRT alone [303].

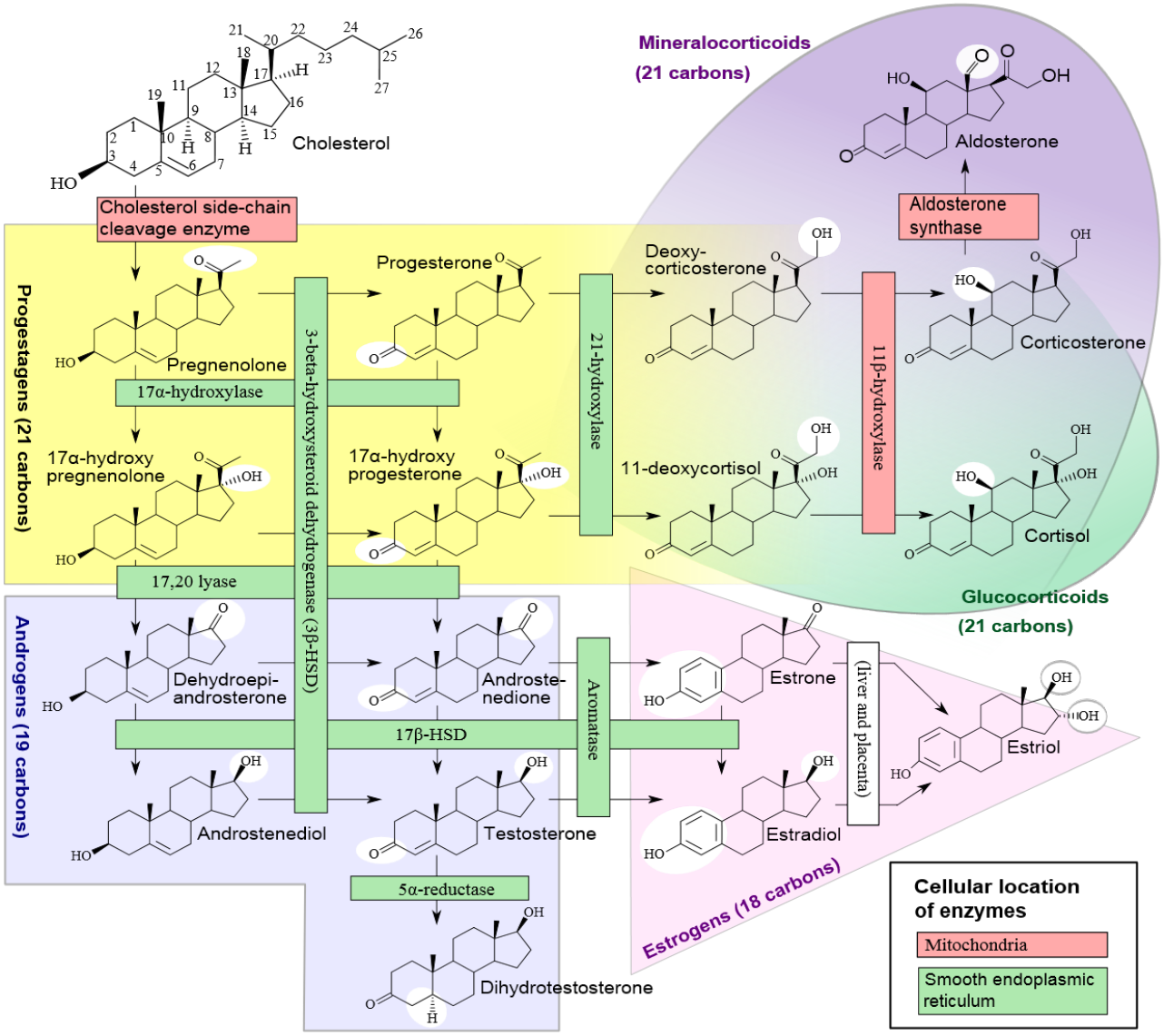


**Figure 5.** Steroidogenesis.

Both controlled experimental and observational human studies demonstrate that intake of alcohol is associated with increased level of female sex-hormone blood levels in pre- and postmenopausal women [276-282], which could explain the link between BC and alcohol consumption. However, the underlying mechanism is not fully understood. One explanation could be through a disruption in hepatic steroid catabolism. In the

liver, the 17β-hydroxysteroid dehydrogenase type 2 (17β-HSD2) enzyme degrades estradiol into estrone and testosterone into androstenedione in an oxidative catalyzed reaction [304] – which is the opposite compared to steroidogenesis (see Figure 5). Alcohol consumption increases the nicotinamide adenine dinucleotide hydroxide (NADH) to nicotinamide adenine dinucleotide (NAD+) ratio, which leads to a shift in the 17β-HSD2 catalyzed reaction in favour of reduction of androstendione to testosterone and estrone to estradiol leading to accumulation of testosterone and estradiol along with decrease in androstendione and estrone levels [305-307]. Alcohol also stimulates the hypothalamic–pituitary–adrenal axis which could lead to secretion of androstenedione and dehydroepiandrosterone [281] and subsequently increase in circulating estrogens. Another possible mechanism for the increased sex hormone levels may be through an increase in aromatase activity induced by alcohol [308]. Aromatase (encoded by *CYP19A1*) is the rate-limiting enzyme that catalyzes the conversion of androstenedione and testosterone to estrone and estradiol, respectively. In postmenopausal women, adipose tissue is the primary site for estrogen synthesis catalyzed by aromatase, when ovarian production has ceased [309]. Indeed, alcohol intake has been demonstrated to increase aromatisation [308,310] and hence estrogen levels. Among postmenopausal women, obesity is also a risk factor for BC [311] due to increased conversion of androgenic precursors to estrogens. Therefore, several factors point to aromatase as a central mediator in alcohol-related postmenopausal breast carcinogenesis through increased biosynthesis of estrogens.

The expression of *CYP19A1* is regulated differently in different tissues with hormonally controlled promoters using alternative splicing of exon I [288,312]. Thus, regulation of aromatase expression varies by tissue site e.g., the human ovary synthesizes primarily estradiol, whereas the placenta synthesizes estriol and adipocytes synthesize estrone. In the adipose tissue, the major source of substrate is circulating androstenedione produced by the adrenal cortex. The aromatase-dependent conversion of androgenic precursors in adipose tissue is the main source of estrogens in postmenopausal women. Several mechanisms may be responsible for aromatase overexpression in BC: (1) Alteration in cellular composition leads to increase in aromatase-expressing cell types using different promoters. (2) Favouring of the binding of transcriptional enhancers instead of inhibitors due to cellular environmental alterations, which results in increased transcriptional activity. (3) Mutations, which cause the aromatase coding region to lie adjacent to constitutively active promoters that normally transcribe other genes, resulting in excessive estrogen production. Indeed, aromatase inhibitors have proven effective in treating BC supporting the role of aromatase as a key factor in carcinogenesis [288,312].

Several polymorphisms in estrogen metabolizing enzymes have been associated with BC risk [222]. Among postmenopausal women, aromatase has major impact on endogenous steroid production and, thus, variation in *CYP19A1* may be of great importance in relation to postmenopausal BC risk. Several studies have demonstrated that genetic variation in *CYP19A1* is associated with differences in circulating estrogen levels [313-316]. However, these differences in hormone levels do not seem to contribute detectably to BC risk [317-319]. If alcohol causes BC by increasing aromatase activity it seems reasonably that variations in *CYP19A1* that are associated with estrogen levels may interact with alcohol intake in relation to BC risk.

So, how does alcohol disrupt aromatisation? PPARγ ligands are inhibitors of *CYP19A1* via the promotors used in both normal [320] and malignant [321] breast adipose stromal cells. A common functional polymorphism in *PPARG* results in a proline to alanine substitution at codon 12 (Pro$^{12}$Ala) located in Exon B. Exon B is only present in the PPARγ2 isoform which is primarily located in adipose tissue - which was the main site of estrogen production in postmenopausal women [163]. The variant Ala-allele has a twofold lower affinity to PPREs compared to the Pro isoform, and a reduced ability to trans-activate target genes [322]. In a Danish prospective cohort study, carriers of the variant Ala-allele of the *PPARG* Pro$^{12}$Ala polymorphism had a 33% lower risk of BC compared to homozygous wild type carriers [323]. Consumption of 10 g alcohol per day was associated with a 20% increased risk of BC among homozygous Pro-carriers (with a high PPARγ activity), whereas alcohol consumption was not associated with BC risk among variant Ala-allele carriers (with a low PPARγ activity) [323]. Two other studies have investigated the effect of *PPARG* Pro$^{12}$Ala in relation to BC with null results, but they did not examine for interaction with alcohol [324,325]. Alcohol consumption is high in the Danish cohort (16.9-18.6 g/day for participants from Aarhus and Copenhagen, respectively) [326], whereas the other two cohort studies have low intake of alcohol of 5.8 g/day [327] and 4.7 g/day [328], respectively. This indicates that alcohol may modify BC risk through a mechanism involving a specific activation of PPARγ2 that differs between the two polymorphic forms of PPARγ2. Indeed, perosixome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), a transcriptional co-activator encoded by *PPARGC1A*, binds to the PPARγ/RXR complex which results in inhibition of transcription of *CYP19A1*. This complex sequesters PGC-1α from forming a complex with liver receptor homolog-1 (LRH-1) [312], which is a specific transcriptional activator of aromatase gene expression in human breast adipocytes [329]. The PPARγ $^{12}$Ala protein is defective in the interaction with PGC-1α [330] and ethanol has been shown to inhibit the PPARγ/PGC-1α complex in a transient transfection assay using the wild type *PPARG* Pro-allele, whereas the Ala-allele was unaffected due to the defective interaction between *PPARG* $^{12}$Ala and PGC-1α [285]. Thus, alcohol may cause BC in a PPARγ and PGC-1α dependent manner in postmenopausal women where ethanol abolishes a PPARγ-dependent inhibition of adipocyte aromatase expression, due to an inhibition of formation of the PPARγ/PGC-1α complex as illustrated in Figure 6. Moreover, functional polymorphisms in *PPARGC1A* interact negatively with *PPARG* Pro$^{12}$Ala in relation to alcohol-related BC [285], confirming that the two proteins are part of the same pathway linking alcohol intake with BC. Investigating gene-gene interactions between functional polymorphisms in *PPARG*, *PPARGCIA* and *CYP19A1* with BC risk and female sex-hormone level would further enlighten this mechanism.

In addition, NSAID use has been shown to interact with *PPARG* Pro$^{12}$Ala in relation to alcohol-related BC so that the protective effect of *PPARG* $^{12}$Ala is absent among NSAID users [323]. Thus, use of NSAIDs, which are known to stimulate PPARγ [175,176], may also interact with *PPARG* Pro$^{12}$Ala in relation to alcohol-induced increase in female sex-hormone synthesis resulting in differences in circulating estrogen levels.

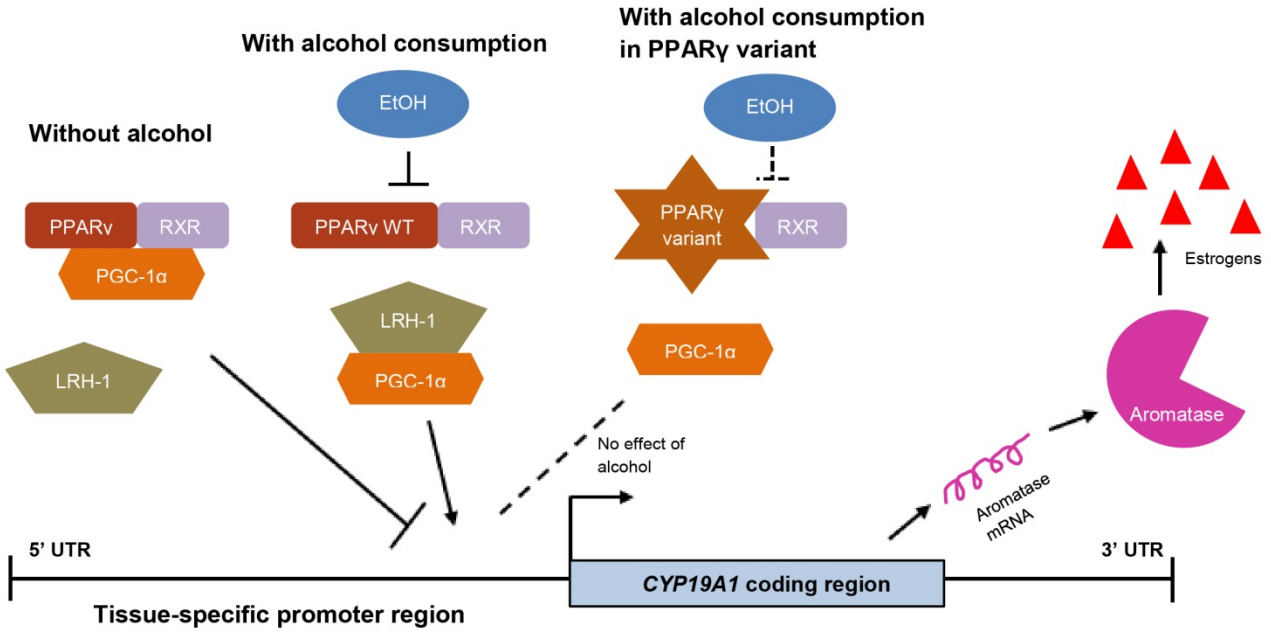


**Figure 6.** A proposed mechanism for alcohol-related breast cancer (BC). In absence of alcohol, peroxisome proliferator-activated receptor gamma (PPARγ) binds to perosixome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) and inhibits transcription of *CYP19A1*, by sequestering PGC-1α from forming a complex with liver receptor homolog-1 (LRH-1) – the co-activator of aromatase transcription. When alcohol is consumed in *PPARG* Pro$^{12}$Ala wild type carriers, ethanol inhibits the PPARγ/PGC-1α complex resulting in binding of PGC-1α to LRH-1 which activates transcription of aromatase. This would increase the risk of BC due to increased level of circulating estrogens. Among *PPARG* Pro$^{12}$Ala variant carriers, alcohol does not affect transcription of aromatase due to the defective interaction between PPARγ $^{12}$Ala and PGC-1α. EtOH: ethanol; RXR: Retinoic X-receptor.

#### *1.6.1.1. Occupational exposure to other organic solvents in relation to BC and PPARG Pro$^{12}$Ala*

As stated above, alcohol consumption is an important isolated risk factor for development of BC, and PPARγ may be a central mediator in the process of carcinogenesis. However, the population is also exposed to other organic solvents besides alcohol that may act via a similar mechanism. Several occupational studies have linked exposure of different chemicals, including organic solvents, to BC [331-335]. The largest study to date encompasses 15 million people aged 30-64 years with 373,361 cases of incident female BC and shows major variation in BC risk related to occupation [332]. Differences in birth pattern, education and physical activity do not explain these differences in risks. However, some of the variation may be explained by the fact that some types of occupations are related to high alcohol consumption, such as among journalists, musicians and hotel and restaurant workers. However, the consumption of alcohol should be considerably higher in a certain occupational field compared to the average consumption among all employees in a population in order for this factor to contribute significantly to an increased risk. Consequently, the differences in risk of BC point to exposure in the working environment [331]. Consistent with this, working with organic solvents for just one year was associated with a 20-70% increased risk of BC in another study [335]. The risk estimates tended to increase with increasing duration of employment. All analyses were adjusted for social class, age at first child, and number of children emphasising the significance of work-related exposure

to organic solvents as a risk factor for BC [335]. Most mammary carcinogens among organic solvents are lipophilic [336-338]. They are hypothesised to cause cancer due to their ability to reside in the adipose tissue surrounding the breast parenchyma and lobules where the solvents and their bioactivated metabolites may exert harmful local effects [336]. However, hydrophilic organic solvents may act differently by inhibition of PPARγ leading to increased aromatisation followed by increased levels of circulating estrogens. Testing hydrophilic organic solvents with known occupational exposure for inhibition of PPARγ in a cell-based screening assay would easily identify solvents with breast carcinogenic potential.

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# Chapter 2

## The “Diet, Cancer and Health” Cohort &

## Selection of polymorphisms

## 2.1. Description of the Danish Diet, Health and Cancer cohort

The Danish, prospective "Diet, Cancer and Health" (DCH) cohort study was initiated after convincing evidence relating lifestyle factors, especially diet, to cancer incidence [1]. The overall aim of the cohort study was to investigate the relations between dietary and lifestyle factors and the incidence of cancer and other chronic diseases, e.g., type 2 diabetes and cardiovascular diseases. Moreover, combining questionnaire data and biological samples from participants, would permit the investigator to examine genetic susceptibility and the interaction with diet and life style factors in relation to the development of cancer.

### 2.1.1. Study population

Between December 1993 and May 1997, 80,996 men and 79,729 women were invited to participate in the DCH study. The criteria for being invited were: 1) age between 50 and 64 years, 2) residency in Copenhagen municipality, or Copenhagen or Aarhus counties, 3) born in Denmark and 4) no diagnosis of cancer registered in the Danish Cancer Registry. Of 160,725 invited individuals, 57,053 (27,178 men and 29,875 women) (35.5%) accepted the invitation. All participants completed a mailed, detailed 192-item semi-quantitative food frequency questionnaire (FFQ) prior to the visit to the study centres. At the study centres, the participants filled in a general questionnaire concerning lifestyle habits, social factors and health status including medical history and reproductive factors. Anthropometrical measures, including height and weight, were obtained by professional staff and biological materials (blood, fat biopsy and toe nails) were collected. The DCH study was approved by the regional Ethical 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.

### 2.1.2. Case ascertainments and selection

The DCH cohort is annually linked to the Danish Cancer Registry, which has recorded cancer incidences in Denmark since 1943 [2]. Record linkage to the Central Population Register provides information on vital status and date of death or emigration. Gleason's score data was obtained from Hospital records. Participants were followed-up until censoring, which was the date of diagnosis of any cancer (except non-melanoma skin cancer), date of death and date of emigration or end of follow-up. PC and BC case were age-matched via incidence density sampling, that is, the controls were alive and at risk of cancer at the time the corresponding case was diagnosed, but could be subsequently diagnosed as a case. In addition, BC controls were matched on use of HRT (current/former/never) and on certainty of postmenopausal status (known/probably postmenopausal) at enrolment. CRC controls or sub-cohort members were randomly selected, which allows the studying of multiple outcomes. In order to correct for differences in age between cases and sub-cohort members, age was used as the underlying time axis in the statistical analyses, which ensured that the estimation procedure was based on comparisons of individuals at the same age and the analyses were corrected for delayed entry, such that persons were considered under risk only from the age at enrolment in the cohort.

### 2.1.3. Assessment of questionnaire data

In the FFQ, participants were asked to report habitual intake of foods and beverages during the last 12 month in 12 predefined categories ranging from "never" to "eight times or more per day". Daily intakes of

foods and nutrients were then calculated for each participant using FoodCalc [3] which uses standardized recipes and portion sizes specifically developed for the DCH cohort population [4,5]. The FFQ originally included 92 foods and recipes predicting the intake of 19 nutrients considered important in human carcinogenesis, and which provided 81% of the average total nutrient supply [6]. Since then, the FFQ was expanded to finally include 192 foods and recipes for the main study.The first FFQ with 92 food items was validated by comparing the data from the FFQ with two times seven day of weighed diet records assessed in a pilot study prior to the DCH study. Correlation between calorie-adjusted intakes from the FFQ and diet records ranged from 0.17 to 0.71 [7]. Red meat was calculated by combining intake of fresh and minced beef, veal, pork, lamb, and offal, whereas processed meat combined intake of bacon, smoked or cooked ham, other cold cuts, salami, frankfurter, Cumberland sausage, and liver pâté. Total dietary fiber was estimated by the method of the Association of Official Analytical Chemists [8], which includes lignin and resistant starch [9]. Intake of cereals was calculated by adding up intake of wholegrain foods (wholegrain bread, rye bread, wholegrain flour, oatmeal, corncobs, müsli, and crispbread) and refined grain foods (white wheat bread, wheat flour, rice flour, potato flour, corn flour/starch, pasta, wheat) and was measured in grams per day. Intake of fish in grams per day was calculated by adding up intake of fresh and processed fish. For fruit, only intake of fresh fruit (as indicated in the FFQ) was examined, whereas intake of vegetables also included estimated contributions from food recipes. 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), 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. Wine included both red and white wine [10]. Smoking intensity was calculated as gram tobacco smoked per day and included information on cigarettes (one cigarette = 1 g tobacco), cigars (one cigar = 4.5 g tobacco), cheroot (one cheroot = 4 g tobacco), and pipe (one pipe = 3 g tobacco). Information on food supplements included questions on brand and doses, and frequency of intake. Information about the contents of micronutrients was retrieved from producers or distributors of the different brands.

In the lifestyle questionnaire, information on NSAID use, HRT and length of school education was assessed. With regards to NSAID use, participants were asked “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 frequent 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, study subjects were classified according to use of ‘any NSAID’ (2 pills per month during one year) at baseline. All women were asked about their use of HRT (never received HRT, previously received HRT, or currently receive HRT), the age at which they began receiving HRT, the duration (in years) of their HRT use, and the brand of HRT product currently used. Time since HRT cessation was calculated using the participant’s age at the start of HRT use and the reported duration of use. In order to determine the women’s postmenopausal status at

baseline (known/probably post-menopausal) the following criteria were set: “Known” postmenopausal status included women that were (1) non-hysterectomized and reporting no menstruation during 12 months before inclusion, (2) reporting bilateral oophorectomy, or (3) reporting age at last menstruation lower than age at hysterectomy. “Probably” post-menopausal women were either (1) reporting menstruation during the 12 months prior to inclusion and current use of HRT (the bleeding was assumedly caused by HRT), (2) reporting hysterectomy with a unilateral oophorectomy or an oophorectomy of unknown laterality, or (3) reporting last menstruation at the same age as age of hysterectomy. The lifestyle questionnaire also provided information regarding duration of schooling (short: ≤ 7 years, medium: 8–10 years, or long: >10 years). At the study centre, the self-administrated questionnaires were scanned optically and checked for missing values and reading errors. Uncertainties were cleared up with the participants by the laboratory technician during the visit.

## 2.2. Selection of polymorphisms

In this PhD thesis, 32 polymorphisms have been examined in relation to PC, CRC and/or BC and relevant dietary and lifestyle factors. In Table 2, a description of each polymorphism is provided.

**Table 2. Description of polymorphisms examined in the present PhD thesis.**

| SNP | Reference SNP ID number | Location on gene | Paper | Function/effect of polymorphism |
|---|---|---|---|---|
| ***COX-2* -1195 A/G** | (rs689466) | Promoter | I | Loss of a potential c-myb binding site with reduced promoter activity and mRNA levels [11]. Associated with reduced risk of CRC [12] and basal cell carcinoma [13]. Interacts with NSAID use in relation to lung cancer [14] and several dietary factors and smoking in relation to CRC [15]. |
| ***COX-2* +8473 T/C** | (rs5275) | Exon 10/3' UTR | I | *COX-2* +8473 T/C interrupts a predicted stem and creates an additional loop [16], which may alter mRNA stability and expression. Haplotype analysis of *COX-2* +8473 T/C and -765 G/C (-765 G/C is in LD with *COX-2* +3496 T/C [17] and used in the present study instead of -765 G/C) reveals an increase in $PGE_2$ and $PGD_2$ by peripheral blood monocytes among variant carriers of both SNPs [18]. The effect is suggestively caused by *COX-2* +8473 T/C alone, which is supported by studies showing increased risk of esophageal adenocarcinoma [19], basal cell carcinoma [13], BC [16] and CRC [15] for variant carriers. *COX-2* +8473 T/C also interacts with dietary factors in relation to CRC [15]. |
| ***COX-2* +3496 T/C** | (rs2066826) | Intronic | I | Please see *COX-2* +8473 T/C: Interaction with *COX-2* +8473 T/C in haplotype analysis [18]. |
| ***COX-2* +202 G/A** | (rs2745557) | Intronic | I | Interacts with NSAID use in relation to risk of PC [20]. |
| ***COX-2* +306 C/G** | (rs5277) | Exon 3 | I | A suggestive risk haplotype defined by variant carriers of the G allele of *COX-2* +306 C/G with the above mentioned *COX-2* SNPs in Caucasians [20]. |
| ***PPARG* Pro[12]Ala C/G** | (rs1801282) | Exon B in PPARγ2 | I, V and VI | Missense mutation. Reduced ability to trans-activate transcription of target genes, e.g., *COX-2* [21,22]. This SNP has been extensively studied and has been associated with reduced risk of type 2 diabetes [23,24], CRC [25] and BC [26,27]. The SNP has opposing effects in relation to alcohol consumptions and risk of BC and CRC, respectively. WT carriers have increased risk of alcohol-related BC [26,27], whereas variant carriers have increased risk of alcohol-related CRC [26]. In addition, NSAID use interacts with PPARG Pro[12]Ala and alcohol intake in relation to BC [26]. |
| ***NFKB1* -94 ins/del ATTG** | (rs28362491)*** | Promoter | I and II | The deletion of four amino acids leads to weaker binding of nuclear proteins and decreased promoter activity, resulting in lower levels of the p50 subunit [28]. *NFKB1* -94 ins/del ATTG is associated with reduced risk of PC [29], but increased risk of CRC [30-32] and IBD [28,33,34]. |
| ***IL1B* -31 T/C** | (rs1143627) | Promoter | I and III** | Higher levels of IL-1β and hence, increased stabilizing of COX-2 mRNA [35,36]. In a haplotype context with the other two *IL1B* SNPs, variant carriers of the C allele of *IL1B* -31 T/C has the highest transcription level of IL-1β in vitro [35] and homozygous variant carriers had higher mucosal IL-1β levels than heterozygous variant carriers and WT carriers in Japanese Helicobacter pylori-infected patients [37]. Associated with increased risk of lung cancer [14], multiple myeloma [38] and CRC [15]. |
| ***IL1B* -1464 G/C** | (rs1143623) | Promoter | I and III** | High transcriptional level of IL1β *in vitro* in a haplotype context with the two other *IL1B* SNPs [35]. Associated with increased risk of CRC [15]. |
| ***IL1B* -3737 G/A** | (rs4848306) | Promoter | I and III** | Abolishes a binding site for the anti-inflammatory NF-κB subunit p50 and results in lower transcriptional activity [35]. Associated with reduced risk of CRC and interacts with vegetables and NSAID use [15]. |
| ***CYP19A1* A/G** | (rs10519297) | Promoter | V | Associated with femoral neck-shaft angle in GWAS - a phenotypic trait in osteoporosis [39]. SNP tagger – force included*. |
| ***CYP19A1* G/A** | (rs749292) | Promoter | V | Associated with increased estrone and estradiol levels in postmenopausal women [40,41]. SNP tagger – force included*. |
| ***CYP19A1*** | (rs1062033) | Exon | V | Increased transcriptional activity by binding of the CCAAT/ enhancer binding protein β [42]. Associated |

| | | | | |
|---|---|---|---|---|
| **C/G** | | I.2/promoter | | with enhanced bone mineral density in postmenopausal women [42,43] and increased aromatase gene expression in adipose tissue [44]. SNP tagger – force included*. |
| ***CYP19A1* 3'UTR A/G** | (rs10046) | Exon 10/3' UTR | V | Considered a low activity genotype (*G-carriers*) [45]. This SNP has been extensively examined and has consistently been associated with decreased levels of circulating estrogens [40,41,46]. Some studies have found a decreased risk of BC [45,47] and decreased risk of ER positive [48] and HER2 positive BC [49]; whereas others have not been able to confirm the association between the SNP and BC [41,46,50]. SNP tagger – force included*. |
| ***CYP19A1* C/A** | (rs4646) | Exon 10/3' UTR | V | Associated with decreased levels of estrogens [40,41] and decreased risk of HER2 positive BC [49]. Others have not been able to confirm association with BC [41]. SNP tagger*. |
| ***CYP19A1* A/G** | (rs6493487) | Intronic | V | SNP tagger*. |
| ***CYP19A1* A/G** | (rs2008691) | Promoter | V | Associated with femoral neck-shaft angle in GWAS - a phenotypic trait in osteoporosis [39]. SNP tagger*. |
| ***CYP19A1* T/C** | (rs3751591) | Promoter | V | Associated with age at natural menopause [51]; and is placed in a recombination spot. SNP tagger*. |
| ***CYP19A1* T/C** | (rs2445762) | Promoter | V | Associated with lower estradiol and higher FSH levels in GWAS [52]. SNP tagger*. |
| ***CYP19A1* C/T** | (rs11070844) | Promoter | V | SNP tagger*. |
| ***ABCB1* Ile$^{1145}$Ile +3435 C/T** | (rs1045642)*** | Exon 26 | III | Synonymous substitution. Extensively studied SNP. Associated with lower levels of mRNA and protein levels [53,54] possibly caused by lower mRNA stability and protein folding [55]. It has also been associated with altered drug and inhibitor interactions resulting in altered response to pharmacotherapy [53,56-58]. Several studies have investigated the association with CRC [59-65] and IBD [66], but the results are inconsistent. In line with these results, studies on intestinal mRNA levels and protein expression levels are not consistent either [53,67-69]. The SNP has been found to interact with meat intake and NSAID use in relation to CRC [70]. |
| ***ABCB1* Gly$^{412}$Gly 1236 C/T** | (rs1128503) | Exon 12 | III | Synonymous substitution. Lowered Pgp/MDR1 activity [58]. Associated with decreased risk of IBD [71-73]. However, these studies were mostly using haplotype combinations with rs1045642 and another SNP not examined in this thesis (rs2032582). |
| ***ABCB1* C/T** | (rs3789243)*** | Intronic | III | Associated with increased risk of IBD [72,74,75] and possibly increased risk of CRC [70]. However, other studies point to no risk of CRC [60,76]. Interacts with intake of meat in relation to CRC [70]. |
| ***ABCC2* Val$^{417}$Ile +1249 G/A or +1286 G/A** | (rs2273697) | Exon 10 | III | Missense mutation. High MRP2 activity [77] which is consistent with a study showing increased intestinal efflux and, as a consequence, decreased bioavailability of the drug β-blocker talinolol [78]. |
| ***ABCC2* Val$^{1188}$Glu +3600 T/A** | (rs17222723) | Exon 25 | III | Missense mutation. High MRP2 expression [79]. |
| ***ABCC2* -24 C/T** | (rs717620) | Exon 1/promoter | III | Decreased mRNA expression [54] and low MRP2 activity [54,77]. Associated with response rate to drugs [80-83]. |
| ***ABCG2* Gln$^{141}$Lys +421 C/A** | (rs2231142)*** | Exon 5 | III | Missense mutation. Decreased expression of BCRP and lower transport activity [69,84,85]. Decreased risk of chronic lymphocytic leukemia [86]; elevated uric acid levels and prevalent gout [87-89]. Associated with altered pharmacokinetic parameters of BCRP substrates [90]. |
| ***ABCG2* Val$^{12}$Met +34 C/G** | (rs2231137) | Exon 2 | III | Missense mutation. Lower mRNA levels possibly due to polymorphic splicing [91]. |
| ***ABCG2*** | (rs2622604) | Intronic | III | Low intestinal BCRP expression [91] |

| | | | | |
|---|---|---|---|---|
| **+1143 G/A** | | | | |
| ***TLR4*** **Asp$^{299}$Gly** **+896 A/G** | (rs4986790) | Exon 4 | II | Missense mutation. Extensively studied SNP. Loss of a negatively charged area at position 299, leading to alteration in the ligand-binding receptor site and decreased sensitivity to Gram-negative LPS [92]. Increased expression and of inflammatory and/or oncological genes and proteins in CRC cells [93]. Increased NF-κB activity in CRC cells [94]. Associated with several diseases e.g., Gram-negative bloodstream infections, sepsis, chronic sarcoidosis, haematogenous and chronic osteomyelitis and IBD [95]. The association with risk of CRC is less consistent [95]. Some studies point to no effect [96-99], whereas others point to increased risk [100-104]. Low MAF of ~4% in Caucasians. |
| ***TLR4*** **+385 G/A** | (rs5030728) | Intronic | II | In partially LD with rs4986790 (D': 1.0; $r^2$: 0.017) and a higher MAF of ~30% in Caucasians. Located near exon 3, which makes it likely to affect messenger RNA processing and stability [105]. Intake of dietary saturated fatty acids is inversely related to blood level of high density lipoprotein cholesterol in individuals homozygous for the TLR4/rs5030728 G-allele [105]. Associated with response to anti-TNF therapy in patients with IBD [34]. Not associated with risk of CRC, but associated with increased survival after diagnosis of colon cancer [102]. |
| ***HMOX1*** **-413 A/T** | (rs2071746)*** | Upstream of gene 5' | IV | Increased expression of HO-1 due to alteration in promoter activity [106]. Associated with beneficial outcome in several diseases e.g., ischemic heart disease [106], hypertension in women [107], sepsis [108] and cardiac function [109]. Additionally, this SNP is in LD with a microsatellite GT-dinucleotide repeat, which has also been associated with various diseases [110-112]. However, no association with risk of CRC and meat intake was found in a sub-cohort DCH study [113]. |

* C*YP19A1* SNPs are tagged using HapMap [114] genotyping data set from Utah residents with ancestry from Northern and Western Europe (CEU, version 3, release 2) in combination with Haploview (version 4.2, Broad Institute, Cambridge USA) [115] in order to evaluate the major variations of *CYP19A1*. Criteria for SNP inclusion was a minor allele frequency (MAF) of minimum 5% and an $r^2$ threshold of 0.1. Tagging of SNPs with an $r^2$ higher than 0.1 resulted in several SNPs being in complete LD. Four functional polymorphisms were force included in the aggressive SNP tagging (rs10046, rs749292, rs1062033 and rs10519297) due to their documented impact on aromatase RNA expression, estrogen levels and/or BC risk (see above).
** *IL1B* SNPS were only investigated in relation to CRC for gene-gene interactions, *not* main effects.
*** These SNPs have been examined in CRC sub-cohorts of the DCH cohort.

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# Chapter 3

*Cancer Genet 2013; 206 (7-8): 266-278*

## Paper I: *Polymorphisms in genes related to inflammation, NSAID use, and the risk of prostate cancer among Danish men*

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**Abstract**

The aetiology of prostate cancer (PC) remains mostly unknown, but increasing evidence suggests that chronic inflammation in the prostate is associated with an increased risk of PC. Epidemiological studies have suggested that use of non-steroidal anti-inflammatory drugs (NSAIDs) may protect against PC. Inborn variations in genes involved in the inflammatory response may modulate the risk of PC and interact with NSAIDs. The aims of the study were 1) to evaluate whether polymorphisms and haplotypes of the inflammation-related genes *COX-2, IL1B, NFKB1* and *PPARG* are associated with risk of PC; 2) to investigate gene-environment interactions between polymorphisms, and NSAID use; and 3) to examine whether the studied polymorphisms were associated with aggressiveness of PC. The study population consisted of 370 cases of PC and 370 risk-set matched (age) controls nested within the prospective Danish "Diet, Cancer and Health" cohort. Carriers of the variant del-allele of *NFKB1* -94ins/delATTG had a tendency towards a reduced risk of PC (incidence rate ratio (IRR) = 0.73; 95% CI= 0.52-1.04). A lowered risk for PC was also found for variant allele carriers of *NFKB1* -94ins/delATTG among non-users of NSAIDs (IRR= 0.68; 95% CI= 0.47-0.99), for non-aggressive disease (IRR= 0.64; 95% CI= 0.42-0.99), and among men with BMI above 30 kg/m$^2$ (IRR= 0.56; 95% CI= 0.27-1.16), although the latter estimate was based on small numbers. A similar pattern was seen for carriage of the variant C-allele of the *COX-2* +8473T→C polymorphism. No apparent associations with PC were observed for the other studied polymorphisms. Our study did not indicate that chronic inflammation is a major risk factor for aggressive PC.

# Introduction

Prostate cancer (PC) is the most frequent non-skin cancer and the second leading cause of cancer-related deaths among men in the Western world [1,2]. The incidence of PC has reached epidemic proportions during the past two decades [1,2]. In Denmark, 3,900 men were diagnosed with PC in 2009, corresponding to an age-standardized incidence rate of 138 per 100,000 persons [3]. The steep increase in PC incidence is mainly due to increasing use of prostate specific antigen (PSA) testing[4]. Although PSA testing has been employed less frequently in Denmark than in other Western countries, the annual number of new cases of PC has doubled from 2000 to 2009 [3]. The only well-established risk factors for PC are increasing age, race, family history, and genetic susceptibility of PC. However, studies of migrant populations clearly indicate that environmental factors are important [5-8].

There is increasing evidence for a role of chronic inflammation in the carcinogenesis of PC [9-13], and dysregulation of several inflammatory mediators have been associated with prostate carcinogenesis [14-19]. In addition, epidemiological studies have suggested that use of non-steroidal anti-inflammatory drugs (NSAIDs) may protect against PC [20-22].

Cyclooxygenase-2 (COX-2) is an inducible, pro-inflammatory enzyme that converts arachidonic acid (AA) to a range of prostaglandins (PGs) and the enzymatic activity of COX-2 is inhibited by NSAIDs [23-25]. An increased level of prostaglandin $E_2$ ($PGE_2$) has been reported to be involved in cellular proliferation, suppression of immune responses, promotion of angiogenesis, inhibition of apoptosis, and stimulation of invasion and motility [23-25]. *COX-2* is expressed at sites of inflammation and neoplasia [24,26], and overexpression of the enzyme has been found in several types of cancer, including PC [14-16]. Thus, *COX-2* upregulation may be an important step in prostate carcinogenesis.

Peroxisome proliferator-activated receptor γ (PPAR-γ) is a member of the nuclear hormone receptor super family and is known to possess anti-inflammatory properties [27]. In addition, PPAR-γ has potential anti-cancer effects, since a lowered expression of the *PPARG* gene has been correlated with a poor prognosis among lung cancer patients [28]. PPAR-γ ligands, which form heterodimers with PPAR-γ, have been shown to reduce *COX-2* expression by interfering with the NF-κB pathway [29] and to inhibit the AP-1-mediated transcriptional activation of COX-2 in human epithelial cells [30] and in cervical cancer cells [31]. Furthermore, *COX-2* and *PPARG* messenger RNA (mRNA) levels are inversely regulated [31], which is in agreement with the findings of a study that reported that *PPARG* seems to be regulated by some PGs in a negative feedback mechanism [29].

The cytokine interleukin 1 (IL-1) is a member of the *IL-1* gene family. IL-1β is secreted upon induction and is found in pathological fluids [32]. Elevated levels of mucosal IL-1β have been detected in animal models of intestinal inflammation and in humans with inflammatory bowel disease [33]. IL-1α and β stabilise the mRNA and induces transcription of *COX-2*[34]. Upon stimulation, COX-2 production remains elevated for several hours and $PGE_2$ concentration increases steeply. Many of the IL-1α- and IL-1β-induced changes are mediated by $PGE_2$, and the use of NSAIDs are therefore potent inhibitors of IL-1-induced activity [32].

Induction of the transcription factor nuclear factor kappa B (NF-κB) is essential for the expression of several immune response genes, e.g., *COX-2* [35] and *IL-1* [36,37]. NF-κB is implicated in carcinogenesis due to its regulation of expression of inflammatory proteins, anti-apoptotic proteins and the cell-cycle regulator cyclin D1 [36,37]. NSAIDs have been shown to inhibit NF-κB independently of COX inhibition [38].

The primary aims of the study were to examine the associations between genetic variations in inflammatory genes, including haplotype analyses, and risk of PC in a nested case-control study within the prospective, Danish "Diet, Cancer and Health" (DCH) cohort. We selected 10 predominantly functional polymorphisms in *COX-2, IL1B, NFKB1* and *PPARG* which allowed us to analyse the haplotypes of *COX-2* and *IL-1B* (Table 1). Secondarily, we investigated interactions between genetic variations and NSAID use, overall and according to aggressiveness of PC.

## Methods

### *Study population*

The study population was drawn from the DCH study that was conducted between December 1993 and May 1997 and has been described in detail elsewhere [39]. Of 160,725 invited individuals, 57,053 (27,178 men and 29,875 women) (35.5%) agreed to participate in the study. The criteria for being invited were: 1) age between 50 and 64 years, 2) residency in one of the two largest cities in Denmark, Copenhagen or Aarhus; and 3) no history of cancer. Each participant completed a detailed 192-item food-frequency questionnaire and a general questionnaire concerning lifestyle, medical history and background factors. Anthropometric measures, including height and weight, were obtained by professional staff and biological materials, including blood samples, were collected.

The DCH cohort is annually linked to the Danish Cancer Registry, which has recorded cancer incidence in Denmark since 1943 [3,40,41]. Record linkage to the Central Population Register provides information on vital status and date of death or emigration of each participant [39,42].

We identified 370 patients with a first diagnosis of PC during the period from entry into the DCH until December 31, 2003. For each case, we chose one male control matched on year of birth, center, length of follow-up, time of blood collection and state of fasting using risk-set sampling; the controls were alive and free of cancer at the time the corresponding case was diagnosed (index date), but they were subsequently eligible as cases. Thereby, the estimated odds ratios (OR) are unbiased estimates of the incidence rate ratio (IRR) [43].

In total, 7 cases (and their corresponding controls) were excluded because of a previous cancer diagnosis, 7 case-control pairs were excluded because of missing information on potential confounders, and 22 pairs either failed the genotype analysis or had no buffy-coats available for analysis. Thus, a total of 334 case-control pairs were included in our analyses.

### Exposure variables

From the general DCH questionnaire, we obtained information on NSAID use, duration of school education, history of diabetes, smoking habits, intake of alcohol, red meat, dietary fat, omega-3 fatty acids, calcium, vitamin D, vitamin E, selenium, and total calories. Each participant's body mass index (BMI) was computed based on measurements of height and weight obtained at enrolment [6,39,44].

**Table 3. Schematic view of the studied single nucleotide polymorphisms (SNPs).**

| *SNP* | *Location on the gene* | *Function/effect of polymorphism* |
|---|---|---|
| *COX-2* -1195A→G (rs689466) | Promoter | Loss of a potential c-myb binding site with reduced promoter activity and mRNA levels [45]. Carriers of the variant G-allele of *COX-2* -1195A→G have lower risks of colorectal cancer [46] and basal cell carcinoma [47] compared with that of carriers of the A-allele. An interaction between *COX-2* -1195A→G and NSAIDs was found in a study of lung cancer [48]. |
| *COX-2* +8473T→C (rs5275) | Exon 10/3' UTR | *COX-2* +8473T→C interrupts a predicted stem and creates an additional loop [49], which may alter mRNA stability and expression. Haplotypeanalysis of *COX-2* +8473T→C and -765G→C (-765G→C is in LD with *COX-2* +3496T→C [50] and used in the present study instead of -765G→C) reveals an increase in PGE2 and PGD2 by peripheral blood monocytes among variant carriers of both SNPs [51]. The effect is suggestively caused by *COX-2* +8473T→C alone, which is supported by studies of esophageal adenocarcinoma [52], basal cell carcinoma [47] and breast cancer [49], where variant carriers of *COX-2* +8473T→C were associated with increased risk of disease compared with that of WT. |
| *COX-2* +3496T→C (rs2066826) | Intron 6 | Please see *COX-2* +8473T→C: Interaction with *COX-2* +8473T→C in haplotype analysis [51]. |
| *COX-2* +202G→A (rs2745557) | Intron 1 | Heterozygous carriers of the A-allele have a lower risk for PC compared with that of WT carriers and a lower risk for PC among homozygous carriers of the G-allele who use NSAIDs, whereas NSAID use has no impact on risk among men carrying the GA/AA genotype [53]. |
| *COX-2* +306C→G (rs5277) | Exon 3 | A suggestive risk haplotype defined by variant carriers of the G-allele of *COX-2* +306C→G with the above mentioned *COX-2* SNPs has been seen in Caucasians [53]. |
| *PPARG* Pro12AlaC→G (rs1801282) | Codon 12 in PPARγ-2 | Reduced ability to activate transcription of target genes e.g. *COX-2* [54,55]. Carriers of the variant Ala-allele have a reduced risk of breast cancer compared with that of WT carriers [56], and weak interactions between alcohol consumption, NSAID use and Pro12Ala in relation to breast cancer risk have been seen [57]. |
| *NFKB1* -94ins/delATTG (rs28362491) | Promoter | Weaker binding of nuclear proteins and decreased promoter activity [58], leading to lower levels of the p50 subunit. Deletion allele carriers have higher plasma levels of CRP, which is transcriptionally regulated by the p50 dimer [59]. A reduced risk of PC is observed for carriers of the variant deletion allele [60]. |
| *IL-1B* -31T→C (rs1143627) | Promoter | Higher levels of IL-1β and hence, increased stabilizing of *COX-2* mRNA [61,62]. In haplotype context with the other two *IL-1β* SNPs, variant carriers of the C-allele of *IL-1β* -31T→C has the highest transcription level of IL-1β *in vitro* [61] and homozygous variant carriers had higher mucosal IL-1β levels than heterozygous variant carriers and WT carriers in Japanese *Helicobacter pylori*-infected patients [63]. Variant allele carriers of *IL-1β* -31T→C are at increased risk of lung cancer [48] and multiple myeloma [64]. |
| *Il-1B* -1464G→C (rs1143623) | Promoter | The variant allele gives lower transcriptional activity. However, a high transcriptional level of IL-1β *in vitro* in a haplotype context with the -31T→C *IL-1β* SNPs was reported among variant carriers of the C-allele [61]. |
| *Il-1B* -3737G→A (rs4848306) | Promoter | Low transcriptional level of IL-1β *in vitro* in haplotype context with the two other *IL-1β* SNPs among variant carriers of the C-allele [61]. |

**NSAID use**

The general DCH questionnaire asked how frequently aspirin, non-aspirin (NA) NSAID, or acetaminophen (paracetamol) had been used during the 12 months prior to enrolment. Frequency of use was assessed as number of pills taken per month, week, or day. Indications for use and dosing schedule were not assessed. We defined *NSAID use* as the weekly use of aspirin or an NA-NSAID at baseline; this threshold was based on the results of a recent study of colorectal cancer within the DCH cohort, which reported that long-term consistent use of aspirin or NA-NSAID appears necessary to achieve a protective effect [65].

**BMI**

BMI was incorporated in the gene-environment analyses, since there is accumulating evidence that BMI is associated with PC risk [66,67]. BMI was classified according to the World Health Oranization standard definition of obesity; a BMI of 18-25 kg/m$^2$ was defined as normal weight, 25-30 kg/m$^2$ as overweight and above 30 kg/m$^2$ as obesity [68]. There were no men with BMI below 18 kg/m$^2$ in the present study group.

**Outcome variable**

*Aggressiveness of PC*

The purpose of stratifying PC by aggressiveness was to evaluate whether genetic variations in inflammatory genes affected the severity of the disease. We hypothesized that the effects of the functional polymorphisms would be more pronounced among aggressive PC cases, because functional genetic variations could potentially promote carcinogenesis, leading to more severe disease. The stratification of PC by aggressiveness was based on the Gleason score [69], which were retrieved from the cases' pathology reports [70]. "Non-aggressive" or "low-grade" PC was defined as Gleason's scores of below 7, whereas "aggressive" or "high-grade" PC was defined as Gleason's scores of 7 or more.

**Genotyping**

At DCH enrolment, 30 ml blood was collected from non-fasting participants, and buffy coat samples were stored at -150 ˚C in liquid nitrogen. DNA was isolated from frozen lymphocytes as described by Miller *et al.* [71]. Genotypes were determined using reverse transcription-polymerase chain reaction (RT-PCR) and allelic discrimination on Applied Biosystems ABI 7900HT instruments (Applied Biosystems, Nærum, Denmark). Generally, 50-300 ng/µl DNA was obtained from $10^7$ lymphocytes, and 20 ng of DNA was genotyped in 5 µl solution containing 50% 2 × Mastermix (Applied Biosystems), 100 nM probes, and 900 nM primers. Controls were included in each reaction and replication of 10% of the samples yielded 100 % identical genotypes. The average genotyping success rate was 99%. Analysis of a small sub-cohort ((N=50, aggressive and non-aggressive) and controls (N=58)) using a genotyping method that differs from the one used in this study has been published previously [72].

For *COX-2* -1195A→G (rs689466) primers and probes were: primers: 5'-GCA CTA CCC ATG ATA GAT GTT AAA CAA A-3' and 5'-TGG AAC ATA GTT GGA TGA GGA ATT AAT-3': probes: A-allele: 5'-

FAM-ATG AAA TTC CA**A** CTG TCA AAA TCT CCC TT-BHQ-1-3' and G-allele: 5'-Yakima Yellow-ATG AAA TTC CA**G** CTG TCA AAA TCT CCC T-BHQ-1-3' [47].

For *COX-2* +8473T→C (rs5275) primers and probes were: primers: 5'-ATG CAC TGA CTG TTT TTG TTT G-3' and 5'-GTT TCC AAT GCA TCT TCC ATG A-3': probes: T-allele: 5'-VIC-TGA CAG AAA AAT **A**AC CAA AA- MGB-3' and C-allele: 5'-FAM-TGA CAG AAA AAT **G**AC CAA A- MGB-3' [73].

For *COX-2* +3496T→C (rs2066826), we used a TaqMan® predesigned single nucleotide polymorphism (SNP) genotyping assay (assay ID: C__11997848_20).

For *COX-2* +306C→G (rs5277), we used a TaqMan® predesigned SNP genotyping assay (assay ID: C__7550198_10).

For *COX-2* +202G→A (rs2745557) primers and probes were: primers: 5'-TTG TGG GAA AGC TGG AAT ATC C-3' and 5'-TGA CTT GGG AAA GAG CTT GGA-3': probes: G-allele: 5'-FAM-CTT TCG **G**AC TCT AGC-MGB-3' and A-allele: 5'-VIC-CTT TCG **A**AC TCT AGC G-MGB-3'.

For *PPARG* Pro12Ala (rs1801282) primers and probes were: primers: 5'-GTT ATG GGT GAA ACT CTG GGA GAT-3' and 5'-GTT ATG GGT GAA ACT CTG GGA GAT-3': probes: C-allele: 5''-FAM-CTC CTA TTG A**C**G CAG AAA GCG ATT C-BHQ-1-3' and G-allele: 5'-Yakima Yellow-TCC TAT TGA CCC A**G**A AAG CGA TTC C-BHQ-1-3' [73].

For *IL-1B* -31T→C (rs1143627) primers and probes were: primers: 5'-CCC TTT CCT TTA ACT TGA TTG TGA-3' and 5'-GGT TTG GTA TCT GCC AGT TTC TC-3': probes: T-allele: 5'-Yakima Yellow-CCT CGC TGT TTT TAT **A**GC TTT CAA AAG CAG A-BHQ-1-3' and C-allele: 5'FAM-TCG CTG TTT TTA TG**G** CTT CTT TCA AAA GCA G-BHQ-1-3' [74].

For *IL-1B* -1464G→C (rs1143623) primers and probes were: primers: 5'-CAG TAG AAC CTA TTT CCC TCG TGT CT-3' and 5'- TCA AAT TAG TAT GTG CCA GGT ATC G-3'. Probes: C-allele: 5'-FAM-CTC CCT TG**C** ATA ATG-MGB-3' and G-allele: 5'-VIC- CTC CCT TG**G** ATA ATG-MGB-3' [75].

For *IL-1B* -3737G→A (rs4848306) primers and probes were: primers: 5'-TGG ATC CAG GTT CTC AGA TTG G-3' and 5'-GCT CAT ATA TCT AAG CCC TCC TTG TT-3'. Probes: A-allele: 5'-FAM-CAA GG**A** ACA TTC C-MGB-3' and G-allele: 5'-VIC-CCA AGG **G**AC ATT CC-MGB-3' [75].

For *NFKB1* -94ins/delATTG (rs28362491) primers and probes were: primers: 5'-CTA TGG ACC GCA TGA CTC TAT CAG-3' and 5'-GGG CTC TGG CTT CCT AGC A-3'; probes: ins-allele: 5'- FAM-ACC **ATT G**AT TGG GCC CGG-BHQ -1-3' and del-allele: 5'-Yakima Yellow- CCG ACC ATT GGG CCC G -BHQ-1-3' [64].

**Statistical methods**

Descriptive statistics were used to characterize the study population by demographic characteristics and baseline levels of potential confounders, including NSAID use and BMI. Conditional logistic regression analysis was used to calculate IRRs for the associations between the exposure variables and PC [76].

Haplotype determination was based on combinatorial analysis. First, all possible haplotypes were identified. Second, homozygous haplotypes were included twice, and heterozygous haplotypes were only included once. In the haplotype analyses, no adjustment for confounders was performed, since the data used in the analysis were not reviewed on a per person level, but on a frequency level. Logistic regression analysis was used to calculate ORs. Wild type (WT) was defined as the allele with the highest frequency in the general population, because of the assumption that there is a selection pressure against the variant carrier. Furthermore, WT was used as reference in all genotype analyses (except for the haplotype analysis of *COX-2* SNPs, in which the predefined risk haplotype was used as reference to compare the results with a similar study by Cheng *et al.* [53]). Heterozygous and homozygous variant carriers were combined in the analyses, since no recessive associations were observed, to achieve a higher statistical precision.

Although the associations between BMI or school education and PC risk were weak, all survival analyses were adjusted for these two characteristics, since the direction of the associations were compatible with the literature [6,66,77]. This also allowed us to compare with the crude risk estimates.

Risk estimates for PC overall applied to the total study population of 334 cases and controls. In the analyses by aggressiveness of PC, the risk estimates for aggressive and non-aggressive PC applied to subsets of case-control pairs with case type of aggressive (n=113) and non-aggressive (n=221) PC, respectively. We used SAS version 9.1 (SAS Institute Inc., Cary, NC, USA) in all statistical analyses.

## Results

The baseline characteristics of PC cases and matched controls are presented in Table 2. BMI was inversely associated with PC risk, in other words, a high BMI was associated with a reduced PC risk (IRR=0.90; 95% CI:0.82-1.00; *P*=0.04). The duration of school education was associated with an increased risk of PC, although not statistically significant (IRR=1.42; 95% CI:0.98-2.06; *P*=0.06 for medium, and IRR=1.33; 95% CI:0.85-2.09; *P*=0.21 for long school education versus short education). The risk estimates for NSAID use and the remaining covariates in Table 2 were close to unity.

### SNP analyses and gene-gene interactions

Among controls, the genotype distributions of all the studied polymorphisms were in Hardy-Weinberg equilibrium (data not shown).

**Table 2. Baseline characteristics of PC cases and controls**

| *Variable* | *Cases (N = 334)* | | *Controls (N = 334)* | | *IRR[a] (95% CI)* |
|---|---|---|---|---|---|
| | *No. (%)* | *Median (5-95%)* | *No. (%)* | *Median (5-95%)* | |
| Age | | 59.0 (51.0-64.0) | | 59.0 (51.0-64.0) | 0.95 (0.59-1.53) |
| School education | | | | | |
| Short (≤7 years) | 113 (33.8) | | 137 (41.0) | | 1.00 (ref.) |
| Medium (8-10 years) | 133 (39.8) | | 122 (36.5) | | 1.42 (0.98-2.06) |
| Long (>10 years) | 88 (26.4) | | 75 (22.5) | | 1.33 (0.85-2.09) |
| BMI (kg/m$^2$) overall | | 26.1 (21.8-31.9) | | 26.8 (21.5-35.2) | 0.90 (0.82-1.00)[d] |
| BMI < 25 | 108 (32.3) | | 100 (29.9) | | |
| BMI 25-30 | 181 (54.2) | | 173 (51.8) | | |
| BMI > 30 | 45 (13.5) | | 61 (18.3) | | |
| Alcohol intake (g/day) | | 18.3 (1.9-81.3) | | 19.8 (2.8-67.7) | 0.99 (0.90-1.08)[e] |
| Smoking status | | | | | |
| Never | 86 (25.8) | | 95 (28.4) | | 1.00 (ref.) |
| Past | 128 (38.3) | | 129 (38.6) | | 1.07 (0.71-1.61) |
| Current | 120 (35.9) | | 110 (32.9) | | 1.25 (0.79-1.98) |
| NSAID use (% ever users)[b] | 56 (16.8) | | 56 (16.8) | | 1.17 (0.76-1.80) |
| Total energy (MJ/day) | | 10.8 (7.2-15.5) | | 11.0 (7.5-15.1) | 0.99 (0.90-1.10)[f] |
| Red meat (g/day) | | 93.6 (43.7-177.7) | | 99.5 (54.0-177.7) | 0.91 (0.79-1.06)[g] |
| Dietary fat (g/day) | | 96.3 (54.1-147.9) | | 97.1 (57.7-145.0) | 0.87 (0.61-1.22)[h] |
| Omega-3 fatty acids (g/day) | | 0.7 (0.3-1.7) | | 0.7 (0.3-1.6) | 1.02 (0.66-1.58)[i] |
| Calcium (mg/day) | | 1,132 (512-2,140) | | 1,063 (538-2,083) | 1.16 (0.93-1.45)[j] |
| D vitamin (µg/day) | | 5.2 (2.6-10.0) | | 5.0 (2.6-9.8) | 1.06 (0.65-1.74)[k] |
| E vitamin (α-TE/day)[c] | | 9.5 (5.3-17.1) | | 9.5 (5.3-16.1) | 1.07 (0.77-1.56)[l] |
| Selenium (µg/day) | | 53.5 (34.3-85.6) | | 53.4 (35.3-86.6) | 1.04 (0.62-1.75)[m] |
| Diabetes Mellitus | | | | | |
| No | 324 (97.0) | | 325 (97.3) | | 1.00 (ref.) |
| Yes | 10 (3.0) | | 9 (2.7) | | 1.32 (0.51-3.4) |

Median values with 5$^{th}$-95$^{th}$ percentiles or fraction of the distribution of potential confounders for PC among PC cases and controls.

[a] IRR for PC - mutually adjusted

[b] NSAID use is defined as ≥ 2 pills per week during the preceding year

[c] 1 α-TE equals 1 mg R,R,R-α-tocopherol (the most active form of E vitamin)

[d] Risk estimate per 2 kg/m$^2$ increment of BMI

[e] Risk estimate for the increment of 10 g alcohol per day

[f] Risk estimate for the increment of 500 kJ energy per day

[g] Risk estimate for the increment of 25 g red meat per day

[h] Risk estimate for the increment of 25 g dietary fat per day

[i] Risk estimate for the increment of 0.5 g omega-2 fatty acids per day

[j] Risk estimate for the increment of 400 mg calcium per day

[k] Risk estimate for the increment of 3 µg D vitamin per day

[l] Risk estimate for the increment of 5 α-TE E vitamin per day

[m] Risk estimate for the increment of 20 µg Selenium per day

Carriers of the variant deletion allele of *NFKB1* -94ins/delATTG had a 27% lowered risk of PC compared with WT carriers (95% CI:0.52-1.04; *P*=0.08) and, similarly, carriers of the variant C-allele of *COX-2* +8473T→C had a 26% lowered PC risk (95% CI:0.53-1.04; *P*=0.08) (Table 3).

**Table 3. IRR for PC in relation to the studied polymorphisms**

| *Genotypes* | $N_{cases}$ *(%) (N=334)* | $N_{controls}$ *(%) (N=334)* | *IRR (95% CI)*[a] | *P-value*[c] | *IRR (95% CI)*[b] | *P-value*[c] |
|---|---|---|---|---|---|---|
| *COX-2* -1195A→G | | | | | | |
| AA | 210 (63) | 210 (63) | 1.00 (ref.) | | 1.00 (ref.) | |
| AG | 111 (33) | 112 (33) | 1.02 (0.74-1.42) | 0.91 | 1.04 (0.74-1.45) | 0.96 |
| GG | 13 (4) | 12 (4) | 0.85 (0.38-1.91) | | 0.92 (0.41-2.10) | |
| AG + GG | 123 (37) | 124 (37) | 1.00 (0.73-1.37) | 1.00 | 1.02 (0.74-1.41) | 0.90 |
| *COX-2* +8473T→C | | | | | | |
| TT | 169 (51) | 146 (44) | 1.00 (ref.) | | 1.00 (ref.) | |
| TC | 130 (39) | 154 (46) | 0.74 (0.53-1.05) | 0.23 | 0.72 (0.51-1.02) | 0.18 |
| CC | 35 (10) | 34 (10) | 0.92 (0.53-1.60) | | 0.84 (0.48-1.49) | |
| TC + CC | 165 (49) | 188 (56) | 0.77 (0.56-1.07) | 0.12 | 0.74 (0.53-1.04) | 0.08 |
| *COX-2* +3496T→C | | | | | | |
| TT | 263 (79) | 274 (82) | 1.00 (ref.) | | 1.00 (ref.) | |
| TC | 67 (20) | 53 (16) | 1.37 (0.90-2.06) | 0.23 | 1.32 (0.86-2.02) | 0.28 |
| CC | 4 (1) | 7 (2) | 0.62 (0.18-2.13) | | 0.58 (0.17-2.01) | |
| TC + CC | 71 (21) | 60 (18) | 1.28 (0.86-1.91) | 0.23 | 1.23 (0.82-1.85) | 0.32 |
| *COX-2* +202G→A | | | | | | |
| GG | 223 (67) | 236 (71) | 1.00 (ref.) | | 1.00 (ref.) | |
| GA | 99 (30) | 91 (27) | 1.14 (0.81-1.62) | 0.40 | 1.11 (0.80-1.58) | 0.41 |
| AA | 12 (3) | 7 (2) | 1.79 (0.70-4.58) | | 1.86 (0.72-4.86) | |
| GA + AA | 111 (33) | 98 (29) | 1.19 (0.85-1.66) | 0.31 | 1.16 (0.83-1.64) | 0.38 |
| *COX-2* +306C→G | | | | | | |
| CC | 227 (68) | 228 (68) | 1.00 (ref.) | | 1.00 (ref.) | |
| CG | 98 (29) | 99 (30) | 0.99 (0.70-1.40) | 0.96 | 0.96 (0.70-1.41) | 0.94 |
| GG | 9 (3) | 7 (2) | 1.16 (0.39-3.49) | | 1.22 (0.40-3.73) | |
| CG + GG | 107 (32) | 106 (32) | 1.00 (0.72-1.40) | 1.00 | 1.01 (0.72-1.42) | 0.97 |
| *PPARG* Pro12Ala | | | | | | |
| CC | 241 (72) | 245 (73) | 1.00 (ref.) | | 1.00 (ref.) | |
| CG | 90 (27) | 87 (26) | 1.14 (0.78-1.65) | 0.80 | 1.10 (0.75-1.60) | 0.89 |
| GG | 3 (1) | 2 (1) | 1.03 (0.15-7.35) | | 0.91 (0.12-6.74) | |
| CG + GG | 93 (28) | 89 (27) | 1.13 (0.78-1.64) | 0.51 | 1.09 (0.75-1.59) | 0.65 |
| *NFKB1* -94ins/delATTG | | | | | | |
| ins/ins | 128 (38) | 109 (33) | 1.00 (ref.) | | 1.00 (ref.) | |
| ins/del | 152 (46) | 161 (48) | 0.81 (0.57-1.15) | 0.36 | 0.74 (0.52-1.06) | 0.21 |
| del/del | 54 (16) | 64 (19) | 0.73 (0.46-1.17) | | 0.71 (0.44-1.15) | |
| ins/del + del/del | 206 (62) | 225 (67) | 0.79 (0.57-1.11) | 0.17 | 0.73 (0.52-1.04) | 0.08 |
| *IL-1B* -31T→C | | | | | | |
| TT | 134 (40) | 148 (44) | 1.00 (ref.) | | 1.00 (ref.) | |
| TC | 178 (53) | 143 (43) | 1.48 (1.06-2.08) | 0.004 | 1.58 (1.11-2.24) | 0.002 |
| CC | 22 (7) | 43 (13) | 0.56 (0.30-1.05) | | 0.56 (0.30-1.07) | |
| TC + CC | 200 (60) | 186 (56) | 1.27 (0.92-1.74) | 0.15 | 1.32 (0.96-1.84) | 0.09 |
| *IL-1B* -1464G→C | | | | | | |
| GG | 170 (51) | 179 (54) | 1.00 (ref.) | | 1.00 (ref.) | |
| GC | 149 (45) | 130 (39) | 1.27 (0.92-1.76) | 0.14 | 1.30 (0.93-1.81) | 0.14 |
| CC | 15 (4) | 25 (7) | 0.69 (0.34-1.41) | | 0.72 (0.35-1.48) | |
| GC + CC | 164 (49) | 155 (46) | 1.18 (0.86-1.62) | 0.30 | 1.21 (0.88-1.67) | 0.24 |
| *IL-1B* -3737G→A | | | | | | |
| GG | 107 (32) | 106 (32) | 1.0 (ref.) | | 1.00 (ref.) | |
| GA | 162 (49) | 172 (51) | 0.88 (0.62-1.26) | 0.60 | 0.87 (0.30-1.25) | 0.69 |
| AA | 65 (19) | 56 (17) | 1.08 (0.68-1.72) | | 0.99 (0.62-1.60) | |
| GA + AA | 227 (68) | 228 (68) | 0.92 (0.66-1.30) | 0.67 | 0.90 (0.63-1.27) | 0.54 |

[a] Crude
[b] Adjusted for BMI and school education at baseline
[c] P-value for trend

Heterozygous variant carriers of *IL-1B* -31T→C had a 1.6-fold higher PC risk (95% CI:1.11-2.24; *P*=0.002) than homozygous WT carriers, whereas homozygous variant carriers had a 44% lowered risk (95% CI:0.30-1.07; *P*=0.002). In combined analysis of heterozygous and homozygous variant carriers, there was no association between *IL-1B* -31T→C and risk of PC (IRR=1.32; 95% CI:0.96-1.84; *P*=0.09).

Haplotype analysis of the five *COX-2* SNPs identified six haplotypes covering 88% of the variation among cases and controls (Table 4). No statistically significant associations with risk of PC were observed. A predefined risk haplotype defined by the variant G-allele of *COX-2* +306C→G [53] was not associated with risk of PC.

**Table 4. Association between *COX-2* haplotypes and PC risk**

| -1195A→G | +202G→A | +306C→G | +3496T→C | +8473T→C | $N_{haplotype}$ in case/controls (%) | OR (95% CI) |
|---|---|---|---|---|---|---|
| A | **A** | C | T | T | (18) 123/103 (15) | 1.00 (ref.) |
| A | G | C | T | T | (14) 92/90 (13) | 0.86 (0.58-1.27) |
| **G** | G | C | T | T | (17) 111/101 (15) | 0.92 (0.63-1.34) |
| A | G | C | T | **C** | (15) 103/122 (18) | 0.71 (0.49-1.02) |
| A | G | **G** | T | T | (17) 116/112 (17) | 0.87 (0.60-1.25) |
| A | G | C | **C** | **C** | (9) 57/52 (8) | 0.92 (0.58-1.45) |

Bold letters indicate the polymorphic bases. The reference haplotype is defined as the AACTT similar to the study by Cheng *et al.* [53].

Haplotype analysis of the *IL-1B* SNPs identified four haplotypes covering 99% of the variation among cases and controls (Table 5). No associations were found between these haplotypes and PC risk.

Stratification according to aggressiveness of PC (Table 6) showed that carriers of the variant deletion allele of *NFKB1* -94ins/delATTG had a lowered risk of non-aggressive PC (IRR=0.64; 95% CI:0.42-0.99; *P*=0.05), but not of aggressive PC (IRR=0.93; 95% CI:0.52-1.67; *P*=0.81).

**Table 5. Association between *IL-1B* haplotypes and PC risk**

| -3737G→A | -1464G→C | -31T→C | $N_{haplotype}$ in case/controls (%) | OR (95% CI) |
|---|---|---|---|---|
| G | G | T | (23) 156/157 (24) | 1.00 (ref.) |
| G | **C** | **C** | (27) 178/175 (26) | 1.02 (0.76-1.39) |
| **A** | G | T | (34) 288/281 (42) | 1.03 (0.78-1.36) |
| G | G | **C** | (6) 42/49 (7) | 0.86 (0.54-1.38) |

Bold letters indicate the polymorphic bases. The reference haplotype is defined as the WT haplotype.

Presence of the *NFKB1* -94/ins/delATTG polymorphism was consistently, although only borderline statistically significantly associated with a reduced risk of PC. A similar pattern was seen for *COX-2* +8473T→C. An interaction analysis revealed an additive effect between *NFKB1* -94ins/delATTG and *COX-2* 8473T→C, resulting in an additionally lowered risk of PC of 46% (95% CI:0.33-0.88) (data not shown), compared with individual risk estimates of 30% for *NFKB1* -94ins/delATTG (95% CI:0.43-1.14) and 30% for *COX-2* +8473T→C (95% CI:0.40-1.21; *P* for interaction=0.77).

**Gene-environment analyses**

We found no interaction between the studied polymorphisms and NSAID use (Table 7). Carriers of the variant allele of *NFKB1* -94ins/delATTG had a lowered risk of PC among non-users of NSAIDs (IRR=0.68; 95% CI:0.47-0.99; *P* for interaction=0.30) only. Moreover, NSAID use was not associated with risk of total PC or non-aggressive PC, whereas a borderline statistically significantly increased risk was observed for aggressive PC (IRR=2.08; 95% CI:0.96-4.52; *P*=0.06) compared with non-use of NSAIDs (data not shown).

**Table 6. The studied polymorphisms in relation to aggressiveness**

| *Genotypes* | $N_{aggr.}$ *(%) (N=113)* | $N_{non\text{-}aggr.}$ *(%) (N=221)* | *Aggressive PC* | | *Non-aggressive PC* | |
|---|---|---|---|---|---|---|
| | | | *IRR (95% CI)*[a] | *IRR (95% CI)*[b] | *IRR (95% CI)*[a] | *IRR (95% CI)*[b] |
| *COX-2* -1195A→G | | | | | | |
| AA | 72 (64) | 138 (62) | 1.00 (ref.) | 1.00 (ref.) | 1.00 (ref.) | 1.00 (ref.) |
| AG + GG | 41 (36) | 83 (38) | 0.92 (0.53-1.61) | 0.96 (0.55-1.70) | 1.04 (0.71-1.52) | 1.04 (0.71-1.54) |
| *COX-2* +8473T→C | | | | | | |
| TT | 56 (50) | 113 (51) | 1.00 (ref.) | 1.00 (ref.) | 1.00 (ref.) | 1.00 (ref.) |
| TC + CC | 57 (50) | 108 (49) | 0.90 (0.54-1.51) | 0.88 (0.51-1.50) | 0.70 (0.47-1.07) | 0.68 (0.44-1.05) |
| *COX-2* +3496T→C | | | | | | |
| TT | 88 (78) | 175 (79) | 1.00 (ref.) | 1.00 (ref.) | 1.00 (ref.) | 1.00 (ref.) |
| TC + CC | 25 (22) | 46 (21) | 1.82 (0.87-3.79) | 1.66 (0.78-3.52) | 1.09 (0.68-1.77) | 1.08 (0.66-1.78) |
| *COX-2* +202G→A | | | | | | |
| GG | 77 (68) | 146 (66) | 1.00 (ref.) | 1.00 (ref.) | 1.00 (ref.) | 1.00 (ref.) |
| GA + AA | 36 (32) | 75 (34) | 1.04 (0.60-1.80) | 0.96 (0.54-1.70) | 1.29 (0.84-1.97) | 1.27 (0.83-1.96) |
| *COX-2* +306C→G | | | | | | |
| CC | 79 (70) | 148 (67) | 1.00 (ref.) | 1.00 (ref.) | 1.00 (ref.) | 1.00 (ref.) |
| CG + GG | 34 (30) | 73 (33) | 0.80 (0.44-1.44) | 0.85 (0.47-1.56) | 1.12 (0.74-1.68) | 1.13 (0.74-1.72) |
| *PPARG* Pro12Ala | | | | | | |
| CC | 76 (67) | 165 (75) | 1.00 (ref.) | 1.00 (ref.) | 1.00 (ref.) | 1.00 (ref.) |
| CG + GG | 37 (33) | 56 (25) | 1.22 (0.66-2.28) | 1.18 (0.62-2.24) | 1.09 (0.69-1.72) | 1.07 (0.67-1.72) |
| *NFKB1*94ins/delATTG | | | | | | |
| ins/ins | 43 (38) | 85 (38) | 1.00 (ref.) | 1.00 (ref.) | 1.00 (ref.) | 1.00 (ref.) |
| ins/del + del/del | 70 (62) | 136 (62) | 1.00 (0.57-1.76) | 0.93 (0.52-1.67) | 0.69 (0.46-1.06) | 0.64[c] (0.42-0.99) |
| *IL-1B* -31T→C | | | | | | |
| TT | 44 (39) | 90 (41) | 1.00 (ref.) | 1.00 (ref.) | 1.00 (ref.) | 1.00 (ref.) |
| TC + CC | 69 (61) | 125 (59) | 1.30 (0.76-2.25) | 1.36 (0.78-2.36) | 1.24 (0.84-1.84) | 1.35 (0.90-2.03) |
| *IL-1B* -1464G→C | | | | | | |
| GG | 54 (48) | 116 (52) | 1.00 (ref.) | 1.00 (ref.) | 1.00 (ref.) | 1.00 (ref.) |
| GC + CC | 59 (52) | 105 (48) | 1.29 (0.76-2.20) | 1.31 (0.76-2.26) | 1.13 (0.76-1.67) | 1.21 (0.81-1.81) |
| *IL-1B* -3737G→A | | | | | | |
| GG | 39 (35) | 68 (31) | 1.00 (ref.) | 1.00 (ref.) | 1.00 (ref.) | 1.00 (ref.) |
| GA + AA | 74 (65) | 153 (69) | 0.83 (0.46-1.51) | 0.76 (0.41-1.41) | 0.98 (0.65-1.48) | 0.94 (0.62-1.44) |

[a] Crude.
[b] Adjusted for BMI and school education at baseline.
[c] P = 0.05

There was a trend towards a reduced risk of PC among obese men (Table 8). For both *NFKB1* -94ins/delATTG and *COX-2* +8473T→C, there seemed to be a consistent additive effect between BMI and genotype (Table 8). Obese carriers of the WT of both polymorphisms exhibited a reduced risk of PC, whereas all men carrying the variant deletion or C-allele experienced a reduced risk. The risk decrease was most pronounced among the obese, albeit this finding was not statistically significant. BMI was also associated with severity of PC (data not shown). Among obese men, the lower risk of total PC was due to a reduction in risk of non-aggressive PC (IRR=0.64; 95% CI:0.35-1.17; *P*=0.12), whereas a risk estimate above unity, although based on small numbers, was observed for aggressive PC (IRR=1.20; 95% CI:0.45-3.19; *P*=0.57).

**Table 7. Interaction between the studied genotypes and NSAID use in relation to the risk of PC**

| *Genotypes* | *NSAID use* $N_{cases}/N_{controls}$ | | *NSAID use* *IRR (95% CI)*[a] | | $P^c$ | *NSAID use* *(IRR 95% CI)*[b] | | $P^c$ |
|---|---|---|---|---|---|---|---|---|
| | No | Yes | No | Yes | | No | Yes | |
| *COX-2* -1195A→G | | | | | | | | |
| AA | 172/172 | 38/38 | 1.00 (ref.) | 1.10 (0.67-1.79) | 0.83 | 1.00 (ref.) | 1.10 (0.67-1.80) | 0.97 |
| AG + GG | 106/106 | 18/18 | 1.01 (0.72-1.43) | 1.02 (0.52-1.98) | | 1.03 (0.72-1.45) | 1.11 (0.56-2.20) | |
| *COX-2* +8473T→C | | | | | | | | |
| TT | 146/127 | 23/19 | 1.00 (ref.) | 1.13 (0.59-2.19) | 0.91 | 1.00 (ref.) | 1.15 (0.59-2.26) | 0.96 |
| TC + CC | 132/151 | 33/37 | 0.78 (0.54-1.11) | 0.83 (0.49-1.43) | | 0.74 (0.51-1.07) | 0.83 (0.48-1.43) | |
| *COX-2* +3496T→C | | | | | | | | |
| TT | 224/227 | 39/47 | 1.00 (ref.) | 0.88 (0.56-1.40) | 0.10 | 1.00 (ref.) | 0.89 (0.56-1.43) | 0.08 |
| TC + CC | 54/51 | 17/9 | 1.11 (0.72-1.71) | 2.31 (0.95-5.66) | | 1.04 (0.67-1.64) | 2.36 (0.95-5.85) | |
| *COX-2* +202G→A | | | | | | | | |
| GG | 181/193 | 42/43 | 1.00 (ref.) | 1.04 (0.64-1.67) | 0.67 | 1.00 (ref.) | 1.06 (0.65-1.73) | 0.66 |
| GA + AA | 97/85 | 14/13 | 1.17 (0.82-1.68) | 1.50 (0.66-3.43) | | 1.15 (0.79-1.66) | 1.52 (0.65-3.53) | |
| *COX-2* +306C→G | | | | | | | | |
| CC | 188/188 | 39/40 | 1.00 (ref.) | 1.06 (0.66-1.72) | 0.97 | 1.00 (ref.) | 1.12 (0.69-1.83) | 0.85 |
| CG + GG | 90/90 | 17/16 | 1.00 (0.70-1.43) | 1.08 (0.51-2.26) | | 1.02 (0.71-1.48) | 1.05 (0.49-2.23) | |
| *PPARG* Pro12Ala | | | | | | | | |
| CC | 198/204 | 43/41 | 1.00 (ref.) | 1.13 (0.71-1.80) | 0.63 | 1.00 (ref.) | 1.15 (0.72-1.86) | 0.64 |
| CG + GG | 80/74 | 13/15 | 1.18 (0.79-1.77) | 1.05 (0.48-2.28) | | 1.13 (0.75-1.71) | 1.03 (0.47-2.29) | |
| *NFKB1*94ins/delATTG | | | | | | | | |
| ins/ins | 107/86 | 21/23 | 1.00 (ref.) | 0.84 (0.43-1.63) | 0.41 | 1.00 (ref.) | 0.80 (0.40-1.58) | 0.30 |
| ins/del + del/del | 171/192 | 35/33 | 0.75 (0.52-1.08) | 0.89 (0.50-1.56) | | 0.68 (0.47-0.99) | 0.85 (0.48-1.52) | |
| *IL-1B* -31T→C | | | | | | | | |
| TT | 112/127 | 22/21 | 1.00 (ref.) | 1.38 (0.72-2.65) | 0.33 | 1.00 (ref.) | 1.38 (0.71-2.67) | 0.41 |
| TC + CC | 166/151 | 34/35 | 1.35 (0.96-1.91) | 1.23 (0.71-2.15) | | 1.41 (0.99-2.00) | 1.35 (0.76-2.40) | |
| *IL-1B* -1464G→C | | | | | | | | |
| GG | 141/154 | 29/25 | 1.00 (ref.) | 1.45 (0.80-2.60) | 0.15 | 1.00 (ref.) | 1.50 (0.82-2.72) | 0.15 |
| GC + CC | 137/124 | 27/31 | 1.32 (0.93-1.87) | 1.06 (0.60-1.85) | | 1.35 (0.95-1.93) | 1.10 (0.62-1.96) | |
| *IL-1B* -3737G→A | | | | | | | | |
| GG | 88/86 | 19/20 | 1.00 (ref.) | 1.00 (0.49-2.04) | 0.83 | 1.00 (ref.) | 1.00 (0.48-2.06) | 0.75 |
| GA + AA | 190/192 | 37/36 | 0.91 (0.62-1.33) | 1.00 (0.59-1.71) | | 0.87 (0.59-1.29) | 1.00 (0.58-1.72) | |

[a] Crude
[b] Adjusted for BMI and school education at baseline
[c] P-value for interaction on a multiple scale

## Discussion

We evaluated whether inflammation is a risk factor for PC by assessing whether genetically determined differences in the inflammatory response were associated with the development of PC. Specifically, we examined associations between polymorphisms in selected inflammatory genes and risk of PC. Analyses of baseline characteristics indicated an inverse association between BMI and risk of PC and a tendency toward and increasing PC risk with increasing length of education. We found no association between the weekly use of NSAID and the risk of PC.

If inflammation is a risk factor for PC, a genetically determined high inflammatory response should be associated with PC, and vice versa. We found that none of the studied polymorphisms were associated with PC risk; however, at tendency toward an associated with PC risk was seen for one polymorphism. Carriers of the variant deletion allele of *NFKB1* -94ins/delATTG had a marginal and not statistically significant 27% reduced risk of PC. Risk reductions were seen for variant carriers of *NFKB1* -94ins/delATTG among non-users of NSAIDs (32%), for non-aggressive PC cases (36%), and among men with a BMI above 30 kg/m$^2$ (44%). Moreover, an additive effect between *NFKB1* -94ins/delATTG and *COX-2* +8473T→C was observed. Carriers of variant alleles of both polymorphisms had a 46% reduced risk of PC. These risk reductions only showed a tendency and were not statistically significant (except for the interaction between *NFKB1* -94ins/delATTG and non-aggressive PC cases), which could be due to the low number of persons in each strata or it could be attributable to chance findings.

An association between *NFKB1* -94ins/delATTG and PC has previously been observed by Zhang *et al*., who reported an 1.5-fold increased risk for PC among WT carriers of *NFKB1* -94ins/delATTG (95% CI:1.03-2.08) compared with variant allele carriers [60]. This finding is compatible with the reduced risk for PC observed among variant carriers in our study. The ins/del *NFKB1* polymorphism was included because of its proven functionality and biological effects [58,59]. Deletion of an ATTG repeat in the promoter region of *NFKB1* abolishes the binding of a nuclear factor and leads to less promoter transcriptional activity and less p50 biosynthesis [58]. The NF-κB p50 dimer activates transcription of C-reactive protein (CRP) with IL-6 and IL-1β, and deletion allele carriers of the *NFKB1* polymorphism have lower baseline plasma CRP levels in an allele-dose-dependent manner [59].

NF-κB is a transcription factor that is a homo- or heterodimer consisting of a number of different subunits p65, p50, p105, C-rel and relB [78,79], and the target gene specificity of NF-κB is determined by the subunits. The p50 subunit encoded by *NFKB1* has both pro- and anti-inflammatory properties. The p65/p50 NF-κB transcription factor complex is pro-inflammatory, controlling transcription of pro-inflammatory cytokines, such as tumor necrosis factor (TNF) and IL-1β [79]. Conversely, p50 has anti-inflammatory properties in the p50 homodimer, which represses transcription of pro-inflammatory cytokines such as TNF and IL-12 and stimulates transcription of the anti-inflammatory cytokine IL-10 and the acute phase protein CRP [59,78,80,81]. The relative abundance of p50/p65 heterodimers and p50 homodimers will therefore determine the magnitude of inflammation by balancing the pro-inflammatory and anti-inflammatory response [78]. We therefore believe that partial depletion of p50 will disfavour the

anti-inflammatory response, because the formation of the pro-inflammatory p65/p50 heterodimer depends on the concentration of p50, whereas the formation of the anti-inflammatory p50 homodimer depends on the concentration of p50 squared [59].

Consequently, the anti-inflammatory response is more affected by the genetically determined partial p50 depletion of the deletion allele than the pro-inflammatory response. Thus, the pro-inflammatory allele (deletion allele) appears to be associated with a lower PC risk.

One might assume that *NFKB1* -94ins/delATTG would be more strongly associated with aggressive PC. However, we found that carriers of the variant deletion allele *of NFKB1* -94ins/delATTG had a lower risk of non-aggressive PC, but not of aggressive PC. Zhang *et al.* found no difference between low-grade (non-aggressive) and high-grade (aggressive) PC. Furthermore, Zhang *et al*., found no difference between localized (low TNM stage) and advanced (high TNM stage) PC.

In the haplotype analyses of the *COX-2* SNPs, the haplotype frequencies were comparable to similar analyses in the study by Cheng *et al*. [53]. We were not able to reproduce the findings regarding the previously found risk haplotype defined by the variant allele of the G-allele of *COX-2* +306C→G. In fact, all the risk estimates in our study were less than unity, in contrast to the estimates by Cheng *et al.* who were above unity.

*NFKB1* -94ins/delATTG may be associated with the risk of non-aggressive PC. However, our results imply that genetically determined low *NFKB1*-mediated anti-inflammatory response in the prostate lowers the risk of non-aggressive PC, but not of aggressive PC.

**Table 8. Interaction between the studied polymorphisms and BMI in relation to the risk of PC**

| *Genotypes* | *BMI (kg/m)²* $N_{cases}/N_{controls}$ | | | *BMI (kg/m²)* *IRR (95% CI)*[a] | | | *P-value*[c] | *BMI (kg/m²)* *IRR (95% CI)*[b] | | | *P-value*[c] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | < 25 | 25-30 | > 30 | < 25 | 25-30 | > 30 | | < 25 | 25-30 | > 30 | |
| *COX-2* -1195A→G | | | | | | | | | | | |
| AA | 70/63 | 113/110 | 27/37 | 1.00 (ref.) | 0.90 (0.57-1.41) | 0.66 (0.35-1.22) | 0.92 | 1.00 (ref.) | 0.93 (0.59-1.47) | 0.69 (0.37-1.30) | 0.88 |
| AG + GG | 38/37 | 68/63 | 18/24 | 0.92 (0.51-1.66) | 0.94 (0.58-1.53) | 0.72 (0.35-1.49) | | 0.90 (0.49-1.64) | 1.01 (0.62-1.65) | 0.77 (0.37-1.61) | |
| *COX-2* +8473T→C | | | | | | | | | | | |
| TT | 49/35 | 95/82 | 25/29 | 1.00 (ref.) | 0.89 (0.52-1.52) | 0.71 (0.35-1.47) | 0.96 | 1.00 (ref.) | 0.96 (0.56-1.66) | 0.77 (0.37-1.62) | 0.97 |
| TC + CC | 59/65 | 86/91 | 20/32 | 0.75 (0.43-1.32) | 0.72 (0.42-1.23) | 0.51 (0.25-1.03) | | 0.76 (0.43-1.34) | 0.75 (0.43-1.31) | 0.54 (0.26-1.11) | |
| *COX-2* +3496T→C | | | | | | | | | | | |
| TT | 86/81 | 140/145 | 37/48 | 1.00 (ref.) | 0.89 (0.60-1.32) | 0.77 (0.44-1.34) | 0.45 | 1.00 (ref.) | 0.95 (0.64-1.42) | 0.83 (0.47-1.47) | 0.44 |
| TC + CC | 22/19 | 41/28 | 8/13 | 1.24 (0.61-2.52) | 1.40 (0.78-2.51) | 0.59 (0.23-1.51) | | 1.22 (0.59-2.51) | 1.41 (0.78-2.55) | 0.60 (0.23-1.53) | |
| *COX-2* +202G→A | | | | | | | | | | | |
| GG | 78/74 | 119/119 | 26/43 | 1.00 (ref.) | 0.91 (0.60-1.38) | 0.61 (0.34-1.08) | 0.66 | 1.00 (ref.) | 0.96 (0.63-1.47) | 0.64 (0.36-1.16) | 0.64 |
| GA + AA | 30/26 | 62/54 | 19/18 | 1.06 (0.58-1.96) | 1.07 (0.65-1.75) | 1.00 (0.47-2.12) | | 1.00 (0.54-1.87) | 1.08 (0.66-1.77) | 1.03 (0.48-2.20) | |
| *COX-2* +306C→G | | | | | | | | | | | |
| CC | 80/77 | 115/116 | 32/35 | 1.00 (ref.) | 0.92 (0.60-1.41) | 0.89 (0.48-1.65) | 0.35 | 1.00 (ref.) | 1.02 (0.66-1.58) | 0.98 (0.52-1.83) | 0.35 |
| CG + GG | 28/23 | 66/57 | 13/26 | 1.08 (0.55-2.14) | 1.06 (0.65-1.73) | 0.51 (0.24-1.08) | | 1.20 (0.60-2.40) | 1.11 (0.68-1.81) | 0.55 (0.26-1.17) | |
| *PPARG* Pro12Ala | | | | | | | | | | | |
| CC | 78/75 | 129/131 | 34/40 | 1.00 (ref.) | 0.93 (0.62-1.40) | 0.87 (0.49-1.54) | 0.32 | 1.00 (ref.) | 0.98 (0.65-1.48) | 0.94 (0.52-1.71) | 0.27 |
| CG + GG | 30/26 | 52/42 | 11/21 | 1.24 (0.65-2.35) | 1.22 (0.70-2.14) | 0.53 (0.23-1.23) | | 1.23 (0.64-2.34) | 1.29 (0.73-2.29) | 0.55 (0.24-1.27) | |
| *NFKB1* -94ins/delATTG | | | | | | | | | | | |
| ins/ins | 43/37 | 66/50 | 19/22 | 1.00 (ref.) | 1.07 (0.59-1-97) | 0.79 (0.35-1.78) | 0.85 | 1.00 (ref.) | 1.11 (0.60-2.04) | 0.86 (0.38-1.97) | 0.87 |
| ins/del + del/del | 65/63 | 115/123 | 26/39 | 0.90 (0.49-1.66) | 0.78 (0.45-1.35) | 0.57 (0.28-1.18) | | 0.84 (0.45-1.57) | 0.79 (0.45-1.37) | 0.56 (0.27-1.16) | |
| *IL-1B* -31T→C | | | | | | | | | | | |
| TT | 45/49 | 72/72 | 17/27 | 1.00 (ref.) | 1.05 (0.61-1.81) | 0.64 (0.29-1.41) | 0.78 | 1.00 (ref.) | 1.18 (0.68-2.07) | 0.72 (0.32-1.61) | 0.64 |
| TC + CC | 63/51 | 109/101 | 28/34 | 1.43 (0.82-2.51) | 1.22 (0.74-2.02) | 0.98 (0.52-1.88) | | 1.55 (0.88-2.75) | 1.34 (0.80-2.24) | 1.10 (0.57-2.12) | |
| *IL-1B* -1464G→C | | | | | | | | | | | |
| GG | 57/57 | 90/87 | 23/35 | 1.00 (ref.) | 1.00 (0.61-1.64) | 0.67 (0.34-1.29) | 0.85 | 1.00 (ref.) | 1.08 (0.66-1.79) | 0.71 (0.36-1.41) | 0.80 |
| GC + CC | 51/43 | 91/86 | 22/26 | 1.27 (0.72-2.25) | 1.11 (0.68-1.79) | 0.93 (0.46-1.89) | | 1.32 (0.74-2.35) | 1.19 (0.73-1.94) | 1.03 (0.50-2.11) | |
| *IL-1B* -3737G→A | | | | | | | | | | | |
| GG | 35/22 | 57/62 | 15/22 | 1.00 (ref.) | 0.52 (0.27-1.02) | 0.39 (0.16-0.95) | 0.10 | 1.00 (ref.) | 0.53 (0.27-1.05) | 0.42 (0.17-1.02) | 0.08 |
| GA + AA | 73/78 | 124/111 | 30/39 | 0.50 (0.26-0.97) | 0.59 (0.32-1.10) | 0.44 (0.21-1.95) | | 0.48 (0.24-0.93) | 0.61 (0.33-1.14) | 0.46 (0.21-0.99) | |

[a] Crude
[b] Adjusted for school education at baseline
[c] P-value for interaction on a multiple scale

We found a statistically significantly increased risk of PC among heterozygous carriers of the variant allele of *IL1B* T-31C, whereas a reduced risk of PC was seen among homozygous carriers of the variant allele. A combined analysis of heterozygous and homozygous variant carriers did not reveal an association between *IL-1B* -31T→C and the risk of PC. However, the discrepant findings could be due to other functional SNPs near the *IL-1B* -31T→C polymorphism. A haplotype analysis with other functional polymorphisms in the *IL-1B* gene might reveal such effects, and we therefore conducted a haplotype analysis with three SNPs in *IL1B,* all with proven functionality [61]. Previous studies have found associations between *IL-1β* -31T→C polymorphism and risk of lung cancer [48] and multiple myeloma [64]; these findings are in agreement with the biological function of the SNP (higher transcriptional levels of *IL-1β* and, hence, a more aggressive inflammatory state). However, the haplotypes defined by the variant C-allele of *IL-1β* -31T→C (GGC) and by the variant C-allele of both *IL-1β* -31T→C and *IL-1β* -1464G→C (GCC) were not associated with increased risk of PC, even though these two haplotypes leads to increased transcriptional levels of *IL-1β* [61]. Therefore, based on the results from our study, there is no indication that a high *IL-1β* expression is a risk factor for PC, and, hence, the discrepant findings for the association between *IL-1B* -31T→C and PC were likely due to chance.

NSAID inhibits inflammation and specifically COX-2 [25]. If inflammation is a risk factor for PC, we would expect that genetically determined differences in inflammatory response would be associated with PC in the absence of NSAID use, but not in the presence of NSAID use. However, no overall association with NSAID use was seen in relation to risk of PC. When stratifying by genotype, we found no apparent interactions between the selected polymorphisms and NSAID use. An interaction between *COX-2* -1195A→G and NSAIDs has been found in relation to breast and lung cancer risk [47,48]. The fact that NSAID use did not protect against PC is consistent with lack of association with PC risk for the functional *COX-2* SNPs. Consistent with this result, a recent study [82] was not able to find associations between CRP and other commonly used markers of inflammation and risk of PC, again indicating that inflammation is not a strong risk factor for PC.

Two recent studies also found an apparent modifying effect of obesity for PC among carriers of certain SNPs of inflammatory genes, including *COX-2* [72,83]. However, the study by Allott *et al.* [66] suggests that the associations between obesity and PC could be partly due to detection bias. The authors argue that digital rectal examination is more difficult and PSA levels are lower in obese men and that obesity therefore may be associated with an impaired detection of PC [66]. In Denmark, however, PSA testing is not used nearly as frequently as in other Western countries, but it remains a possibility that our findings on obesity and PC are biased due to increasing use of PSA tests.

We observed a tendency towards elevated risk estimates for PC among men with eight or more years of formal education. A similar pattern has been reported for women with breast cancer [57]. It is possible that people with a higher educational background have a higher risk of PC and breast cancer. However, these findings may also be the result of a detection bias; individuals with higher education may visit the doctor more often and insist on participation in screening measures. For PC, PSA has been implemented as a

screening test in several countries, resulting in an increased incidence of PC, but with little impact on PC mortality [4].

Our cases and controls were selected from the same population-based cohort, all with almost complete follow-up of the participants, which should minimize the risk for selection bias. For all participants, information on lifestyle factors was collected at enrolment, which minimized the risk for differential exposure misclassification between the cases and controls.

Our study group has limited statistical power, with only 334 matched cases and controls. However, we have previously found associations between the polymorphisms we investigated and risk of cancer in similarly powered studies nested within the DCH cohort. For example, among 304 basal cell carcinoma (BCC) case-control pairs, variant allele carriers of *COX-2* A11905G with genetically determined low COX-2 activity had a reduced risk of BCC (IRR=0.65; 95% CI:0.47-0.89), and homozygous carriers of *COX-2* T8473C, with genetically determined high COX-2 activity, were at increased risk of BCC (IRR=2.27; 95% CI:1.31-3.92) [47]. Assuming Hardy–Weinberg equilibrium and the allele frequency of the control group of *IL-1β* -31T→C, we had an 84% chance of detecting an OR of 1.6 for variant allele carriers at a 5% significance level. Similarly, we had 87% chance of detecting an OR of 1.7 for variant allele carriers of *Il-1β* -3737G→A. However, considering the number of statistical tests and the obtained *P* values, chance also remains a possible explanation for our findings.

Another limitation of our study is that we relied solely on Gleason scores in the categorization of PC aggressiveness. Recent evidence indicates that intermediate PC risk [84] - for example, PC with Gleason score 7 - has increasing aggressive potential with increasing quantities of Gleason score 4 components, in other words, from 3+4 to 4+3 [85]. Unfortunately, we did not have access to these pathological details. Further, we were not able to supplement the Gleason scores with data on clinical stage from the Cancer Registry because of the heavy underreporting of this information [86].

In conclusion, this study did not indicate that inflammation is a major risk factor for aggressive PC.

*Acknowledgements*

We thank Katja Boll for excellent technical support. The study was supported by a Mobility PhD grant (09-06 7572) from the Danish Research Council.

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# Chapter 4

*Accepted for publication in PLoS One*

## Paper II: *Polymorphisms in NFKB1 and TLR4 and interaction with dietary and life style factors in relation to colorectal cancer in a Danish prospective case-cohort study*

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**Abstract**

Maintenance of a balance between commensal bacteria and the mucosal immune system is crucial and intestinal dysbiosis may be a key event in the pathogenesis of colorectal cancer (CRC). The toll-like receptor 4 (TLR4) is an important pattern-recognition receptor that regulates inflammation and barrier function in the gut by a mechanism that involves activation of the nuclear factor–κB (NF-κB) transcription factor. Dietary and life style factors may impact these functions. We therefore used a Danish prospective case-cohort study of 1010 CRC cases and 1829 randomly selected participants from the Danish Diet, Cancer and Health cohort to investigate three polymorphisms in *NFKB1* and *TLR4* and their possible interactions with diet and life style factors in relation to risk of CRC. Homozygous carriage of the variant allele of the *TLR4*/rs5030728 polymorphism was associated with increased risk of CRC (incidence rate ratio (IRR)=1.30; 95% confidence interval (CI): 1.05-1.60; *P*=0.02 (gene-dose model); IRR=1.24; 95%CI: 1.01-1.51; *P*=0.04 (recessive model)). Del-carriers of the *NFKB1*/rs28362491 polymorphism had a 17% (95%CI: 1.03-1.34; *P*=0.02) increased risk of CRC compared to homozygous carriers of the ins-allele. However, none of these risk estimates withstood adjustment for multiple comparisons. We found no strong gene-environment interactions between the examined polymorphism and diet and life style factors in relation to CRC risk.

## Introduction

Colorectal cancer (CRC) is the third most common cancer type in men and the second in women worldwide [1]. Hereditary factors are estimated to contribute to only 35% of the risk [2] emphasizing the importance of environmental factors in the etiology of CRC. Indeed, dietary and lifestyle factors have been intensively studied for their role in colorectal carcinogenesis; and alcohol, smoking, obesity and high meat intake are now established risk factors for CRC [3-5]. Intake of red and processed meat has the potential of inducing cancer by chemical carcinogens formed during cooking of meat at high temperatures [6], production of toxic fermentation compounds [7-9] or by inducing inflammation due to changes in bacterial composition [10]. Conversely, fibre, fruit and vegetables provide short-chain fatty acids (SCFAs) to the colonic epithelium by fermentation of unabsorbed dietary fibre and starch [11-15]. The SCFA butyrate is important for colonic integrity [14], inhibits growth of cancer cells *in vitro* [16,17] and has anti-inflammatory properties mainly by the inhibition of nuclear factor–κB (NF-κB) activation [18].

Toll-like receptors (TLR) are important pattern-recognition receptors that regulate inflammation and barrier function in the gut thereby maintaining a balance between commensal bacteria and the mucosal immune system [19,20]. The receptor for gram-negative bacterial lipopolysaccharide (LPS), TLR4, regulates cell proliferation in response to cell injury through induction of cyclooxygenase 2 expression [21] in a cascade that involves activation of NF-κB and epidermal growth factor [22] suggesting that TLR4 is an important element in the transition from inflammation to neoplasia [19]. Indeed, increased expression of TLR4 has been linked to development of inflammation-associated neoplasia [23-26]. In addition, we have previously found evidence that inflammation may contribute to CRC carcinogenesis. Thus, genetically determined high IL-1β and COX-2 levels were associated with increased risk of CRC [27].

Dysbiosis in the gut may be a key event in the pathogenesis of both inflammatory bowel diseases (IBD) and CRC. Using functional single nucleotide polymorphisms (SNPs) and their interaction with diet and life style may reveal important pathways for colorectal carcinogenesis [28]. Since *TLR* polymorphisms have been associated with IBD [29], we aimed to examine a possible mutual mechanism for IBD and CRC.

We have previously shown that carriage of the variant del-allele of the functional ins/del *NFKB1*/rs28362491 polymorphism is associated with increased risk of CRC and interacted with meat intake in a subset of the current study group [30] in agreement with results from two other studies also reporting increased risk of CRC among variant carriers of the polymorphism among a Malaysian [31] and a Swedish population [32], but not a Chinese study group [32]. A functional SNP in *TLR4* (rs4986790) has been extensively studied and has been associated with neoplastic progression *in vitro* [33], aggressive human colon cancer [33], IBD [34-37] and CRC [38-40]. However others were not able to find an association between CRC and the SNP [41-43]. In a Canadian study [44], intake of dietary saturated fatty acids was inversely related to blood level of high density lipoprotein cholesterol in individuals homozygous for the *TLR4*/rs5030728 G-allele. *TLR4*/rs5030728 may therefore interact with dietary components in the gut.

Thus, we expected that inflammation is an important factor in colorectal carcinogenesis and thus examined whether diet and life style factors (non-steroidal anti-inflammatory drugs (NSAID) and smoking) modify CRC risk by altering the mucosal immune response in the gut via interacting with TLR4 and NF-κB. We therefore evaluated three polymorphisms in *NFKB1* and *TLR4* and their possible interaction with diet and life style factors in a prospective cohort of 1010 CRC cases and 1829 randomly selected participants from the Danish Diet, Cancer and Health Study.

## Methods

### Studied subjects

The Diet, Cancer and Health study is an ongoing Danish cohort study designed to investigate the relation between diet, lifestyle and cancer risk [45]. The cohort consists of 57,053 persons, recruited between December 1993 and May 1997. All the subjects were born in Denmark, and the individuals were 50 to 64 years of age and had no previous cancers at study entry. Blood samples and questionnaire data on diet and lifestyle were collected at study entry.

### Follow-up and endpoints

Follow-up was based on population-based cancer registries. Between 1994 and 31th December 2009, 1010 CRC cases were diagnosed. A sub-cohort of 1829 persons was randomly selected within the cohort. 28 persons were both cases and sub-cohort due to the used study design [46]. 245 with missing genotype data and 16 with missing data on risk factors were excluded. All information on genotypes and diet and lifestyle factors was available for 915 CRC cases and 1719 sub-cohort members.

### Dietary and lifestyle questionnaire

Information on diet, lifestyle, weight, height, medical treatment, environmental exposures, and other socio-economic factors were collected at enrolment using questionnaires and interviews and has been described in details elsewhere [27,47-49]. In short, the food-frequency questionnaire, diet 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 FoodCalc [45]. Smoking status was classified as never, past or current. Persons smoking at least 1 cigarette daily during the last year were classified as smokers. NSAID use ("Aspirin", "Paracetamol", "Ibuprofen", or "Other pain relievers) was assessed as ≥ 2 pills per month during one year at baseline.

### Genotyping

Buffy coat preparations were stored at minus 150°C until use. DNA was extracted as described [50]. The DNA was genotyped by LGC KBioscience (LGC KBioscience, Hoddesdon, United Kingdom) by PCR-based KASP™ genotyping assay (http:// www.lgcgenomics.com/). *NFKB1*/rs28362491 was analysed and reported for a subset of the current study group [30]. Two of the polymorphisms (*NFKB1*/rs28362491 and *TLR4*/rs4986790) were chosen based on known functionality and their association with CRC [30-33] and IBD [34-37] from a literature search. The *TLR4*/rs5030728 polymorphism, on the other hand, has no known

functionality. However, *TLR4*/rs4986790 is tightly linked with *TLR4*/rs5030728 (D': 1.0; $r^2$: 0.017) using Haploview version 4.2 (Broad Institute of MIT and Harvard, Cambridge) [51] with HapMap3 Genome Browser release #2 (Phase 3) [52]; and since *TLR4*/rs5030728 has a higher minor allele frequency in Caucasians than *TLR4*/rs4986790 (0.305 vs. 0.035), this polymorphism is more suitable for gene-environment interaction analyses. To confirm reproducibility, genotyping was repeated for 10% of the samples yielding 100% identity.

**Statistical analysis**

Deviation from Hardy-Weinberg equilibrium was assessed using a Chi-square test.

Incidence rate ratios (IRR) and 95% Confidence Interval (CI) were calculated according to the principles for analysis of case-cohort studies using an un-weighted approach [46]. Age was used as the time scale in the Cox regression models. Tests and confidence intervals were based on Wald's tests using the robust estimate of the variance-covariance matrix for the regression parameters in the Cox regression models [53] as previously described [27,46,48,54-61].

All models were adjusted for baseline values of risk factors for colorectal cancer such as body mass index (BMI) (kg/$m^2$, continuous), use of hormone replacement therapy (HRT) (never/past/current, among women), intake of dietary fibre (g/day, continuous), and red meat and processed meat (g/day, continuous) and in addition to suspected risk factors such as NSAID use (yes/no) and smoking status (never/past/current). Cereals, fibre, fruit and vegetables were also entered linearly. All analyses were stratified by gender, so that the basic (underlying) hazards were gender specific. For all the polymorphisms, IRR was calculated separately for heterozygous and homozygous variant allele carriers. For *TLR4*/rs4986790 and *NFKB1*/rs28362491, variant allele carriers were subsequently grouped for interaction analyses since no recessive effects were observed. *TLR4/*rs5030728 was inferred both in a gene-dose and a recessive mode in the subsequent analyses.

Moreover, we assessed weekly use of NSAID based on the results of a study of colorectal cancer within the Diet, Cancer and Health cohort [62] reporting that long-term consistent use of Aspirin or Non-Aspirin NSAID appears necessary to achieve a protective effect. However, there were no differences in risk estimates between monthly or weekly use, consequently, to maintain the statistical power in the strata; we used monthly NSAID use in the analyses.

The likelihood ratio test was used for interaction analyses between the studied polymorphisms and intake of red and processed meat, dietary fibre, cereals, fish, fruits, vegetables, alcohol intake, smoking status and NSAID use. In interaction analyses where the dietary factors were entered as categorical variables, tertile cutpoints were based on the empirical distribution among male and female cases, respectively. The possible interactions were investigated using the likelihood ratio test.

All analyses were performed using SAS version 9.3 (SAS Institute Inc., Cary, NC). A $p<0.05$ was considered to be significant. Moreover, to test for multiple comparisons, Bonferroni correction was used.

**Ethics statement**

All participants gave verbal and written informed consent. The Diet, Cancer and Health study was approved by the National Committee on Health Research Ethics (journal nr. (KF) 01-345/93) and the Danish Data Protection Agency.

## Results

Baseline characteristics of the study population are presented in Table 1. Among sub-cohort members, the genotype distribution of the SNPs did not deviate from Hardy-Weinberg equilibrium (results not shown).

**Table 1. Baseline characteristics of the study participants by selected demographic and established CRC risk factors.**

| *Variable* | *Cases* | | *Sub-cohort* | | *IRR[a] (95% CI)* |
|---|---|---|---|---|---|
| | *n (%)* | *Median (5-95%)* | *n (%)* | *Median (5-95%)* | |
| Total | 915 (100) | | 1719 (100) | | |
| Sex | | | | | |
| Men | 515 (56) | | 920 (54) | | |
| Women | 400 (44) | | 799 (46) | | |
| Age at inclusion (years) | | 58 (51-64) | | 56 (50-64) | |
| BMI (kg/m$^2$) | | 26.3 (20.7-34.3) | | 25.6 (20.5-33.0) | 1.03 (1.00-1.06)[d] |
| Food intake (g/day) | | | | | |
| Alcohol[b] | | 15.1 (1.0-71.6) | | 14.2 (1.2-65.3) | 1.03 (1.00-1.07)[e] |
| Dietary fibre | | 19.9 (10.8-32.9) | | 20.7 (10.7-34.2) | 0.88 (0.80-0.97)[f] |
| Red and processed meat | | 113.1 (47.4-233.4) | | 108.9 (41.5-235.4) | 1.03 (1.00-1.06)[g] |
| Smoking status | | | | | |
| Never | 274 (30) | | 572 (33) | | 1.00 (ref.) |
| Past | 280 (31) | | 513 (30) | | 1.04 (0.88-1.23) |
| Current | 361 (39) | | 634 (37) | | 1.11 (0.94-1.30) |
| NSAID use[c] | | | | | |
| No | 632 (69) | | 1174 (68) | | 1.00 (ref.) |
| Yes | 283 (31) | | 545 (32) | | 0.99 (0.86-1.14) |
| HRT use among women | | | | | |
| Never | 246 (62) | | 418 (52) | | 1.00 (ref.) |
| Past | 50 (13) | | 126 (16) | | 0.66 (0.49-0.90) |
| Current | 104 (26) | | 255 (32) | | 0.74 (0.59-0.93) |

Values are expressed as medians (5th and 95th percentiles) or as fractions (%).
[a]IRRs for CRC – mutually adjusted.
[b]Among current drinkers.
[c]NSAID use is defined as ≥ 2 pills per month during one year.
[d]Risk estimate per 2 kg/m$^2$ increment of BMI.
[e]Risk estimate for the increment of 10 g alcohol per day.
[f]Risk estimate for the increment of 10 g dietary fibres per day.
[g]Risk estimate for the increment of 25 g red and processed meat per day.

*Associations between polymorphisms and CRC*

Homozygous variant carriers of the *TLR4*/rs5030728 polymorphism were at 1.30-fold (95%CI: 1.05-1.60) increased risk of CRC in a gene-dose model and at 1.24-fold (95%CI: 1.01-1.51) increased risk of CRC compared to wild type and heterozygous carriers in a recessive model (Table 2). Moreover, carriers of the *NFKB1* del-allele had a 17% (95%CI: 1.03-1.34) increased risk of CRC compared to homozygous carriers of the ins-allele (Table 2). These risk estimates did not, however, reach statistically significance after Bonferroni correction. There was no interaction between the two risk genotypes *TLR4*/rs5030728 and *NFKB1*/rs28362491 but on the other hand, there was no additive effect of being homozygous carrier of both variant alleles (Table S1).

**Table 2. IRR for CRC in relation to the studied polymorphisms.**

| | | $n_{cases}$ (%) | $n_{sub\text{-}cohort}$ (%) | IRR[a] (95% CI) | IRR[b] (95% CI) | P-value[c] |
|---|---|---|---|---|---|---|
| *TLR4* | rs4986790 | | | | | |
| | AA | 839 (92) | 1577 (92) | 1.00 (ref.) | 1.00 (ref.) | - |
| | GA | 76 (8) | 141 (8) | 0.99 (0.79-1.25) | 1.00 (0.79-1.26) | 0.98 |
| | GG | 0 (0) | 1 (0) | - | - | - |
| | GA+GG | 76 (8) | 142 (8) | 0.99 (0.79-1.25) | 1.00 (0.79-1.26) | 0.97 |
| | rs5030728 | | | | | |
| | GG | 405 (44) | 826 (48) | 1.00 (ref.) | 1.00 (ref.) | - |
| | GA | 399 (44) | 731 (43) | 1.10 (0.96-1.26) | 1.11 (0.96-1.27) | 0.16 |
| | AA | 111 (12) | 162 (9) | 1.30 (1.06-1.61) | 1.30 (1.05-1.60) | 0.02 |
| | GA+AA | 510 (56) | 398 (23) | 1.14 (1.00-1.30) | 1.14 (1.00-1.30) | 0.05 |
| | AA vs. GG+GA | 111 (12) | 162 (9) | 1.24 (1.02-1.52) | 1.24 (1.01-1.51) | 0.04 |
| *NFKB1* | rs28362491 | | | | | |
| | Ins/Ins | 320 (35) | 679 (60) | 1.00 (ref.) | 1.00 (ref.) | - |
| | Ins/Del | 449 (49) | 787 (46) | 1.19 (1.03-1.37) | 1.19 (1.03-1.37) | 0.02 |
| | Del/Del | 146 (16) | 253 (15) | 1.13 (0.93-1.37) | 1.14 (0.94-1.38) | 0.18 |
| | Ins/Del+Del/Del | 595 (65) | 1040 (61) | 1.17 (1.02-1.34) | 1.17 (1.03-1.34) | 0.02 |

[a] Crude – adjusted for age and sex.
[b] In addition, adjusted for smoking status, alcohol intake , HRT status (women only), BMI, use of NSAID, intake of red and processed meat, and dietary fibre.
[c] P-value for the adjusted estimate

*Gene-environment analyses*

We found no interaction between any of the dietary factors and the studied polymorphisms in relation to risk of CRC in the linear analyses (Table S2). In the tertile analyses, there was weak interaction between *TLR4*/rs5030728 and intake of vegetables (Gene-dose model: P-value for interaction ($P_{int}$)=0.05; Recessive model: $P_{int}$=0.03) (Table 3). Moreover, a borderline statistically significant interaction between meat intake and the *NFKB1*/rs28362491 polymorphism ($P_{int}$=0.06) was found (Table 3). For ins-carriers, risk estimates were comparable across tertiles of meat intake. Conversely, among del-carriers, intake of meat in the second (IRR=1.46; 95%CI: 1.17-1.83) and third tertile (IRR=1.24; 95%CI: 0.99-1.56) was associated with risk of CRC, whereas del-allele carriers were not at risk in the first tertile with low meat intake (IRR=0.98; 95%CI: 0.78-1.23). With regard to alcohol, variant carriers of *NFKB1*/rs28362491 and homozygous A-allele carriers of *TLR4*/rs5030728 were associated with CRC risk compared to the homozygous wild type carriers among participants with a low intake of alcohol. Furthermore, for variant carriers of all three polymorphisms, a low intake (first tertile) of alcohol was associated with the highest CRC risk compared with moderate (second tertile) intake - which was associated with the lowest risk - and high alcohol intake (third tertile) (Table 3).

**Table 3. IRR for CRC for tertiles of intake of dietary factors for the studied polymorphisms.**

| | *1.tertile* | | *2.tertile* | | *3.tertile* | | *1.tertile* | *2.tertile* | *3.tertile* | *P-value*[b] | *1.tertile* | | *2.tertile* | | *3.tertile* | | *1.tertile* | *2.tertile* | *3.tertile* | *P-value*[b] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *Nc* | *Ns* | *Nc* | *Ns* | *Nc* | *Ns* | *IRR (95%CI)*[a] | *IRR (95%CI)*[a] | *IRR (95%CI)*[a] | | *Nc* | *Ns* | *Nc* | *Ns* | *Nc* | *Ns* | *IRR (95%CI)*[a] | *IRR (95%CI)*[a] | *IRR (95%CI)*[a] | |
| *TLR4*/rs4986790 | | | | | | | ***Red and processed meat*** | | | | | | | | | | ***Fish*** | | | |
| AA | 273 | 598 | 248 | 457 | 288 | 522 | 1.00 (ref.) | 1.31 (1.11-1.55) | 1.22 (1.04-1.54) | | 279 | 534 | 286 | 496 | 274 | 547 | 1.00 (ref.) | 1.02 (0.86-1.20) | 0.92 (0.77-1.10) | |
| GA+GG | 29 | 49 | 27 | 48 | 20 | 45 | 1.15 (0.77-1.72) | 1.22 (0.85-1.76) | 0.98 (0.62-1.55) | 0.49 | 27 | 48 | 21 | 46 | 28 | 48 | 1.06 (0.70-1.60) | 0.85 (0.55-1.32) | 0.93 (0.63-1.37) | 0.72 |
| | | | | | | | ***Dietary cereal*** | | | | | | | | | | ***Dietary fibre*** | | | |
| AA | 282 | 445 | 277 | 534 | 280 | 598 | 1.00 (ref.) | 0.88 (0.74-1.05) | 0.94 (0.75-1.17) | | 279 | 479 | 277 | 464 | 283 | 634 | 1.00 (ref.) | 0.99 (0.84-1.16) | 0.81 (0.69-0.96) | |
| GA+GG | 19 | 44 | 30 | 42 | 27 | 56 | 0.74 (0.46-1.19) | 1.01 (0.69-1.47) | 0.96 (0.64-1.46) | 0.33 | 21 | 48 | 29 | 36 | 26 | 58 | 0.78 (0.49-1.22) | 1.29 (0.88-1.90) | 0.72 (0.49-1.07) | 0.19 |
| | | | | | | | ***Fruit*** | | | | | | | | | | ***Vegetables*** | | | |
| AA | 275 | 487 | 287 | 535 | 277 | 555 | 1.00 (ref.) | 0.96 (0.81-1.14) | 0.95 (0.77-1.16) | | 275 | 466 | 278 | 549 | 286 | 562 | 1.00 (ref.) | 1.02 (0.86-1.21) | 1.08 (0.89-1.32) | |
| GA+GG | 25 | 39 | 22 | 52 | 29 | 51 | 1.04 (0.69-1.56) | 0.81 (0.52-1.27) | 0.97 (0.66-1.44) | 0.75 | 28 | 34 | 26 | 59 | 22 | 49 | 1.17 (0.79-1.72) | 0.91 (0.60-1.38) | 0.95 (0.62-1.47) | 0.55 |
| | | | | | | | ***Alcohol*** | | | | | | | | | | | | | |
| AA | 288 | 517 | 277 | 578 | 274 | 482 | 1.00 (ref.) | 0.92 (0.78-1.08) | 1.10 (0.93-1.31) | | | | | | | | | | | |
| GA+GG | 33 | 42 | 22 | 53 | 21 | 47 | 1.23 (0.86-1.78) | 0.80 (0.52-1.22) | 0.89 (0.57-1.40) | 0.28 | | | | | | | | | | |
| *TLR4*/rs5030728 | | | | | | | ***Red and processed meat*** | | | | | | | | | | ***Fish*** | | | |
| GG | 134 | 295 | 134 | 255 | 137 | 276 | 1.00 (ref.) | 1.19 (0.94-1.51) | 1.08 (0.86-1.36) | | 127 | 284 | 139 | 262 | 139 | 280 | 1.00 (ref.) | 1.08 (0.86-1.37) | 1.00 (0.78-1.27) | |
| GA | 133 | 284 | 134 | 210 | 132 | 237 | 1.00 (0.79-1.27) | 1.34 (1.06-1.69) | 1.26 (1.00-1.60) | | 137 | 240 | 131 | 227 | 131 | 264 | 1.21 (0.94-1.54) | 1.13 (0.89-1.44) | 1.03 (0.81-1.32) | |
| AA | 35 | 68 | 37 | 40 | 39 | 54 | 1.06 (0.73-1.55) | 1.70 (1.19-2.43) | 1.54 (1.09-2.17) | 0.73 | 42 | 58 | 37 | 53 | 32 | 51 | 1.39 (0.99-1.96) | 1.32 (0.92-1.90) | 1.23 (0.83-1.81) | 0.90 |
| GG+GA | 267 | 579 | 268 | 465 | 269 | 513 | 1.00 (ref.) | 1.26 (1.06-1.49) | 1.16 (0.98-1.37) | | 264 | 524 | 270 | 489 | 270 | 544 | 1.00 (ref.) | 1.01 (0.85-1.20) | 0.93 (0.78-1.11) | |
| AA | 35 | 68 | 37 | 40 | 39 | 54 | 1.06 (0.74-1.52) | 1.70 (1.21-2.38) | 1.54 (1.11-2.13) | 0.55 | 42 | 58 | 37 | 53 | 32 | 51 | 1.27 (0.92-1.75) | 1.20 (0.85-1.70) | 1.12 (0.77-1.63) | 0.96 |
| | | | | | | | ***Dietary cereal*** | | | | | | | | | | ***Dietary fibre*** | | | |
| GG | 125 | 233 | 136 | 275 | 144 | 318 | 1.00 (ref.) | 0.97 (0.76-1.24) | 1.00 (0.76-1.31) | | 130 | 254 | 131 | 234 | 144 | 338 | 1.00 (ref.) | 1.05 (0.83-1.33) | 0.83 (0.66-1.05) | |
| GA | 126 | 211 | 145 | 244 | 128 | 276 | 1.10 (0.85-1.41) | 1.07 (0.84-1.37) | 1.07 (0.81-1.42) | | 126 | 223 | 142 | 217 | 131 | 291 | 1.10 (0.86-1.40) | 1.14 (0.91-1.44) | 0.89 (0.70-1.14) | |
| AA | 50 | 45 | 26 | 57 | 35 | 60 | 1.54 (1.12-2.13) | 0.89 (0.58-1.36) | 1.34 (0.90-2.00) | 0.34 | 44 | 50 | 33 | 49 | 34 | 63 | 1.35 (0.96-1.90) | 1.22 (0.84-1.78) | 1.11 (0.76-1.62) | 0.98 |
| GG+GA | 251 | 444 | 281 | 519 | 272 | 594 | 1.00 (ref.) | 0.97 (0.81-1.17) | 0.99 (0.79-1.23) | | 256 | 477 | 273 | 451 | 275 | 629 | 1.00 (ref.) | 1.05 (0.89-1.24) | 0.82 (0.69-0.98) | |
| AA | 50 | 45 | 26 | 57 | 35 | 60 | 1.48 (1.09-1.99) | 0.85 (0.57-1.28) | 1.28 (0.88-1.87) | 0.10 | 44 | 50 | 33 | 49 | 34 | 63 | 1.30 (0.94-1.78) | 1.17 (0.82-1.67) | 1.06 (0.74-1.52) | 0.79 |
| | | | | | | | ***Fruit*** | | | | | | | | | | ***Vegetables*** | | | |
| GG | 139 | 264 | 135 | 275 | 131 | 287 | 1.00 (ref.) | 0.98 (0.77-1.24) | 0.91 (0.70-1.18) | | 132 | 250 | 130 | 289 | 143 | 287 | 1.00 (ref.) | 1.03 (0.80-1.31) | 1.16 (0.90-1.50) | |
| GA | 126 | 212 | 136 | 260 | 137 | 259 | 1.10 (0.87-1.40) | 0.98 (0.78-1.25) | 1.07 (0.83-1.39) | | 136 | 187 | 129 | 269 | 134 | 275 | 1.28 (1.01-1.62) | 1.06 (0.83-1.35) | 1.15 (0.88-1.50) | |
| AA | 35 | 50 | 38 | 52 | 38 | 60 | 1.26 (0.88-1.82) | 1.24 (0.87-1.76) | 1.21 (0.83-1.75) | 0.92 | 35 | 63 | 45 | 50 | 31 | 49 | 1.02 (0.70-1.47) | 1.75 (1.26-2.44) | 1.44 (0.96-2.15) | 0.05 |

| | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| GG+GA | 265 | 476 | 271 | 353 | 268 | 546 | 1.00 (ref.) | 0.94 (0.79-1.12) | 0.94 (0.77-1.15) | | 268 | 437 | 259 | 558 | 277 | 562 | 1.00 (ref.) | 0.93 (0.78-1.11) | 1.03 (0.84-1.26) | |
| AA | 35 | 50 | 38 | 52 | 38 | 60 | 1.21 (0.85-1.71) | 1.18 (0.85-1.65) | 1.15 (0.81-1.65) | 0.98 | 35 | 63 | 45 | 50 | 31 | 49 | 0.90 (0.64-1.28) | 1.56 (1.15-2.13) | 1.28 (0.88-1.88) | 0.03 |
| | | | | | | | ***Alcohol*** | | | | | | | | | | | | | |
| GG | 133 | 267 | 135 | 306 | 137 | 253 | 1.00 (ref.) | 0.98 (0.78-1.25) | 1.23 (0.97-1.57) | | | | | | | | | | | |
| GA | 150 | 246 | 128 | 261 | 121 | 224 | 1.23 (0.97-1.54) | 1.07 (0.84-1.36) | 1.21 (0.94-1.55) | | | | | | | | | | | |
| AA | 38 | 46 | 36 | 64 | 37 | 52 | 1.70 (1.19-2.42) | 1.14 (0.79-1.65) | 1.37 (0.95-1.98) | 0.41 | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| GG+GA | 283 | 513 | 263 | 567 | 258 | 477 | 1.00 (ref.) | 0.92 (0.78-1.09) | 1.10 (0.93-1.31) | | | | | | | | | | | |
| AA | 38 | 46 | 36 | 64 | 37 | 52 | 1.53 (1.10-2.14) | 1.03 (0.73-1.46) | 1.24 (0.88-1.75) | 0.33 | | | | | | | | | | |
| *NFKB1*/rs28362491 | | | | | | | ***Red and processed meat*** | | | | | | | | | | ***Fish*** | | | |
| I/I | 112 | 238 | 102 | 225 | 106 | 216 | 1.00 (ref.) | 1.01 (0.78-1.31) | 1.06 (0.82-1.37) | | 99 | 228 | 116 | 214 | 105 | 237 | 1.00 (ref.) | 1.09 (0.84-1.41) | 0.95 (0.72-1.26) | |
| I/D+D/D | 190 | 409 | 203 | 280 | 202 | 351 | 0.98 (0.78-1.23) | 1.46 (1.17-1.83) | 1.24 (0.99-1.56) | 0.06 | 207 | 354 | 191 | 328 | 197 | 358 | 1.25 (0.98-1.58) | 1.20 (0.94-1.53) | 1.13 (0.88-1.44) | 0.76 |
| | | | | | | | ***Dietary cereal*** | | | | | | | | | | ***Dietary fibre*** | | | |
| I/I | 105 | 187 | 101 | 212 | 114 | 280 | 1.00 (ref.) | 0.87 (0.66-1.15) | 0.91 (0.68-1.22) | | 104 | 202 | 112 | 201 | 104 | 276 | 1.00 (ref.) | 1.05 (0.81-1.36) | 0.75 (0.58-0.99) | |
| I/D+D/D | 196 | 302 | 206 | 364 | 193 | 374 | 1.10 (0.87-1.39) | 1.04 (0.81-1.32) | 1.13 (0.85-1.50) | 0.76 | 196 | 325 | 194 | 299 | 205 | 416 | 1.14 (0.90-1.44) | 1.16 (0.92-1.46) | 0.97 (0.77-1.23) | 0.66 |
| | | | | | | | ***Fruit*** | | | | | | | | | | ***Vegetables*** | | | |
| I/I | 113 | 218 | 107 | 216 | 100 | 245 | 1.00 (ref.) | 0.96 (0.74-1.25) | 0.88 (0.66-1.17) | | 105 | 199 | 114 | 240 | 101 | 240 | 1.00 (ref.) | 1.16 (0.89-1.50) | 1.11 (0.83-1.49) | |
| I/D+D/D | 187 | 308 | 202 | 371 | 206 | 361 | 1.15 (0.91-1.45) | 1.07 (0.85-1.35) | 1.12 (0.87-1.45) | 0.70 | 198 | 301 | 190 | 368 | 207 | 371 | 1.31 (1.04-1.65) | 1.19 (0.94-1.52) | 1.33 (1.03-1.71) | 0.37 |
| | | | | | | | ***Alcohol*** | | | | | | | | | | | | | |
| I/I | 104 | 230 | 111 | 254 | 105 | 195 | 1.00 (ref.) | 1.08 (0.83-1.40) | 1.32 (1.01-1.73) | | | | | | | | | | | |
| I/D+D/D | 217 | 329 | 188 | 377 | 190 | 334 | 1.45 (1.15-1.83) | 1.16 (0.92-1.48) | 1.36 (1.08-1.73) | 0.09 | | | | | | | | | | |

[a] Analysis adjusted for smoking status, alcohol intake, HRT status (women only), BMI, use of NSAID, intake of red and processed meat, and dietary fibre.
[b] P-value for interaction between polymorphisms and dietary factors for the adjusted estimates

Women: Tertiles of red and processed meat (<74.7773 g, 74.7773 g < and < 102.086 g, >102.086 g), fish (<27.2196 g, 27.2196 g < and < 43.6676 g, > 43.6676 g), dietary fibre (<16.9701 g, 16.9701 g < and < 22.0983 g, > 22.0983 g), cereals (<135.539 g, 135.539 g < and < 190.481 g, > 190.481 g), fruit (<141.858 g, 141.858 g < and < 266.488 g, > 266.488 g), vegetables (<121.870 g, 121.870 g < and < 213.721 g, > 213.721 g), laktose (<7.54871 g, 7.54871 g < and < 17.1355 g, > 17.1355 g), dairy products (<218.144 g, 218.144 g < and < 454.235 g, > 454.235 g), alcohol (<4.31931 g, 4.31931 g < and < 12.9957 g, > 12.9957 g).

Men: Tertiles of red and processed meat (<116.935 g, 116.935 g < and < 159.387 g, >159.387 g), fish (<33.3477 g, 33.3477 g < and < 52.7767 g, > 52.7767 g), dietary fibre (<17.5748 g, 17.5748 g < and < 22.4931 g, > 22.4931 g), cereals (<166.378 g, 166.378 g < and < 233.859 g, > 233.859 g), fruit (<90.9913 g, 90.9913 g < and < 193.509 g, > 193.509 g), vegetables (<105.532 g, 105.532 g < and < 186.459 g, > 186.459 g), laktose (<7.93777 g, 7.93777 g < and < 17.2082 g, > 17.2082 g), dairy products (<217.360 g, 217.360 g < and < 461.449 g, > 461.449 g), alcohol (<14.4960 g, 14.4960 g < and < 37.1134 g, > 37.1134 g).

There was no interaction between NSAID use or smoking status and the studied genotypes (Table S3 and S4). Among non-smokers (Table S4), however, the *TLR4*/rs5030728 polymorphism demonstrated gene-dose effect which is comparable with the results seen among participants with low intake of alcohol (Table 3). In a model where the risk of CRC was inferred per 25 g intake of meat per day subdivided by NSAID use, the risk of CRC by meat intake increased in a dose-dependent manner among variant allele carriers of *TLR4*/rs5030728 in the absence of NSAID use, but not among NSAID-users (Table S5). Thus, meat intake was not associated with risk among homozygous carriers of the wild type allele, whereas meat intake was associated with a 4% increased risk per 25 g meat/day (95%CI: 1.00-1.09) among heterozygotes and 11% increased risk among homozygous variant allele carriers (95%CI: 1.02-1.22). However, there were no statistically significant interactions (Table S5).

## Discussion

In the present study, we found that homozygous variant carriage of *TLR4*/rs5030728 and variant carriage of the *NFKB1*/rs28362491 polymorphism were associated with increased risk of CRC, but not after correction for multiple testing. We only found weak interactions with a few dietary factors and, thus, we were not able to reproduce the previously found interaction between the *NFKB1*/rs28362491 polymorphism and meat intake. The lack of association between *TLR4*/rs4986790 and CRC found in the present study could possibly be due to the very low variant allele frequency in the Danish population. Only one person was homozygous variant allele carrier. We therefore cannot exclude that the functional effect of this SNP affects colorectal carcinogenesis.

The *NFKB1*/rs28362491 polymorphism has rather consistently been associated with CRC risk [30-32], and to some extend also IBD [29,63,64]. As previously described [65,66], *NFKB1* encodes the p50/p105 subunits of the transcription factor NF-κB. NF-κB consists of homo- or heterodimers of a number of different subunits p65, p50, p105, C-rel and relB [67,68] and the combination determines target gene specificity. As a p65/p50 heterodimer, the complex is pro-inflammatory [68], whereas the p50 homodimer has anti-inflammatory properties [65,67,69,70]. The relative abundance of p50/p65 heterodimers and p50 homodimers will therefore determine the magnitude of inflammation by balancing the pro-inflammatory and anti-inflammatory response [67]. The *NFKB1*/rs28362491 polymorphism generates a deletion of four nucleotides in the promoter region causing lowered transcription levels and consequently partial depletion of p50 [63]. In agreement with this, it was found that the mRNA levels of *NFKB1* were lower in colon biopsies of healthy tissue from homozygous del-carriers compared to heterozygotes [71]. This disfavours the anti-inflammatory response since the formation of the pro-inflammatory p65/p50 heterodimer depends on the concentration of p50, whereas the formation of the anti-inflammatory p50 homodimer depends on the concentration of p50 squared [65].

The *TLR4*/rs5030728 polymorphism has not yet been linked to CRC [20] and its function is unknown [72]. *TLR4*/rs4986790, which has been associated with IBD and CRC, is tightly linked with *TLR4*/rs5030728. It is therefore not clear which of the two polymorphisms is the biologically relevant one. However, our results indicate that carriage of *TLR4*/rs4986790 was not associated with risk of CRC, whereas carriage of *TLR4*/rs5030728 was associated with risk. This suggests that the risk conferred by *TLR4*/rs5030728 carriage

was not caused by linkage with *TLR4*/rs4986790. Interestingly, variant carriage of the *TLR4*/rs5030728 A-allele has been associated with beneficial response to anti-TNF therapy among patients with IBD [73], implying that these patients may have a higher baseline activity or expression of TLR4.

The two *TLR4* SNP are present on several commonly used GWAS arrays (https://www.broadinstitute.org/mpg/snap/ldsearch.php) whereas the ins/del *NFKB1*/rs28362491 polymorphism is not monitored linkage in GWAS [65]. None of the two *TLR4* SNPs were associated with CRC in GWAS. However, our main focus was to search for gene-environment interactions, rather than identifying loci with strong associations to CRC. Gene-environment interactions are rarely assessed in GWAS.

We did not find any strong indications of gene-environment interactions. For variant carriers of *NFKB1*/rs28362491 and homozygous A-allele carriers of *TLR4*/rs5030728, risk of CRC among low meat consumers was lower compared to medium and high meat consumers, who had risk estimates between 1.24 and 1.70 indicating a stronger role of meat in colorectal carcinogenesis among subjects with genetically determined high inflammatory response. Alternatively, meat intake covaries with other life style factors that *per se* induce an inflammatory response that we have not been able to adjust for.

The found interaction with vegetables and *TLR4*/rs5030728 in the present study is not directly interpretable and could be due to small groups in the tertiles. However, vegetables seemed to slightly increase the risk of CRC in the present study, which should be addressed in other prospective studies.

We had limited statistical power to detect gene-environment interactions. However, the prospective study design used in this study is well suited for gene-environment interaction analyses due to the collection of dietary and life style factors before diagnosis, eliminating the risk of recall bias. Changing in dietary and life style habits during follow-up is, however, possible, but is not expected to result in differential misclassification between cases and the comparison group. In addition, the present study group is homogenous consisting of Danes and two of the studied polymorphisms have high allele frequencies. Using the present study group, we have previously found gene-environment interactions between diet and *IL10* rs3024505 ($P_{int}$;meat=0.04, fish=0.007, fibre=0.0008, vegetables=0.0005), *IL1B* C-3737T ($P_{int}$; NSAID use=0.040), *PTGS2* G-765C ($P_{int}$; meat=0.006, fibre=0.0003, fruit 0.004), and *PTGS2* T8473C ($P_{int}$; fruit=0.03) and *PTGS2* A-1195G ($P_{int}$;fibre 0.020 and current smoking=0.046) [27]. We adjusted risk estimates for suspected risk factors and carefully selected the polymorphisms based on function and/or previously findings on association with dietary factors, CRC or IBD. However, none of the analyses withstood adjusting for multiple testing. Thus, we cannot rule out that our findings are due to chance and they should therefore not be considered as significant associations.

In conclusion, this study was not able to demonstrate associations between the studied polymorphisms in the inflammatory mediator genes *NFKB1* and *TLR4* as none of the found associations withstood adjustment for multiple comparisons. We found no strong gene-environment interactions between the examined polymorphism and diet and life style factors in relation to CRC risk.

*Acknowledgements*

We thank Nick Martinussen and Katja Boll for technical assistance with data managing; and Jane Christensen for statistical consulting. The project was supported by a PhD stipend from the Danish Research Councils in the programme 'An integrated approach to risk-benefit assessment of human health effects of food and food contaminants' (Forskeruddannelse 2009-10), and by a Mobility PhD grant (09-06 7572) from the Danish Council for Independent Research (www.ufm.dk) and as part of the project: "Beef versus pork consumption in the etiology of cancers in the colon and rectum: investigations performed within the Diet, Cancer and Health cohort" also from the Danish Council for Independent Research; Medical Sciences (grant no. 09-073597) (www.ufm.dk). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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# Chapter 5

*Submitted to Cancer Genetics*

## Paper III: *Polymorphisms in ABC transporter genes and interaction with diet and life style factors in relation to colorectal cancer in a Danish prospective case-cohort study*

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**Abstract**

The ATP-binding cassette (ABC) transporter family transports various molecules across the enterocytes in the gut protecting the intestine against potentially harmful substances. Moreover, ABC transporters are involved in mucosal immune defence through the control of ABC transporter expression by cytokines. We used a Danish prospective case-cohort study of 1010 CRC cases and 1829 randomly selected participants from the Danish Diet, Cancer and Health cohort to 1) examine whether polymorphisms in *ABCB1*, *ABCC2* and *ABCG2* were associated with risk of CRC; 2) investigate possible interactions with diet and life style factors (smoking and use of non-steroidal anti-inflammatory drugs (NSAIDs)) in relation to CRC risk; and 3) examine interactions between ABC gene, IL10 and IL1B polymorphisms in relation to CRC risk (retrieved from Andersen et al.; PLoS One 2013; 8(10):e78366). None of the polymorphisms were associated with CRC *per se*. Cereals and fibre interacted with *ABCB1*/rs1045642 (*P*-value for interaction ($P_{int}$)=0.001 and 0.01, respectively) and fruit interacted with *ABCC2*/rs2273697 ($P_{int}$=0.01). Combinations of ABC transporter gene polymorphisms and IL10 polymorphisms interacted with intake of fibre ($P_{int}$=0.0007 and 0.009). A pattern where NSAID use was associated with decreased risk of CRC among carriers of low activity variants of the three ABC transporter genes was seen. Additionally, interaction with NSAID use was found for combinations of *ABCB1*/rs1045642, *ABCG2*/rs2231137 and *IL10*/rs3024505 ($P_{int}$=0.007 and 0.02, respectively). In conclusion, we found indication of interaction between ABC transporter polymorphisms and intake of fibre, cereals, fruit and NSAID use in relation to CRC risk suggesting that these factors may affect CRC risk by a mechanism involving the ABC transporters. We also found indication of involvement of the immune defence system in this mechanism. These results should be replicated in other cohorts to rule out chance findings.

## Introduction

Colorectal cancer (CRC) is one of the most frequent cancer types in the world. Environmental factors, such as alcohol, smoking, obesity and high meat intake have major impact on the carcinogenesis in combination with inheritable genetic factors [1-3]. Modulation of the exposure to food carcinogens in the intestines by enzymes involved in transport or metabolism of the carcinogenic substances may modify risk of CRC. Identifying gene-environment interactions may provide insight into the underlying mechanism of action because an interaction places gene or pathways and environmental factors in the same carcinogenic pathway [4].

The ATP-binding cassette transporter family (ABCs) transports various molecules across extra- and intra-cellular membranes controlling absorption, distribution, metabolism and excretion of a wide variety of exogenous and endogenous substrates including numerous drugs, but also natural food constituents such as flavonoids, lipids and mycotoxins [5-8] and "human-made" food constituents like pesticides [9], insecticides [9], the carcinogens polycyclic aromatic hydrocarbons (PAHs) [10] and heterocyclic amines (HCAs) [11-13] including 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIPs) [10,11,14]. Moreover, the ABC transporters are implicated in mucosal immune defence by the modulating of expression of the ABC transporters by several cytokines [15-18] suggesting a mechanism involving inflammation. Indeed, *mdr1a* -/- mice develop colitis spontaneously due to a defective intestinal epithelial barrier [19]. Some studies also suggest that cytokines are substrates for the ABC transporters [20-22].

The three ABC transporter proteins P-glycoprotein (Pgp)/multidrug resistance 1 (MDR1) (encoded by *ABCB1*), multidrug-resistance-associated protein 2 (MRP2) (encoded by *ABCC2*) and breast cancer resistance protein (BCRP) (encoded by *ABCG2*) are all efflux transporters. They are expressed in various tissues including the apical surface of enterocytes where they serve to restrict the body from exposure to potentially harmful substances [5,7,8,23]. Genetic variations in these ABC transporters have various effects on their expression, mRNA stability, protein folding, intracellular localization, degradation, substrate binding, and/or transport kinetics [5,8,24,25], resulting in variation in the exposure of the intestinal epithelial barrier to these substances.

The formation of PAHs and HCAs during cooking of meat at high temperatures could explain the higher risk of CRC associated with red meat consumption [2,3,26]. We have previously found interaction between two polymorphisms in *ABCB1* and red meat intake in relation to risk of CRC [27] which could be caused by a change in binding affinity or transport activity for PAHs and HCAs. Moreover, we found interaction between use of non-steroidal anti-inflammatory drugs (NSAID) and an *ABCB1* polymorphism which is in line with studies showing that several NSAIDs modulate expression of ABC transporters [28-33] or function as substrates for the ABC transporters [28]. In another study using the same study group as the present, we found interaction between several dietary factors and polymorphisms in genes encoding the cytokines interleukin (IL) 1β and IL-10 in relation to CRC risk [34]. Since cytokines control expression of the ABC transporters, examination of gene-gene and gene-environment interactions may reveal important pathways implicated in colorectal carcinogenesis.

In the present study, we aimed to update our findings on meat intake, use of NSAID and *ABCB1* polymorphisms in relation to CRC in a prospective cohort of 1010 CRC cases and 1829 randomly selected participants from the Danish Diet, Cancer and Health Study. Additionally, we wanted to investigate whether other dietary and life style factors interact with other ABC transporter polymorphisms in relation to CRC risk; and to investigate gene-gene interactions between the previously studied polymorphisms in *IL1B* and *IL10* and ABC transporter genes in relation to different dietary factors and CRC risk.

## Methods

### Studied subjects

The Diet, Cancer and Health Study is an ongoing Danish cohort study designed to investigate the relation between diet, lifestyle and cancer risk [35]. The cohort consists of 57,053 persons, recruited between December 1993 and May 1997. All the subjects were born in Denmark, and the individuals were 50 to 64 years of age and had no previous cancers at study entry. Blood samples and questionnaire data on diet and lifestyle were collected at study entry. Informed consent was obtained from all patients for being included in the study. The Diet, Cancer and Health study was approved by the National Committee on Health Research Ethics (journal no (KF) 01-345/93), and the Danish Data Protection Agency.

### Follow-up and endpoints

Follow-up was based on population-based cancer registries. Between 1994 and 31th December 2009, 1010 CRC cases were diagnosed. A sub-cohort of 1829 persons was randomly selected within the cohort. 28 persons were both cases and sub-cohort due to the used study design [36]. In total, 120 with missing genotype data were excluded. All information on genotypes and diet and lifestyle factors was available for 959 CRC cases and 1799 sub-cohort members. The present study group was previously described [34].

### Dietary and lifestyle questionnaire

Information on diet, lifestyle, weight, height, medical treatment, environmental exposures, and other socio-economic factors were collected at enrolment using questionnaires and interviews and has been described in details elsewhere [34,37-39]. In short, the food-frequency questionnaire, diet 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 [35]. Smoking status was classified as never, past or current. Persons smoking at least 1 cigarette daily during the last year were classified as smokers. NSAID use ("Aspirin", "Paracetamol", "Ibuprofen", or "Other pain relievers) was assessed as ≥ 2 pills per month during one year at baseline.

### Genotyping

Buffy coat preparations were stored at minus 150°C until use. DNA was extracted as described [40]. The DNA was genotyped by LGC KBioscience (LGC KBioscience, Hoddesdon, United Kingdom) by PCR-based KASP™ genotyping assay (http:// www.lgcgenomics.com/). To confirm reproducibility, genotyping was repeated for 10 % of the samples yielding 100% identity.

*ABCB1*/rs1045652 [41-44], *ABCB1*/rs1128503 [45], *ABCG2*/rs2231142 [46-48], *ABCG2*/rs2231137 [49], *ABCG2*/rs2622604 [49], *ABCC2*/rs2273697 [50], *ABCC2*/17222723 [51] and *ABCC2*/rs717620 [50] were all selected based on their documented functionality from a literature search; and *ABCG2*/rs3789243 was chosen based on its association with inflammatory bowel disease [52], CRC [27] and low mRNA levels in morphologically normal intestinal tissue from patients with adenoma [53].

*ABCB1*/rs1045652, *ABCB1*/rs3789243, *ABCG2*/rs2231142, *ABCC2*/rs2273697 and *ABCC2*/rs717620 have been studied previously in a subset of the present cohort [27,54]. *IL10*/rs1800872, *IL10*/rs3024505, *IL1B*/rs4848306, *IL1B*/rs1143623 and *IL1B*/rs1143627 have also been determined and reported in the same cohort as the present [34] and were only included in interaction analyses with ABC transporter polymorphisms based on findings from the study by Andersen *et al.,* 2013a.

**Statistical analysis**

Deviation from Hardy-Weinberg equilibrium was assessed using a Chi-square test.

The data were sampled according to the case-cohort design and the unweighted case-cohort approach was used for analyses [36] Incidence rate ratios (IRR) for CRC were estimated by the Cox proportional hazards model. Age was used as the underlying time axis, which ensured that the estimation procedure was based on comparisons of individuals at the same age and the analyses were corrected for delayed entry, such that persons were considered under risk only from the age at enrolment in the cohort. Tests and 95% confidence intervals (CI) were based on Wald's tests using the robust estimate of the variance-covariance matrix for the regression parameters in the Cox regression models [55] as previously described [27,34,37,54,56-62].

All models were adjusted for baseline values of suspected risk factors for colorectal cancer such as body mass index (BMI) (kg/m$^2$, continuous), NSAID use (yes/no), use of hormone replacement therapy (HRT) (never/past/current, among women), smoking status (never/past/current), intake of dietary fibre (g/day, continuous), and red meat and processed meat (g/day, continuous). Cereals, fibre, fruit and vegetables were also entered linearly. All analyses were stratified by gender, so that the basic (underlying) hazards were gender specific. For all the polymorphisms, IRR was calculated separately for heterozygous and homozygous variant allele carriers. For all the single nucleotide polymorphisms (SNP) except for *ABCG2/*rs2622604, *ABCC2/*rs2273697 and *ABCB1*/rs1045642, all variant allele carriers were subsequently grouped for interaction analyses since no recessive effects were observed. For *ABCG2/*rs2622604 and *ABCC2/*rs2273697, a recessive mode was used in the subsequent analyses. The frequencies of the *ABCB1*/rs1045642 polymorphism vary across ethnical populations [63]. Mostly, the T-allele has been considered as the variant allele. In this study population, however, the T-allele is the most frequent and should therefore be considered as the wild type. However, we found different effects in the interaction analyses. Therefore, we analysed the *ABCB1*/rs1045642 polymorphism in three different ways by analysing all three genotypes separately; by grouping T-allele carriers; and by grouping C-allele carriers.

We also assessed weekly use of NSAID based on the results of a study of colorectal cancer within the Diet, Cancer and Health cohort [64] reporting that regular use of Aspirin or Non-Aspirin NSAID appears necessary to

achieve a protective effect. However, there were no differences in risk estimates between monthly or weekly use, consequently, to maintain the statistical power in the strata; we used monthly NSAID use in the analyses.

Haplotypes of *ABCB1* and *ABCG2* were inferred manually as done previously [27,34,65,66].

The likelihood ratio test was used for interaction analyses between the studied polymorphisms and intake of meat, dietary fibre, cereals, fish, fruits, vegetables, alcohol, smoking status and NSAID use. In interaction analyses where the dietary factors were entered as categorical variables, tertile cutpoints were based on the empirical distribution among cases divided by gender. The possible interactions were investigated using the likelihood ratio test.

All analyses were performed using SAS version 9.3 (SAS Institute Inc., Cary, NC). A $P<0.05$ was considered to be significant. Moreover, to test for multiple comparisons, Bonferroni correction was used.

**Ethics statement**

All participants gave verbal and written informed consent. The Diet, Cancer and Health study was approved by the National Committee on Health Research Ethics (journal nr. (KF) 01-345/93) and the Danish Data Protection Agency.

## Results

Baseline characteristics of CRC cases and sub-cohort members are presented in Table 1 as published previously [34]. Among sub-cohort members, the genotype distribution of the SNPs did not deviate from Hardy-Weinberg equilibrium (results not shown), except for the *ABCC2*/rs17222723 which was therefore excluded from further analyses.

### *Associations between polymorphisms and CRC*

Risk estimates for the association between the studied SNPs and risk of CRC are presented in Table 2. No statistically significant associations between genotype distribution and risk of CRC were found. Haplotype analysis of the three *ABCB1* polymorphisms revealed that the CCC/TCT combination (T-rs1045642C, C-rs1128503T, C-rs3789243T) was associated with a 41% increased risk of CRC (95%CI: 1.01-1.95; $P=0.04$) compared to the reference haplotype TTC/TTC (Table S1). Having two copies of the CCC haplotype was associated with a 1.77-fold increased risk of CRC (95%CI: 1.00-3.13; $P=0.05$) (Table S1). Conversely, having a GTC/GGT combination of the three *ABCG2* polymorphisms (G-rs2231137A, G-rs2231142T, C-rs2622604T) was associated with a 39% decreased risk of CRC (IRR=0.61; 95%CI: 0.44-0.86; $P=0.004$) compared to the reference haplotype GGC (Table S2). We found no interaction with ABC transporter polymorphisms and *IL1B* or *IL10* SNPs in relation to CRC (results not shown).

**Table 1: Baseline characteristics of the study participants by selected demographic and established CRC risk factors.**

| *Variable* | *Cases* | | *Sub-cohort* | | *IRR[a] (95% CI)* |
|---|---|---|---|---|---|
| | *n (%)* | *Median (5-95%)* | *n (%)* | *Median (5-95%)* | |
| Total | 959 (100) | | 1799 (100) | | |
| Sex | | | | | |
| Men | 539 (56) | | 967 (54) | | |
| Women | 420 (44) | | 832 (46) | | |
| Age at inclusion (years) | | 58 (51-64) | | 56 (50-64) | |
| BMI (kg/m$^2$) | | 26.3 (20.6-34.4) | | 25.6 (20.5-33.0) | 1.03 (1.00-1.06)[d] |
| Food intake (g/day) | | | | | |
| Alcohol[b] | | 15.0 (1.2-71.4) | | 14.1 (1.1-66.1) | 1.03 (1.00-1.06)[e] |
| Dietary fibre | | 20.0 (10.6-32.9) | | 20.6 (10.8-34.2) | 0.88 (0.80-0.97)[f] |
| Red and processed meat | | 113.0 (47.1-233.4) | | 109.2 (41.5-237.2) | 1.02 (1.00-1.05)[g] |
| Smoking status | | | | | |
| Never | 286 (30) | | 599 (33) | | 1.00 (ref.) |
| Past | 295 (31) | | 528 (29) | | 1.05 (0.89-1.24) |
| Current | 378 (39) | | 672 (37) | | 1.10 (0.94-1.28) |
| NSAID use[c] | | | | | |
| No | 667 (70) | | 1239 (69) | | 1.00 (ref.) |
| Yes | 292 (30) | | 560 (31) | | 0.99 (0.87-1.14) |
| HRT use among women | | | | | |
| Never | 256 (61) | | 437 (53) | | 1.00 (ref.) |
| Past | 54 (12) | | 131 (16) | | 0.68 (0.50-0.91) |
| Current | 110 (26) | | 264 (32) | | 0.76 (0.60-0.95) |

Values are expressed as medians (5th and 95th percentiles) or as fractions (%).
[a]IRRs for CRC – mutually adjusted.
[b]Among current drinkers.
[c]NSAID use is defined as ≥ 2 pills per month during one year.
[d]Risk estimate per 2 kg/m$^2$ increment of BMI.
[e]Risk estimate for the increment of 10 g alcohol per day.
[f]Risk estimate for the increment of 10 g dietary fibres per day.
[g]Risk estimate for the increment of 25 g red and processed meat per day.

*Gene-environment analyses*

**Meat, fish, vegetables and alcohol:** We found no associations between ABC transporter SNPs and meat, fish, vegetables and alcohol intake (Table 3).

**Cereals, fruits and fibre:** *ABCB1* rs1045642 C-allele carriers were at 13% increased risk of CRC per 50 g cereals per day (95%CI: 1.03-1.25) whereas TT carriers had a slightly non-significantly reduced risk (*P*-value for interaction ($P_{int}$)=0.001) (Table 3). These findings were supported by the tertile analyses (Table S3), where the risk of CRC increased with increasing intake of cereals, whereas the opposite effect was found for C-allele carriers ($P_{int}$=0.01) (Table S3). A diet low in cereals was associated with a 1.37 increased risk of CRC among carriers of the C-allele (95%CI: 1.06-1.79). Furthermore, intake of 10 g fibre per day decreased the risk of CRC with 20% among those carrying the *ABCB1*/rs1045642 C-allele (IRR=0.80; 95%CI: 0.71-0.90; $P_{int}$=0.01) whereas TT-carriers had no risk reduction for similar intake (Table 3). In the tertile analyses, a high intake of fibre was only associated with a protective effect against CRC among C-allele carriers, whereas absence of fibre was associated with increased risk of CRC ($P_{int}$=0.006) (Table S3).

**Table 2: IRR for CRC in relation to the studied polymorphisms.**

| | | $n_{cases}$ (%) | $n_{sub\text{-}cohort}$ (%) | IRR[a] (95% CI) | IRR[b] (95% CI) | P-value[c] |
|---|---|---|---|---|---|---|
| *ABCB1* | rs1045642 | | | | | |
| | TT | 300 (32) | 563 (32) | 1.00 (ref.) | 1.00 (ref.) | - |
| | TC | 457 (49) | 855 (49) | 1.01 (0.87-1.16) | 1.02 (0.88-1.18) | 0.82 |
| | CC | 178 (19) | 320 (18) | 1.07 (0.89-1.29) | 1.05 (0.87-1.27) | 0.59 |
| | TC+CC | 635 (68) | 1175 (68) | 1.02 (0.89-1.17) | 1.03 (0.90-1.28) | 0.70 |
| | | | | | | |
| | CC | 178 (19) | 320 (18) | 1.00 (ref.) | 1.00 (ref.) | - |
| | TC | 457 (49) | 855 (49) | 0.94 (0.79-1.12) | 0.97 (0.81-1.15) | 0.71 |
| | TT | 300 (32) | 563 (32) | 0.94 (0.78-1.13) | 0.95 (0.79-1.14) | 0.59 |
| | TC+TT | 757 (81) | 1418 (81) | 0.94 (0.80-1.11) | 0.96 (0.82-1.13) | 0.63 |
| | rs1128503 | | | | | |
| | CC | 300 (32) | 573 (33) | 1.00 (ref.) | 1.00 (ref.) | - |
| | CT | 460 (49) | 840 (48) | 1.00 (0.86-1.16) | 1.00 (0.87-1.16) | 0.96 |
| | TT | 176 (19) | 343 (20) | 0.96 (0.80-1.15) | 0.95 (0.80-1.15) | 0.62 |
| | CT+TT | 636 (68) | 1183 (67) | 0.99 (0.86-1.13) | 0.99 (0.86-1.14) | 0.88 |
| | rs3789243 | | | | | |
| | CC | 239 (26) | 482 (28) | 1.00 (ref.) | 1.00 (ref.) | - |
| | CT | 485 (52) | 836 (48) | 1.14 (0.98-1.33) | 1.14 (0.98-1.33) | 0.09 |
| | TT | 206 (22) | 434 (25) | 0.99 (0.82-1.20) | 1.00 (0.83-1.20) | 0.99 |
| | CT+TT | 691 (74) | 1270 (72) | 1.09 (0.94-1.26) | 1.10 (0.95-1.27) | 0.22 |
| *ABCG2* | rs2231142 | | | | | |
| | GG | 747 (80) | 1368 (78) | 1.00 (ref.) | 1.00 (ref.) | - |
| | TG | 172 (18) | 373 (21) | 0.88 (0.74-1.03) | 0.88 (0.75-1.04) | 0.14 |
| | TT | 15 (2) | 24 (1) | 1.24 (0.77-2.00) | 1.27 (0.78-2.06) | 0.33 |
| | TG+TT | 187 (20) | 397 (22) | 0.90 (0.77-1.05) | 0.91 (0.77-1.06) | 0.22 |
| | rs2231137 | | | | | |
| | GG | 872 (93) | 1641 (93) | 1.00 (ref.) | 1.00 (ref.) | - |
| | GA | 62 (7) | 121 (7) | 0.99 (0.76-1.29) | 1.01 (0.77-1.31) | 0.97 |
| | AA | 1 (0) | 2 (0) | 1.23 (0.18-8.34) | 1.13 (0.16-8.10) | 0.90 |
| | GA+AA | 63 (7) | 123 (7) | 0.99 (0.76-1.29) | 1.01 (0.78-1.31) | 0.96 |
| | rs2622604 | | | | | |
| | CC | 514 (55) | 921 (53) | 1.00 (ref.) | 1.00 (ref.) | - |
| | TC | 364 (39) | 707 (40) | 0.96 (0.84-1.10) | 0.98 (0.86-1.12) | 0.78 |
| | TT | 53 (6) | 125 (7) | 0.79 (0.59-1.06) | 0.81 (0.61-1.09) | 0.16 |
| | TC+TT | 417 (45) | 832 (47) | 0.94 (0.82-1.07) | 0.96 (0.84-1.09) | 0.50 |
| | TT vs. CC+TC | 53 (6) | 125 (7) | 0.80 (0.60-1.07) | 0.82 (0.62-1.09) | 0.17 |
| *ABCC2* | rs2273697 | | | | | |
| | GG | 592 (63) | 1130 (65) | 1.00 (ref.) | 1.00 (ref.) | - |
| | AG | 297 (32) | 557 (32) | 1.01 (0.88-1.16) | 1.00 (0.87-1.15) | 0.97 |
| | AA | 46 (5) | 61 (3) | 1.27 (0.96-1.68) | 1.23 (0.93-1.64) | 0.15 |
| | AG+AA | 343 (37) | 618 (35) | 1.04 (0.91-1.18) | 1.03 (0.90-1.17) | 0.68 |
| | AA vs. GG+AG | 46 (5) | 61 (3) | 1.26 (0.96-1.66) | 1.23 (0.93-1.63) | 0.14 |
| | rs17222723 | | | | | |
| | TT | 877 (93) | 1645 (93) | 1.00 (ref.) | 1.00 (ref.) | - |
| | AT | 63 (7) | 112 (6) | 1.06 (0.82-1.38) | 1.05 (0.81-1.36) | 0.73 |
| | AA | 3 (0) | 7 (0) | 0.82 (0.26-2.56) | 0.79 (0.25-2.49) | 0.69 |
| | AT+AA | 66 (7) | 119 (7) | 1.05 (0.81-1.35) | 1.03 (0.80-1.33) | 0.80 |
| | rs717620 | | | | | |
| | GG | 626 (67) | 1137 (65) | 1.00 (ref.) | 1.00 (ref.) | - |
| | AG | 270 (29) | 542 (31) | 0.93 (0.81-1.07) | 0.93 (0.80-1.07) | 0.28 |
| | AA | 37 (4) | 67 (4) | 1.08 (0.77-1.51) | 1.05 (0.75-1.47) | 0.78 |
| | AG+AA | 307 (33) | 609 (35) | 0.95 (0.83-1.09) | 0.94 (0.82-1.08) | 0.36 |

[a] Crude – adjusted for age and sex.
[b] In addition, adjusted for smoking status, alcohol, HRT status (women only), BMI, use of NSAID, intake of red and processed meat, and dietary fibre.
[c] P-value for the adjusted estimates.

Intake of fruit interacted with the *ABCC2*/rs2273697 polymorphism ($P_{int}$=0.01) so that heterozygous carriers of the G-allele had a 2% decreased risk of CRC per 50 gram per day (IRR=0.98; 95%CI: 0.95-1.01),

whereas homozygous variant carriers of the A-allele had a 5% increased risk of CRC per 50 gram fruit per day (IRR=1.05; 95%CI: 0.99-1.11).

Carriers of different combinations of polymorphisms in ABC transporter genes and *IL10* interacted with fibre intake (Table 4 and 5). Homozygous carriers of the *ABCB1*/rs1045642 T-allele, who were also heterozygous carriers of the *IL10*/rs3024505 T-allele had a 1.31-fold increased risk of CRC per 10 gram fibre per day (95%CI: 1.04-1.65), whereas heterozygous carriers of the *ABCB1*/rs1045642 C-allele, who were also wild type carriers of the *IL10*/rs3024505 C-allele, had a 25% reduced risk of CRC for similar fibre intake (IRR=0.75; 95%CI: 0.65-0-87) ($P_{int}$=0.0007) (Table 4). Wild type carriers of both the *IL10*/rs3024505 and the *ABCG2*/rs2231137 polymorphism had an 18% decreased risk of CRC per 10 gram fibre per day (IRR=0.82; 95%CI: 0.72-0.92). If the CC-carriers of the *IL10*/rs3024505 polymorphism also were heterozygous carriers of the variant *ABCG2*/rs2231137 A-allele, the risk of CRC was additionally reduced for identical fibre intake (IRR=0.51; 95%CI: 0.30-0.86) ($P_{int}$=0.009) (Table 5).

**NSAID use:** Generally, a pattern of intake of NSAIDs associated with decreased risk of CRC among carriers of low activity variants of the ABC transporter genes was seen (Table 6). Increased risk of CRC was found among users of NSAID, who were homozygous carriers of either *ABCB1*/rs1045642 C-allele (IRR=1.39; 95%CI: 1.03-1.87; $P_{int}$=0.02), *ABCB1*/rs1128503 C-allele (IRR=1.21; 95%CI: 0.96-1.52; $P_{int}$=0.04) or *ABCC2*/rs2273697 A-allele (IRR=1.75; 95%CI: 1.12-2.72; $P_{int}$=0.06), whereas risk reduction was found for variant T-allele carriers of the *ABCG2*/rs2231142 polymorphism among NSAID users (IRR=0.74; 95%CI: 0.55-1.00; $P_{int}$=0.03) (Table 6). Carrying two of either of these polymorphisms did not increase or decrease risk of CRC significantly (results not shown). Interaction with NSAID use was found for combinations of *ABCB1*/rs1045642, *ABCG2*/rs2231137 and *IL10*/rs3024505 polymorphisms (Table S4 and S5). Homozygous carriers of the *ABCB1*/rs1045642 C-allele, who were also wild type carriers of the *IL10*/rs3024505 polymorphisms, had a 77% increased risk of CRC if they used NSAIDs (95%CI: 1.27-1.48; $P_{int}$=0.007) (Table S4). Interaction with *ABCG2*/rs2231137 and *IL10*/rs3024505 was also found ($P_{int}$=0.02), but none of the risk estimates were statistically significant (Table S5).

**Smoking:** There was no interaction with smoking status and any of the ABC transporter polymorphisms in relation to CRC risk (Table S6).

## Discussion

In the present study, we show that none of the ABC transporter polymorphisms *per se* are associated with CRC risk. In the haplotype analysis of *ABCB1,* the CCC/TCT combination was associated with a 41% increased risk of CRC; and carriage of two copies of the *ABCB1* CCC haplotype further increased the risk. The opposite association was found for *ABCG2* where a GTC/GGT haplotype combination was associated with reduced risk of CRC.

**Table 3. Interaction between dietary factors and the studied polymorphisms in relation to CRC risk.**

| | | *IRR[a] (95% CI)* | *IRR[b] (95% CI)* | *P[c]* | *IRR[a] (95% CI)* | *IRR[b] (95% CI)* | *P[c]* | *IRR[a] (95% CI)* | *IRR[b] (95% CI)* | *P[c]* | *IRR[a] (95% CI)* | *IRR[b] (95% CI)* | *P[c]* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ***Red and processed meat per 25 g/day*** | | | ***Fish per 25 g/day*** | | | ***Dietary cereal per 50 g/day*** | | | ***Alcohol per 10 g/day[d]*** | | |
| ***ABCB1*** | rs1045642 | | | | | | | | | | | | |
| | TT | 1.01 (0.97-1.05) | 1.01 (0.96-1.05) | 0.19 | 0.91 (0.82-1.01) | 0.92 (0.82-1.03) | 0.41 | 1.05 (0.97-1.13) | 1.13 (1.03-1.25) | 0.001 | 1.02 (0.97-1.07) | 1.01 (0.96-1.06) | 0.25 |
| | TC+CC | 1.04 (1.00-1.07) | 1.04 (1.01-1.08) | | 0.96 (0.90-1.04) | 0.97 (0.90-1.05) | | 0.91 (0.86-0.96) | 0.97 (0.90-1.05) | | 1.05 (1.01-1.09) | 1.04 (1.01-1.08) | |
| | TT | 1.01 (0.97-1.05) | 1.01 (0.96-1.05) | | 0.91 (0.82-1.01) | 0.92 (0.82-1.03) | | 1.05 (0.97-1.13) | 1.13 (1.03-1.25) | | 1.02 (0.97-1.07) | 1.01 (0.96-1.06) | |
| | TC | 1.04 (1.01-1.08) | 1.04 (1.01-1.08) | 0.40 | 0.96 (0.88-1.05) | 0.96 (0.88-1.05) | 0.63 | 0.92 (0.86-0.98) | 0.98 (0.90-1.07) | 0.006 | 1.06 (1.02-1.10) | 1.06 (1.02-1.10) | 0.24 |
| | CC | 1.02 (0.96-1.09) | 1.03 (0.97-1.11) | | 0.98 (0.86-1.11) | 1.00 (0.88-1.13) | | 0.88 (0.79-0.98) | 0.95 (0.84-1.07) | | 1.01 (0.94-1.08) | 1.01 (0.94-1.08) | |
| | CC | 1.02 (0.96-1.09) | 1.03 (0.97-1.11) | 0.85 | 0.98 (0.86-1.11) | 1.00 (0.88-1.13) | 0.46 | 0.88 (0.79-0.98) | 0.95 (0.83-1.07) | 0.13 | 1.01 (0.94-1.08) | 1.01 (0.94-1.08) | 0.47 |
| | TC+TT | 1.03 (1.00-1.06) | 1.03 (1.00-1.06) | | 0.94 (0.88-1.01) | 0.95 (0.88-1.02) | | 0.97 (0.92-1.01) | 1.04 (0.96-1.11) | | 1.04 (1.01-1.08) | 1.04 (1.00-1.07) | |
| | rs1128503 | | | | | | | | | | | | |
| | CC | 1.01 (0.96-1.06) | 1.02 (0.97-1.08) | 0.83 | 0.96 (0.87-1.07) | 0.97 (0.87-1.08) | 0.61 | 0.94 (0.87-1.02) | 1.01 (0.92-1.11) | 0.57 | 1.06 (1.01-1.12) | 1.06 (1.00-1.11) | 0.20 |
| | CT+TT | 1.03 (1.00-1.06) | 1.03 (1.00-1.06) | | 0.93 (0.86-1.00) | 0.94 (0.87-1.02) | | 0.97 (0.92-1.02) | 1.04 (0.96-1.12) | | 1.03 (0.99-1.06) | 1.02 (0.98-1.05) | |
| | rs3789243 | | | | | | | | | | | | |
| | CC | 1.00 (0.96-1.04) | 1.00 (0.96-1.05) | 0.33 | 0.91 (0.81-1.01) | 0.91 (0.82-1.02) | 0.47 | 0.96 (0.90-1.03) | 1.00 (0.93-1.07) | 0.95 | 1.03 (0.97-1.08) | 1.01 (0.96-1.07) | 0.44 |
| | CT+TT | 1.03 (1.00-1.06) | 1.03 (1.00-1.06) | | 0.95 (0.88-1.02) | 0.96 (0.89-1.03) | | 0.95 (0.90-1.00) | 1.00 (0.93-1.08) | | 1.05 (1.01-1.08) | 1.04 (1.01-1.08) | |
| ***ABCG2*** | rs2231142 | | | | | | | | | | | | |
| | GG | 1.01 (0.98-1.04) | 1.01 (0.98-1.04) | 0.45 | 0.91 (0.86-0.98) | 0.92 (0.85-0.98) | 0.07 | 0.94 (0.90-0.99) | 0.99 (0.93-1.05) | 0.40 | 1.03 (1.00-1.06) | 1.02 (0.99-1.06) | 0.17 |
| | TG+TT | 1.04 (0.98-1.10) | 1.04 (0.98-1.10) | | 1.04 (0.90-1.20) | 1.06 (0.92-1.23) | | 0.98 (0.89-1.08) | 1.03 (0.93-1.14) | | 1.07 (1.01-1.14) | 1.07 (1.01-1.13) | |
| | rs2231137 | | | | | | | | | | | | |
| | GG | 1.02 (1.00-1.05) | 1.03 (1.00-1.06) | 0.90 | 0.94 (0.88-1.00) | 0.94 (0.88-1.01) | 0.13 | 0.97 (0.92-1.01) | 1.04 (0.97-1.11) | 0.14 | 1.03 (1.00-1.07) | 1.03 (1.00-1.06) | 0.21 |
| | GA+AA | 1.02 (0.92-1.14) | 1.02 (0.92-1.13) | | 1.02 (0.88-1.19) | 1.04 (0.93-1.17) | | 0.84 (0.72-0.99) | 0.91 (0.77-1.09) | | 1.13 (1.00-1.27) | 1.11 (0.98-1.25) | |
| | rs2622604 | | | | | | | | | | | | |
| | CC+TC | 1.02 (0.99-1.05) | 1.02 (0.99-1.05) | 0.39 | 0.95 (0.89-1.01) | 0.96 (0.90-1.02) | 0.66 | 0.96 (0.92-1.00) | 1.01 (0.95-1.07) | 0.56 | 1.03 (1.00-1.06) | 1.03 (1.00-1.06) | 0.08 |
| | TT | 1.05 (0.96-1.16) | 1.06 (0.97-1.17) | | 0.94 (0.75-1.17) | 0.91 (0.72-1.14) | | 0.90 (0.75-1.07) | 0.96 (0.80-1.14) | | 1.16 (1.01-1.32) | 1.16 (1.01-1.33) | |
| ***ABCC2*** | rs2273697 | | | | | | | | | | | | |
| | GG+AG | 1.03 (1.00-1.05) | 1.03 (1.00-1.06) | 0.91 | 0.95 (0.89-1.01) | 0.96 (0.90-1.02) | 0.53 | 0.97 (0.93-1.01) | 1.02 (0.96-1.08) | 0.62 | 1.04 (1.01-1.07) | 1.03 (1.00-1.06) | 0.84 |
| | AA | 1.04 (0.92-1.17) | 1.04 (0.92-1.17) | | 1.04 (0.75-1.43) | 1.06 (0.77-1.47) | | 0.91 (0.71-1.16) | 0.95 (0.73-1.24) | | 1.05 (0.94-1.18) | 1.04 (0.93-1.17) | |
| | rs717620 | | | | | | | | | | | | |
| | GG | 1.03 (1.00-1.06) | 1.03 (1.00-1.07) | 0.47 | 0.98 (0.91-1.05) | 0.99 (0.91-1.06) | 0.12 | 0.94 (0.89-1.00) | 1.01 (0.94-1.09) | 0.53 | 1.05 (1.02-1.09) | 1.05 (1.01-1.08) | 0.31 |
| | AG+AA | 1.01 (0.96-1.06) | 1.01 (0.97-1.07) | | 0.87 (0.78-0.97) | 0.89 (0.79-0.99) | | 0.97 (0.90-1.05) | 1.04 (0.95-1.15) | | 1.02 (0.97-1.07) | 1.01 (0.96-1.07) | |
| | | ***Dietary fibre per 10 g/day*** | | | ***Fruit per 50 g/day*** | | | ***Vegetables per 50 g/day*** | | | | | |
| ***ABCB1*** | rs1045642 | | | | | | | | | | | | |
| | TT | 0.99 (0.85-1.15) | 1.01 (0.87-1.18) | 0.01 | 0.98 (0.95-1.01) | 0.99 (0.96-1.03) | 0.28 | 0.98 (0.92-1.04) | 1.01 (0.95-1.09) | 0.84 | | | |
| | TC+CC | 0.79 (0.70-0.88) | 0.80 (0.71-0.90) | | 0.96 (0.93-0.99) | 0.97 (0.94-1.00) | | 0.98 (0.95-1.02) | 1.02 (0.98-1.07) | | | | |
| | TT | 0.99 (0.85-1.15) | 1.01 (0.87-1.18) | | 0.98 (0.95-1.01) | 0.99 (0.96-1.03) | | 0.98 (0.92-1.04) | 1.01 (0.95-1.09) | | | | |
| | TC | 0.77 (0.67-0.88) | 0.77 (0.67-0.89) | 0.04 | 0.94 (0.91-0.97) | 0.95 (0.92-0.99) | 0.08 | 0.98 (0.94-1.03) | 1.02 (0.97-1.07) | 0.95 | | | |
| | CC | 0.84 (0.68-1.04) | 0.86 (0.69-1.07) | | 1.00 (0.95-1.05) | 1.01 (0.96-1.06) | | 0.99 (0.92-1.06) | 1.03 (0.95-1.11) | | | | |
| | CC | 0.84 (0.68-1.04) | 0.86 (0.69-1.07) | 0.95 | 1.00 (0.95-1.05) | 1.01 (0.96-1.06) | 0.15 | 0.99 (0.92-1.06) | 1.03 (0.95-1.11) | 0.77 | | | |
| | TC+TT | 0.85 (0.77-0.95) | 0.87 (0.78-0.96) | | 0.96 (0.94-0.98) | 0.97 (0.94-1.00) | | 0.98 (0.95-1.02) | 1.02 (0.97-1.06) | | | | |
| | rs1128503 | | | | | | | | | | | | |
| | CC | 0.81 (0.68-0.95) | 0.82 (0.70-0.97) | 0.35 | 0.97 (0.93-1.00) | 0.98 (0.94-1.02) | 0.92 | 0.99 (0.94-1.05) | 1.03 (0.97-1.10) | 0.63 | | | |
| | CT+TT | 0.89 (0.80-1.00) | 0.90 (0.81-1.01) | | 0.97 (0.94-0.99) | 0.98 (0.95-1.01) | | 0.98 (0.94-1.02) | 1.01 (0.97-1.06) | | | | |
| | rs3789243 | | | | | | | | | | | | |
| | CC | 0.95 (0.84-1.07) | 0.95 (0.84-1.08) | 0.12 | 0.99 (0.95-1.02) | 0.99 (0.96-1.03) | 0.22 | 1.01 (0.95-1.07) | 1.03 (0.97-1.10) | 0.38 | | | |
| | CT+TT | 0.82 (0.73-0.91) | 0.83 (0.74-0.93) | | 0.96 (0.93-0.98) | 0.97 (0.94-1.00) | | 0.97 (0.93-1.01) | 1.00 (0.96-1.05) | | | | |
| ***ABCG2*** | rs2231142 | | | | | | | | | | | | |
| | GG | 0.86 (0.78-0.94) | 0.87 (0.79-0.95) | 0.70 | 0.97 (0.94-0.99) | 0.98 (0.95-1.00) | 0.74 | 0.98 (0.95-1.02) | 1.01 (0.97-1.06) | 0.55 | | | |
| | TG+TT | 0.90 (0.74-1.09) | 0.90 (0.74-1.10) | | 0.96 (0.92-1.01) | 0.97 (0.92-1.01) | | 0.96 (0.90-1.03) | 0.99 (0.92-1.06) | | | | |
| | rs2231137 | | | | | | | | | | | | |
| | GG | 0.89 (0.81-0.98) | 0.90 (0.82-0.99) | 0.06 | 0.97 (0.95-0.99) | 0.98 (0.96-1.01) | 0.40 | 0.99 (0.96-1.02) | 1.02 (0.98-1.07) | 0.32 | | | |
| | GA+AA | 0.59 (0.41-0.85) | 0.63 (0.44-0.90) | | 0.92 (0.85-1.01) | 0.95 (0.87-1.03) | | 0.92 (0.81-1.04) | 0.96 (0.84-1.09) | | | | |
| | rs2622604 | | | | | | | | | | | | |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | CC+TC | 0.89 (0.82-0.97) | 0.90 (0.83-0.98) | 0.44 | 0.97 (0.95-0.99) | 0.98 (0.95-1.00) | 0.54 | 0.98 (0.95-1.02) | 1.01 (0.98-1.05) | 0.84 |
| | TT | 0.77 (0.53-1.12) | 0.78 (0.53-1.13) | | 0.95 (0.88-1.03) | 0.95 (0.88-1.03) | | 0.97 (0.85-1.11) | 1.00 (0.87-1.15) | |
| ***ABCC2*** | rs2273697 | | | | | | | | | |
| | GG+AG | 0.90 (0.83-0.98) | 0.91 (0.84-0.99) | 0.94 | 0.97 (0.95-0.99) | 0.98 (0.95-1.01) | 0.01 | 0.99 (0.96-1.02) | 1.02 (0.98-1.06) | 0.63 |
| | AA | 0.90 (0.61-1.32) | 0.90 (0.60-1.34) | | 1.04 (0.98-1.09) | 1.05 (0.99-1.11) | | 0.95 (0.82-1.09) | 0.99 (0.85-1.15) | |
| | rs717620 | | | | | | | | | |
| | GG | 0.84 (0.75-0.94) | 0.85 (0.76-0.96) | 0.54 | 0.97 (0.95-1.00) | 0.99 (0.96-1.02) | 0.51 | 0.97 (0.93-1.01) | 1.00 (0.96-1.05) | 0.30 |
| | AG+AA | 0.89 (0.76-1.04) | 0.91 (0.78-1.06) | | 0.96 (0.93-1.00) | 0.97 (0.93-1.01) | | 1.00 (0.95-1.05) | 1.04 (0.98-1.10) | |

[a] Crude – adjusted for age and sex.
[b] In addition, adjusted for smoking status, alcohol intake, HRT status (women only), BMI, use of NSAID, intake of red and processed meat, and dietary fibre.
[c] P-value for interaction for the adjusted estimates.
[d] Among current drinkers only

**Table 4: IRR for CRC in relation to combinations of *ABCB1*/rs1045642 and *IL10*/rs3024505 genotypes per 10 g fibre per day**

| *Genotype* | *IL10*/rs3024505 | | *IL10*/rs3024505 | | *IL10*/rs3024505 | | *P-value*[c] |
|---|---|---|---|---|---|---|---|
| | CC<br>$n_{cases}/n_{sub\text{-}cohort}$ | CT+TT<br>$n_{cases}/n_{sub\text{-}cohort}$ | CC<br>IRR (95% CI)[a] | CT+TT<br>IRR (95% CI)[a] | CC<br>IRR (95% CI)[b] | CT+TT<br>IRR (95% CI)[b] | |
| *ABCB1*/rs1045642 | | | | | | | |
| TT | 202/382 | 93/178 | 0.83 (0.69-1.01) | 1.28 (1.02-1.61) | 0.85 (0.70-1.03) | 1.31 (1.04-1.65) | 0.0007 |
| TC+CC | 430/789 | 198/383 | 0.74 (0.64-0.86) | 0.89 (0.74-1.08) | 0.75 (0.65-0.87) | 0.90 (0.75-1.10) | |

[a] Crude – adjusted for age and sex.
[b] In addition, adjusted for smoking status, alcohol intake, HRT status (women only), BMI, use of NSAID and intake of red and processed meat.
[c] P-value for comparison of the adjusted risk estimates.

**Table 5: IRR for CRC in relation to combinations of *ABCG2*/rs2231137 and *IL10*/rs3024505 genotypes per 10 g fibre per day**

| *Genotype* | *IL10*/rs3024505 | | *IL10*/rs3024505 | | *IL10*/rs3024505 | | *P-value*[c] |
|---|---|---|---|---|---|---|---|
| | CC<br>$n_{cases}/n_{sub\text{-}cohort}$ | CT+TT<br>$n_{cases}/n_{sub\text{-}cohort}$ | CC<br>IRR (95% CI)[a] | CT+TT<br>IRR (95% CI)[a] | CC<br>IRR (95% CI)[b] | CT+TT<br>IRR (95% CI)[b] | |
| *ABCG2*/rs2231137 | | | | | | | |
| GG | 587/1108 | 269/528 | 0.81 (0.71-0.91) | 1.05 (0.50-1.23) | 0.82 (0.72-0.92) | 1.06 (0.91-1.24) | 0.009 |
| GA+AA | 42/82 | 21/40 | 0.48 (0.28-0.81) | 0.78 (0.48-1.26) | 0.51 (0.30-0.86) | 0.82 (0.49-1.35) | |

[a] Crude – adjusted for age and sex.
[b] In addition, adjusted for smoking status, alcohol intake, HRT status (women only), BMI, use of NSAID and intake of red and processed meat.
[c] P-value for comparison of the adjusted risk estimates.

Cereals and fibre interacted with the *ABCB1*/rs1045642 polymorphism and fruit interacted with *ABCC2*/rs2273697. Moreover, different combinations of ABC transporter and *IL10* polymorphisms interacted with fibre intake. Use of NSAID changed the risk estimates in four of the nine studied ABC SNPs. Combining the *ABCB1*/rs1045642 and *ABCG2*/rs2231137 polymorphisms with *IL10*/rs3024505 increased the differences in risk estimates for NSAID intake. However, none of the associations withstood adjustment for multiple testing. We found no interaction with intake of meat, fish, vegetables, alcohol or smoking status for the studied genotypes.

The risk *ABCB1* haplotype (CCC) encompasses two high activity alleles (T-rs1045642C and C-rs1128503T), whereas the third (C-rs3789243T) has no known functionality, suggesting that inherent high activity of Pgp/MDR1 is associated with increased risk of CRC. The biological effect of the *ABCG2* haplotype with the GTC/GGT combination is less clear. The reference *ABCG2* haplotype (GGC) consists of three high activity alleles (G-rs2231137A, G-rs2231142T, C-rs2622604T), whereas the protective haplotype combination encompasses a combination of both high and low activity alleles. The found associations could thus be caused by LD with other neighbouring functional SNPs.

We were not able to reproduce our previous findings on interaction between *ABCB1*/rs1045642 and *ABCB1*/rs3789243 and meat intake in this updated study. We hypothesized that difference in Pgp/MDR1 activity would influence the transport of meat carcinogens across the intestinal barrier and hence the exposure to these carcinogens which again would increase risk of CRC. We used a combination of red meat and processed meat intake as a proxy for intake of meat carcinogens. However, meat is prepared in many different methods resulting in varying amounts of carcinogens, such as HCAs, and PAHs. Variables that could more accurately estimate the intake of these carcinogens could elucidate a possible mechanism involving Pgp/MDR1.

It is well documented that dietary fibre protects against CRC [67]. In the intestines, plant polysaccharides from ingested fibres increase stool mass which lower the exposure of the enterocytes to carcinogens through reduction in transit time [68]. Moreover, dietary fibres are fermented by the commensal bacteria to short-chain fatty acids (SCFAs), which are important for the colonic integrity and for its anti-tumorigenic and anti-inflammatory properties [68]. Butyrate is one of the most important SCFAs since it is the primary colonic energy source [69,70]. Butyrate has been shown to induce Pgp/MDR1 expression [71] and is used as a substrate for BCRP [72]. We have previously found interaction between dietary fibre and two polymorphisms in *IL10* [34,37] suggesting a role of anti-inflammatory cytokines in the protective effect of dietary fibre. In the present study, we now extend this mechanism to include some of the ABC efflux transporters. Cytokines have been shown to modulate expression of ABC transporter genes [15-18] and also suggested to function as substrates [21,22]. Interestingly, the release of several cytokines from peripheral blood mononuclear cells was significantly decreased for cells with a haplotype encompassing the T-allele of the *ABCB1*/rs1045642 polymorphism, although this was not observed for IL-10 [20]. However, the present study does not clarify how the exact underlying mechanism is; only that IL-10, Pgp/MDR1 and BCRP are part of the same biological mechanism underlying the protective effect of dietary fibre on CRC risk.

**Table 6: Interaction between NSAID use and the studied polymorphisms in relation to CRC risk.**

| | | *NSAID use* $n_{cases}/n_{sub\text{-}cohort}$ | | *NSAID use IRR (95% CI)*[a] | | *NSAID use IRR (95% CI)*[b] | | *P-value*[c] | *Activity (references)* |
|---|---|---|---|---|---|---|---|---|---|
| | | *No* | *Yes* | *No* | *Yes* | *No* | *Yes* | | |
| *ABCB1* | rs1045642 | | | | | | | | |
| | TT | 215/391 | 85/172 | 1.00 (ref.) | 0.89 (0.70-1.14) | 1.00 (ref.) | 0.88 (0.69-1.13) | 0.25 | **T=**↓ [41-44] |
| | TC+CC | 435/804 | 200/371 | 0.97 (0.83-1.14) | 1.03 (0.86-1.25) | 0.97 (0.83-1.15) | 1.03 (0.85-1.24) | | |
| | | | | | | | | | |
| | TT | 215/391 | 85/172 | 1.00 (ref.) | 0.89 (0.70-1.14) | 1.00 (ref.) | 0.88 (0.69-1.13) | | |
| | TC | 329/593 | 128/262 | 0.99 (0.83-1.17) | 0.94 (0.76-1.16) | 1.00 (0.84-1.19) | 0.93 (0.75-1.16) | 0.05 | |
| | CC | 106/211 | 72/109 | 0.92 (0.73-1.16) | 1.27 (0.98-1.65) | 0.90 (0.71-1.14) | 1.25 (0.96-1.62) | | |
| | | | | | | | | | |
| | CC | 106/211 | 72/109 | 1.00 (ref.) | 1.38 (1.03-1.86) | 1.00 (ref.) | 1.39 (1.03-1.87) | 0.02 | |
| | TC+TT | 435/804 | 200/371 | 1.08 (0.88-1.33) | 1.00 (0-79-1.26) | 1.11 (0.90-1.38) | 1.01 (0.80-1.28) | | |
| | rs1128503 | | | | | | | | |
| | CC | 192/384 | 108/189 | 1.00 (ref.) | 1.21 (0.96-1.52) | 1.00 (ref.) | 1.21 (0.96-1.52) | 0.04 | **T=**↓ [45] |
| | CT+TT | 458/819 | 178/364 | 1.08 (0.92-1.28) | 0.98 (0.80-1.20) | 1.09 (0.92-1.30) | 0.97 (0.79-1.19) | | |
| | rs3789243 | | | | | | | | |
| | CC | 162/333 | 77/149 | 1.00 (ref.) | 1.07 (0.82-1.41) | 1.00 (ref.) | 1.05 (0.80-1.38) | 0.70 | unknown |
| | CT+TT | 481/879 | 210/400 | 1.12 (0.94-1.33) | 1.11 (0.91-1.36) | 1.12 (0.94-1.33) | 1.10 (0.90-1.35) | | |
| *ABCG2* | rs2231142 | | | | | | | | **T=**↓ [46-48] |
| | GG | 503/943 | 244/425 | 1.00 (ref.) | 1.10 (0.95-1.29) | 1.00 (ref.) | 1.09 (0.93-1.27) | 0.03 | |
| | TG+TT | 142/272 | 45/125 | 1.01 (0.84-1.22) | 0.73 (0.55-0.99) | 1.01 (0.84-1.22) | 0.74 (0.55-1.00) | | |
| | rs2231137 | | | | | | | | |
| | GG | 601/1125 | 271/516 | 1.00 (ref.) | 1.03 (0.89-1.18) | 1.00 (ref.) | 1.02 (0.88-1.17) | 0.30 | **A=**↓ [49] |
| | GA+AA | 46/85 | 17/38 | 1.07 (0.79-1.46) | 0.85 (0.52-1.38) | 1.10 (0.81-1.50) | 0.83 (0.51-1.35) | | |
| | rs2622604 | | | | | | | | |
| | CC+TC | 608/1119 | 270/509 | 1.00 (ref.) | 1.01 (0.88-1.17) | 1.00 (ref.) | 1.00 (0.87-1.15) | 0.70 | **T=**↓ [49] |
| | TT | 37/86 | 16/39 | 0.82 (0.58-1.17) | 0.78 (0.48-1.26) | 0.85 (0.60-1.21) | 0.76 (0.47-1.23) | | |
| *ABCC2* | rs2273697 | | | | | | | | |
| | GG+AG | 625/1159 | 264/528 | 1.00 (ref.) | 0.98 (0.85-1.13) | 1.00 (ref.) | 0.96 (0.83-1.11) | 0.06 | **A=**↑ [50] |
| | AA | 27/45 | 19/16 | 1.04 (0.73-1.49) | 1.77 (1.15-2.72) | 1.01 (0.70-1.44) | 1.75 (1.12-2.72) | | |
| | rs717620 | | | | | | | | |
| | GG | 427/776 | 199/361 | 1.00 (ref.) | 1.05 (0.89-1.24) | 1.00 (ref.) | 1.04 (0.88-1.23) | 0.35 | **A=**↓ [50] |
| | AG+AA | 222/427 | 85/182 | 0.98 (0.84-1.16) | 0.90 (0.72-1.13) | 0.98 (0.83-1.15) | 0.88 (0.70-1.11) | | |

[a] Crude – adjusted for age and sex.
[b] In addition, adjusted for smoking status, alcohol intake, HRT status (women only), BMI, intake of red and processed meat, and dietary fibre.
[c] P-value for interaction for the adjusted estimates.

Consumption of cereals has also been associated with a protective effect in relation to CRC, mostly due to the high content of fibre [73]. However, in this study, T-allele carriers of the *ABCB1*/rs1045642 polymorphism were at an increased risk of CRC per 50 gram intake of cereal per day. Cereals are often contaminated with mycotoxins produced by fungi of the genera *Aspergillus*, *Fusarium*, and *Penicillium* that grow on food crops [74]. These mycotoxins are very toxic and carcinogenic [74]. Mycotoxins or their metabolites are substrates to Pgp/MDR1 [75-77] and other ABC transporters [78,79] and are known to interact with several drugs [6]. It is possible that carriage of the low activity T-allele of *ABCB1*/rs1045642 causes detrimental enterocytic accumulation of mycotoxins from cereals.

As with mycotoxins, flavoniods from fruits are substrates for and modulate the function of the ABC transporters [5-7] which could explain the differences in risk estimates for the *ABCC2*/rs2273697 polymorphism seen in the present study. The flavonoids are also well-known for interaction with several drugs due to their binding to the ABC transporters; and for their anticancer, anti-inflammatory, antioxidant, anti-angiogenic and free radical scavenger properties [6]. The increased risk of CRC per 50 gram of fruits for carriers of the high activity A-allele of the *ABCC2*/rs2273697 polymorphism could be a result of the flavonoids not being able to exert their beneficial effect due to the high efflux activity of the MRP2 transporter.

Several studies have shown that NSAID modulate expression of the ABC transporters [28-33] and possibly function as substrates [28]. The present study supports these findings and suggests that carriers of polymorphisms that are associated with lower ABC transport activity benefit from the anti-inflammatory effect of the NSAIDs. Conversely, carriers of high activity ABC transporter polymorphisms, have a tendency towards increased risk of CRC if they use NSAID, which indicates that the drugs do not exert their anti-inflammatory effect in these persons due to enhanced efflux of the drugs. Indeed, several clinical studies rapport that drug resistance is caused by high activity variants of these transporter genes [24,42,80-82]. We also found indications of involvement of IL-10 in this mechanism, however since the *IL10*/rs3024505 polymorphisms is a marker polymorphisms, which is strongly associated with inflammatory bowel disease [83,84] but with no known biological function, it is difficult to elucidate the underlying mechanism.

This prospective cohort has limited statistical power for studying gene-environment interactions. On the other hand, the study design has some advantages. Study participants were middle age (50-64 years) at entry which reduces the likely-hood of substantial change in dietary patterns during follow up. However, if it happens, it is not expected to result in differential misclassification. The Danish cohort is very homogenous eliminating population specific genetics and dietary patterns seen in larger multicentre studies. Intake of meat in this cohort is very high, which makes it suitable for testing the effect of meat on colorectal carcinogenesis. Consumption of alcohol among women is the highest in Europe [85], which also makes it suitable for mechanistic investigating. We are well aware of the risk of change findings due to the large number of tests. None of the results withstood Bonferroni correction and could therefore theoretically be due to change. However, since all our tests are based on *a priori* hypotheses and previous findings, we believe that the risk of chance findings is reduced [86].

In conclusion, we found indication of interaction between ABC transporter polymorphisms and intake of fibre, cereals, fruit and NSAID use in relation to CRC risk suggesting that these dietary and lifestyle factors affect CRC risk by a mechanism involving ABC transporters. We also found interaction with polymorphisms in *IL10* suggesting that the immune system is involved supporting the theory of inflammation as an important risk factor for CRC. These proposed mechanisms need to be replicated in other cohorts.

*Acknowledgements*

We thank Nick Martinussen and Katja Boll for technical assistance with data managing; and Jane Christensen for statistical consulting. The project was supported by a PhD stipend from the Danish Research Councils in the program 'An integrated approach to risk-benefit assessment of human health effects of food and food contaminants' (Forskeruddannelse 2009-10), and by a Mobility PhD grant (09-06 7572) from the Danish Council for Independent Research (www.ufm.dk) and as part of the project: "Beef versus pork consumption in the etiology of cancers in the colon and rectum: investigations performed within the Diet, Cancer and Health cohort" also from the Danish Council for Independent Research; Medical Sciences (grant no. 09-073597) (www.ufm.dk). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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# Chapter 6

*Accepted for publication in International Journal of Molecular Sciences*

## Paper IV: *No association between HMOX1 and risk of colorectal cancer and no interaction with diet and lifestyle factors in a prospective Danish case-cohort study*

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**Abstract**

Red meat is a risk factor for colorectal cancer (CRC). We wanted to evaluate whether a functional polymorphism in the *HMOX1* gene encoding heme oxygenase modifies risk of CRC or interacts with diet or lifestyle factors because this would identify heme or heme iron as a risk factor of CRC. The *HMOX1* A-413T (rs2071746) was assessed in relation to risk of CRC and interactions with diet (red meat, fish, fibre, cereals, fruit and vegetables) and lifestyle (use of non-steroidal anti-inflammatory drug and smoking status) were assessed in a case-cohort study of 928 CRC cases and a comparison group of 1726 randomly selected participants from a prospective study of 57,053 persons. No association between *HMOX1* A-413T and CRC risk was found (TT vs AA+TA; IRR=1.15, 95%CI: 0.98-1.36, *P*=0.10 for the adjusted estimate). No interactions were found between diet or lifestyle and *HMOX1* A-413T. *HMOX1* A-413T was not associated with CRC risk and no interactions with diet or lifestyle were identified in this large, prospective cohort with high meat intake. The results reproduced the previous findings from the same cohort and did not support a link between heme or heme iron and CRC. These results should be sought replicated in other well-characterized cohorts with high meat intake.

## Introduction

Heme oxygenase (HO-1, encoded by *HMOX1*) is the inducible, rate-limiting enzyme converting heme to iron, carbon monoxide (CO) and biliverdin which is subsequently converted into bilirubin. HO-1 activity reduces cellular oxidative stress by removing the pro-oxidant heme and at the same time produces pro-inflammatory iron. Thus, HO-1 activity may help discriminate the underlying mechanisms involved in meat-related colorectal cancer (CRC) carcinogenesis. The *HMOX1* A-413T and a microsatellite GT-dinucleotide repeat $(GT)_n$ polymorphisms are in strong linkage and haplotype analyses indicated that the *HMOX1* A-413T genotype was likely to be the biologically functional polymorphism by modifying the promoter activity [1]. *HMOX1* polymorphisms have been found to be associated with various diseases and interaction with diet has been suggested [2]. We have previously evaluated the *HMOX1* A-413T polymorphism in a prospective Danish study of 383 cases of CRC and a sub-cohort of 763 participants [3]. We now extended this study in a larger cohort with power to assess gene-environment interaction. The aim of the present study was to evaluate whether the *HMOX1* polymorphism modifies risk of CRC or interacts with diet or lifestyle factors in a larger updated study from the same cohort because this would identify heme or heme iron as risk factors for CRC.

## Methods

### Studied Subjects

The Diet, Cancer and Health Study is an ongoing Danish cohort study designed to investigate the relation between diet, lifestyle and cancer risk [4]. The cohort consists of 57,053 persons, recruited between December 1993 and May 1997. All the subjects were born in Denmark, and the individuals were 50 to 64 years of age and had no previous cancers at study entry. Blood samples and questionnaire data on diet and lifestyle were collected at study entry.

### Follow-up and endpoints

Follow-up was based on population-based cancer registries. Between 1994 and 31th December 2009, 970 CRC cases were diagnosed. A sub-cohort of 1897 persons was randomly selected within the cohort. Of these, 213 with missing genotype data were excluded. 28 sub-cohort members developed CRC during follow-up and they are thus both sub-cohort members (prior to the diagnosis) and cases (after diagnosis) as a consequence of the case-cohort design. All information on genotypes and diet and lifestyle factors was available for 928 CRC cases and 1726 sub-cohort members.

### Dietary and lifestyle questionnaire

Information on diet, lifestyle, weight, height, medical treatment, environmental exposures, and other socio-economic factors were collected at enrolment using questionnaires and interviews. In the food-frequency questionnaire, diet 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 [4]. The study has been described in details elsewhere [3-14].

Smoking status was classified as never, past or current. Persons smoking at least 1 cigarette daily during the last year were classified as smokers.

The lifestyle questionnaire included information on the frequency regarding of use of "Aspirin", "Paracetamol", "Ibuprofen", or "Other pain relievers"). Based on all records, we classified study subjects according to use of "any NSAID" (≥ 2 pills per month during one year) at baseline.

**Genotyping**

Buffy coat preparations were stored at minus 150$^0$C until use. DNA was extracted as described [15]. *HMOX1* A-413T (rs2071746) was genotyped by KBioscience (KBioscience, Hoddesdon, United Kingdom) by PCR-based KASP™ genotyping assay (http://www.lgcgenomics.com/). To confirm reproducibility, genotyping was repeated for 10% of the samples yielding 100% identity. Laboratory staff was blinded for the status of the samples.

**Statistical Analysis**

Deviation from Hardy-Weinberg equilibrium was assessed using a Chi square test.

Incidence rate ratios (IRR) and 95% Confidence Interval (95%CI) were calculated according to the principles for analysis of case-cohort studies using an un-weighted approach [16]. Age was used as the time scale in the Cox regression models. Tests and confidence intervals were based on Wald's tests using the robust estimate of the variance-covariance matrix for the regression parameters in the Cox regression models [17] as previously described in [3-14].

All models were adjusted for baseline values of suspected risk factors for CRC such as body mass index (BMI) (kg/m$^2$, continuous), NSAID (yes/no), use of hormone replacement therapy (HRT) (never/past/current, among women), smoking status (never/past/current), intake of dietary fibre (g/day, continuous), and red meat and processed meat (g/day, continuous). Cereals, fibre, fruit and vegetables were also entered linearly. All analyses were stratified by gender, so that the basic (underlying) hazards were gender specific. IRR was calculated separately for heterozygous and homozygous variant allele carriers. Since recessive effects were observed a recessive mode was used in the subsequent analyses.

We investigated possible interactions between the polymorphism and intake of meat, dietary fibre, cereals, fish, fruit, vegetables, smoking status and use of non-steroid anti-inflammatory drug (NSAID) using the likelihood ratio test [3,5-9,14].

In another set of interaction analyses between the polymorphism and the dietary intake subdivided in tertiles, dietary intake was entered as a categorical variable. Tertile cut-points were based on the empirical distribution among cases. The possible interactions were investigated using the likelihood ratio test.

The procedure PHREG in SAS (release 9.3; SAS Institute., Cary, NC, USA) was used for the statistical analyses. A $P<0.05$ was considered to be significant.

The power to detect a dominant effect with an odds ratio of 1.5 and 1.4 was more than 98% and more than 92%, respectively [18].

The power to detect interaction was calculated. The average meat intake among controls was 109 g/day in the present study group, which is associated with a 10% increased risk of CRC. The power was 80% to detect a 3-fold difference in effect of meat intake with a primary effect of meat intake of 0.1 and an allele frequency of 0.41 [19].

**Ethics Statement**

All participants gave verbal and written informed consent. The Diet, Cancer and Health study was approved by the National Committee on Health Research Ethics (journal nr. (KF) 01-345/93) and the Danish Data Protection Agency.

**Table 1: Baseline characteristics of the study participants by selected demographic and established CRC risk factors.**

| *Variable* | *Cases* | | *Sub-cohort* | | *IRR[a] (95% CI)* |
|---|---|---|---|---|---|
| | *n (%)* | *Median (5-95%)* | *n (%)* | *Median (5-95%)* | |
| Total | 928 (100) | | 1726 (100) | | |
| Sex | | | | | |
| Men | 521 (56) | | 922 (53) | | |
| Women | 407 (44) | | 804 (47) | | |
| Age at inclusion (years) | | 58 (51-64) | | 56 (50-64) | |
| BMI (kg/m$^2$) | | 26.3 (20.7-34.0) | | 25.6 (20.5-32.9) | 1.03 (1.00-1.06)[d] |
| Food intake (g/day) | | | | | |
| Alcohol[b] | | 15.0 (1.2-71.6) | | 14.0 (1.1-65.3) | 1.04 (1.01-1.07)[e] |
| Dietary fibre | | 19.9 (10.6-32.7) | | 20.6 (10.7-34.1) | 0.87 (0.80-0.97)[f] |
| Red and processed meat | | 113.0 (47.1-233.4) | | 109.2 (41.7-236.1) | 1.02 (0.99-1.05)[g] |
| Smoking status | | | | | |
| Never | 277 (30) | | 571 (33) | | 1.00 (ref.) |
| Past | 284 (31) | | 508 (29) | | 1.03 (0.87-1.22) |
| Current | 367 (39) | | 647 (37) | | 1.08 (0.92-1.27) |
| NSAID use[c] | | | | | |
| No | 643 (69) | | 1185 (69) | | 1.00 (ref.) |
| Yes | 285 (31) | | 541 (31) | | 1.00 (0.87-1.15) |
| HRT use among women | | | | | |
| Never | 250 (61) | | 425 (53) | | 1.00 (ref.) |
| Past | 50 (13) | | 124 (15) | | 0.68 (0.50-0.93) |
| Current | 107 (26) | | 255 (32) | | 0.76 (0.61-0.96) |

Values are expressed as medians (5th and 95th percentiles) or as fractions (%).
[a]IRRs for CRC – mutually adjusted.
[b]Among current drinkers.
[c]NSAID use is defined as ≥ 2 pills per month during one year.
[d]Risk estimate per 2 kg/m$^2$ increment of BMI.
[e]Risk estimate for the increment of 10 g alcohol per day.
[f]Risk estimate for the increment of 10 g dietary fibres per day.
[g]Risk estimate for the increment of 25 g red and processed meat per day.

# Results

Baseline characteristics of the study participants are shown in Table 1. Median intake of fiber was statistically significantly lower and median intake of alcohol was statistically higher among cases compared to members of the sub-cohort. Also, a significantly lower proportion of the cases were past or current users of hormone

replacement therapy compared to the members of the sub-cohort. The genotype distribution of the polymorphisms in the comparison group did not deviate from Hardy-Weinberg equilibrium (results not shown). The variant allele frequency in the sub-cohort was 0.41.

**Table 2: IRR for CRC in relation to *HMOX1* A-413T (rs2071746).**

| | | $n_{cases}$ (%) | $n_{sub\text{-}cohort}$ (%) | IRR[a] (95% CI) | IRR[b] (95% CI) | P-value[c] |
|---|---|---|---|---|---|---|
| *HMOX1* | rs2071746 | | | | | |
| | AA | 310 (33) | 587 (34) | 1.00 (ref.) | 1.00 (ref.) | - |
| | TA | 446 (40) | 864 (50) | 1.01 (0.87-1.16) | 1.00 (0.86-1.15) | 0.94 |
| | TT | 172 (19) | 275 (16) | 1.16 (0.97-1.40) | 1.15 (0.95-1.38) | 0.15 |
| | TA+TT | 618 (67) | 1139 (66) | 1.04 (0.91-1.20) | 1.03 (0.90-1.18) | 0.64 |
| | TT vs. AA+TA | 172 (19) | 275 (16) | 1.16 (0.98-1.37) | 1.15 (0.98-1.36) | 0.10 |

[a] Crude – adjusted for age and sex.
[b] In addition, adjusted for smoking status, alcohol, HRT status (women only), BMI, use of NSAID, intake of red and processed meat, and dietary fibre.
[c] P-value for the adjusted estimates.

As shown in Table 2, no association between *HMOX1* A-413T and CRC risk was found (TA vs AA; IRR=1.00 and 95%CI: 0.86-1.15, *P*=0.94, TT vs AA; IRR=1.15, 95%CI: 0.95-1.38, *P*=0.15, and TT vs AA+TA; IRR=1.15, 95%CI: 0.98-1.36, *P*=0.10, respectively, for the fully adjusted estimates (Table 2). A potential recessive effect was found and therefore AA and AT carriers were grouped versus TT carriers to maximize the statistical power.

**Table 3. Interaction between dietary factors and *HMOX1* A-413T (rs2071746) in relation to CRC risk**.

| *HMOX1*/ rs2071746 | IRR[a] (95% CI) | IRR[b] (95% CI) | P-value[c] | IRR[a] (95% CI) | IRR[b] (95% CI) | P-value[c] |
|---|---|---|---|---|---|---|
| | ***Red and processed meat per 25 g/day*** | | | ***Fish per 25 g/day*** | | |
| AA+TA | 1.02 (0.99-1.05) | 1.02 (0.99-1.05) | 0.97 | 0.96 (0.90-1.03) | 0.97 (0.91-1.04) | 0.18 |
| TT | 1.02 (0.96-1.09) | 1.02 (0.96-1.09) | | 0.85 (0.72-1.00) | 0.86 (0.73-1.02) | |
| | ***Dietary fibre per 10 g/day*** | | | ***Fruit per 50 g/day*** | | |
| AA+TA | 0.87 (0.79-0.97) | 0.89 (0.80-0.99) | 0.69 | 0.97 (0.95-1.00) | 0.98 (0.96-1.01) | 0.57 |
| TT | 0.84 (0.68-1.04) | 0.85 (0.69-1.05) | | 0.96 (0.92-1.01) | 0.97 (0.93-1.02) | |
| | ***Dietary cereal per 50 g/day*** | | | ***Vegetables per 50 g/day*** | | |
| AA+TA | 0.95 (0.90-1.00) | 1.01 (0.94-1.09) | 0.58 | 1.00 (0.96-1.04) | 1.04 (0.99-1.08) | 0.14 |
| TT | 0.98 (0.89-1.09) | 1.04 (0.93-1.17) | | 0.93 (0.85-1.02) | 0.97 (0.88-1.06) | |

[a] Crude – adjusted for age and sex.
[b] In addition, adjusted for smoking status, alcohol, HRT status (women only), BMI, use of NSAID, intake of red and processed meat, and dietary fibre.
[c] P-value for interaction for adjusted risk estimates.

No interactions were found between diet and *HMOX1* A-413T using the continuous scale (*P*-value for interaction ($P_{int}$); meat=0.97, fish=0.18, cereal=0.58, fibre=0.69, fruit=0.57, vegetables=0.14) (Table 3). This analysis describes the increased risk per 25 gram meat per day (Table 3, footnotes). Similarly, no interactions with diet were found using the tertile analysis (Table 4). No interactions were found between life style and *HMOX1* A-413T (use of NSAID *P*=0.11, smoking status *P*=0.75) (TT vs AA+TA; IRR=1.15, 95%CI: 0.98-1.36, *P*=0.10 for the adjusted estimate) (Table 5-6).

**Table 4. IRR for CRC for tertiles of intake of dietary factors for *HMOX1* A-413T (rs2071746).**

| | *1.tertile* | | *2.tertile* | | *3.tertile* | | *1.tertile* | *2.tertile* | *3.tertile* | *P-value*[b] |
|---|---|---|---|---|---|---|---|---|---|---|
| | *Nc*[c] | *Ns*[c] | *Nc*[c] | *Ns*[c] | *Nc*[c] | *Ns*[c] | *IRR (95%CI)*[a] | *IRR (95%CI)*[a] | *IRR (95%CI)*[a] | |
| **Red and processed meat** | | | | | | | | | | |
| AA+TA | 251 | 548 | 257 | 462 | 248 | 441 | 1.00 (ref.) | 1.25 (1.05-1.50) | 1.28 (1.06-1.56) | |
| TT | 57 | 98 | 55 | 101 | 60 | 76 | 1.32 (0.98-1.78) | 1.21 (0.92-1.61) | 1.52 (1.13-2.04) | 0.33 |
| **Dietary cereal** | | | | | | | | | | |
| AA+TA | 252 | 421 | 259 | 514 | 245 | 516 | 1.00 (ref.) | 0.91 (0.76-1.10) | 0.96 (0.76-1.21) | |
| TT | 61 | 97 | 52 | 91 | 59 | 87 | 1.07 (0.80-1.42) | 0.99 (0.72-1.35) | 1.27 (0.92-1.75) | 0.51 |
| **Fruit** | | | | | | | | | | |
| AA+TA | 253 | 452 | 252 | 460 | 251 | 539 | 1.00 (ref.) | 1.00 (0.83-1.20) | 0.92 (0.74-1.13) | |
| TT | 60 | 90 | 59 | 89 | 53 | 96 | 1.20 (0.90-1.60) | 1.13 (0.85-1.50) | 1.02 (0.74-1.51) | 0.93 |
| **Fish** | | | | | | | | | | |
| AA+TA | 257 | 528 | 255 | 417 | 244 | 506 | 1.00 (ref.) | 1.12 (0.94-1.33) | 0.93 (0.77-1.12) | |
| TT | 58 | 87 | 55 | 86 | 59 | 102 | 1.32 (0.98-1.78) | 1.21 (0.91-1.61) | 1.01 (0.76-1.34) | 0.57 |
| **Dietary fibre** | | | | | | | | | | |
| AA+TA | 253 | 436 | 253 | 437 | 250 | 578 | 1.00 (ref.) | 0.97 (0.82-1.16) | 0.81 (0.68-0.97) | |
| TT | 60 | 98 | 59 | 75 | 53 | 102 | 1.05 (0.79-1.40) | 1.33 (1.01-1.76) | 0.89 (0.65-1.18) | 0.37 |
| **Vegetables** | | | | | | | | | | |
| AA+TA | 249 | 439 | 252 | 501 | 255 | 511 | 1.00 (ref.) | 1.06 (0.88-1.27) | 1.12 (0.91-1.37) | |
| TT | 64 | 78 | 57 | 97 | 51 | 100 | 1.35 (1.02-1.80) | 1.21 (0.90-1.62) | 1.09 (0.79-1.49) | 0.28 |

[a] Analysis adjusted for smoking status, alcohol, HRT status (women only), BMI, use of NSAID, intake of red and processed meat, and dietary fibre.
[b] P-value for interaction between polymorphisms and dietary factors for the adjusted estimates.
[c] c: cases, s: sub-cohort
Tertiles of red and processed meat (<94.2266 g, 94.2266 g < and < 139.266 g, >139.266 g), fish (<30.0189 g, 30.0189 g < and < 48.0483 g, > 48.0483 g), dietary fibre (<17.2846 g, 17.2846 g < and < 22.4505 g, > 22.4505 g), cereals (<152.202 g, 152.202 g < and < 218.014 g, > 218.014 g), fruit (<115.622 g, 115.622 g < and < 224.174 g, > 224.174 g), vegetables (<113.755 g, 113.755 g < and < 197.250 g, > 197.250 g).

## Discussion

In the present candidate gene study, we extended our previous study of *HMOX1 A-413T* polymorphism in relation to diet, lifestyle and colorectal carcinogenesis in a study group of 383 CRC cases and 763 members of the comparison group to a larger cohort encompassing 928 number of cases and 1726 members of the comparison group and included more dietary factors. We found no association between *HMOX1* A-413T and CRC risk and no interactions between diet and lifestyle and *HMOX1* A-413T. Although no statistically significant interactions were found, we found that meat intake was associated with increased risk of CRC among carriers of both genotypes. Also, fibre intake was associated with lowered risk of CRC among carriers of both genotypes. We thus reproduced the previous finding that the studied *HMOX1* polymorphism was not associated with risk of CRC [3]. Our analyses suggest that genetically determined variation in heme oxygenase activity does not modify risk of CRC. Furthermore, we observed no interaction with meat intake. Thus, the results do not support that intake of heme or heme iron from meat is linked to CRC. The investigated *HMOX1* A-413T polymorphism was selected based on the role of the gene product in heme

metabolism and the functional effect of the polymorphism. Of the two strongly linked *HMOX1* promoter polymorphisms, the $(GT)_n$ repeats and the A-413T, the A-413T genotype was most likely responsible for the effect on the promoter activity [1]. Accordingly, this polymorphism has been found to be associated with various diseases [1,20,21]. To the best of our knowledge, apart from our previous analysis, no other large studies of heme oxygenase in relation to CRC have been performed to date [3].

**Table 5: Interaction between NSAID use and *HMOX1* A-413T (rs2071746) in relation to CRC risk.**

| *HMOX1* | *NSAID use* $n_{cases}/n_{sub\text{-}cohort}$ | | *NSAID use IRR (95% CI)*[a] | | *NSAID use IRR (95% CI)*[b] | | *P-value*[c] |
|---|---|---|---|---|---|---|---|
| | *No* | *Yes* | *No* | *Yes* | *No* | *Yes* | |
| rs2071746 | | | | | | | |
| AA+TA | 539/1004 | 217/447 | 1.00 (ref.) | 0.95 (0.81-1.11) | 1.00 (ref.) | 0.94 (0.80-1.10) | 0.11 |
| TT | 104/181 | 68/94 | 1.05 (0.85-1.30) | 1.32 (1.03-1.70) | 1.04 (0.84-1.29) | 1.30 (1.01-1.66) | |

[a] Crude – adjusted for age and sex.
[b] In addition, adjusted for smoking status, alcohol, HRT status (women only), BMI, intake of red and processed meat, and dietary fibre.
[c] P-value for interaction for the adjusted estimates.

The study design has several strengths. The study design used in this study is well suited for analysing interactions between genes and meat intake due to the collection of dietary and life style factors before diagnosis, the cases and controls being drawn from the same population, the large sample size and the relatively high meat intake [3,5-9,14] which may contribute to differential association pattern among populations with high and low meat intakes [22]. Study limitation includes limited power for detecting interactions. This study has more than 92% to detect a dominant effect of 1.4 had it been there [18]. The average meat intake among controls was 109 g/day in the present study group, which is associated with a 10% increased risk of CRC. The present study has approximately 80% chance of detecting a 3-fold difference in effect of meat intake with a primary effect of meat intake of 0.1 and an allele frequency of 0.41 [19]. Intake of meat may be associated with other CRC risk factors such as low fibre intake. However, we have adjusted for known risk factors in this cohort to minimize potential confounding.

The results reproduced our previous findings and do not support a link between heme or heme iron and colorectal cancer. The result should be evaluated in prospective cohorts with high meat intake.

*Acknowledgments*

Staff at the Libraries, Regional Hospital Viborg and Hospital of Southern Jutland, are thanked for help. The project has been funded by Danish Council for Independent Research | Medical Sciences (FSS) (09-073597).

**Table 6: Interaction between smoking status and *HMOX1*/rs2071746 in relation to risk of colorectal cancer.**

| *HMOX1* | *Never smokers* | *Past smokers* | *Current smokers* | *Never smokers* | *Past smokers* | *Current smokers* | *Never smokers* | *Past smokers* | *Current smokers* | P-value[c] |
|---|---|---|---|---|---|---|---|---|---|---|
| | $n_{cases}/n_{sub\text{-}cohort}$ | $n_{cases}/n_{sub\text{-}cohort}$ | $n_{cases}/n_{sub\text{-}cohort}$ | *IRR (95%CI)*[a] | *IRR (95%CI)*[a] | *IRR (95%CI)*[a] | *IRR (95%CI)*[b] | *IRR (95%CI)*[b] | *IRR (95%CI)*[b] | |
| rs2071746 | | | | | | | | | | |
| AA+TA | 228/484 | 232/416 | 296/551 | 1.00 (ref.) | 1.07 (0.89-1.28) | 1.12 (0.94-1.33) | 1.00 (ref.) | 1.06 (0.88-1.27) | 1.08 (0.90-1.28) | 0.75 |
| TT | 49/87 | 52/92 | 71/96 | 1.24 (0.90-1.69) | 1.11 (0.83-1.50) | 1.34 (1.02-1.75) | 1.20 (0.88-1.64) | 1.10 (0.82-1.48) | 1.30 (0.98-1.71) | |

[a] Crude – adjusted for age and sex.
[b] In addition, adjusted for alcohol, HRT status (women only), BMI, use of NSAID, intake of red and processed meat, and dietary fibre.
[c] P-value for interaction for adjusted risk estimates.

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# Chapter 7

*Manuscript in preparation*

## Paper V: *Alcohol-related breast cancer in postmenopausal women – effect of CYP19A1, PPARG and PPARGC1A polymorphisms on female sex-steroid levels and interaction with alcohol consumption and NSAID usage*

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**Abstract**

Alcohol consumption is associated with increased risk of breast cancer (BC), and the underlying mechanism is thought to be sex-hormone driven. *In vitro* and observational studies have suggested that a mechanism involving peroxisome proliferator-activated receptor gamma (PPARγ) in a complex with peroxisome proliferator-activated receptor gamma coactivator (PGC) 1α and interaction with aromatase (encoded by *CYP19A1*) is involved in alcohol-related increase in circulating female sex-hormone levels eventually leading to BC. Additionally, use of non-steroidal anti-inflammatory drugs (NSAIDs) seems to be an effect modifier in alcohol-related BC due to their interaction with PPARγ. In the present study we assess whether genetic variation in *CYP19A1* is associated with hormone levels and risk of BC in a case-control study group nested within the Danish "Diet, Cancer and Health" cohort and search for gene-gene interaction with *PPARG* and *PPARGC1A* and gene-alcohol and gene-NSAID interactions. Furthermore, we perform a pilot human intervention trial to determine the effect of the *PPARG* Pro$^{12}$Ala polymorphism and the PPARγ stimulator, Ibuprofen, on sex-hormone levels following alcohol intake in postmenopausal women. Our results show that variations in *CYP19A1* are associated with hormone levels and interact with alcohol intake in relation to hormone levels. These polymorphisms were not, however associated with BC risk. Acute intake of alcohol decreased blood estrone, estrone sulphate and SHBG levels, but Ibuprofen intake and *PPARG* Pro12Ala variant allele carrier status did not affect the found sex-hormone levels. These studies suggest that acute and chronic alcohol consumption have different effect on biosynthesis and metabolism of sex-hormones, and we were therefore not able to put PPARγ and the aromatase in the same pathway linking alcohol-related BC.

## Introduction

Alcohol is a well-known risk factor for breast cancer (BC) [1-3], and observational studies have shown that intake of alcohol is associated with 7-10% increased risk of BC for every 10 gram alcohol consumed per day [4-9]. It is believed that at least part of the underlying mechanism is sex-hormone driven [1,10,11]. Several controlled experimental and observational human studies demonstrate associations between alcohol intake and increased female sex-hormone blood concentrations in pre- and postmenopausal women [12-25]. Additionally, alcohol is more strongly associated to hormone-sensitive BCs than hormone-insensitive subtypes [26]. Increased aromatization [27,28], impairment of the metabolism of estrogens in the liver [27] or stimulation of adrenal steroidogenesis [17] are possible mechanisms by which alcohol increases sex-hormone concentrations in women.

The use of genetic epidemiology in BC research may elucidate the involved molecular pathways and define subpopulations of women being more sensitive to alcohol consumption in relation to BC. Indeed, in the Danish prospective study "Diet, Cancer and Health" (DCH), variant carriers of the *PPARG* Pro$^{12}$Ala (rs1801282) polymorphism had a 20% increased risk of BC per 10 gram of alcohol consumed per day, whereas carriage of the wild type was not associated with alcohol-related BC [29], thus implicating peroxisome proliferator-activated receptor gamma (PPARγ) in alcohol-related breast carcinogenesis. In an updated study including 798 BC cases, the risk estimate was increased by 13% per 10 gram alcohol per day [30]. PPARγ is a transcription factor regulating adipocyte differentiation and expression of several adipocyte specific genes by binding to regulatory response elements in target genes as a heterodimer with retinoid X receptor (RXR) [31]. The single nucleotide polymorphism (SNP), *PPARG* Pro$^{12}$Ala, only exists in the PPARγ2 isoform, which is primarily expressed in adipose tissue [31]. The *PPARG* Pro$^{12}$Ala substitution causes a 30% reduction in target gene transcription [32]. In postmenopausal women, estrogens are primarily synthesized in adipose tissue, where aromatase (encoded by *CYP19A1*) catalyses the biosynthesis of estrogens [33]. Intake of alcohol increases the expression of aromatase in rat adipocytes [34], and aromatase is negatively regulated by PPARγ at the transcriptional level [35,36] by a mechanism involving binding of the PPARγ-RXR complex to peroxisome proliferator-activated receptor gamma coactivator (PGC) 1α [37]. An *in vitro* study has shown that ethanol inhibits the PPARγ-PGC-1α complex at physiologically relevant concentrations [30]. Moreover, Petersen *et al.* also illustrated that PGC-1α dependent co-activation of the PPARγ-complex is compromised for the rarer variant of *PPARG* Pro$^{12}$Ala. Thus, it was proposed that alcohol inhibits PPARγ-mediated inhibition of aromatase transcription, thus resulting in increased aromatase transcription and increased levels of sex hormones.

PPARγ interacts with commonly used non-steroidal anti-inflammatory drugs (NSAIDs) [38] which are suspected to function as endocrine disruptors [39,40]. In the DCH cohort study, interaction between use of NSAIDs and the *PPARG* Pro$^{12}$Ala polymorphism was observed [29]. NSAID use did not modify the risk of alcohol-related BC among *PPARG* Pro$^{12}$Ala wild type carriers, however, among variant carriers, only users of NSAIDs were at risk. This study indicates that NSAIDs activate the less active PPARγ $^{12}$Ala variant so that it has the same effect as the wild type PPARγ Pro$^{12}$ transcription factor. In a meta-analysis, the use of NSAIDs

has been associated with decreased risk of BC [41]. However, in the DCH cohort, female NSAID users, with an intake of more than 13 gram alcohol per day had a 1.60 fold increased risk of BC compared to non-users of NSAIDs who consumed less than 3 gram of alcohol per day [42], indicating that alcohol consumption modifies BC risk among users of NSAID.

In the present study we further pursue the proposed mechanism of action of alcohol-related BC. We assess whether genetic variation in *CYP19A1* is associated with hormone levels and risk of BC in a case-control study group nested within the DCH cohort and search for gene-gene interactions with *PPARG* and *PPARGC1A* and gene-alcohol and gene-NSAID interactions. Furthermore, we perform a pilot intervention study to determine the effect of *PPARG* Pro[12]Ala and the PPARγ stimulator, Ibuprofen [38], on sex-hormone levels following alcohol intake in postmenopausal women.

# Methods

### DHC cohort study

*Participants*

The subjects were selected from the ongoing Danish DCH cohort study. The present study group has been described previously [8,30]. In short, 79,729 women aged 50–64 years, born in Denmark, living in the Copenhagen or Aarhus areas and having no previous cancers at the time of invitation were invited to participate in the study between December 1993 and May 1997. A total of 29,875 women accepted the invitation, corresponding to 37% of the invited women.

Study participants were followed up for diagnosis of BC from date of entry until either the date of diagnosis of cancer using record linkage to the Danish Cancer Registry until 2003 and afterwards by linkage to the Danish Pathology Databank, date of death, date of emigration, or April 27th, 2006, whichever came first. A total of 975 women were diagnosed with BC during the follow-up period. For each case, one matched control was selected [8,30]. The control was cancer-free at the exact age at diagnosis of the case and was further matched on age at inclusion into the cohort (half-year intervals), use of hormone replacement therapy (HRT) (current/former/never) and on certainty of postmenopausal status (known/probably postmenopausal) upon inclusion into the cohort [8,30]. 72 individuals were excluded because of missing information about one or more of the potential confounding variables. Additionally 201 individuals were excluded because of failed genotyping or no buffy coat was available. 265 individuals were excluded because of a missing partner in the case-control pair, due to the above mentioned exclusions leaving 687 pairs for data analyses.

*Data on covariates*

From the baseline questionnaires we obtained information on duration of school education, smoking status, HRT use, birth pattern (number of births and age at first birth) and alcohol intake. BMI was computed based on measurements of height and weight at enrolment. Intake of alcohol was inferred from the food-frequency questionnaire and life-style questionnaire as described in details in [8,30]. Abstainers were defined as those who reported no intake of alcohol on the food-frequency questionnaire and no drinking occasions on the

lifestyle questionnaire. 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 frequent 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. Findings on all of these known risk factors have been reported previously for both the entire DCH cohort, for a subset of the present study, and for the present study group [5,8,29,42-44].

**Human intervention study**

*Participants*

The human intervention study was conducted at the Department of Nutrition, Exercise and Sports, University of Copenhagen, Denmark. The participants were recruited by advertisements in the Copenhagen area, in local newspapers and at the webpage www.forsogsperson.dk. To be eligible, women had to meet the following requirements: 1) aged 50-70 years and postmenopausal (last menses at least 1 year earlier); 2) having no history of hysterectomy *before* last menses *with* preservation of both ovaries (unless a medical confirmation for the postmenopausal status exists or the participant is 60 years or older); 3) having no major health problems, such as ulcers, heart diseases, diabetes or cancer; 4) having a weekly alcohol use of less than 14 drinks, but not being an abstainer and having no history of alcohol abuse; 5) not using HRT; 6) not taking prescription medications that could interfere with the study (i.e. daily use of NSAIDs and/or medication that interact with PPARγ e.g. cholesterol lowering medicine); 7) having a BMI of 18-35; 8) not being allergic to alcohol and/or Ibuprofen; 9) being a non-smoker. The research protocol was approved by The National Committee on Health Research Ethics for the Capital Region of Denmark, protocol number: H-3-2013-056. All participants received oral and written information concerning the study before they gave their written consent.

Fourty-three postmenopausal women were enrolled in the initial screening procedure which included anthropometric measures, completing of questionnaires (regarding alcohol habits, medical history and medicine use) and donating a blood sample for genotyping. Power calculation showed that we had an 80% change of finding a 10% change in estradiol level if 11 participants were included in each group (α=5%). In order to take drop out into account, we decided to enrol 18 women in each group. However, only 7 (16%) women were *PPARG* $^{12}$Ala carriers, and they were all included in the study as well as 18 homozygous wild type carriers of the *PPARG* Pro$^{12}$ allele. The 18 wild type carriers were chosen based on their questionnaires data; that is, those that did not take any form of medication, were preferred. All 25 participants, 7 *PPARG* $^{12}$Ala carriers and 18 *PPARG* Pro$^{12}$ carriers completed the study.

*Study design*

The study was performed as a randomised, double-blinded, placebo controlled 2x24 h crossover study. The volunteers were randomised to 1 of 2 groups and served an alcoholic drink and a placebo tablet (treatment

1) or an alcoholic drink and an Ibuprofen tablet (treatment 2). The two treatments were separated by a 5-7 week washout period. Alcohol was supplied as 96% ethanol (Navimer, G.D.C., Jumet, Belgium) in an 8% solution with 1:8 Rose's Lime Flavour Cordial Mixer® (Dr Pepper Snapple Group Inc., Plano, Texas), 1:8 Rose's Sugar Cane Flavour Cordial Mixer® (Dr Pepper Snapple Group Inc., Plano, Texas) and water. Placebo and Ibuprofen tablets looked identical, and were supplied by The Pharmacy of the Capital Region of Denmark (Herlev, Denmark), and Ibuprofen by Nycomed ApS (Roskilde, Denmark).

Fourty-eight hours before each intervention, participants were asked to refrain from alcohol consumption and any form of painkillers. The participants showed up fasting and were served lunch at the University's dining facility two hours before drinking (Figure 1). The lunch consisted of a sandwich, which was identical for each participant at each intervention, and was eaten within 30 minutes. All blood samples were collected following 10 minutes supine resting. The first blood sample was drawn 40 minutes before serving the drink. Just after this blood collection and 30 minutes prior to drinking, participants had their Ibuprofen or placebo tablet administered. This time point was chosen in order to capture the plasma concentration peak of Ibuprofen which is 1-2 hours after administration [45,46]. After another 30 minutes, the alcoholic beverage was administered and consumed within 30 minutes under the surveillance of a research assistant to ensure that the drink was ingested slowly over the entire period. The participants were offered ice cubes to put in the drink and water ad libitum during the whole day. After 30, 60 and 90 minutes, blood was collected. These time points were selected based on two other studies reporting peak in blood estradiol concentrations between 30 and 60 minutes after alcohol consumption [16,20]. After the last blood collection and after checking blood alcohol level in an alcometer, the participants went home. The next morning, they showed up again for the last blood collection. This time point was chosen to enable the comparison of our results with those obtained in long term intervention or cohort studies where blood typically is collected the day after having consumed alcohol. Since estrone sulphate has a half-life of 5-7 hours [17], an alcohol induced change in estrone sulphate caused by disruption in estrogen biosynthesis would be measurable in this morning blood sample.

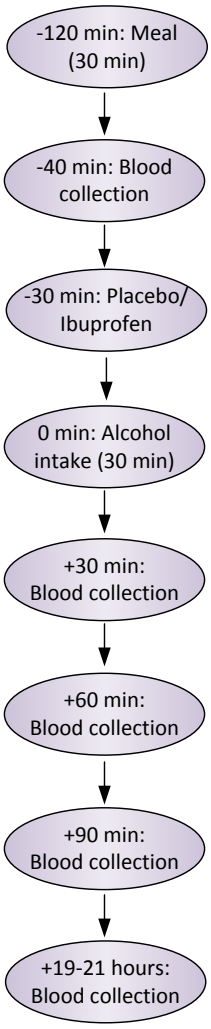


**Figure 1. The study course for each participant at each intervention.**

*Blood sampling and storage*

For the genotype screening, blood was collected in 6 ml EDTA BD Vacutainer® Blood Collection Tubes (Becton, Dickinson and Company) and buffy coat was separated by centrifugation (4000 rpm, 4°C) and frozen at -80°C. During the intervention, blood was collected in 10 ml silicone-coated serum BD Vacutainer® Blood Collection Tubes (Becton, Dickinson and Company) for ethanol and hormone measurements. The tubes were left at room temperature for >30 minutes, centrifuged (4000 rpm, 4°C) and aliquots were frozen at -80°C. To avoid ethanol evaporation from the tubes during storage, serum for ethanol analysis was kept in tubes with airtight caps.

**Laboratory methods**

*DCH cohort study*

DNA from the DCH participants was extracted from frozen lymphocytes as described [47]. *PPARG* Pro[12]Ala (rs1801282), *PPARGC1A* Gly[482]Ser (rs8192678) and *PPARGC1A* Thr[612]Met (rs3736265) were determined as previously described [30] and have been published previously [29,30]. Ten tagging SNPs of the *CYP19A1* gene were selected using publicly available HapMap [48] genotyping data set from Utah residents with ancestry from Northern and Western Europe (CEU, version 3, release 2) in combination with Haploview (version 4.2, Broad Institute, Cambridge USA) [49] comprising the major variations in *CYP19A1*. Criteria for SNP inclusions were a minor allele frequency (MAF) of minimum 5% and an $r^2$ threshold of 0.1. Tagging of SNPs with an $r^2$ higher than 0.1 resulted in several SNPs being in complete linkage disequilibrium. Moreover, our intention was to include only major variations of the gene. Four polymorphisms were force included in the aggressive SNP tagging (rs10046, rs749292, rs1062033 and rs10519297) due to their documented impact on aromatase RNA expression, blood hormone levels and/or BC risk [11,50-57]. Moreover, one of the tag SNPs (rs4646) has been associated with BC [57] and blood estrogen levels [51,54,58]; another tag SNP (rs2445762) was associated with low estradiol levels in GWAS [59]; and rs3751591 is located in a recombination spot between two haplotype blocks (Figure S1).

Four of the polymorphisms (rs10046, rs749292, rs1062033 and rs10519297) were determined using the ABI 7900HT RT-PCR system (Applied Biosystems, Nærum, Denmark): rs10046 and rs1062033 were determined using the TaqMan® Pre-designed SNP genotyping; assay ID C_8234732_30 and C_8794680_30, respectively (Applied Biosystems, Nærum, Denmark). rs749292: Primers: F: 5'-GCT TCT GCC AGT CCT TCT TCA-3', R: 5'-GCT TAG GGC CTG ATA GAA ATT GTG-3' (TAGCopenhagen, Copenhagen, Denmark), G-allele: 5'-FAM-CTC GGA GTC GAG GAT-MGB-3', A-allele: 5'-VIC-TCG GAG TCA AGG ATT-MGB-3' (Applied Biosystems, Nærum, Denmark). rs10519297: Primers: F: 5'CCT TGC CTG AGC CAT CTC TT-3', R: 5'- CTT GGC AGT CAA AAG CAG TAG TAG TC-3' (TAGCopenhagen, Copenhagen, Denmark), G-allele: 5'-FAM-CTC CGA CAT GGG TC-MGB-3', A-allele: 5'-VIC-TCT CCG ACA TAG GTC-MGB-3' (Applied Biosystems, Nærum, Denmark).

The remaining six *CYP19A1* polymorphisms (rs2008691, rs2445762, rs3751591, rs4646, rs6493487 and rs749292) were genotyped by KBioscience (KBioscience, Hoddesdon, United Kingdom) by PCR-based KASP™ genotyping assay (http://www.lgcgenomics.com/). To confirm reproducibility, genotyping was repeated for 10 % of the samples yielding 100% identical genotypes.

Serum levels of estrone, estrone sulphate and sex-hormone binding globulin (SHBG) were previously determined for a subset of the DCH study (434 participants) in blood samples collected at entry into the DCH cohort [60]. Estrone and estrone sulphate were measured by radioimmunoassay and SHBG by use of immunofluorometric analysis.

*Human intervention study*

DNA was isolated from frozen lymphocytes as described by Miller et al. [47]. Genotypes were determined using RT-PCR and allelic discrimination on ABI 7900HT instruments (Applied Biosystems, Nærum, Denmark). Generally, 40-200 ng/µl DNA was obtained and 10 ng of DNA was genotyped in six µl containing 50% 2 × Mastermix (Applied Biosystems, Nærum, Denmark), 100 nM probes, and 900 nM primers. *PPARG* Pro[12]Ala (rs1801282) primers and probes were: primers: 5'-GTT ATG GGT GAA ACT CTG GGA GAT-3' and 5'-GTT ATG GGT GAA ACT CTG GGA GAT-3': probes: C-allele: 5''-FAM-CTC CTA TTG ACG CAG AAA GCG ATT C-BHQ-1-3' and G-allele: 5'-Yakima Yellow-TCC TAT TGA CCC AGA AAG CGA TTC C-BHQ-1-3'. Serum ethanol concentration was measured by the enzymatic rate assay (kat. no. 11776312190, Roche Diagnostics GmbH, Mannheim, Germany) using the ABX Pentra 400 (Horiba Medical, Brøndby, Denmark) instrument with inter- and intra assay variation of <4%. All samples were measured in duplicates in random order and in the same batch to decrease analytical variation.

Analyses of estrogens and SHBG levels were performed at the Department of Clinical Biochemistry and Immunology, Statens Serum Institute, Copenhagen, Denmark. Estrogens were extracted by Solid-Phase Extraction and the extracts were analyzed using Liquid-Chromatography (LC) coupled to Mass-Spectrometry (MS). All samples were analyzed in duplicate and the average of both measurements was used. Within the working range of the method (20 pmol/L to 50 nmol/L) the CV's for all analytes are 10% for estrone, estrone sulphate and 17β-estradiol and the CV's increase below the working range and reach 20% at the limit of detection. Due to the low recovery rate of estradiol (60%), these measurements were excluded from statistical analyses. SHBG was determined on an Abbott Architect using Abbotts SHBG kit (Abbott, Abbott Park, Illinois). The LOD and the intra- and inter-assay coefficients of variation were 0.1 nmol/l, 4% and 6%, respectively.

**Statistical methods**

*DCH cohort study*

Deviation from Hardy-Weinberg equilibrium was assessed using a Chi square test.

The analysis of the association between the exposure variables and the BC incidence rate ratios (IRR) was based on a conditional logistic regression analysis corresponding to a Cox Proportional Hazard model due to the used study design [61]. Age was used as the time axis. Two-sided 95% confidence intervals (CI) for the IRR were calculated on the basis of Wald's test of the regression parameter, that is, on the log rate ratio scale. All models were adjusted for baseline values of risk factors for BC such as parity (entered as two variables; parous/nulliparous and number of births), age at first birth, length of school education (low, medium and high), duration of HRT use, and BMI. Analyses not concerning interaction between alcohol intake and the polymorphisms were further adjusted for alcohol intake of 10 gram per day. For the different genetic variations, we investigated different interactions with alcohol intake and use of NSAID, using the likelihood ratio test. IRR was calculated separately for heterozygous and homozygous variant allele carriers. For all the SNPs except for rs3751591, variant allele carriers were subsequently grouped for interaction

analyses since no recessive effects were observed. For rs3751591, a recessive mode was used in the subsequent analyses. Haplotypes of *CYP19A1* were inferred manually as done previously [62-64].

For the analyses with sex-hormone levels as outcome, multiple log-linear regression analyses of the association between *CYP19A1* genotypes and serum levels of estrone, estrone sulphate and SHBG were performed with adjustment for potential confounders i.e., age (linear), smoking (categorical: never, past and current) and BMI (linear). All values of hormone concentrations were log-transformed to correct for left-skewed distribution. The statistical analyses were carried out using SAS (release 9.3, SAS Institute, Inc, Cary, North Carolina, USA).

*Human intervention study*

This study was divided up into two types of analyses using two different models. Model A only involved the time at the beginning of the study period and the end (time=0 and time=1200 minutes) whereas model B involved the time 0, 30, 60, and 90 minutes after start. Since the response was not linear with the time over the whole period (0 to 1200 minutes) it made more sense to divide the analysis up in this way.

Model A used a mixed model with *id* as the random effect nested with genotype and order (*geno*order)*. The explanatory categorical variables were treatment (*treat),* order in which they were given treatment A or B first *(order),* genotype (*geno),* and *time* (0 and1200). *BMI* was included as a covariate.

Model B also used a mixed model with *id* as the random effect nested with genotype and order (*geno*order).* The explanatory categorical variables were treatment (*treat),* order in which they were given treatment A or B first *(order),* genotype (*geno),* and *time* (0, 30, 60, 90). *BMI* was included as a covariate. *Time* was also included as repeated measurement with *id(geno*treat*order)* as the subject.

The response variables = estrone sulphate was log-transformed to correct for right-skewed distribution. The response variables = estrone and SHBG were not log-transformed, since the conditional residuals were not or only very little skewed.

# Results

**DCH cohort study**

We assessed 10 tag *CYP19A1* SNPs including two functional polymorphisms in relation to BC risk in a nested, prospective case-control cohort of 687 BC cases with matched controls within the prospective DCH Study.

**Table 1: Baseline characteristics of the DCH study participants by selected demographic and established BC risk factors.**

| *Variable* | *Cases* | | *Controls* | | *IRR[a] (95% CI)* |
|---|---|---|---|---|---|
| | *n (%)* | *Median (5-95%)* | *n (%)* | *Median (5-95%)* | |
| Women | 687 (100) | | 687 (100) | | |
| Age at inclusion, years | | 57 (51-64) | | 57 (51-64) | |
| School education | | | | | |
| Short | 198 (29) | | 257 (37) | | 1.0 (ref.) |
| Medium | 344 (50) | | 316 (46) | | 1.39 (1.07-1.79) |
| Long | 145 (21) | | 114 (17) | | 1.59 (1.13-2.24) |
| Body mass index, kg/m$^2$ | | 25 (20-34) | | 25 (20-34) | 1.01 (0.96-1.07)[b] |
| Nulliparous | 102 (15) | | 78 (11) | | 1.02 (0.64-1.60)[c] |
| Number of births | | 2 (1-4) | | 2 (1-4) | 0.92 (0.79-1.06) |
| Age at first birth, years | | 23 (18-31) | | 23 (18-32) | 1.07 (0.91-1.25)[d] |
| Use of HRT, years[e] | | 6 (0.5-19) | | 5 (0.5-20) | 1.00 (0.87-1.15)[f] |
| Abstainers | 15 (2) | | 22 (3) | | 0.80 (0.40-1.61)[g] |
| Alcohol intake, g/day | | 11 (1-43) | | 9 (1-40) | 1.12 (1.04-1.21)[h] |
| NSAID use[i] | 286 (42) | | 239 (35) | | 1.33 (1.07-1.66) |

Values are expressed as medians (5th and 95th percentiles) or as fractions (%).
a The risk estimates for breast cancer are mutually adjusted.
b The risk is estimated per additional 2 kg/m$^2$.
c The risk is estimated for nulliparous versus one birth at age 35.
d The risk is estimated per additional 5 years.
e Among ever users of HRT.
f The risk is estimated per additional 5-year of HRT use.
g The risk for abstainers compared to the increment of 10 g alcohol per day.
h Among current drinkers, risk estimate is estimated for the increment of 10 g alcohol per day.
i NSAID use is defined as ≥ 2 pills per month during one year.

The aim was to assess the association of *CYP19A1* SNPs with sex-hormone levels and BC risk and to assess a possible interaction with polymorphisms in *PPARG* and *PPARGC1A*, alcohol intake and use of NSAIDs in relation to risk of BC in order to evaluate the hypothesis that alcohol intake causes BC by increasing circulating levels of estrogens mediated by interaction between PPARγ/PGC-1α and *CYP19A1*. Baseline characteristics of the present study group including BC risk factors are presented in Table 1 as published previously [8,30]. Among the controls, the genotype distributions of the studied polymorphisms were in Hardy–Weinberg equilibrium (results not shown).

*Associations with hormone levels*

Serum estrone, estrone sulphate and SHBG have been measured for 434 women from the DCH cohort. The hormone levels differed significantly for five of the ten polymorphisms. Variant T-carriers of the *CYP19A1*/rs11070844 polymorphism had 17% higher estrone levels (*P*=0.009) and 14% higher estrone sulphate levels (*P*=0.01) than the wild type (Table 2). SHBG levels were 37% higher among CC-carriers of the *CYP19A1*/rs3751591 polymorphism compared to T-carriers (*P*=0.03). Carriers of the variant alleles of the two *CYP19A1* polymorphisms rs749292 and rs1062033 had decreased levels of 12% of estrone sulphate compared to the wild type (*P*=0.004 and 0.007, respectively), whereas variant carriers of the *CYP19A1*/rs10519297 polymorphism had 12% increased levels of estrone sulphate compared to the wild type (*P*=0.03). Thus, several of the studied SNPs were associated with hormone levels.

**Table 2: Plasma levels of estrone, estrone sulphate and SHBG among 339 never and past users of hormone replacement therapy as percentage change in hormonal measurements in relation to *CYP19A1* polymorphisms.**

| *Genotype* | *n (%)* | *Estrone* | *P-value*[b] | *Estrone sulphate* | *P-value*[b] | *SHBG* | *P-value*[b] |
|---|---|---|---|---|---|---|---|
| | *n=339* | *Δ (95% CI)*[a] | | *Δ (95% CI)*[a] | | *Δ (95% CI)*[a] | |
| rs10519297 | | | | | | | |
| AA | 81 (24) | 0 (ref.) | | 0 (ref.) | | 0 (ref.) | |
| AG | 187 (55) | 8 (-4;21) | 0.35 | 10 (-1;21) | 0.05 | -5 (-14;6) | 0.66 |
| GG | 71 (21) | 1 (-13;16) | | 16 (3;31) | | -2 (-14;11) | |
| AG+GG | 258 (56) | 6 (-6;18) | 0.34 | 12 (1;23) | 0.03 | -4 (-13;6) | 0.44 |
| rs749292 | | | | | | | |
| GG | 101 (30) | 0 (ref.) | | 0 (ref.) | | 0 (ref.) | |
| AG | 169 (50) | -5 (-15;6) | 0.62 | -11 (-19;-2) | 0.007 | -3 (-12;7) | 0.07 |
| AA | 69 (20) | -5 (-17;9) | | -16 (-26;-6) | | 11 (-2;26) | |
| AG+AA | 238 (70) | -5 (-14;5) | 0.33 | -12 (-20;-4) | 0.004 | 1 (-8;11) | 0.82 |
| rs1062033 | | | | | | | |
| CC | 88 (26) | 0 (ref.) | | 0 (ref.) | | 0 (ref.) | |
| CG | 180 (53) | -2 (-13;10) | 0.53 | -10 (-19;-1) | 0.01 | -1 (-11;9) | 0.45 |
| GG | 71 (21) | -7 (-20;6) | | -16 (-26;-6) | | 6 (-7;21) | |
| CG+GG | 251 (74) | -4 (-14;7) | 0.51 | -12 (-20;-3) | 0.007 | 1 (-9;11) | 0.88 |
| rs10046 | | | | | | | |
| AA | 93 (27) | 0 (ref.) | | 0 (ref.) | | 0 (ref.) | |
| AG | 177 (52) | 2 (-9;15) | 0.58 | 4 (-5;15) | 0.26 | -1 (-10;10) | 0.94 |
| GG | 69 (20) | -4 (-17;10) | | 11 (-2;25) | | -2 (-14;11) | |
| AG+GG | 246 (73) | 0 (-10;12) | 0.93 | 6 (-3;16) | 0.21 | -1 (-10;9) | 0.82 |
| rs4646 | | | | | | | |
| CC | 186 (55) | 0 (ref.) | | 0 (ref.) | | 0 (ref.) | |
| CA | 133 (39) | 3 (-7;14) | 0.68 | 5 (-3;15) | 0.38 | -4 (-13;5) | 0.59 |
| AA | 20 (6) | -6 (-23;16) | | 10 (-8;31) | | -5 (-21;15) | |
| CA+AA | 153 (45) | 2 (-8;12) | 0.73 | 6 (-3;15) | 0.18 | -4 (-13;4) | 0.30 |
| rs6493487 | | | | | | | |
| AA | 203 (60) | 0 (ref.) | | 0 (ref.) | | 0 (ref.) | |
| GA | 127 (38) | -5 (-14;5) | 0.27 | 4 (-4;14) | 0.63 | -5 (-13;4) | 0.37 |
| GG | 9 (3) | -19 (-40;9) | | 0 (-23;29) | | -14 (-34;13) | |
| GA+GG | 136 (41) | -6 (-15;4) | 0.23 | 4 (-4;13) | 0.36 | -5 (-13;3) | 0.22 |
| rs2008691 | | | | | | | |
| AA | 220 (65) | 0 (ref.) | | 0 (ref.) | | 0 (ref.) | |
| GA | 109 (32) | 4 (-6;15) | 0.35 | 3 (-6;12) | 0.83 | 2 (-7;12) | 0.17 |
| GG | 10 (3) | 21 (-9;60) | | 0 (-21;28) | | 27 (-1;64) | |
| GA+GG | 119 (35) | 5 (-4;16) | 0.29 | 2 (-6;12) | 0.57 | 4 (-5;14) | 0.37 |
| rs3751591 | | | | | | | |
| TT | 241 (71) | 0 (ref.) | | 0 (ref.) | | 0 (ref.) | |
| TC | 90 (27) | 2 (-9;13) | 0.55 | 1 (-8;10) | 0.50 | 1 (-9;11) | 0.09 |
| CC | 8 (2) | 19 (-13;63) | | 18 (-10;54) | | 38 (4;83) | |
| CC vs. TT+TC | 8 (2) | 18 (-14;62) | 0.29 | 17 (-10;53) | 0.24 | 37 (4;82) | 0.03 |
| rs2445762 | | | | | | | |
| TT | 175 (52) | 0 (ref.) | | 0 (ref.) | | 0 (ref.) | |
| TC | 142 (42) | -3 (-12;7) | 0.71 | -1 (-9;8) | 0.71 | -3 (-19;16) | 0.53 |
| CC | 22 (6) | -7 (-24;13) | | -7 (-21;10) | | -5 (-13;4) | |
| TC+CC | 164 (48) | -3 (-12;6) | 0.47 | -2 (-10;6) | 0.63 | -5 (-13;4) | 0.27 |
| rs11070844 | | | | | | | |
| CC | 267 (79) | 0 (ref.) | | 0 (ref.) | | 0 (ref.) | |
| TC | 68 (20) | 17 (4;31) | 0.03 | 13 (2;25) | 0.04 | 6 (-5;18) | 0.20 |
| TT | 4 (1) | 16 (-26;79) | | 23 (-16;79) | | 36 (-9;103) | |
| TC+TT | 72 (21) | 17 (4;31) | 0.009 | 14 (3;25) | 0.01 | 7 (-3;19) | 0.18 |

Δ Percentage change in hormonal measurements compared to WT.
[a] Adjusted for age, smoking (never, past, current), alcohol intake (increment of 10 g per day) and BMI (kg/m$^2$) at baseline.
[b] *P*-value for interaction.
SHBG: Sex-hormone binding globulin.

Three of the ten *CYP19A1* polymorphisms had alcohol dependent changes in hormone levels according to genotype (Table 3). Carriers of the *CYP19A1*/rs2008691 and *CYP19A1*/rs1062033 polymorphism had 3% and 1% higher estrone sulphate levels, respectively, than the wild type (*P*=0.02 and 0.03, respectively) per

10 gram alcohol intake per day. Variant T-carriers of the *CYP19A1*/rs11070844 polymorphism had 3% decreased levels of SHBG compared to the wild type carriers (*P*=0.03) per 10 gram daily alcohol intake.

**Table 3: Plasma levels of estrone, estrone sulphate and SHBG among 325 never and past users of hormone replacement therapy as percentage change in hormonal measurements per 10 g/day in alcohol intake.**

| *Genotype* | *n (%)* *n=325* | *Estrone* *Δ (95% CI)*[a] | *P-value*[b] | *Estrone sulphate* *Δ (95% CI)*[a] | *P-value*[b] | *SHBG* *Δ (95% CI)*[a] | *P-value*[b] |
|---|---|---|---|---|---|---|---|
| rs10519297 | | | | | | | |
| AA | 74 (23) | 2 (-4;9) | | 3 (-3;9) | | -5 (-11;0) | |
| AG | 182 (56) | 3 (-1;6) | 0.82 | 3 (0;7) | 0.17 | -6 (-9;-3) | 0.23 |
| GG | 69 (21) | 1 (-5;6) | | 4 (0;9) | | -3 (-7;2) | |
| AG+GG | 251 (77) | 2 (-1;5) | 0.54 | 4 (1;6) | 0.08 | -5 (-8;-2) | 0.27 |
| rs749292 | | | | | | | |
| GG | 98 (30) | 3 (-2;7) | | 4 (1;8) | | -5 (-8;-1) | |
| AG | 165 (51) | 1 (-3;6) | 0.96 | 3 (0;7) | 0.38 | -6 (-9;-2) | 0.94 |
| AA | 62 (19) | 2 (-5;10) | | -1 (-7;6) | | 0 (-7;6) | |
| AG+AA | 227 (70) | 1 (-2;5) | 0.85 | 3 (-1;6) | 0.15 | -5 (-8;-1) | 0.95 |
| rs1062033 | | | | | | | |
| CC | 86 (26) | 1 (-3;6) | | 4 (0;8) | | -5 (-9;-1) | |
| CG | 175 (54) | 3 (-1;7) | 0.76 | 4 (0;7) | 0.11 | -5 (-8;-2) | 0.67 |
| GG | 64 (20) | 1 (-6;8) | | 1 (-4;7) | | -4 (-9;2) | |
| CG+GG | 239 (74) | 2 (-1;6) | 0.45 | 3 (0;7) | 0.03 | -5 (-8;-2) | 0.90 |
| rs10046 | | | | | | | |
| AA | 85 (26) | 3 (-3;10) | | 4 (-2;10) | | -7 (-12;-1) | |
| AG | 173 (53) | 2 (-1;7) | 0.89 | 3 (0;7) | 0.29 | -6 (-9;-3) | 0.06 |
| GG | 67 (21) | 1 (-4;5) | | 3 (0;8) | | -3 (-7;1) | |
| AG+GG | 240 (74) | 2 (-1;5) | 0.64 | 3 (1;6) | 0.22 | -5 (-7;-2) | 0.24 |
| rs4646 | | | | | | | |
| CC | 177 (54) | 3 (-2;7) | | 4 (0;7) | | -4 (-8;-1) | |
| CA | 129 (40) | 2 (-2;6) | 0.49 | 3 (0;7) | 0.30 | -5 (-8;-2) | 0.87 |
| AA | 19 (6) | -6 (-18;7) | | 6 (-5;19) | | -8 (-18;4) | |
| CA+AA | 148 (46) | 1 (-2;5) | 0.41 | 3 (0;7) | 0.14 | -5 (-8;-2) | 0.59 |
| rs6493487 | | | | | | | |
| AA | 192 (59) | 3 (-1;8) | | 3 (-1;7) | | -5 (-9;-1) | |
| GA | 125 (39) | 2 (-2;5) | 0.79 | 4 (1;8) | 0.64 | -5 (-8;-2) | 0.60 |
| GG | 8 (2) | -1 (-11;11) | | -1 (-10;9) | | -5 (-14;5) | |
| GA+GG | 133 (41) | 1 (-2;5) | 0.79 | 4 (1;7) | 0.97 | -5 (-8;-2) | 0.33 |
| rs2008691 | | | | | | | |
| AA | 210 (65) | 2 (-2;6) | | 5 (2;8) | | -6 (-9;-3) | |
| GA | 106 (32) | 2 (-2;6) | 0.67 | 1 (-2;5) | 0.03 | -4 (-8;0) | 0.41 |
| GG | 9 (3) | 12 (-4;31) | | 10 (-3;26) | | 6 (-7;22) | |
| GA+GG | 115 (35) | 2 (-2;7) | 0.70 | 2 (-2;6) | 0.02 | -4 (-7;0) | 0.31 |
| rs3751591 | | | | | | | |
| TT | 232 (72) | 2 (-1;6) | | 4 (1;7) | | -5 (-8;-2) | |
| TC | 85 (26) | 1 (-3;6) | 0.78 | 2 (-2;6) | 0.70 | -5 (-8;-1) | 0.90 |
| CC | 8 (2) | 11 (-7;31) | | 9 (-6;26) | | 10 (-6;28) | |
| CC vs. TT+TC | 317 (98) | 2 (-1;5) | 0.81 | 3 (1;6) | 0.74 | -5 (-8;-2) | 0.86 |
| rs2445762 | | | | | | | |
| TT | 166 (51) | 2 (-1;6) | | 3 (0;6) | | -4 (-7;-1) | |
| TC | 139 (43) | 1 (-3;6) | 0.94 | 5 (1;9) | 0.19 | -6 (-10;-2) | 0.72 |
| CC | 20 (6) | 3 (-9;16) | | -2 (-11;8) | | -8 (-17;2) | |
| TC+CC | 159 (49) | 1 (-3;6) | 1.00 | 4 (0;8) | 0.17 | -6 (-10;-3) | 0.61 |
| rs11070844 | | | | | | | |
| CC | 255 (78) | 1 (-2;5) | | 3 (0;6) | | -4 (-7;-1) | |
| TC | 66 (21) | 5 (-1;11) | 0.73 | 7 (1;12) | 0.92 | -7 (-12;-3) | 0.06 |
| TT | 4 (1) | 4 (-20;37) | | 15 (-9;45) | | 11 (-13;42) | |
| TC+TT | 70 (22) | 5 (-1;11) | 0.42 | 7 (2;12) | 0.74 | -7 (-12;-2) | 0.03 |

ΔPercentage change in hormonal measurements per 10 g/day change in alcohol intake.
[a]Adjusted for age, smoking (never, past, current) and BMI (kg/m$^2$) at baseline.
[b]P-value for interaction
SHBG: Sex-hormone binding globulin.

In general, estrone and estrone sulphate levels increased whereas SHBG levels decreased for every 10 gram alcohol consumed per day irrespectively of genotype (Table 3). Estrone sulphate levels differed significantly according to *CYP19A1*/rs3751591 genotype for NSAID users and non-users, respectively (*P*=0.008) (Table S1). Carriers of the CC genotype had 48% higher levels of estrone sulphate when using NSAID compared to T-carriers who did not use NSAID (95% CI: 3;114), whereas T-carriers who were also NSAID users had 13% decreased levels of estrone sulphate (95% CI; -20;-5). However, these estimates were based on very small numbers. There was also a borderline statistically significant interaction between NSAID usage and the *CYP19A1*/rs6493487 polymorphism in relation to SHBG levels (*P*=0.05). Overall, NSAID users had higher levels of SHBG and lower levels of estrone and estrone sulphate (Table S1). Thus, some of the studied *CYP19A1* SNPs were associated with hormone levels and a few other SNPs interacted with alcohol intake in relation to hormone levels. *CYP19A1*/rs1062033 was associated to both hormone levels and to alcohol-dependent differences in hormone levels.

*Associations with BC risk*

Homozygous variant carriers of the *CYP19A1*/rs3751591 polymorphism were at 2.12-fold increased risk of BC (95% CI: 1.02-4.43; *P*=0.04) compared to wild-type carriers (Table 4). Carriers of the haplotype combination GGG/GAG (*CYP19A1*/A-rs10046-G, A-rs6493487-G, A-rs10519297-G) were at 56% increased risk of BC (IRR=1.56; 95% CI: 1.02-2.40) (Table S2). Thus, *CYP19A1*/rs3751591 was both associated with SHBG levels and with risk of BC such that homozygous variant allele carriers had increased levels of serum SHBG and were at increased risk of BC.

None of the *CYP19A1* polymorphisms interacted with alcohol (Table S3) or NSAID usage (Table S4) in relation to BC risk. All risk estimates showed increased risk of BC of 10-66% per 10 gram alcohol per day regardless of genotype (Table S3). NSAID usage also increased BC risk compared to non-users irrespectively of genotype (Table S4). There was no interaction between any of the *CYP19A1* polymorphisms and being carrier of either of the *PPARG* $Pro^{12}Ala$ alleles (Table S5). However, we found interaction between *CYP19A1*/rs3751591 and *PPARGC1A* $Gly^{482}Ser$ (*P*=0.02) in relation to BC risk (Table S6); and interaction between *CYP19A1*/rs4646 and *PPARGC1A* $Thr^{612}Met$ (*P*=0.002) in relation to BC risk (Table S7). Wild type carriers of *CYP19A1*/rs4646, who were also variant Met-carriers of *PPARGC1A* $Thr^{612}Met$ were at 2.06-fold increased risk of BC (95% CI: 1.17-3.65). Conversely, variant *CYP19A1*/rs4646-carriers, who also carry the variant *PPARGC1A* $Thr^{612}Met$ allele had a 38% decreased risk of BC (IRR=0.62; 95% CI: 0.36-1.08) (Table S7). When including alcohol in the model as 10 gram alcohol per day, practically all *CYP19A1* polymorphisms interacted with *PPARG* $Pro^{12}Ala$ (*P*-values between 0.03-0.10) (Table S8). Only *PPARG* $Pro^{12}Ala$ wild type carriers were at significantly increased risk of BC. Neither *PPARGC1A* $Gly^{482}Ser$ nor *PPARGC1A* $Thr^{612}Met$ interacted with any of the *CYP19A1* polymorphisms when inferring the BC risk per 10 gram alcohol per day (Tables S9 and S10). There were no interactions with NSAID use for combinations of *CYP19A1* genotypes for 10 gram alcohol per day (Table S11).

**Table 4: IRR for BC in relation to the studied polymorphisms.**

| | $n_{cases}$ (%) (n=687) | $n_{control}$ (%) (n=687) | IRR[a] (95% CI) | IRR[b] (95% CI) | P-value[c] |
|---|---|---|---|---|---|
| rs10519297 | | | | | |
| AA | 170 (25) | 174 (25) | 1.00 (ref.) | 1.00 (ref.) | |
| AG | 341 (50) | 361 (53) | 0.98 (0.76-1.27) | 0.94 (0.72-1.23) | 0.15 |
| GG | 176 (25) | 152 (22) | 1.23 (0.90-1.68) | 1.25 (0.91-1.72) | |
| AG+GG | 511 (75) | 513 (54) | 1.05 (0.82-1.34) | 1.03 (0.80-1.32) | 0.83 |
| rs749292 | | | | | |
| GG | 216 (31) | 203 (30) | 1.00 (ref.) | 1.00 (ref.) | |
| AG | 332 (48) | 352 (51) | 0.89 (0.69-1.14) | 0.89 (0.69-1.16) | 0.62 |
| AA | 139 (20) | 132 (19) | 0.97 (0.71-1.33) | 0.99 (0.72-1.37) | |
| AG+AA | 471 (68) | 484 (70) | 0.91 (0.72-1.15) | 0.92 (0.72-1.17) | 0.50 |
| rs1062033 | | | | | |
| CC | 203 (30) | 186 (27) | 1.00 (ref.) | 1.00 (ref.) | |
| CG | 333 (48) | 354 (52) | 0.85 (0.66-1.10) | 0.85 (0.65-1.11) | 0.46 |
| GG | 151 (22) | 147 (21) | 0.92 (0.67-1.25) | 0.94 (0.68-1.28) | |
| CG+GG | 484 (70) | 501 (73) | 0.87 (0.68-1.11) | 0.88 (0.68-1.12) | 0.30 |
| rs10046 | | | | | |
| AA | 182 (27) | 188 (28) | 1.00 (ref.) | 1.00 (ref.) | |
| AG | 346 (50) | 353 (51) | 1.02 (0.79-1.31) | 0.97 (0.75-1.26) | 0.52 |
| GG | 159 (23) | 146 (21) | 1.16 (0.85-1.57) | 1.15 (0.84-1.57) | |
| AG+GG | 505 (73) | 499 (72) | 1.06 (0.84-1.34) | 1.02 (0.80-1.31) | 0.86 |
| rs4646 | | | | | |
| CC | 372 (54) | 371 (54) | 1.00 (ref.) | 1.00 (ref.) | |
| CA | 265 (39) | 262 (38) | 1.00 (0.80-1.25) | 0.97 (0.77-1.21) | 0.95 |
| AA | 50 (7) | 54 (8) | 0.96 (0.63-1.45) | 1.00 (0.65-1.53) | |
| CA+AA | 315 (46) | 316 (46) | 0.99 (0.81-1.22) | 0.97 (0.78-1.20) | 0.78 |
| rs6493487 | | | | | |
| AA | 407 (59) | 430 (62) | 1.00 (ref.) | 1.00 (ref.) | |
| GA | 245 (36) | 218 (32) | 1.23 (0.98-1.55) | 1.24 (0.98-1.58) | 0.16 |
| GG | 35 (5) | 39 (6) | 0.96 (0.60-1.54) | 0.88 (0.54-1.44) | |
| GA+GG | 280 (40) | 257 (38) | 1.19 (0.95-1.48) | 1.18 (0.94-1.48) | 0.16 |
| rs2008691 | | | | | |
| AA | 479 (70) | 470 (68) | 1.00 (ref.) | 1.00 (ref.) | |
| GA | 179 (26) | 198 (29) | 0.88 (0.69-1.11) | 0.88 (0.69-1.12) | 0.25 |
| GG | 29 (4) | 19 (3) | 1.43 (0.80-2.57) | 1.45 (0.80-2.64) | |
| GA+GG | 208 (30) | 217 (32) | 0.93 (0.74-1.17) | 0.93 (0.74-1.18) | 0.57 |
| rs3751591 | | | | | |
| TT | 479 (70) | 498 (72) | 1.00 (ref.) | 1.00 (ref.) | |
| TC | 182 (26) | 176 (26) | 1.07 (0.84-1.36) | 1.07 (0.83-1.37) | 0.60 |
| CC | 26 (4) | 13 (2) | 2.13 (1.04-4.39) | 2.12 (1.02-4.43) | 0.04 |
| TC+CC | 208 (30) | 189 (28) | 1.13 (0.89-1.42) | 1.13 (0.89-1.44) | 0.31 |
| CC vs. TT+TC[d] | 26 (4) | 13 (2) | 2.09 (1.02-4.29) | 2.09 (1.00-4.34) | 0.05 |
| rs2445762 | | | | | |
| TT | 359 (52) | 365 (53) | 1.00 (ref.) | 1.00 (ref.) | |
| TC | 278 (40) | 276 (40) | 1.02 (0.81-1.27) | 1.04 (0.83-1.31) | 0.74 |
| CC | 50 (8) | 46 (7) | 1.10 (0.72-1.69) | 1.19 (0.76-1.85) | |
| TC+CC | 328 (48) | 322 (47) | 1.03 (0.83-1.27) | 1.06 (0.85-1.32) | 0.61 |
| rs11070844 | | | | | |
| CC | 552 (80) | 556 (81) | 1.00 (ref.) | 1.00 (ref.) | |
| TC | 129 (19) | 125 (18) | 1.06 (0.801-1.39) | 1.06 (0.80-1.40) | 0.88 |
| TT | 6 (1) | 6 (1) | 1.01 (0.33-3.13) | 0.86 (0.27-2.73) | |
| TC+TT | 135 (20) | 131 (19) | 1.06 (0.81-1.38) | 1.05 (0.80-1.38) | 0.73 |

[a] Crude.
[b] Adjusted for parous/nulliparous, number of births, age at first birth, length of school education (low, medium, high), duration of HRT use (years), BMI (kg/m$^2$) and alcohol intake (10 g/day).
[c] P-value for trend.
[d] CC versus TT and TC.

**Human intervention study**

We assessed the impact of *PPARG* Pro[12]Ala on female sex-hormone concentrations after consumption of alcohol and the possible interaction with the NSAID Ibuprofen in postmenopausal women. Baseline characteristics of the study participants are presented in Table 5, and mean hormone, ethanol and SHBG concentrations in Table S12. Baseline measurements (time=0) did not differ between the two treatment groups (results not shown). Intake of Ibuprofen and inherent variation according to *PPARG* Pro[12]Ala genotype did not affect hormone or SHBG concentrations (*treat* was not significant - neither in model A nor in model B). However, there was a statistically significant effect of *time* on the hormone measurements (model B); that is, estrone, estrone sulphate and SHBG concentrations declined over the time period from 0 to 90 minutes (Figure 2-4), whereas ethanol concentration increased as expected (Figure 5). Results from model A showed that *time* was not significant from the beginning of the study to the end except from estrone that ended at a higher level than at the beginning. BMI affected the analyses with SHBG and ethanol and were nearly significant in the analyses with estrone sulphat (*P*=0.07) and estrone (*P*=0.09) so that women with high BMI also had high blood concentrations of hormones, SHBG and ethanol.

In the analyses of estrone sulphat an outlier was removed from the data set. Id=5650 was very dominating and had a high Cook's D value and a relatively large residual value.

**Table 5. Characteristics of the study participants in the human intervention study.**

| *Characteristic* | *PPARγ2* Pro[12] (n=18) | | *PPARγ2* [12]Ala (n=7) | | *P*-values[d] |
|---|---|---|---|---|---|
| | Mean±SD | | Mean±SD | | |
| Age[a], y | 59.4±6.2 | | 56.4±6.0 | | 0.28 |
| Weight, kg | 66.0±9.0 | | 61.9±5.1 | | 0.27 |
| BMI, kg/m$^2$ | 23.5±2.4 | | 21.9±2.0 | | 0.14 |
| Years since last menses | 8.6±6.9 | | 6.4±4.6 | | 0.46 |
| Alcohol intake/week | 7.3±4.9 | | 6.5±3.6 | | 0.70 |
| *Characteristic* | *PPARγ2* Pro[12] (n=18) | | *PPARγ2* [12]Ala (n=7) | | |
| | No. | % | No. | % | |
| Menopause type | | | | | |
| Natural[b] | 17 | 94.4 | 6 | 85.7 | |
| Hysterectomy[c] | 1 | 6.6 | 1 | 14.3 | |
| Smoking status | | | | | |
| Never | 10 | 55.6 | 5 | 71.4 | |
| Former | 8 | 44.4 | 2 | 28.6 | |

Characteristics of participants from the human intervention study divided by genotype.
[a] One participant was only 48 years, but had not had her menses for 6 years. All other participants were older than 50 years.
[b] One participant had had a unilateral oophorectomy.
[c] One participant had a combined hysterectomy and oophorectomy; and one participant had a hysterectomy before last menses, but was older than 60 years.
[d] *P*-values for comparison of baseline characteristics using Student's t-test.

## Discussion

In the present study, we find that polymorphisms in *CYP19A1* are associated with circulating blood levels of female sex-hormones and interact with alcohol consumption. These same polymorphisms are not, however, associated with BC risk. In addition we find indications of interaction between use of NSAID, *CYP19A1* polymorphisms and levels of circulating female sex-hormones. We show that *CYP19A1* polymorphisms

interact with polymorphisms in *PPARGC1A* in relation to risk of BC, but alcohol intake does not interact. Additionally, we show that acute alcohol consumption affects circulating blood hormone and SHBG levels, but Ibuprofen intake and being a carrier of either of the *PPARG* Pro$^{12}$Ala alleles do not affect the found sex-hormone concentrations.

In the prospective study, we were able to show that inherent variations in *CYP19A1* predict female sex-hormone levels, but these genetically determined changes were not associated with BC risk. These results support several other studies showing association between *CYP19A1* polymorphisms and estrogens [51,53,54,65], but not BC [66-68]. It is, however, evident that alcohol consumption, and to some extent also NSAID use, has major impact on the circulating sex-hormones. Nevertheless, *CYP19A1*/rs3751591 and the GGG/GAG (*CYP19A1*/A-rs10046-G, A-rs6493487-G, A-rs10519297-G) haplotype combination were associated with BC risk, but both results were based on very low number of cases and should therefore be interpreted with caution and must be replicated in other, larger studies along with the other findings on this specific polymorphism. *CYP19A1*/rs3751591 has no known function; it was chosen as a tag SNP and is located in a recombination spot.

We found no evidence of interaction between aromatase and PPARγ. When alcohol was included in the model, we found that only the *PPARG* Pro$^{12}$Ala wild type carriers were at significantly increased risk of BC irrespective of *CYP19A1* polymorphisms. This indicates that the effect of the *PPARG* Pro$^{12}$Ala polymorphism is very strong and that the *CYP19A1* polymorphisms only have minor influence on the BC risk. We did observe a possible interaction between aromatase and PGC-1α, however, none of the *PPARGC1A* polymorphisms interacted with *CYP19A1* polymorphisms in the presence of alcohol. Almost all risk estimates were above unity indicating a strong effect of alcohol regardless of genotype combination. We have previously found evidence of *PPARG* and *PPARGC1A* being involved in alcohol-related BC [30]; however, based on the present findings, we cannot extend this mechanism to involve aromatase.

After acute ingestion of alcohol, estrone, estrone sulphate and SHBG levels declined significantly and correlated inversely with ethanol blood levels. Both controlled acute trials [16,20-23,69], controlled trials with fixed amounts of alcohol over longer periods [17,18] and observational studies [12-15,24,25] have found increased levels of estrogens after consumption of alcohol. Most acute studies have only measured estradiol levels, which have been consistently increased after consumption of alcohol [16,19-23], whereas estrone has only been measured in two acute studies in women using oral contraceptives [23] and HRT [20], respectively. In the study by Sarkola *et al.,* estradiol increased after alcohol administration, but alcohol intake had no effect on estrone levels. However, the estradiol-to-estrone ratio was significantly increased. In the other study by Ginsburg *et al.*, estrone declined after alcohol consumption, whereas estradiol increased. Long-term interventional and observational studies most consistently report either increased levels of estradiol [13,15,18,25,70-72] and/or estrone [12,13,18,71,72] and/or estrone sulphate [14,17,71] and decreased SHBG levels [12,71-73] among alcohol drinkers in both pre- and postmenopausal women. However, estradiol and estrone have very short half-lives of 35 minutes, whereas estrone sulphate has a half-life of 5-7 hours [17].

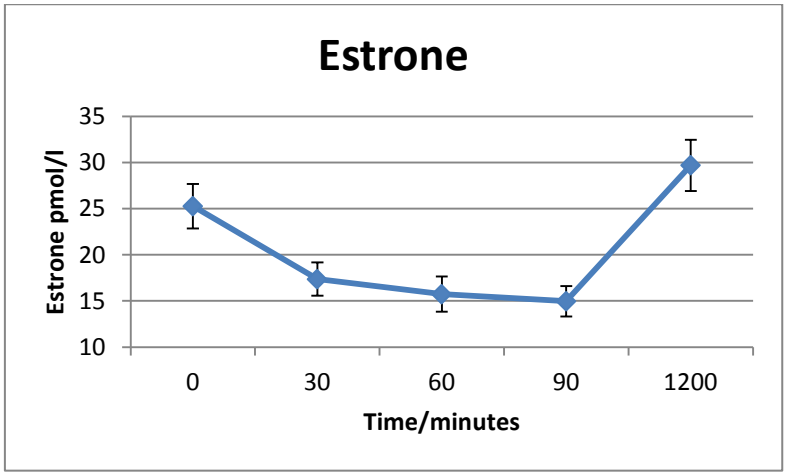


**Figure 2.** Estrone concentrations as a function of time. Values represent pooled mean measurements ± SEM (n=50). $P_{time}$ (0-90minutes)=<0.001; $P_{time}$ (0-1200minutes)=0.02.

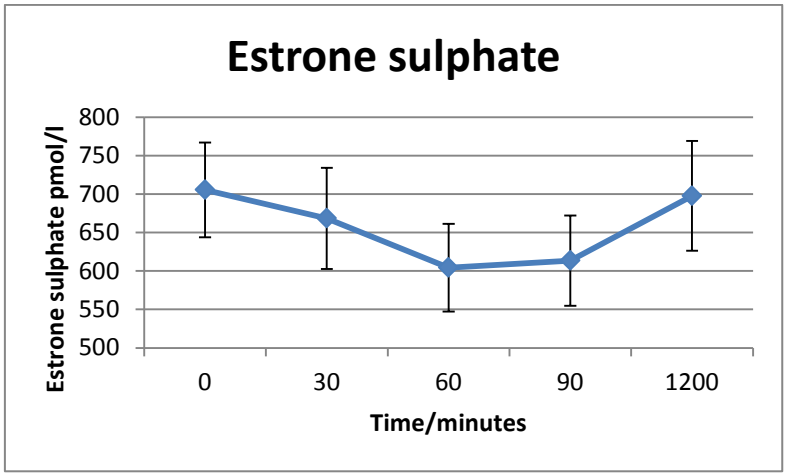


**Figure 3.** Estrone sulphate concentrations as a function of time. Values represent pooled mean measurements ± SEM (n=50). $P_{time}$ (0-90minutes)=<0.001.

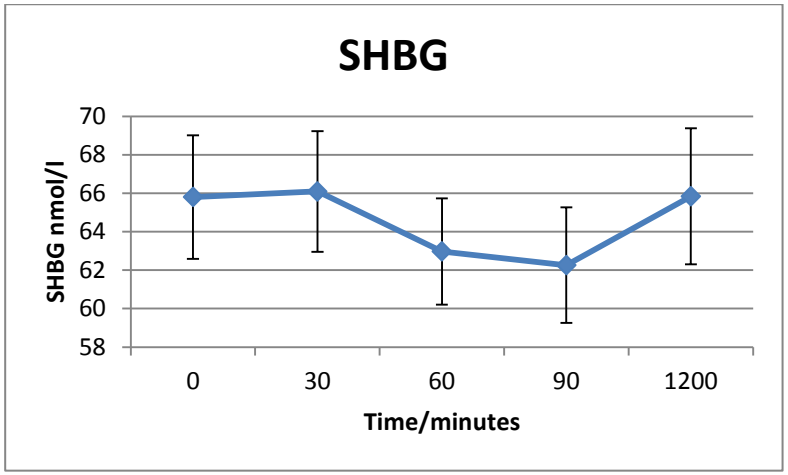


**Figure 4.** SHBG concentrations as a function of time. Values represent pooled mean measurements ± SEM (n=50). $P_{time}$ (0-90minutes)=0.009.

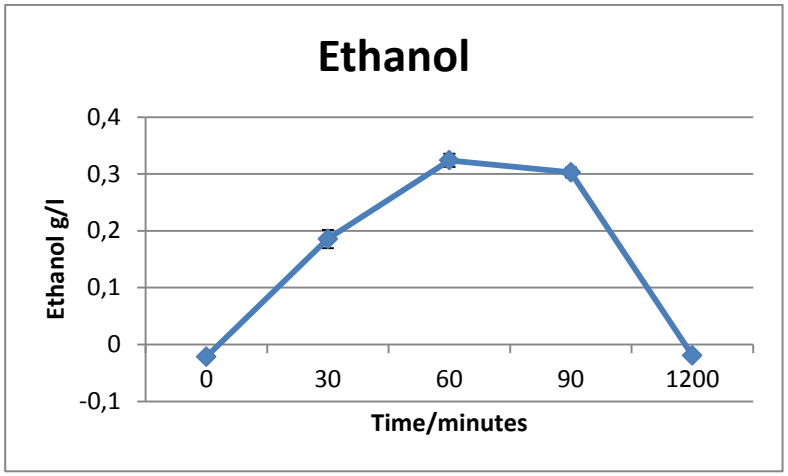


**Figure 5.** Ethanol concentrations as a function of time. Values represent pooled mean measurements ± SEM (n=50). $P_{time}$ (0-90minutes)=<0.001.

Therefore, only acute studies, where blood is collected immediately after alcohol ingestion, are able to correctly measure the effect of alcohol on estradiol and estrone blood levels. On the other hand, acute ingestion of alcohol may have a different effect on sex-steroids than chronic alcohol consumption, as illustrated by studies on alcohol consumption and immune effects[74]. It has been suggested that acute ingestion of alcohol affects catabolism of the hormones in the liver rather than synthesis [22,23,75,76]. In the liver, alcohol consumption increases the [NADH]:[NAD+] ratio which leads to a decreased catabolism of sex-hormones mediated by 17b-hydroxysteroid dehydrogenase type 2 enzyme resulting in increased levels of testosterone and estradiol and decreased levels of androstendione and estrone. Furthermore, it has been shown that only long-term chronic ingestion of alcohol induces aromatase [22,34]. We also detected a decline in estrone sulphate levels shortly after ingestion of alcohol, supporting that acute alcohol intake affects metabolism of female sex-hormone. Therefore, if acute intake of alcohol only affects hormone metabolism and not synthesis, which involves the interaction between aromatase and PPARγ, then we would not be able to detect neither the *PPARG* Pro[12]Ala genotype effect, nor the effect of Ibuprofen, which is a known PPARγ stimulator [38].

We used a nested case-control design for the prospective study, which together with complete follow-up minimizes selection bias. In addition, information on life style factors was collected at enrolment, which minimizes the risk for differential misclassification between cases and controls. The study is fairly large to study main effects, it is homogenous and alcohol consumption is very high in the DCH cohort [77] making it suitable for studying gene-environment interactions. However, we are aware that there are several limitations in studying gene-environment interaction with NSAID use including the limited power. The information on NSAID use retrieved from the FFQ may not necessarily reflect a long-term chronic use which is considered necessary to confer an effect on carcinogenesis [78]. Moreover, use of NSAID was defined as a combined group of different types of pain killers including paracetamol, aspirin and Ibuprofen, which have different pharmacological effects and also different effect in relation to BC risk [42]. The genes were carefully selected based on their role in steroidogenesis and alcohol-related BC. The *CYP19A1* polymorphisms were mainly tag SNPs, whereas the *PPARG* and *PPARGC1A* polymorphisms were functional. However, only the interaction between *CYP19A1*/rs4646 and *PPARGC1A* Thr[612]Met, and the effect of *CYP19A1A*/rs749292 on estrone sulphate levels withstood correction for multiple analyses. Therefore, some of the results based on the prospective study may be due to chance.

The human intervention trial also has several limitations. Our aim was to conduct a pilot study to examine whether the *PPARG* Pro[12]Ala polymorphisms had influence on the blood hormone level after consumption of alcohol with and without concurrent intake of Ibuprofen. Based on a controlled long-term feeding study [17], we should have an 80% chance of detecting a change in hormone levels of 10% on a 5% significance level (α=0.05) with 11 participants in each group. However, we were only able to recruit 7 *PPARG* [12]Ala variant-carriers. Nevertheless, we found statistically significant decreases in estrone, estrone sulphate and SHBG levels. We did not include an alcohol placebo group because our main aim was to examine the effect of concurrent use of Ibuprofen and alcohol consumption on circulating hormone levels. Therefore, the hormone effect could potentially be an effect of the ingredients in the alcoholic drink. However, other interventional

studies have used similar ingredients in the alcoholic test drink e.g. different types of fruit juices [17,18,20,22,23,69,79] or pure glucose [16] without an effect on hormone levels. In order to verify the results from the present study, a new study should preferably include a placebo group, and if feasible for ethical reasons should also consider altered effects over a longer exposure period. Moreover, other steroid hormones should be included to examine other effects of alcohol consumption on steroidogenesis and metabolism. The method used to measure hormones differs from all the other studies mentioned in this paper. We determined the hormones by LC-MS because of its documented specificity [80] and to avoid overestimation due to lack of specificity of antibodies, which is a well-known challenge with conventional radioimmunoassays [80,81]. However, MS methods suffer from lack of sensitivity and, consequently, we were not able to include results on estradiol measurements and 11% of the estrone measurements had levels below zero which further decreased the statistical power.

In conclusion, our results show that alcohol consumption and inherent variations in *CYP19A1* influence the level of circulating sex-hormones. In addition, our results indicate that acute and chronic alcohol consumption may affect metabolism and biosynthesis of estrogens differently. However, we were not able to confirm our primary hypothesis that PPARγ and the aromatase is part of the same pathway in alcohol-related BC or that NSAID (Ibuprofen) use has major impact on steroidogenesis.

*Acknowledgements*

We thank Lene Svensson, Annette Landin, Ümmühan Celik, Susan Svane Laursen, Nick Martinussen and Katja Boll for excellent technical support; and Jane Christensen for statistical consulting on the DCH cohort study data.

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# Chapter 8

*Accepted for publication in Human & Experimental Toxicology*

## Paper VI: *In vitro screening of inhibition of PPARγ activity as a first step in identification of potential breast carcinogens*

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**Abstract**

Alcohol consumption and increased estrogen levels are major risk factors for breast cancer (BC); and peroxisome proliferator-activated receptor γ (PPARγ) plays an important role in alcohol-induced BC. PPARγ activity is inhibited by ethanol, leading to increased aromatase activity and estrogen biosynthesis ultimately leading to BC. If other organic solvents inhibit PPARγ activity, they should also lead to increased estrogen biosynthesis, and thus be potential breast carcinogens. 10 commonly used hydrophilic organic solvents were first tested in a cell-based screening assay for inhibitory effects on PPARγ transactivation. The chemicals shown to inhibit PPARγ were tested with vectors encoding PPARγ with deleted AB domains and only the LBD domain to rule out unspecific toxicity. Next, the effects on biosynthesis of estradiol, testosterone and estrone sulphate were measured in the H295R steroidogenesis assay after incubation with the chemicals. Ethylene glycol, ethyl acetate and DMSO inhibited PPARγ transactivation in a dose-dependent manner. The inhibitory effect on PPARγ was specific for PPARγ since the AB domain of PPARγ was required for the inhibitory effect. In the second step, ethylene glycol significantly increased production of estradiol by 19% ($P$<0.05) and ethyl acetate inhibited production of testosterone ($P$<0.05). We here show that screening of 10 commonly used organic solvents for the ability to inhibit PPARγ transactivation followed by a well-established steroidogenesis assay for production of sex hormones in exposed H295R cells may provide a screening tool for potential breast carcinogens. This initial screening thus identified ethylene glycol and possibly ethyl acetate as potential breast carcinogens.

## Introduction

Breast cancer (BC) is the most common form of cancer among women worldwide, with the highest incidence in the industrialised countries [1]. The incidence of BC has doubled over the last 30 years [1]. Both environmental and genetic factors are known to contribute to the aetiology of BC [2]. The majority of the well-established risk factors are associated with prolonged exposure to increased levels of estrogens e.g. early menarche, late menopause, few or no full-term pregnancies, short or no post-partum lactation, and late childbirth [2,3]. Alcohol consumption is a well-known risk factor for BC [4-6] and intake of one drink per day is associated with 7-10% increased risk of BC [2,7-11]. Controlled human and observational studies have shown that alcohol consumption is associated with increased blood levels of several sex hormones in pre- and postmenopausal women [12-18].

A single nucleotide polymorphism in the *PPARG2* gene has been associated with alcohol-related BC in postmenopausal women [19]. Wild type allele carriers of *PPARG2* Pro[12]Ala had a 20% increased risk of BC per 10 g alcohol (corresponding to one drink) per day, whereas variant allele carriers had no risk of alcohol-related BC [19]. This indicates that the peroxisome proliferator-activated receptor γ (PPARγ), which is encoded by *PPARG*, is an important modulator in alcohol-related breast carcinogenesis. PPARγ regulates the expression of many adipose-specific genes via binding of the heterodimer PPARγ/Retinoic X receptor (RXR) to specific DNA response elements (PPREs) in target gene promoters [20]. PPARγ is a negative regulator of aromatase expression in a complex with the co-activator peroxisome proliferator-activated receptor γ co-activator (PGC-1α) [21,22]. Estrogen is synthesised from testosterone in the biosynthesis step catalysed by aromatase [23]. We have shown that ethanol inhibits the PPARγ-PGC-1α complex and inhibits PPARγ activity [24]. Animal studies have demonstrated that ethanol intake leads to increased aromatase transcription [25] which, in turn, leads to increased estrogen biosynthesis. In support of this notion, we have confirmed that ethanol increases estrogen excretion in the human H295R cell line [24]. These experiments collectively suggest that alcohol increases BC risk by inhibition of PPARγ activity, resulting in obviation of a PPARγ-mediated inhibition of aromatase expression, leading to an alcohol-dependent increase in female sex hormone synthesis and thus increased BC risk [24]. Inhibitors of PPARγ activity should therefore be considered as potential breast carcinogens.

We hypothesized that other chemicals which inhibit PPARγ activity may cause BC by a mechanism similar to alcohol. Consequently, screening chemicals for the ability to inhibit PPARγ activity may provide a tool for identifying breast carcinogens. Indeed, several occupational studies have linked exposure of different chemicals, including organic solvents, to BC [26-30]. The largest study to date encompassing 15 million people aged 30-64 years with 373,361 cases of incident female BC, shows major occupational variation in BC risk [27]. Some of the variation may be explained by differences in birth pattern, education and physical activity, however, as Hansen and Meersohn discuss in their Danish study, the differences in risk of BC point to exposure in the working environment [26]. Consistent with this, working with organic solvents for just one year was associated with a 20-70% increased risk of BC in another study by Hansen [30]. The risk estimates tended to increase with increasing duration of employment. All analyses were adjusted for social class, age at first

child, and number of children emphasising the significance of work-related exposure to organic solvents as an isolated risk factor for BC [30].

Most mammary carcinogens among organic solvents are lipophilic [31-33]. They are hypothesized to cause cancer due to their ability to reside in the adipose tissue surrounding the breast parenchyma and lobules where the solvents and their bioactivated metabolites may exert harmful local effects [31]. In the present study, we hypothesize that organic solvents may inhibit PPARγ in a manner similar to the organic solvent ethanol. Furthermore, we hypothesize that such solvents will also lead to increased estrogen biosynthesis in the H295R assay. To test this hypothesis, we screened 10 chemicals for their ability to inhibit PPARγ transactivation in a widely used HEK293 cell-based screening assay [34,35]. The examined organic solvents were selected based on the following criteria: miscibility with water (>3% in water), widely used in Denmark with "very probable exposure", according to the Danish SPIN2000 database [36] , suspected as being a mammary carcinogen [32,33] and/or found in human breast milk with a milk/plasma ratio above 1 [31]. Afterwards, the positive candidates from the initial screening were assessed for estrogen biosynthesis in an OECD validated method for testing of chemicals and their effect on androgen or estrogen steroid hormone production [37]. We show that exposure to some widely used hydrophilic organic solvents increases female sex hormone levels possibly by a PPARγ-dependent mechanism and may thereby increase the risk of female BC.

## Methods

### Test compounds

12 organic solvents were initially included in the study (CAS number in brackets): Methanol (67-56-1), Dimethyl sulfoxide (DMSO) (67-68-5), isopropanol (67-63-0), ethylene glycol (107-21-1), 1-Propanol (71-23-8), acetone (67-64-1), 1,4-dioxane (123-91-1), benzaldehyde (100-52-7) (Sigma-Aldrich®, Copenhagen, Denmark), acetonitrile (75-05-8) (Merck®, Darmstadt; Germany), N,N-dimethylformamide (68-12-2) (Fluka®, Copenhagen, Denmark ), ethyl acetate (141-78-6) and diethyl ether (60-29-7) (Rathburn Chemicals Ltd., Højbjerg, Denmark). Diethyl ether, however, cannot be pipetted in small amounts due to its high vapour pressure; and benzaldehyde was extremely cytotoxic, even in small doses. Consequently, these two chemicals were excluded, leaving 10 organic solvents in the study.

### Transient transfection

Human Embryonic Kidney (HEK293) cells [38] were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (62.5 µg/ml penicillin and 100 µg/ml streptomycin). HEK293 cells were transfected using 0.11 µl Metafectene®Easy+ (Biontex) per well in a 96-well plate. The plasmids used were the PPARγ-responsive luciferase reporter plasmid [39], pcDNA3.1 (Invitrogen) based expression constructs containing the full-length human PPARγ2 cDNA (wild type) and pRL-CMV normalization vector (Promega). All organic solvents tested positive, by giving indication of an inhibitory or inducing effect, were tested with 1) The same assay as above except that the pcDNA3 expression construct contains human PPARγ2 with deleted AB domains (N-terminal region)

(pcDNA3hPPARγΔAB containing aa 110-475 (PPARγ1 coordinates)) [40]; and 2) An assay with the following plasmids: A Gal4 responsive photinus luciferase expressing reporter plasmid (UAS-Gal), a pM1 based expression construct containing the ligand binding domain (LBD) (aa 174-475 (hPPARγ1 coordinates)) of human PPARγ1 (donated by Dr. Jan Fleckner, Novo Nordic, Denmark) and the pRL-CMV normalization vector. For each well in a 96-well dish, 30 ng reporter, 15 ng expression vector and 5.0 ng normalization vector were used. Six hours after transfection, the cells were supplemented with fresh media containing vehicle, rosiglitazone (1 µM) and test substance (0.1, 0.3, 1 or 3%). Some of the solvents showed severe sign of cytotoxicity at 3% and the top dose was consequently reduced to 1%. Cells were harvested after 18 h and Photinus and Renilla luciferase activities were measured directly in the plate using EnVision 2104 Multilabel Reader (PerkinElmer). Photinus luciferase values were normalized to Renilla luciferase values to correct for differences in transfection efficiency and cytotoxicity or other cellular stress. All experiments were performed with four replicates and repeated twice. Each experiment represents one data point (mean value) leading to n=3 in the data analyses.

**Cell culture**

Human adrenocortical carcinoma NCI-H295R cells (ATCC CRL-2128) [37] were cultured in Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 Ham without phenol red (Sigma) supplemented with 1% ITS Plus premix (BD Biosciences), 2.5% NuSerum (VWR), 1% L-glutamine (Gibco) and 1% antibiotic/antimycotic (Gibco). The cells were cultured as monolayers in a humidified environment at 37 °C with 5% $CO_2$

**Analysis of hormone production**

NCI-H295R cells were cultured as described above and used to investigate if treatment with ethylene glycol, ethyl acetate, acetone or DMSO altered the production of estradiol, testosterone and/or estrone sulphate. The cytotoxic effect of each compound was assayed using the CellTiter 96 AQueous One kit (Promega). The hormone production assay was then performed with the highest concentration of each compound, which gave rise to a cell viability decrease of maximum 10% in four independent determinations leading to n=4.

NCI-H295R cells were plated in 24 well plates and cultured for 48 hours. The cell culture medium was then renewed and the cells were treated with ethylene glycol, ethyl acetate, acetone or DMSO or left untreated (control) for 40 hours. The culture medium was then collected and the hormone levels analysed. Production of estradiol, testosterone and estrone sulphate was measured by enzyme-linked immunosorbent assay (ELISA). The ELISA kits (DE4399, DE1559, and DEV9933) were purchased from Demeditec Diagnostics GmbH, Germany. The analyses were performed in accordance with the manufacturer's recommendations.

**Statistical methods**

Transient transfection: One-way analysis of variance (ANOVA) was performed followed by a Dunnett's post-test to examine differences between exposed groups and controls using GraphPad Prism 5.

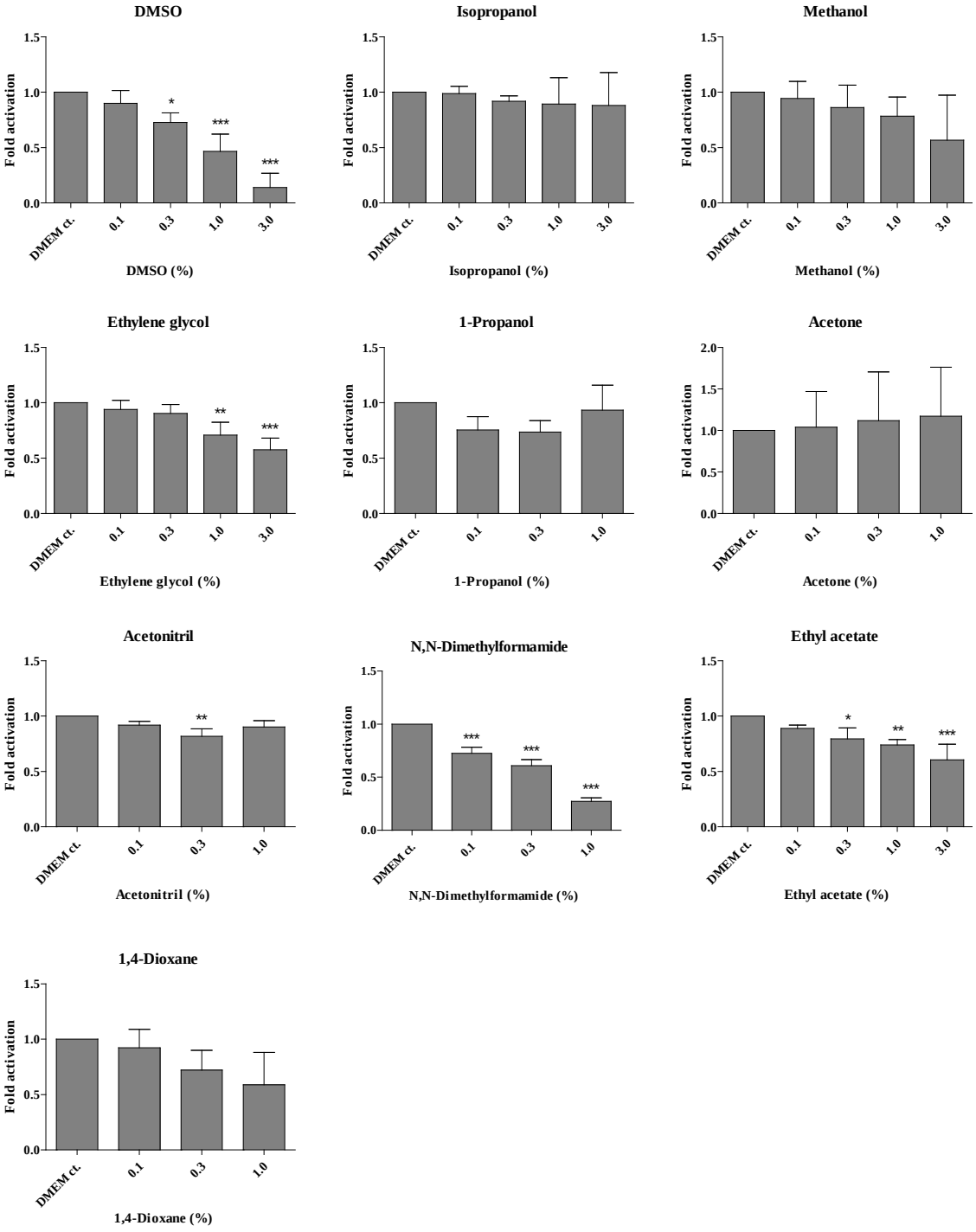


**Fig 1.** Transient transcription activation of PPARγ in a luciferase reporter gene in the presence of 10 different organic solvents. The full-length PPARγ2 cDNA was transiently transfected into HEK293 cells in combination with a PPRE luciferase reporter construct and PPARγ mediated transcription activation was measured. Reporter activity was normalized to pRL-CMV values. Some of the experiments showed cytotoxicity at 3% and the top dose was consequently reduced to 1%. All transfections were performed independently three times in four replicates (i.e. n=3). Results are presented as mean±SD. * denotes significant difference between treated and non-treated samples (*: P<0.05; **: P<0.01; ***: P<0.001)

Hormone production: Kruskal-Wallis test was used to compare mean concentrations of the hormones. When results of the Kruskal-Wallis test were statistically significant, the groups were reanalysed using the Mann–Whitney test. These tests were conducted in Minitab 16.

Data were assessed for normal distribution and homogeneity of variances. The criteria for statistical significance were *P*=0.05, *P*=0.01, and *P*=0.001 leading to the marking *, **, and ***, respectively.

## Results

A transient transfection screening assay using a PPARγ2 expression construct [24] was performed in order to measure a possible inhibitory effect on PPARγ transactivation of 10 frequently used hydrophilic organic solvents.

Four of the tested solvents (ethyl acetate, ethylene glycol, DMSO and N,N-dimethylformamide) inhibited PPARγ- mediated transcriptional activation statistically significantly in a dose-dependent manner (Fig. 1). However, the apparent inhibitory effect of N,N-dimethylformamide was caused by an increased expression of the pRL-CMV normalization vector, rather than by inhibition of PPARγ induced transcription. Consequently, N,N-dimethylformamide was excluded from further analyses. Ethyl acetate, ethylene glycol, DMSO and 1,4-dioxane were also able to inhibit rosiglitazone-induced PPARγ transactivation (Fig. 2). Rosiglitazone is a selective PPARγ ligand which was included in the assays to assess potential inhibition during ligand dependent activation of PPARγ.

In order to clarify whether inhibition of PPARγ was related to the region encompassing the PPARγ $Pro^{12}Ala$ substitution (i.e. the N terminal part of the AB domain) and to ensure that the observed inhibitory effect was not caused by general toxicity; we performed additional transactivation assays with vectors encoding PPARγ with deleted AB domains and PPARγ encompassing only the LBD domain, respectively.

Ethylene glycol and ethyl acetate did not inhibit transcriptional activation by hPPARγ(ΔAB) nor hPPARγ(LBD) (Fig. 3 and 4). However, they did inhibit the rosiglitazone-induced transcription mediated by both constructs at 3% (Fig. 3 and 4) and ethyl acetate also inhibited the rosiglitazone-induced transcription by the hPPARγ(ΔAB) at 1% (Fig. 4). DMSO inhibited hPPARγ(ΔAB) mediated expression at 1%, but not hPPARγ(LBD) expression (Fig. 5). DMSO was able to inhibit the rosiglitazone-induced expression via both the hPPARγ(ΔAB) and the hPPARγ(LBD) construct, but only at the highest dose (3%) (Fig. 5). This indicates that the effect is PPARγ specific and that the AB domain was required for the inhibitory effects of ethylene glycol and ethyl acetate; and to some extent also of DMSO. Furthermore, in concordance with previous observations [40], the ligand rosiglitazone only enhanced transactivation by full-length hPPARγ or hPPARγ(ΔAB) by 4-6 and 6-8-fold, respectively, whereas ligand-dependent activation of the hPPARγ(LBD) construct was up to 30-fold .

We hypothesized that inhibition of PPARγ transactivation would lead to increased aromatase activity and thus increased estrogen biosynthesis.

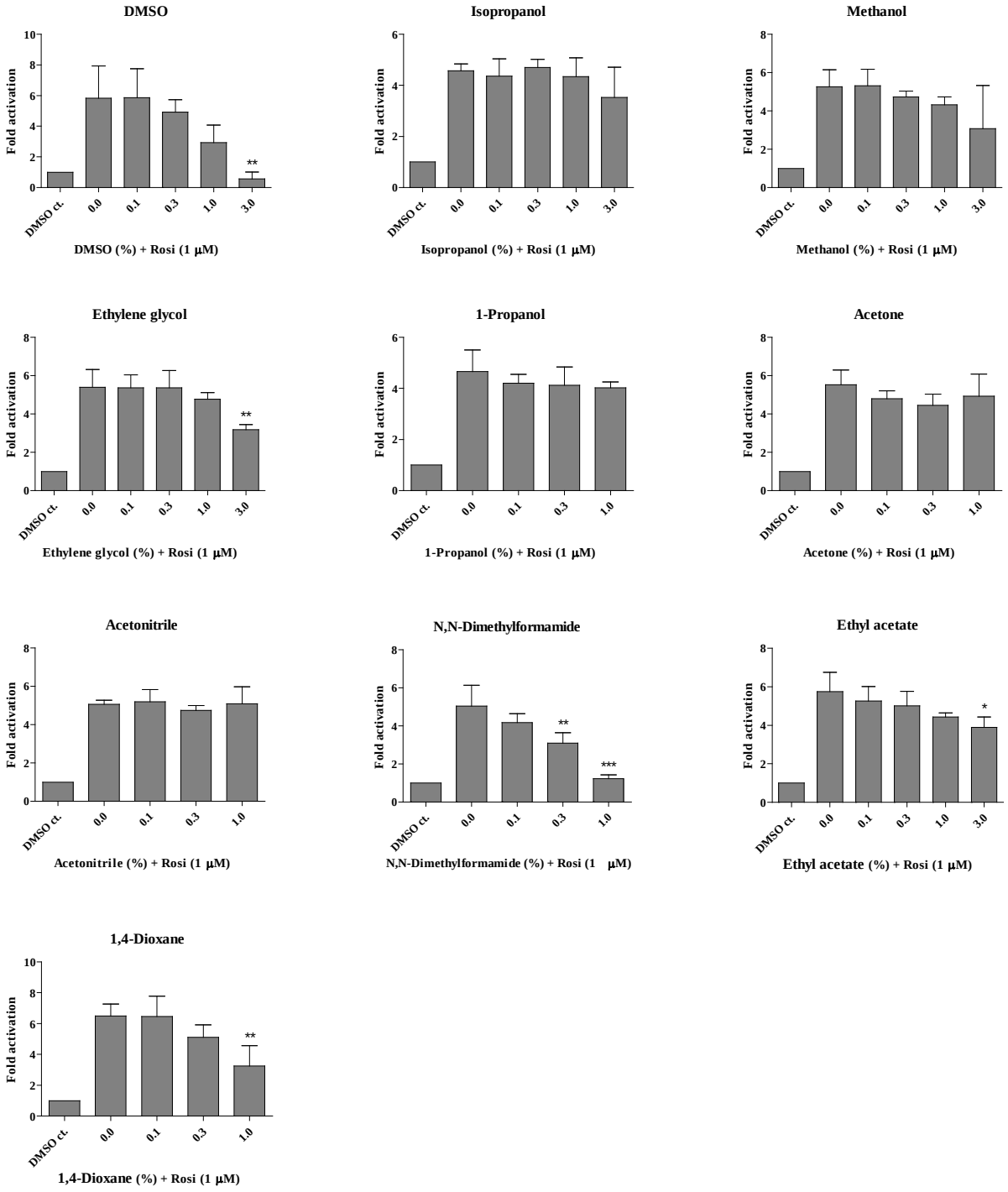


**Fig 2.** The same experiment as in figure 1, but with 1 µM Rosiglitazone added in combination with the test compound. Rosiglitazone contains 0.1 % DMSO; therefore, a negative DMSO containing control is added to each experiment in order to isolate the DMSO effect. All transfections were performed independently three times in four replicates (i.e. n=3). Results are presented as mean±SD. * denotes significant difference between non-treated samples (i.e. with Rosiglitazone, but no test compound) and treated samples (*: P<0.05; **: P<0.01; ***: P<0.001).

In order to examine a direct effect on sex hormone levels, estradiol, testosterone and estrone sulphate

production were measured in H295R cells after incubation with ethylene glycol, DMSO and ethyl acetate for 40 hours. Moreover, acetone was included as a “negative control” since acetone appeared to have no effect on PPARγ transcriptional activation. The highest concentration with maximum 10% effect on cell viability was considered as the no observed effect concentration for toxicity, and was used in the test. A concentration of 0.3% ethylene glycol significantly increased production of estradiol with 19% ($P$<0.05) (Fig. 6); and 3% ethyl acetate significantly inhibited production of testosterone ($P$<0.05) (Fig. 7). None of the tested solvents had any effect on estrone sulphate production (result not shown). All results are provided in Table S1 as numerically values.

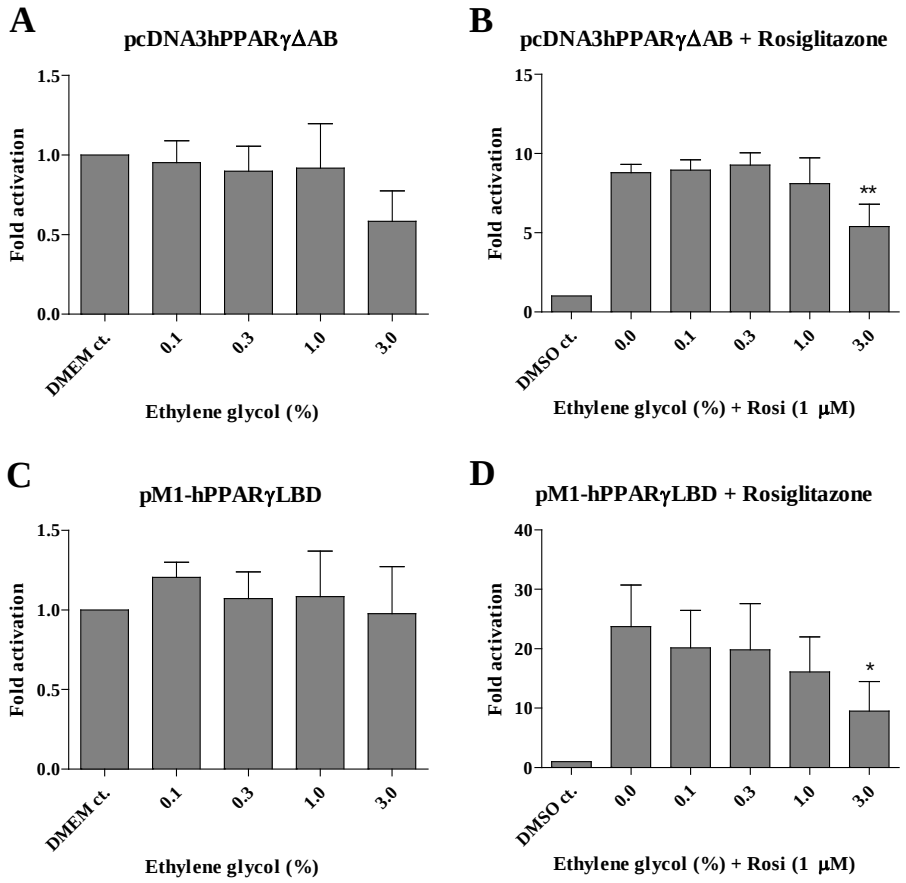


**Fig. 3.** Effect of Ethylene glycol on PPARγ constructs by transient transfections in HEK293 cells using either vectors encoding PPARγ with deleted AB domains (A and B) or pM1-PPARγ(LBD) (C and D) fusions with the appropriate luciferase reporter constructs. In B and D, Rosiglitazone is added to all wells except for DMSO controls. Reporter activity was normalized to pRL-CMV values. All transfections were performed independently three times in four replicates (i.e. n=3). Results are presented as mean±SD. * denotes significant difference between treated and non-treated samples (*: P<0.05; **: P<0.01).

## Discussion

Alcohol inhibits PPARγ-mediated inhibition of aromatase activity, which leads to increasing levels of circulating female sex hormones potentially increasing the risk of BC. We hypothesized that exposure to other organic solvents may cause BC by a similar mechanism. To test this hypothesis, we designed a two-

step screening. We used an *in vitro* transactivation assay to screen for inhibition of PPARγ transactivation, and as a second step, screened candidates for increased estrogen biosynthesis. Ten different hydrophilic organic solvents were selected for the screening based on their widespread use in Denmark and, hence, exposure potential. Moreover, 1,4 dioxane is a known mammary carcinogen [32] and methanol has been found in human breast milk [31] indicating exposure.

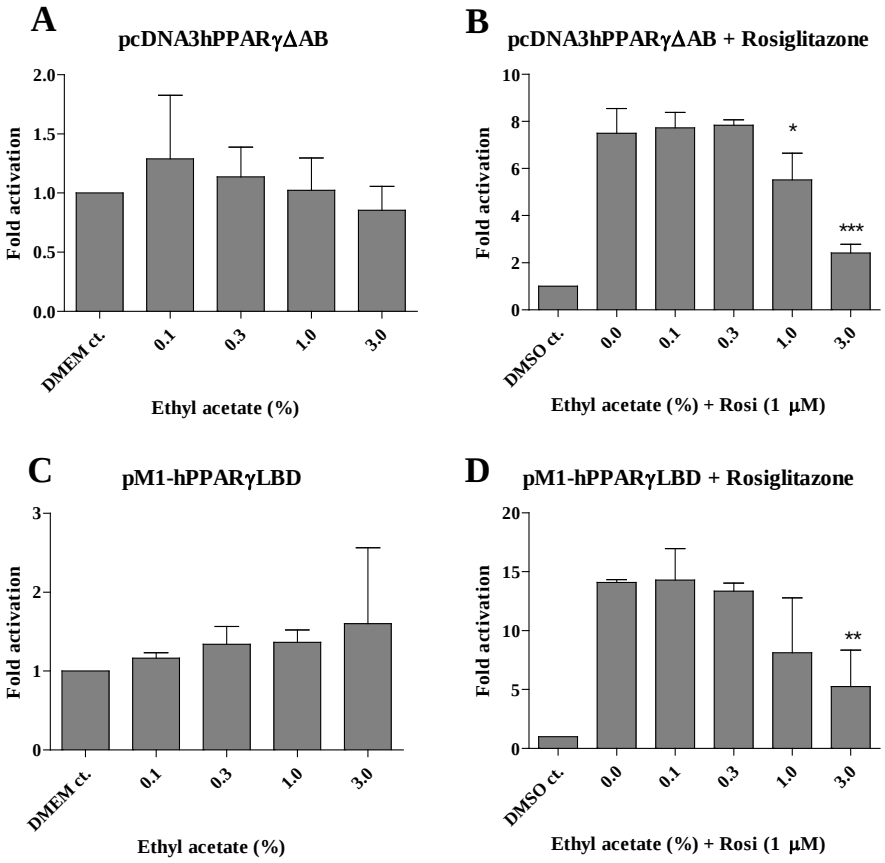


**Fig. 4.** Effect of Ethyl acetate on PPARγ constructs by transient transfections in HEK293 cells using either vectors encoding PPARγ with deleted AB domains (A and B) or pM1-PPARγ(LBD) (C and D) fusions with the appropriate luciferase reporter constructs. In B and D, Rosiglitazone is added to all wells except for DMSO controls. Reporter activity was normalized to pRL-CMV values. All transfections were performed independently three times in four replicates (i.e. n=3). Results are presented as mean±SD. * denotes significant difference between treated and non-treated samples (*: P<0.05; **: P<0.01; ***: P<0.001).

We show that three out of 10 tested chemicals inhibit PPARγ transactivation in a dose-dependent manner using transient transfections with plasmids expressing three different PPARγ constructs – full length PPARγ2, PPARγ with deleted AB domains and a construct consisting of only the LBD of PPARγ. The AB domain which encompasses the PPARγ $Pro^{12}Ala$ polymorphism seems to be essential for the inhibitory effect, especially for ethylene glycol and ethyl acetate where inhibition was confined to full-length PPARγ2. This confirms that the observed inhibition of PPARγ is not caused by general toxic mechanisms such as denaturation of all enzymes. In the second screening step, ethylene glycol, ethyl acetate, DMSO and acetone were tested in the H295R steroidogenesis assay for production of estradiol, testosterone and

estrone sulphate production to determine whether inhibition of PPARγ results in increased female sex hormone levels. Acetone was included as a non PPARγ inhibitor. Ethylene glycol increased production of estradiol statistically significantly at a concentration of only 0.3% and ethyl acetate inhibited production of testosterone with statistical significance at a concentration of 3%. Increased estradiol levels could be a result of an increased synthesis from testosterone catalysed by aromatase or to inhibition of the subsequent metabolism of estradiol to sulphate conjugate, catalysed by SULT1E1. Inhibition of SULT1E1 would result in accumulation of both estradiol and estrone. None of the tested chemicals affected estrone sulphate production (results not shown). Thus, the results were consistent with the interpretation that ethylene glycol inhibited PPARγ transactivation and caused increased estradiol synthesis in H295R cells.

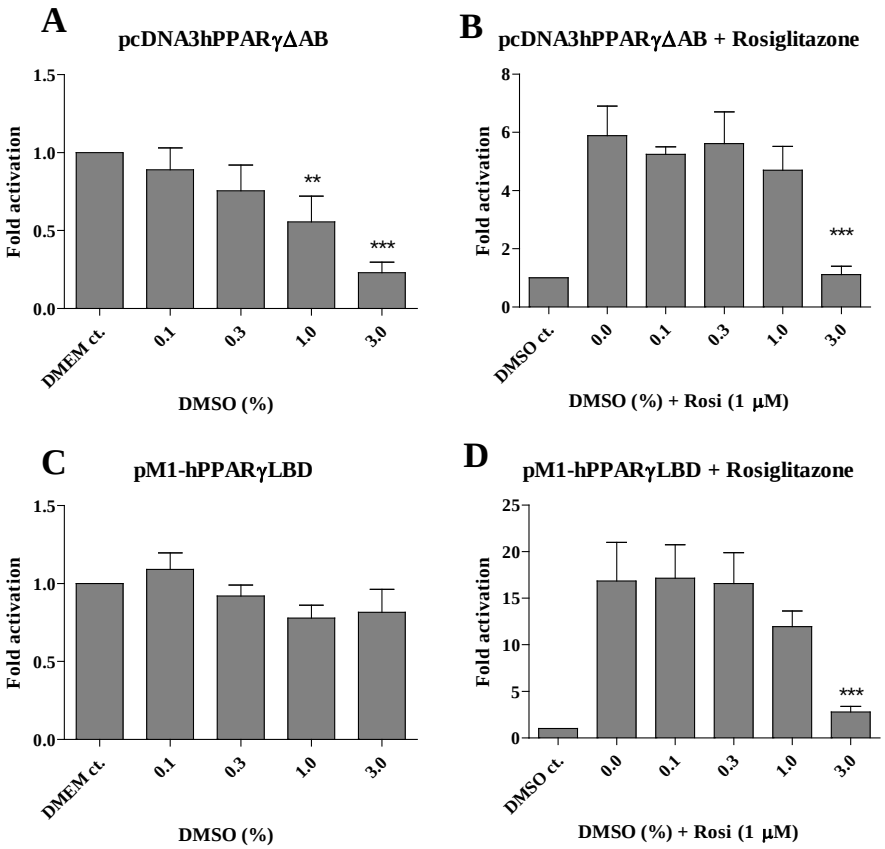


**Fig. 5.** Effect of DMSO on PPARγ constructs by transient transfections in HEK293 cells using either vectors encoding PPARγ with deleted AB domains (A and B) or pM1-PPARγ(LBD) (C and D) fusions with the appropriate luciferase reporter constructs. In B and D, Rosiglitazone is added to all wells except for DMSO controls. Reporter activity was normalized to pRL-CMV values. All transfections were performed independently three times in four replicates (i.e. n=3). Results are presented as mean±SD. * denotes significant difference between treated and non-treated samples (**: P<0.01; ***: P<0.001).

PPARγ is an important modulator of alcohol-induced BC [19,24] and the current study shows that PPARγ may be an important regulator of chemical-induced hormone changes. Thus, many chemicals have been shown to lower sex hormone production by stimulating PPARγ activity, leading to female infertility and male genitalia malformations [41-43]. Interestingly, PPARγ agonists have been suggested to have therapeutic utility in the treatment of postmenopausal BC due to their anti-estrogenic properties [23]. Here, we screen for chemicals

with the opposite, inhibitory effects on PPARγ transactivation activity, leading to increased estrogen levels and BC risk.

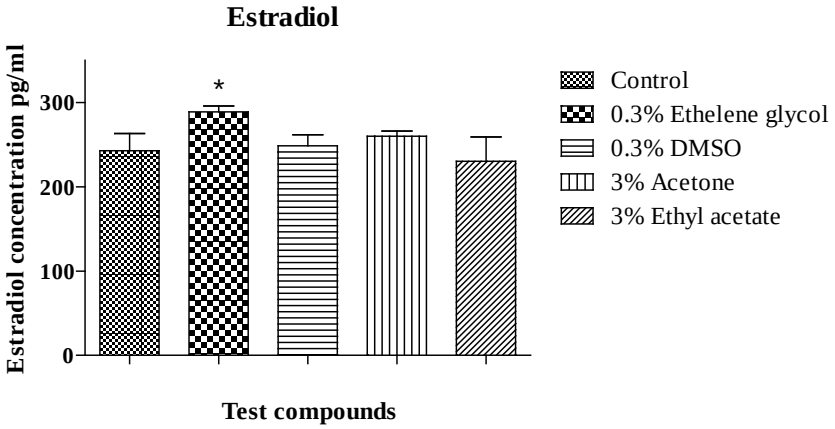


**Fig. 6.** NCI-H295R cells were cultured for 48 hours and treated with ethylene glycol, ethyl acetate, acetone or DMSO or left untreated (control) for 40 hours. The culture medium was then collected and the estradiol production was measured by enzyme-linked immunosorbent assay. The hormone production assay was performed with the highest concentration of each compound, which gave rise to a cell viability decrease of maximum 10%. Results are presented as mean±SD, n=4. * denotes significant difference between treated and non-treated samples (*: $P<0.05$).

The decreased testosterone production after exposure to ethyl acetate could be caused by interference with other targets located prior to testosterone synthesis in steroidogenesis. However, if the substrate for estradiol production (i.e. testosterone) is low, then the level of estradiol could also become low. That indicates that there is indeed an increase in estradiol production, but due to the low concentration of the substrate, the level of estradiol is comparable with that of the control. Therefore, ethyl acetate and ethylene glycol may act via a similar mechanism. Our results therefore indicate that ethyl acetate also interferes with other targets in steroidogenesis. Indeed, it is a well-known phenomenon that chemicals affect more than one target in steroidogenesis [44].

It has previously been demonstrated that ethylene glycol is weakly estrogenic in rainbow trout due to induction of the estrogen receptor [45], however, to our knowledge, no other studies have investigated the effect of ethylene glycol or ethyl acetate on steroidogenesis. DMSO, conversely, has proven to induce a variety of effects including numerous effects on lipid metabolism (summarized in [46]). Our results on DMSO are in agreement with these observations, since PPARγ is a major regulator of genes involved in lipid and lipoprotein metabolism [20].

The present study was intended as an initial screening of commonly used hydrophilic organic solvents and their ability to 1) inhibit PPARγ in a well-established high-throughput cell-based screening assay; and 2) increase endogen estrogen levels in the H295R steroidogenesis assay. Since the H295R steroidogenesis assay is far more time-consuming and expensive, but has been proven to correlate with *in vivo* mechanisms, we use the initial screening comprising of the HEK293 cells which are easy to grow and transfect very readily to select appropriate candidates for this assay [47]. However, since organic solvents possess different

properties, some of the negative findings in the transient transfection assay may simply be due to the chemicals not being able to enter the cells. Moreover, there may be discrepancies in the effect of absorption and metabolism of the test chemicals in different cell lines. The H295R steroidogenesis assay is designed to identify substances that affect targets in steroidogenesis from cholesterol to the production of the terminal steroid hormones, that is, not substances that affect steroidogenesis via a mechanism that involves the hypothalamic-pituitary-gonadal axis [37]. Therefore, stimulation of the aromatase activity caused by inhibition of PPARγ is a plausible mechanism for the effects observed in the present study, but we did not assess PPARγ activity in the H295R assay; nor did we examine aromatase mRNA expression. However, H295R cells are of adrenal gland origin, which indicates that this specific mechanism is not limited to adipocytes. The genotype of the H295R cell line have previously been genotyped to carry the *PPARG* Pro$^{12}$Ala wild-type [24], proving that the H295R cell assay is capable of PPARγ-PGC-1α interaction as the PGC-1α-dependent co-activation of the PPARγ-complex is compromised for the PPARγ Ala$^{12}$ variant.

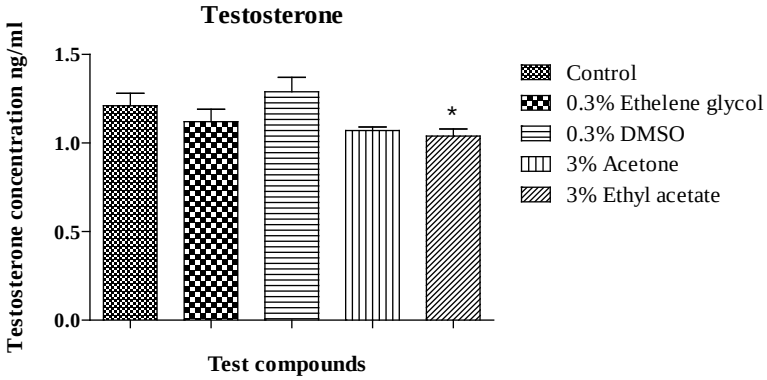


**Fig. 7.** NCI-H295R cells were cultured for 48 hours and treated with ethylene glycol, ethyl acetate, acetone or DMSO or left untreated (control) for 40 hours. The culture medium was then collected and the testosterone production was measured by enzyme-linked immunosorbent assay. The hormone production assay was performed with the highest concentration of each compound, which gave rise to a cell viability decrease of maximum 10%. Results are presented as mean±SD, n=4. * denotes significant difference between treated and non-treated samples (*: P<0.05).

It is highly important to identify human carcinogens as a mean to prevent future exposures. Mutagenic carcinogens are identified in a number of *in vitro* [48,49] and *in vivo* screening tests [50]. However, non-mutagenic carcinogens are more difficult to identify. We have previously provided evidence that ethanol causes BC by a PPARγ-dependent increase in aromatase activity [24]. Based on this proposed mechanism-of-action, we here propose a screening approach for identification of breast carcinogens. We demonstrate that an *in vitro* screening of inhibition of PPARγ transactivation can be used to identify chemicals which may be breast carcinogens. As the second screening step, we used the H295R steroidogenesis assay to confirm an activation of aromatase activity. We show that at least one of three chemicals, ethylene glycol, leads to increased estradiol synthesis in H295R cells. The next screening step would be to show a similar effect *in vivo* in rodent models or in epidemiological studies. It has previously been demonstrated that ethanol intake increased aromatase activity and transcription levels in adipose tissue in orally dosed rats [25]. Conversely, human studies like register-based epidemiological studies are difficult to interpret since exposure often is a

mixture of chemicals and they often lack information of important potential confounders such as cigarette smoking, alcohol intake and hormone replacement therapy [51].

Furthermore, our results indicate that some of the widely used organic solvents may possess endocrine disrupting properties possibly via a PPARγ-dependent mechanism. 0.3% ethylene glycol caused a 19% increase in estradiol biosynthesis. It is not clear whether the observed inhibitory effect will cause increased hormone levels at occupationally relevant exposure levels. However, it is possible that mixed exposure to several organic solvents, including alcohol consumption, may contribute to BC risk by the proposed mechanism, and as such, explains the observed association between exposure to organic solvents and BC risk [30].

Based on knowledge of the mechanism of action of ethanol-mediated breast carcinogenesis, we propose a screening method for breast carcinogens. We show that screening of chemicals for the ability to inhibit PPARγ transactivation followed by a screening for increased estradiol biosynthesis in exposed H295R cells may provide a screening for potential breast carcinogens. This initial screening identified ethylene glycol and possibly ethyl acetate as potential breast carcinogens. These candidates should be tested in rodent model systems for their effect on aromatase expression and activity in adipose tissues.

*Acknowledgements*

The study was supported by a Mobility PhD grant (09-06 7572) from the Danish Council for Independent Research.

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# Chapter 9

## Summarizing and supplementary discussion

## Summarizing and supplementary discussion

In the present PhD thesis, several biological mechanisms have been investigated in relation to three major cancer types. For PC and CRC, the main mechanism investigated involved inflammation as a precursor to neoplastic progression, whereas the effect of alcohol consumption and exposure to other organic solvents on circulating female sex-hormone levels was the focus in relation to BC.

Based on the results from *Paper I*, inflammation does not seem to be a major risk factor for aggressive PC, whereas the results on non-aggressive PC were equivocal. In contrast, *Paper II* and *III* indicated that the immune system is indeed involved in the carcinogenesis of CRC. Carriage of the pro-inflammatory del-allele of *NFKB1* -94Ins/delATTG, was not associated with aggressive PC risk, but was associated to lowered risk of non-aggressive PC, and increased the risk of CRC. Even though none of the results were strong statistically, they demonstrate that cancer is a very heterogeneous disease; and indicated that inflammation may not be a risk factor for (aggressive) PC, whereas in relation to CRC, inflammation seems important. Indeed, inflammation is already an established risk factor for CRC [1-3], but the underlying mechanisms are not yet established. In addition, replication of results is important in genetic epidemiology in order to eliminate population-specific effects. In *Paper III*, a new possible mechanism involving ABC transporters, IL-10 and dietary fibre in relation to protection against inflammation-induced CRC was identified through gene-gene-environment interaction. Furthermore, use of NSAIDs seemed to interact with the ABC transporters and IL-10.

A diet high in red meat has consistently been associated with risk of CRC [4] and several hypotheses have been proposed and were tested in this thesis. First, high meat intake may disrupt the balance between commensal bacteria and the mucosal immune system by interacting with TLR4 – the pattern-recognition receptor that regulates inflammation and barrier function in the gut – and NF-κB, which is activated by TLR4. Second, intestinal exposure to the meat-related mutagens and animal carcinogens, HCAs and PAHs, may interact with enzymes involved in transport of these potentially harmful substances modifying the risk of CRC. Third, heme iron, which is abundant in red meat, is metabolized by HO-1. HO-1 activity reduces cellular oxidative stress by removing the pro-oxidant heme and at the same time produces pro-inflammatory iron. Thus inherent variations in the genes encoding TLR4, NF-κB, the ABC transporters and HO-1 may interact with different components in the meat modifying the risk of CRC.

However, red and processed meat intake did not interact with any of the polymorphisms assessed in this thesis. In addition, studies of polymorphisms in the N-acetyltransferase genes, *NAT1* and *NAT2*, which encode the enzymes that activates HCAs and PAHs, do not consistently support that high intake of processed meat is associated with CRC risk either [5-7]. On the other hand, in *Paper II*, high intake of red meat combined with an inherently increased inflammatory response, was associated with increased risk of CRC. Moreover, in a recent study using the same CRC cohort study group [8], meat intake interacted with a polymorphism in *COX-2* increasing the risk of CRC among variant carriers. This finding is consistent with a Chinese prospective, population-based cohort study showing a positive association between this specific *COX-2* SNP and CRC among subjects with high n-6 PUFA intake (which is abundant in meat). Therefore, a

diet high in meat may induce inflammation in the gut by a mechanism involving COX-2, NF-κB and TLR4, which may contribute to meat-related colorectal carcinogenesis.

There may also be another explanation for the association between red meat intake and CRC. Several studies have shown that a high intake of red and processed meat is associated with a generally unhealthy lifestyle both with respect to diet: high intake of refined sugar and alcohol, low intake of fruits, vegetables, fibre and whole grain; and behavioral factors: low physical activity, smoking and obesity [9-11]. Consistent with this, adherence to five lifestyle recommendations i.e, no smoking, physically activity, low alcohol intake, low waist circumference and healthy dietary intake (≥600 g fruit and vegetables/day, ≤500 g of red and processed meat/week, ≥3 g dietary fibre per MJ of dietary energy and ≤30% of the total energy from fat) was considerably associated with reduced risk of CRC [12]. Furthermore, calculations suggested that 23% of the CRC case could have been prevented had the participants followed the five recommendations [12]. In order to study the independent effect of meat on CRC risk, all these factors should be adjusted for. In the present thesis, most of these factors were included in the models; however, that may not have been sufficient. In addition, it has also been shown that the effect meat has on colorectal carcinogenesis may differ by anatomic tumor site, by gender, the type of meat consumed [9,13] and the preparation method [14]. All analyses in this thesis were adjusted for gender, but dividing the analyses by tumor site, the type of meat consumed and preparation method would have decreased the statistical power drastically.

In the Danish study by Andersen *et al.,* 2013 dietary fibre intake interacted with polymorphisms in the inflammatory genes *COX-2* and *IL10*, indicating a protective effect of fibre that involves these proteins [8]. In *Paper III*, intake of fibre also interacted with a functional polymorphism in *ABCB1*, suggesting that carriers of the low activity allele did not benefit from fibre intake. This result is in line with a study illustrating decreased transport of cytokines among carriers of the same allele [15]. The fact that there was interaction between the polymorphisms in *IL10* and *ABCB1*, suggests that fibre interferes with cytokine transport in the gut, which again have impact on the intestinal inflammatory state. Conversely, since intake of fibre, especially from whole grain, is associated with a healthy life style – opposite to red meat intake [10,11]; these results needs to be further investigated in order to clarify whether the fibre has a functional effect or intake of fibre is just a marker of a healthy life style that reduces inflammation in the colon and rectum.

*Paper V* illustrated that genetic variations in *CYP19A1* predicts circulating hormone levels in post-menopausal women, and that alcohol intake affects female sex-hormone concentrations. However, we were not able to put PPARγ and the aromatase in the same pathway in alcohol-related BC; and the possible effect modification of concurrent use of NSAIDs and alcohol consumption was not confirmed. Nevertheless, results from *Paper VI*, indicated that exposure to commonly used organic solvents may act via PPARγ modulating female sex-hormone levels. However, whether there is a common mechanism linking the aromatase and PPARγ, and also whether these differences in hormone levels increases risk of BC, still needs to be elucidated. Furthermore, these studies illustrated that acute and chronic alcohol consumption may have different effects on sex-hormone biosynthesis and metabolism, and that it is not straightforward to compare epidemiological studies with experimental studies.

It is worth mentioning that the *PPARG* Pro[12]Ala has opposite effects on BC and CRC, respectively, in relation to alcohol intake. Variant Ala-carriers are at increased risk of CRC when drinking alcohol, whereas wild type carriers are not at risk of developing CRC [16]. The exact opposite is seen in relation to BC, where only wild type carriers have increased risk of BC for 10 gram consumed alcohol per day [17]. Additionally, HRT use also seems to have opposite effects on BC and CRC; thus, whereas use of HRT is an established risk factor for BC, HRT use is associated with lowered risk of CRC [18]. In line with this, men have higher incidence of CRC than women, which supports the evidence that estrogens protect against CRC [18,19]. Consequently, these findings indicate that PPARγ is certainly involved in hormone-related BC, but the underlying mechanism is not yet clarified.

External validity of the DCH cohort has been examined [20], where information regarding socioeconomic factors of all 160,725 invited participants was obtained from statistical registers in Statistics Denmark [21]. Participants from the DCH study had higher educational levels, had a higher taxable income, had been employed for longer times and had bigger houses compared to non-participants. Moreover, participants were more often married compared to non-participants [20]. However, even though there are clearly differences in several socioeconomic factors between participants and non-participants, which could also have impact on diet and life style factors, this should not have impact on the internal validity and, hence, the studies used in the present PhD thesis since nested case-control (*Paper I* and *VI*) or case-cohort designs (*Paper II-IV*) have been used.

Prospective data has the advantage of minimizing the introduction of selection and information bias contrary to data from classical, retrospective case-control studies. Though, prospective cohort studies need to be very large to study rare diseases and gene-environment interactions. In this PhD thesis, the studied cancer types are amongst the most common in Denmark which increases the statistical power [20]. Other important factors for the statistical power are the effect size and allele frequency [22,23]. In the DCH cohort, both meat intake and alcohol consumption is high [24,25], and the effect of these factors on cancer risk has proven considerable. Most of the studied polymorphisms in the present PhD thesis have an allele frequency above 0.20 which should be sufficient to detect gene-environment interactions in the used study groups even when the main effect is modest [22,23,26]. Thus, there may not have been enough power to detect gene-environment interactions among some of the low frequency polymorphisms and environmental factors with low exposure and low effect size. To overcome the problem with limited power, multicentre studies and GWAS have been used to study gene-environment interactions. However, these types of studies suffer from heterogeneity of both the environmental exposures, such as dietary and life style factors, and genetics. GWAS also lack information on the functionality of the identified loci. In the present PhD project, the study groups were very homogenous consisting of Danes aged 50-64 years eliminating population specific genetics and dietary patterns. Additionally, many of the polymorphisms were functional allowing interpretation of the biological mechanism.

Overall, this PhD thesis has shown that genetic epidemiology can be used to study biological mechanisms in combination with other mechanistic studies. However, there are several limitations in conducting these types

of studies which include missing knowledge of confounders and limited power to study gene-environment interactions.

Future research could establish whether and how dietary fibre, IL-10 and ABC transporters are connected in reducing the risk of CRC. Correspondingly, whether red meat *per se*, specific preparation methods or the life style associated with high red meat intake are carcinogenic should be clarified in future studies. The acquired knowledge would improve the current dietary recommendations regarding red meat and fibre intake.

There also seem to be several yet unknown effects of NSAID usage that need to be clarified. Information of these potential (side) effects would lead to better medication regimens and, hence, improved public health. Also, further knowledge of the harmful health effects related to alcohol consumption, including the potential effect modification with concurrent use of NSAIDs, would lead to improved public preventive strategies.

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# Appendices

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## Supplementary material for Paper II

**Table S1. IRR for CRC in relation to combinations of *NFKB1*/rs2836249 and *TLR4*/rs5030728 genotypes.**

| *Genotype* | *NFKB1/rs2836249* | | *NFKB1/rs2836249* | | *NFKB1/rs2836249* | | *P-value*[c] |
|---|---|---|---|---|---|---|---|
| | Ins/Ins $n_{cases}/n_{sub\text{-}cohort}$ | Ins/Del+Del/Del $n_{case}/n_{sub\text{-}cohort}$ | Ins/Ins IRR (95% CI)[a] | Ins/Del+Del/Del IRR (95% CI)[a] | Ins/Ins IRR (95% CI)[b] | Ins/Del+Del/Del IRR (95% CI)[b] | |
| *TLR4*/rs5030728 | | | | | | | |
| GG | 145/335 | 260/491 | 1.00 (ref.) | 1.22 (1.00-1.49) | 1.00 (ref.) | 1.22 (1.00-1.49) | 0.55 |
| GA | 131/281 | 268/450 | 1.11 (0.88-1.40) | 1.32 (1.08-1.61) | 1.12 (0.89-1.41) | 1.33 (1.09-1.62) | |
| AA | 44/63 | 67/99 | 1.53 (1.10-2.14) | 1.43 (1.07-1.91) | 1.50 (1.08-2.10) | 1.44 (1.08-1.93) | |
| GG+GA | 276/616 | 528/941 | 1.00 (ref.) | 1.21 (1.05-1.40) | 1.00 (ref.) | 1.21 (1.05-1.40) | 0.27 |
| AA | 44/63 | 67/99 | 1.46 (1.07-2.00) | 1.37 (1.04-1.78) | 1.43 (1.04-1.96) | 1.37 (1.05-1.80) | |

[a] Crude – adjusted for age and sex.
[b] In addition, adjusted for smoking status, alcohol intake, HRT status (women only), BMI, use of NSAID, intake of red and processed meat, and dietary fibre.
[c] P-value for interaction for the adjusted risk estimates.

**Table S2. Interaction between dietary factors and the studied polymorphisms in relation to CRC risk.**

| | | *IRR[a] (95% CI)* | *P-value[b]* | *IRR[a] (95% CI)* | *P-value[b]* | *IRR[a] (95% CI)* | *P-value[b]* | *IRR[a] (95% CI)* | *P-value[b]* |
|---|---|---|---|---|---|---|---|---|---|
| | | ***Red and processed meat per 25 g/day*** | | ***Fish per 25 g/day*** | | ***Dietary cereal per 50 g/day*** | | ***Dietary fibre per 10 g/day*** | |
| *TLR4* | rs4986790 | | | | | | | | |
| | AA | 1.03 (1.00-1.06) | 0.56 | 0.96 (0.89-1.03) | 0.96 | 1.02 (0.95-1.10) | 0.61 | 0.88 (0.80-0.97) | 0.86 |
| | GA+GG | 0.99 (0.87-1.13) | | 0.95 (0.75-1.20) | | 1.06 (0.91-1.25) | | 0.85 (0.60-1.21) | |
| | rs5030728 | | | | | | | | |
| | GG | 1.01 (0.97-1.06) | 0.29 | 1.00 (0.90-1.10) | 0.24 | 1.05 (0.96-1.14) | 0.44 | 0.88 (0.77-1.00) | 0.96 |
| | GA+AA | 1.04 (1.01-1.08) | | 0.92 (0.85-1.01) | | 1.01 (0.93-1.10) | | 0.88 (0.77-1.00) | |
| *NFKB1* | rs28362491 | | | | | | | | |
| | Ins/Ins | 1.01 (0.97-1.06) | 0.46 | 0.95 (0.86-1.05) | 0.84 | 1.02 (0.92-1.12) | 0.63 | 0.89 (0.76-1.04) | 0.83 |
| | Ins/Del+Del/Del | 1.04 (1.00-1.07) | | 0.96 (0.89-1.04) | | 1.04 (0.96-1.12) | | 0.87 (0.77-0.98) | |
| | | ***Fruit per 50 g/day*** | | ***Vegetables per 50 g/day*** | | ***Alcohol per 10 g/day[c]*** | | | |
| *TLR4* | rs4986790 | | | | | | | | |
| | AA | 0.98 (0.96-1.01) | 0.58 | 1.02 (0.98-1.07) | 0.36 | 1.04 (1.01-1.07) | 0.29 | | |
| | GA+GG | 0.96 (0.90-1.03) | | 0.96 (0.85-1.09) | | 0.98 (0.88-1.09) | | | |
| | rs5030728 | | | | | | | | |
| | GG | 0.97 (0.94-1.01) | 0.63 | 1.02 (0.96-1.07) | 0.91 | 1.05 (1.01-1.10) | 0.31 | | |
| | GA+AA | 0.98 (0.95-1.02) | | 1.02 (0.97-1.07) | | 1.02 (0.98-1.06) | | | |
| *NFKB1* | rs28362491 | | | | | | | | |
| | Ins/Ins | 0.98 (0.94-1.02) | 0.97 | 1.03 (0.97-1.09) | 0.58 | 1.07 (1.02-1.12) | 0.10 | | |
| | Ins/Del+Del/Del | 0.98 (0.95-1.01) | | 1.01 (0.96-1.06) | | 1.02 (0.98-1.05) | | | |

[a] Adjusted for age, sex, smoking status, alcohol, HRT status (women only), BMI, use of NSAID, intake of red and processed meat, and dietary fibre.
[b] P-value for interaction for adjusted risk estimates.
[c] Among current drinkers only.

**Table S3. Interaction between NSAID use and the studied polymorphisms in relation to CRC risk.**

| | | *NSAID use* $n_{cases}/n_{sub\text{-}cohort}$ | | *NSAID use IRR (95% CI)*[a] | | *NSAID use IRR (95% CI)*[b] | | *P-value*[c] |
|---|---|---|---|---|---|---|---|---|
| | | *No* | *Yes* | *No* | *Yes* | *No* | *Yes* | |
| *TLR4* | rs4986790 | | | | | | | |
| | AA | 604/1051 | 261/500 | 1.00 (ref.) | 0.98 (0.85-1.13) | 1.00 (ref.) | 0.97 (0.84-1.12) | 0.94 |
| | GA+GG | 51/100 | 25/42 | 0.95 (0.72-1.27) | 0.98 (0.66-1.43) | 0.96 (0.72-1.28) | 0.95 (0.64-1.42) | |
| | rs5030728 | | | | | | | |
| | GG | 556/556 | 122/256 | 1.00 (ref.) | 0.95 (0.77-1.17) | 1.00 (ref.) | 0.95 (0.77-1.17) | |
| | GA | 291/489 | 117/233 | 1.10 (0.94-1.29) | 1.01 (0.92-1.25) | 1.11 (0.94-1.30) | 1.00 (0.81-1.24) | 0.26 |
| | AA | 67/106 | 47/53 | 1.15 (0.88-1.50) | 1.50 (1.11-2.03) | 1.14 (0.88-1.49) | 1.47 (1.08-2.00) | |
| | GG+GA | 847/1649 | 239/489 | 1.00 (ref.) | 0.94 (0.81-1.09) | 1.00 (ref.) | 0.93 (0.80-1.07) | 0.11 |
| | AA | 67/106 | 47/53 | 1.10 (0.85-1.42) | 1.43 (1.07-1.92) | 1.09 (0.84-1.40) | 1.40 (1.04-1.88) | |
| *NFKB1* | rs28362491 | | | | | | | |
| | Ins/Ins | 236/455 | 93/215 | 1.00 (ref.) | 0.90 (0.71-1.14) | 1.00 (ref.) | 0.89 (0.70-1.13) | 0.37 |
| | Ins/Del+Del/Del | 419/696 | 240/327 | 1.12 (0.96-1.32) | 1.16 (0.96-1.40) | 1.13 (0.96-1.32) | 1.15 (0.95-1.38) | |

[a] Crude – adjusted for age and sex.
[b] In addition, adjusted for smoking status, alcohol, HRT status (women only), BMI, intake of red and processed meat, and dietary fibre.
[c] P-value for interaction for the adjusted estimates.

**Table S4. Interaction between smoking status and the studied polymorphisms in relation to risk of CRC.**

| *Gene* | *Genotype* | *Never smokers* | *Past smokers* | *Current smokers* | *Never smokers* | *Past smokers* | *Current smokers* | *Never smokers* | *Past smokers* | *Current smokers* | *P-value*[c] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $n_{cases}/n_{sub\text{-}cohort}$ | $n_{cases}/n_{sub\text{-}cohort}$ | $n_{cases}/n_{sub\text{-}cohort}$ | *IRR (95%CI)*[a] | *IRR (95%CI)*[a] | *IRR (95%CI)*[a] | *IRR (95%CI)*[b] | *IRR (95%CI)*[b] | *IRR (95%CI)*[b] | |
| *TLR4* | rs4986790 | | | | | | | | | | |
| | AA | 249/524 | 258/470 | 332/583 | 1.00 (ref.) | 1.05 (0.88-1.25) | 1.15 (0.98-1.36) | 1.00 (ref.) | 1.05 (0.88-1.25) | 1.11 (0.94-1.31) | |
| | GA+GG | 25/48 | 22/43 | 29/51 | 1.08 (0.72-1.62) | 0.99 (0.64-1.52) | 1.11 (0.76-1.61) | 1.04 (0.69-1.58) | 0.99 (0.63-1.53) | 1.11 (0.76-1.62) | 0.94 |
| | rs5030728 | | | | | | | | | | |
| | GG | 113/279 | 130/251 | 162/296 | 1.00 (ref.) | 1.17 (0.91-1.50) | 1.31 (1.03-1.67) | 1.00 (ref.) | 1.18 (0.92-1.51) | 1.28 (1.01-1.63) | |
| | GA | 124/237 | 114/216 | 161/278 | 1.29 (1.00-1.66) | 1.19 (0.92-1.54) | 1.35 (1.07-1.71) | 1.30 (1.01-1.68) | 1.22 (0.94-1.58) | 1.32 (1.04-1.67) | |
| | AA | 37/56 | 36/46 | 38/60 | 1.56 (1.07-2.29) | 1.53 (1.05-2.24) | 1.46 (1.02-2.09) | 1.59 (1.09-2.31) | 1.53 (1.05-2.25) | 1.40 (0.98-2.01) | 0.50 |
| | GG+GA | 237/516 | 244/467 | 323/574 | 1.00 (ref.) | 1.04 (0.87-1.25) | 1.18 (1.00-1.39) | 1.00 (ref.) | 1.05 (0.88-1.26) | 1.14 (0.96-1.36) | |
| | AA | 37/56 | 36/46 | 38/60 | 1.38 (0.97-1.98) | 1.36 (0.95-1.93) | 1.29 (0.92-1.80) | 1.39 (0.98-1.99) | 1.35 (0.94-1.93) | 1.23 (0.88-1.73) | 0.57 |
| *NFKB1* | rs28362491 | | | | | | | | | | |
| | Ins/Ins | 97/221 | 85/212 | 138/246 | 1.00 (ref.) | 0.91 (0.68-1.21) | 1.24 (0.96-1.60) | 1.00 (ref.) | 0.91 (0.68-1.22) | 1.21 (0.94-1.57) | |
| | Ins/Del+Del/Del | 177/351 | 195/301 | 223/388 | 1.17 (0.91-1.49) | 1.29 (1.01-1.64) | 1.27 (1.00-1.61) | 1.17 (0.92-1.50) | 1.30 (1.02-1.66) | 1.24 (0.97-1.57) | 0.13 |

[a] Crude – adjusted for age and sex.
[b] In addition, adjusted for alcohol intake, HRT status (women only), BMI, use of NSAID, intake of red and processed meat, and dietary fibre.
[c] P-value for interaction for adjusted risk estimates.

**Table S5. Interaction between NSAID use and the studied polymorphisms per 25 g red and processed meat intake per day in relation to CRC risk.**

| *Gene* | *Genotype* | *NSAID use* $n_{cases}/n_{sub\text{-}cohort}$ | | *NSAID use* *IRR (95% CI)*[a] | | *P-value*[b] |
|---|---|---|---|---|---|---|
| | | *No* | *Yes* | *No* | *Yes* | |
| *TLR4* | rs4986790 | | | | | |
| | AA | 604/1051 | 261/500 | 1.03 (1.00-1.07) | 1.03 (0.98-1.08) | 0.84 |
| | GA+GG | 51/100 | 25/42 | 1.03 (0.89-1.18) | 0.89 (0.65-1.22) | |
| | rs5030728 | | | | | |
| | GG | 556/556 | 122/256 | 1.00 (0.95-1.05) | 1.04 (0.98-1.10) | |
| | GA | 291/489 | 117/233 | 1.04 (1.00-1.09) | 1.02 (0.95-1.10) | 0.39 |
| | AA | 67/106 | 47/53 | 1.11 (1.02-1.22) | 0.98 (0.85-1.12) | |
| | GG+GA | 847/1649 | 239/489 | 1.02 (0.99-1.06) | 1.03 (0.98-1.08) | 0.30 |
| | AA | 67/106 | 47/53 | 1.11 (1.02-1.22) | 0.98 (0.85-1.12) | |
| *NFKB1* | rs28362491 | | | | | |
| | Ins/Ins | 236/455 | 93/215 | 1.00 (0.94-1.05) | 1.06 (0.98-1.16) | 0.27 |
| | Ins/Del+Del/Del | 419/696 | 240/327 | 1.05 (1.01-1.09) | 1.00 (0.95-1.06) | |

[a] Adjusted for age, sex, smoking status, alcohol intake, HRT status (women only), BMI, and dietary fibre.
[b] P-value for interaction for the adjusted estimates.

# Supplementary material for Paper III

**Table S1: Risk estimates for different combinations of *ABCB1* haplotypes in relation to risk of CRC.**

| Haplotype[a] | T**T**C | **C**C**T** | TC**T** | **C**CC | TCC | **CT**C | T**TT** |
|---|---|---|---|---|---|---|---|
| T**T**C | 1.00 (ref.) (132/273) | 1.03 (0.85-1.25) (234/432) | 1.13 (0.89-1.45) (97/159) | 0.99 (0.73-1.34) (53/113) | 0.90 (0.56-1.45) (18/35) | 1.11 (0.67-1.83) (15/24) | 1.04 (0.63-1.73) (15/26) |
| **C**C**T** | | 0.99 (0.77-1.28) (88/172) | 0.93 (0.71-1.22) (75/168) | 1.18 (0.88-1.60) (54/93) | * | 1.30 (0.75-2.26) (14/20) | 1.15 (0.68-1.93) (16/27) |
| TC**T** | | | 0.84 (0.49-1.42) (12/30) | 1.41 (1.01-1.95) (41/58) | 1.06 (0.48-2.34) (6/10) | * | 1.14 (0.43-2.98) (5/10) |
| **C**CC | | | | 1.77 (1.00-3.13) (10/8) | 1.06 (0.46-2.45) (5/7) | 0.24 (0.04-1.58) (1/7) | * |
| TCC | | | | | - (0/2) | Som nr. 9 | * |
| **CT**C | | | | | | - (1/0) | 1.29 (0.64-2.60) (5/8) |
| T**TT** | | | | | | | - (0/1) |

IRR (95% CI) for BC for different combinations of haplotypes. The number of cases and controls in each cell is listed. Variant alleles are bold. Adjusted for smoking status, alcohol, HRT status (women only), BMI, use of NSAID, intake of red and processed meat, and dietary fibre. *Dublicate.
[a]Haplotype sequence: rs1045642, rs1128503, rs3789243

**Table S2: Risk estimates for different combinations of *BCRP/ABCG2* haplotypes in relation to risk of CRC.**

| Haplotype[a] | GGC | GG**T** | G**T**C | **A**GC |
|---|---|---|---|---|
| GGC | 1.00 (ref.) (327/583) | 1.02 (0.87-1.18) (295/534) | 0.94 (0.76-1.15) (117/231) | 0.94 (0.64-1.38) (29/56) |
| GG**T** | | 0.79 (0.58-1.08) (48/113) | 0.61 (0.44-0.86) (37/118) | 0.89 (0.52-1.50) (16/37) |
| G**T**C | | | 1.19 (0.72-1.98) (14/23) | 1.10 (0.59-2.06) (9/13) |
| **A**GC | | | | 1.40 (0.18-10.73) (1/1) |

IRR (95% CI) for CRC for different combinations of haplotypes. The number of cases and controls in each cell is listed. Variant alleles are bold. Adjusted for smoking status, alcohol intake, HRT status (women only), BMI, use of NSAID, intake of red and processed meat, and dietary fibre.
[a]Haplotype sequence: rs2231137, rs2231142, rs2622604.

**Table S3: IRR for CRC for tertiles of intake of dietary factors for the studied polymorphisms.**

| | *1.tertile* | | *2.tertile* | | *3.tertile* | | *1.tertile* | *2.tertile* | *3.tertile* | *P-value*[b] | *1.tertile* | | *2.tertile* | | *3.tertile* | | *1.tertile* | *2.tertile* | *3.tertile* | *P-value*[b] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *Nc* | *Ns* | *Nc* | *Ns* | *Nc* | *Ns* | *IRR (95%CI)*[a] | *IRR (95%CI)*[a] | *IRR (95%CI)*[a] | | *Nc* | *Ns* | *Nc* | *Ns* | *Nc* | *Ns* | *IRR (95%CI)*[a] | *IRR (95%CI)*[a] | *IRR (95%CI)*[a] | |
| *ABCB1*/rs1045642 (TT as wild type) | | | | | | | | Red and processed meat | | | | | | | | | | Fish | | |
| TT | 93 | 206 | 99 | 147 | 108 | 210 | 1.00 (ref.) | 1.33 (1.01-1.77) | 1.12 (0.85-1.49) | | 101 | 173 | 97 | 181 | 102 | 209 | 1.00 (ref.) | 0.91 (0.69-1.20) | 0.80 (0.60-1.07) | |
| TC+CC | 216 | 451 | 208 | 360 | 211 | 364 | 1.00 (0.78-1.28) | 1.27 (0.99-1.63) | 1.27 (0.99-1.62) | 0.53 | 210 | 410 | 215 | 374 | 210 | 391 | 0.92 (0.72-1.17) | 0.96 (0.75-1.22) | 0.89 (0.69-1.15) | 0.55 |
| | | | | | | | | Dietary cereal | | | | | | | | | | Dietary fibre | | |
| TT | 79 | 164 | 106 | 183 | 115 | 216 | 1.00 (ref.) | 1.17 (0.87-1.58) | 1.29 (0.93-1.79) | | 84 | 179 | 104 | 166 | 112 | 218 | 1.00 (ref.) | 1.24 (0.93-1.65) | 1.11 (0.83-1.48) | |
| TC+CC | 234 | 335 | 206 | 399 | 195 | 441 | 1.37 (1.06-1.79) | 1.11 (0.84-1.46) | 1.10 (0.81-1.50) | 0.01 | 228 | 350 | 209 | 345 | 198 | 480 | 1.33 (1.03-1.71) | 1.26 (0.97-1.63) | 0.91 (0.71-1.18) | 0.006 |
| | | | | | | | | Fruit | | | | | | | | | | Vegetables | | |
| TT | 91 | 190 | 105 | 186 | 104 | 187 | 1.00 (ref.) | 1.12 (0.84-1.49) | 1.13 (0.83-1.54) | | 101 | 165 | 111 | 202 | 88 | 196 | 1.00 (ref.) | 1.05 (0.80-1.38) | 0.93 (0.69-1.27) | |
| TC+CC | 219 | 335 | 209 | 414 | 207 | 426 | 1.26 (0.98-1.61) | 1.06 (0.82-1.37) | 1.04 (0.79-1.38) | 0.10 | 211 | 335 | 196 | 422 | 228 | 418 | 1.03 (0.81-1.31) | 0.93 (0.73-1.20) | 1.12 (0.86-1.45) | 0.19 |
| | | | | | | | | Alcohol | | | | | | | | | | | | |
| TT | 171 | 96 | 114 | 210 | 90 | 182 | 1.00 (ref.) | 0.96 (0.73-1.25) | 0.92 (0.69-1.22) | | | | | | | | | | | |
| TC+CC | 232 | 398 | 188 | 423 | 215 | 354 | 1.00 (0.79-1.26) | 0.85 (0.66-1.08) | 1.12 (0.88-1.43) | 0.16 | | | | | | | | | | |
| *ABCB1*/rs1045642 (gene-dose model) | | | | | | | | Red and processed meat | | | | | | | | | | Fish | | |
| TT | 93 | 206 | 99 | 147 | 108 | 210 | 1.00 (ref.) | 1.34 (1.01-1.79) | 1.12 (0.85-1.49) | | 101 | 173 | 97 | 181 | 102 | 209 | 1.00 (ref.) | 0.91 (0.69-1.20) | 0.80 (0.60-1.07) | |
| TC | 149 | 327 | 150 | 265 | 158 | 263 | 0.96 (0.74-1.25) | 1.26 (0.98-1.64) | 1.29 (0.99-1.66) | 0.75 | 147 | 288 | 158 | 276 | 152 | 291 | 0.91 (0.70-1.18) | 0.96 (0.74-1.24) | 0.87 (0.67-1.14) | 0.83 |
| CC | 67 | 124 | 58 | 95 | 53 | 101 | 1.09 (0.80-1.49) | 1.29 (0.92-1.81) | 1.22 (0.87-1.71) | | 63 | 122 | 57 | 98 | 58 | 100 | 0.93 (0.67-1.29) | 0.95 (0.68-1.32) | 0.96 (0.68-1.33) | |
| | | | | | | | | Dietary cereal | | | | | | | | | | Dietary fibre | | |
| TT | 79 | 164 | 106 | 183 | 115 | 216 | 1.00 (ref.) | 1.17 (0.87-1.58) | 1.29 (0.93-1.78) | | 84 | 179 | 104 | 166 | 112 | 218 | 1.00 (ref.) | 1.24 (0.94-1.65) | 1.11 (0.83-1.48) | |
| TC | 162 | 239 | 144 | 286 | 151 | 330 | 1.32 (1.00-1.74) | 1.10 (0.82-1.47) | 1.12 (0.82-1.54) | 0.05 | 163 | 237 | 150 | 257 | 144 | 361 | 1.37 (1.05-1.79) | 1.23 (0.94-1.62) | 0.89 (0.67-1.16) | 0.02 |
| CC | 72 | 96 | 62 | 113 | 44 | 111 | 1.51 (1.08-2.09) | 1.12 (0.80-1.58) | 1.03 (0.69-1.53) | | 65 | 113 | 59 | 88 | 54 | 119 | 1.24 (0.89-1.72) | 1.33 (0.95-1.86) | 1.01 (0.72-1.41) | |
| | | | | | | | | Fruit | | | | | | | | | | Vegetables | | |
| TT | 91 | 190 | 105 | 186 | 104 | 187 | 1.00 (ref.) | 1.12 (0.85-1.49) | 1.14 (0.84-1.55) | | 101 | 165 | 111 | 202 | 88 | 196 | 1.00 (ref.) | 1.05 (0.81-1.38) | 0.93 (0.69-1.27) | |
| TC | 161 | 234 | 152 | 298 | 144 | 323 | 1.31 (1.01-1.70) | 1.08 (0.82-1.41) | 0.96 (0.72-1.29) | 0.05 | 155 | 546 | 137 | 305 | 165 | 304 | 1.02 (0.80-1.32) | 0.91 (0.69-1.19) | 1.11 (0.85-1.47) | 0.47 |
| CC | 58 | 101 | 57 | 116 | 63 | 103 | 1.13 (0.81-1.56) | 1.02 (0.73-1.43) | 1.31 (0.92-1.85) | | 56 | 89 | 59 | 117 | 63 | 114 | 1.04 (0.75-1.45) | 0.99 (0.71-1.38) | 1.11 (0.80-1.54) | |
| | | | | | | | | Alcohol | | | | | | | | | | | | |
| TT | 171 | 96 | 114 | 210 | 90 | 182 | 1.00 (ref.) | 0.96 (0.73-1.25) | 0.92 (0.69-1.22) | | | | | | | | | | | |
| TC | 168 | 292 | 132 | 317 | 157 | 246 | 1.00 (0.77-1.28) | 0.80 (0.62-1.04) | 1.16 (0.90-1.49) | 0.23 | | | | | | | | | | |
| CC | 64 | 106 | 56 | 106 | 58 | 108 | 1.00 (0.73-1.37) | 0.98 (0.70-1.37) | 1.04 (0.75-1.44) | | | | | | | | | | | |
| *ABCB1*/rs1045642 (CC as wild type) | | | | | | | | Red and processed meat | | | | | | | | | | Fish | | |
| CC | 67 | 124 | 58 | 95 | 53 | 101 | 1.00 (ref.) | 1.19 (0.83-1.69) | 1.12 (0.78-1.60) | | 63 | 122 | 57 | 98 | 58 | 100 | 1.00 (ref.) | 1.02 (0.71-1.46) | 1.03 (0.72-1.48) | |

| | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| TC+TT | 242 | 533 | 249 | 412 | 266 | 473 | 0.90 (0.68-1.17) | 1.18 (0.90-1.55) | 1.11 (0.85-1.46) | 0.75 | 248 | 461 | 255 | 457 | 254 | 500 | 1.01 (0.77-1.34) | 1.01 (0.77-1.33) | 0.91 (0.68-1.20) | 0.82 |
| | | | | | | | | Dietary cereal | | | | | | | | | | Dietary fibre | | |
| CC | 72 | 96 | 62 | 113 | 44 | 111 | 1.00 (ref.) | 0.75 (0.53-1.06) | 0.68 (0.46-1.02) | | 65 | 113 | 59 | 88 | 54 | 119 | 1.00 (ref.) | 1.07 (0.75-1.54) | 0.81 (0.57-1.17) | |
| TC+TT | 241 | 403 | 250 | 469 | 266 | 546 | 0.79 (0.61-1.04) | 0.75 (0.57-0.99) | 0.79 (0.58-1.07) | 0.13 | 247 | 416 | 254 | 423 | 256 | 579 | 0.98 (0.74-1.30) | 1.00 (0.76-1.32) | 0.78 (0.59-1.04) | 0.23 |
| | | | | | | | | Fruit | | | | | | | | | | Vegetables | | |
| CC | 58 | 101 | 57 | 116 | 63 | 103 | 1.00 (ref.) | 0.91 (0.63-1.31) | 1.16 (0.80-1.69) | | 56 | 89 | 59 | 117 | 63 | 114 | 1.00 (ref.) | 0.95 (0.65-1.38) | 1.07 (0.74-1.55) | |
| TC+TT | 252 | 424 | 257 | 484 | 248 | 510 | 1.05 (0.79-1.39) | 0.97 (0.73-1.29) | 0.91 (0.67-1.24) | 0.18 | 256 | 411 | 248 | 507 | 253 | 500 | 0.97 (0.73-1.30) | 0.93 (0.69-1.25) | 1.00 (0.73-1.37) | 0.85 |
| | | | | | | | | Alcohol | | | | | | | | | | | | |
| CC | 64 | 106 | 56 | 106 | 58 | 108 | 1.00 (ref.) | 0.98 (0.68-1.40) | 1.04 (0.73-1.49) | | | | | | | | | | | |
| TC+TT | 264 | 463 | 246 | 527 | 247 | 428 | 1.00 (0.76-1.31) | 0.86 (0.66-1.14) | 1.06 (0.80-1.40) | 0.75 | | | | | | | | | | |
| *ABCB1*/rs1128503 | | | | | | | | Red and processed meat | | | | | | | | | | Fish | | |
| CC | 110 | 212 | 101 | 187 | 89 | 174 | 1.00 (ref.) | 1.13 (0.86-1.48) | 1.16 (0.88-1.54) | | 97 | 209 | 106 | 179 | 97 | 185 | 1.00 (ref.) | 1.15 (0.87-1.52) | 1.04 (0.78-1.38) | |
| CT+TT | 199 | 449 | 211 | 327 | 226 | 407 | 0.91 (0.72-1.15) | 1.27 (1.01-1.60) | 1.11 (0.88-1.40) | 0.35 | 216 | 378 | 208 | 383 | 212 | 422 | 1.14 (0.89-1.45) | 1.07 (0.84-1.36) | 0.96 (0.75-1.24) | 0.31 |
| | | | | | | | | Dietary cereal | | | | | | | | | | Dietary fibre | | |
| CC | 109 | 167 | 100 | 204 | 91 | 202 | 1.00 (ref.) | 0.75 (0.57-0.99) | 0.87 (0.64-1.19) | | 108 | 184 | 97 | 166 | 95 | 223 | 1.00 (ref.) | 0.94 (0.71-1.24) | 0.77 (0.58-1.01) | |
| CT+TT | 201 | 343 | 214 | 389 | 221 | 451 | 0.84 (0.66-1.06) | 0.86 (0.68-1.09) | 0.88 (0.67-1.15) | 0.15 | 201 | 355 | 220 | 354 | 215 | 474 | 0.91 (0.71-1.15) | 1.00 (0.80-1.26) | 0.77 (0.61-0.98) | 0.25 |
| | | | | | | | | Fruit | | | | | | | | | | Vegetables | | |
| CC | 100 | 177 | 109 | 194 | 91 | 202 | 1.00 (ref.) | 1.02 (0.78-1.35) | 0.91 (0.67-1.23) | | 98 | 164 | 96 | 204 | 106 | 205 | 1.00 (ref.) | 0.94 (0.70-1.25) | 1.13 (0.84-1.51) | |
| CT+TT | 209 | 356 | 207 | 410 | 220 | 417 | 1.03 (0.81-1.30) | 0.92 (0.73-1.17) | 0.96 (0.74-1.25) | 0.52 | 213 | 346 | 215 | 424 | 208 | 413 | 1.00 (0.79-1.27) | 1.01 (0.79-1.28) | 1.02 (0.78-1.33) | 0.65 |
| | | | | | | | | Alcohol | | | | | | | | | | | | |
| CC | 109 | 198 | 91 | 195 | 100 | 180 | 1.00 (ref.) | 0.94 (0.71-1.25) | 1.15 (0.88-1.52) | | | | | | | | | | | |
| CT+TT | 225 | 379 | 212 | 445 | 199 | 359 | 1.09 (0.87-1.38) | 0.93 (0.73-1.17) | 1.06 (0.83-1.34) | 0.78 | | | | | | | | | | |
| *ABCB1*/rs3789243 | | | | | | | | Red and processed meat | | | | | | | | | | Fish | | |
| CC | 81 | 186 | 83 | 121 | 75 | 175 | 1.00 (ref.) | 1.42 (1.04-1.92) | 1.04 (0.76-1.43) | | 77 | 163 | 90 | 145 | 72 | 174 | 1.00 (ref.) | 1.19 (0.87-1.61) | 0.88 (0.63-1.23) | |
| CT+TT | 228 | 475 | 224 | 386 | 239 | 409 | 1.07 (0.83-1.39) | 1.34 (1.03-1.73) | 1.33 (1.03-1.73) | 0.26 | 237 | 424 | 220 | 412 | 234 | 434 | 1.19 (0.91-1.56) | 1.10 (0.84-1.44) | 1.06 (0.81-1.40) | 0.19 |
| | | | | | | | | Dietary cereal | | | | | | | | | | Dietary fibre | | |
| CC | 71 | 139 | 83 | 158 | 85 | 185 | 1.00 (ref.) | 1.02 (0.74-1.41) | 1.02 (0.72-1.45) | | 68 | 151 | 86 | 146 | 85 | 185 | 1.00 (ref.) | 1.22 (0.89-1.67) | 0.99 (0.71-1.37) | |
| CT+TT | 235 | 363 | 232 | 434 | 224 | 473 | 1.17 (0.89-1.54) | 1.06 (0.80-1.40) | 1.12 (0.82-1.52) | 0.81 | 239 | 383 | 226 | 372 | 226 | 515 | 1.29 (0.98-1.69) | 1.27 (0.96-1.67) | 0.99 (0.75-1.31) | 0.16 |
| | | | | | | | | Fruit | | | | | | | | | | Vegetables | | |
| CC | 76 | 160 | 74 | 153 | 89 | 169 | 1.00 (ref.) | 1.04 (0.75-1.43) | 1.10 (0.80-1.53) | | 75 | 163 | 82 | 148 | 82 | 171 | 1.00 (ref.) | 1.32 (0.97-1.81) | 1.15 (0.82-1.60) | |
| CT+TT | 229 | 370 | 241 | 451 | 221 | 449 | 1.24 (0.95-1.61) | 1.13 (0.86-1.47) | 1.09 (0.82-1.46) | 0.41 | 236 | 349 | 225 | 471 | 230 | 450 | 1.31 (1.01-1.70) | 1.15 (0.88-1.51) | 1.32 (0.99-1.76) | 0.09 |
| | | | | | | | | Alcohol | | | | | | | | | | | | |
| CC | 80 | 151 | 87 | 187 | 72 | 144 | 1.00 (ref.) | 0.87 (0.64-1.18) | 0.97 (0.71-1.33) | | | | | | | | | | | |

| | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CT+TT | 249 | 422 | 211 | 455 | 231 | 393 | 1.04 (0.81-1.34) | 0.92 (0.71-1.18) | 1.14 (0.88-1.47) | 0.51 | | | | | | | | | | | |
| *ABCG2*/rs2231142 | | | | | | | | Red and processed meat | | | | | | | | | | | Fish | | |
| GG | 240 | 524 | 252 | 401 | 255 | 443 | 1.00 (ref.) | 1.29 (1.07-1.54) | 1.21 (1.01-1.45) | | 244 | 465 | 256 | 419 | 247 | 484 | 1.00 (ref.) | 1.09 (0.91-1.31) | 0.90 (0.74-1.09) | | |
| TG+TT | 71 | 139 | 55 | 111 | 61 | 147 | 0.97 (0.75-1.26) | 1.18 (0.88-1.57) | 1.03 (0.78-1.36) | 0.99 | 67 | 130 | 58 | 144 | 62 | 123 | 0.99 (0.75-1.29) | 0.75 (0.57-1.00) | 0.99 (0.74-1.31) | 0.05 | |
| | | | | | | | | Dietary cereal | | | | | | | | | | | Dietary fibre | | |
| GG | 251 | 400 | 253 | 457 | 243 | 511 | 1.00 (ref.) | 0.93 (0.77-1.12) | 0.94 (0.75-1.18) | | 241 | 416 | 259 | 410 | 247 | 542 | 1.00 (ref.) | 1.06 (0.89-1.26) | 0.82 (0.68-0.98) | | |
| TG+TT | 59 | 107 | 58 | 138 | 70 | 152 | 0.89 (0.67-1.18) | 0.74 (0.55-0.99) | 0.98 (0.73-1.32) | 0.26 | 67 | 122 | 56 | 114 | 64 | 161 | 0.93 (0.71-1.23) | 0.87 (0.66-1.16) | 0.78 (0.59-1.02) | 0.40 | |
| | | | | | | | | Fruit | | | | | | | | | | | Vegetables | | |
| GG | 247 | 404 | 251 | 477 | 249 | 487 | 1.00 (ref.) | 0.93 (0.78-1.12) | 0.91 (0.74-1.13) | | 240 | 406 | 255 | 488 | 252 | 474 | 1.00 (ref.) | 1.01 (0.84-1.21) | 1.09 (0.88-1.34) | | |
| TG+TT | 64 | 132 | 63 | 127 | 60 | 138 | 0.89 (0.68-1.18) | 0.85 (0.65-1.11) | 0.83 (0.61-1.13) | 0.80 | 71 | 104 | 47 | 146 | 59 | 147 | 1.05 (0.81-1.35) | 0.86 (0.64-1.16) | 0.89 (0.66-1.20) | 0.44 | |
| | | | | | | | | Alcohol | | | | | | | | | | | | | |
| GG | 265 | 443 | 242 | 506 | 240 | 419 | 1.00 (ref.) | 0.87 (0.73-1.04) | 1.01 (0.85-1.21) | | | | | | | | | | | | |
| TG+TT | 65 | 135 | 58 | 138 | 64 | 124 | 0.85 (0.65-1.12) | 0.75 (0.57-0.99) | 1.01 (0.77-1.33) | 0.52 | | | | | | | | | | | |
| *ABCG2*/rs2231137 | | | | | | | | Red and processed meat | | | | | | | | | | | Fish | | |
| GG | 289 | 622 | 290 | 477 | 293 | 542 | 1.00 (ref.) | 1.30 (1.10-1.53) | 1.20 (1.02-1.42) | | 294 | 553 | 289 | 521 | 289 | 567 | 1.00 (ref.) | 0.99 (0.84-1.16) | 0.90 (0.76-1.07) | | |
| GA+AA | 20 | 43 | 20 | 39 | 23 | 41 | 1.05 (0.64-1.72) | 1.22 (0.77-1.96) | 1.23 (0.82-1.84) | 0.88 | 18 | 40 | 26 | 44 | 19 | 39 | 0.82 (0.59-1.38) | 1.12 (0.77-1.63) | 0.95 (0.58-1.55) | 0.56 | |
| | | | | | | | | Dietary cereal | | | | | | | | | | | Dietary fibre | | |
| GG | 289 | 473 | 287 | 552 | 296 | 616 | 1.00 (ref.) | 0.91 (0.76-1.08) | 1.01 (0.81-1.25) | | 285 | 504 | 286 | 485 | 301 | 652 | 1.00 (ref.) | 1.05 (0.89-1.24) | 0.88 (0.74-1.04) | | |
| GA+AA | 19 | 36 | 27 | 41 | 17 | 46 | 1.02 (0.61-1.70) | 1.21 (0.81-1.82) | 0.72 (0.44-1.19) | 0.14 | 22 | 33 | 29 | 40 | 12 | 50 | 1.40 (0.88-2.22) | 1.23 (0.83-1.82) | 0.47 (0.27-0.82) | 0.01 | |
| | | | | | | | | Fruit | | | | | | | | | | | Vegetables | | |
| GG | 285 | 501 | 298 | 560 | 289 | 580 | 1.00 (ref.) | 0.97 (0.82-1.15) | 0.94 (0.77-1.15) | | 288 | 479 | 291 | 593 | 293 | 569 | 1.00 (ref.) | 0.98 (0.83-1.17) | 1.08 (0.89-1.31) | | |
| GA+AA | 23 | 34 | 17 | 45 | 23 | 44 | 1.16 (0.76-1.79) | 0.77 (0.47-1.27) | 1.01 (0.64-1.61) | 0.44 | 22 | 32 | 20 | 39 | 21 | 52 | 1.14 (0.74-1.76) | 1.05 (0.66-1.72) | 0.91 (0.57-1.45) | 0.60 | |
| | | | | | | | | Alcohol | | | | | | | | | | | | | |
| GG | 313 | 540 | 275 | 601 | 284 | 500 | 1.00 (ref.) | 0.84 (0.72-1.00) | 1.04 (0.88-1.23) | | | | | | | | | | | | |
| GA+AA | 17 | 41 | 24 | 42 | 22 | 40 | 0.74 (0.45-1.21) | 1.09 (0.71-1.68) | 1.07 (0.68-1.66) | 0.16 | | | | | | | | | | | |
| *ABCG2*/rs2622604 | | | | | | | | Red and processed meat | | | | | | | | | | | Fish | | |
| CC+TC | 290 | 618 | 293 | 475 | 295 | 535 | 1.00 (ref.) | 1.30 (1.10-1.53) | 1.21 (1.03-1.43) | | 298 | 548 | 293 | 518 | 287 | 562 | 1.00 (ref.) | 1.00 (0.85-1.18) | 0.91 (0.77-1.08) | | |
| TT | 18 | 41 | 17 | 39 | 18 | 45 | 1.00 (0.61-1.64) | 0.97 (0.58-1.64) | 0.88 (0.55-1.42) | 0.97 | 16 | 40 | 17 | 45 | 20 | 40 | 0.90 (0.52-1.57) | 0.77 (0.48-1.25) | 0.73 (0.46-1.17) | 0.83 | |
| | | | | | | | | Dietary cereal | | | | | | | | | | | Dietary fibre | | |
| CC+TC | 293 | 479 | 293 | 548 | 292 | 601 | 1.00 (ref.) | 0.93 (0.78-1.11) | 0.99 (0.80-1.22) | | 293 | 499 | 298 | 488 | 287 | 641 | 1.00 (ref.) | 1.04 (0.88-1.22) | 0.82 (0.70-0.97) | | |
| TT | 16 | 28 | 21 | 45 | 16 | 52 | 1.01 (0.61-1.67) | 0.77 (0.48-1.24) | 0.68 (0.40-1.15) | 0.50 | 16 | 34 | 14 | 33 | 23 | 58 | 0.95 (0.56-1.62) | 0.72 (0.43-1.22) | 0.69 (0.44-1.07) | 0.45 | |
| | | | | | | | | Fruit | | | | | | | | | | | Vegetables | | |
| CC+TC | 293 | 487 | 296 | 569 | 289 | 572 | 1.00 (ref.) | 0.91 (0.77-1.08) | 0.90 (0.74-1.10) | | 296 | 478 | 288 | 578 | 294 | 572 | 1.00 (ref.) | 0.96 (0.81-1.14) | 1.05 (0.87-1.28) | | |

| | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| TT | 15 | 42 | 18 | 33 | 20 | 50 | 0.69 (0.40-1.20) | 0.91 (0.56-1.46) | 0.72 (0.44-1.16) | 0.43 | 15 | 31 | 18 | 46 | 20 | 48 | 0.86 (0.50-1.48) | 0.86 (0.53-1.40) | 0.77 (0.48-1.24) | 0.80 |
| | | | | | | | | Alcohol | | | | | | | | | | | | |
| CC+TC | 307 | 529 | 281 | 592 | 290 | 507 | 1.00 (ref.) | 0.88 (0.74-1.03) | 1.06 (0.90-1.25) | | | | | | | | | | | |
| TT | 21 | 46 | 19 | 47 | 13 | 32 | 0.82 (0.52-1.30) | 0.76 (0.48-1.22) | 0.79 (0.44-1.42) | 0.30 | | | | | | | | | | |
| *ABCC2*/rs2273697 | | | | | | | | Red and processed meat | | | | | | | | | | Fish | | |
| GG+AG | 292 | 636 | 298 | 495 | 299 | 556 | 1.00 (ref.) | 1.30 (1.10-1.53) | 1.21 (1.03-1.43) | | 298 | 571 | 303 | 538 | 288 | 578 | 1.00 (ref.) | 1.02 (0.86-1.20) | 0.89 (0.75-1.06) | |
| AA | 17 | 25 | 10 | 18 | 19 | 18 | 1.38 (0.87-2.19) | 1.24 (0.71-2.17) | 1.60 (1.01-2.53) | 0.70 | 16 | 21 | 11 | 18 | 19 | 22 | 1.25 (0.76-2.07) | 0.92 (0.54-1.54) | 1.39 (0.85-2.17) | 0.27 |
| | | | | | | | | Dietary cereal | | | | | | | | | | Dietary fibre | | |
| GG+AG | 295 | 486 | 297 | 564 | 297 | 637 | 1.00 (ref.) | 0.91 (0.77-1.09) | 0.94 (0.76-1.17) | | 294 | 507 | 293 | 502 | 302 | 678 | 1.00 (ref.) | 1.01 (0.86-1.19) | 0.82 (0.69-0.96) | |
| AA | 17 | 19 | 15 | 23 | 14 | 19 | 1.31 (0.82-2.09) | 0.99 (0.61-1.60) | 1.27 (0.76-2.14) | 0.63 | 19 | 27 | 18 | 19 | 9 | 15 | 1.16 (0.74-1.83) | 1.23 (0.79-1.91) | 1.10 (0.61-1.97) | 0.67 |
| | | | | | | | | Fruit | | | | | | | | | | Vegetables | | |
| GG+AG | 293 | 506 | 298 | 584 | 298 | 597 | 1.00 (ref.) | 0.92 (0.77-1.09) | 0.92 (0.76-1.13) | | 290 | 488 | 293 | 604 | 306 | 595 | 1.00 (ref.) | 0.98 (0.83-1.17) | 1.07 (0.88-1.30) | |
| AA | 16 | 26 | 18 | 19 | 12 | 16 | 0.89 (0.55-1.44) | 1.45 (0.94-2.24) | 1.35 (0.79-2.30) | 0.21 | 22 | 21 | 15 | 22 | 9 | 18 | 1.38 (0.91-2.09) | 1.21 (0.75-1.95) | 1.08 (0.56-2.06) | 0.69 |
| | | | | | | | | Alcohol | | | | | | | | | | | | |
| GG+AG | 315 | 551 | 286 | 621 | 288 | 515 | 1.00 (ref.) | 0.87 (0.74-1.03) | 1.06 (0.89-1.25) | | | | | | | | | | | |
| AA | 14 | 22 | 15 | 19 | 17 | 20 | 1.03 (0.62-1.70) | 1.23 (0.77-1.97) | 1.36 (0.84-2.20) | 0.21 | | | | | | | | | | |
| *ABCC2*/rs717620 | | | | | | | | Red and processed meat | | | | | | | | | | Fish | | |
| GG | 202 | 450 | 213 | 333 | 211 | 354 | 1.00 (ref.) | 1.38 (1.13-1.67) | 1.30 (1.07-1.59) | | 216 | 387 | 201 | 371 | 209 | 379 | 1.00 (ref.) | 0.97 (0.80-1.17) | 0.94 (0.77-1.15) | |
| AG+AA | 108 | 211 | 94 | 176 | 105 | 222 | 1.08 (0.85-1.37) | 1.21 (0.94-1.55) | 1.14 (0.89-1.44) | 0.36 | 99 | 200 | 110 | 184 | 98 | 225 | 0.96 (0.75-1.23) | 1.00 (0.80-1.26) | 0.78 (0.61-1.01) | 0.34 |
| | | | | | | | | Dietary cereal | | | | | | | | | | Dietary fibre | | |
| GG | 201 | 327 | 218 | 371 | 207 | 439 | 1.00 (ref.) | 1.01 (0.83-1.24) | 0.94 (0.74-1.19) | | 209 | 349 | 212 | 331 | 205 | 457 | 1.00 (ref.) | 1.01 (0.83-1.23) | 0.79 (0.65-0.97) | |
| AG+AA | 111 | 173 | 94 | 217 | 102 | 219 | 1.06 (0.84-1.34) | 0.75 (0.58-0.97) | 0.99 (0.74-1.31) | 0.04 | 101 | 183 | 100 | 186 | 106 | 240 | 0.91 (0.71-1.16) | 0.93 (0.73-1.19) | 0.78 (0.62-0.99) | 0.50 |
| | | | | | | | | Fruit | | | | | | | | | | Vegetables | | |
| GG | 218 | 355 | 210 | 400 | 198 | 382 | 1.00 (ref.) | 0.91 (0.75-1.11) | 0.93 (0.74-1.16) | | 220 | 324 | 210 | 422 | 196 | 391 | 1.00 (ref.) | 0.88 (0.73-1.07) | 0.95 (0.76-1.18) | |
| AG+AA | 91 | 171 | 102 | 202 | 114 | 236 | 0.92 (0.72-1.19) | 0.88 (0.69-1.12) | 0.87 (0.67-1.12) | 0.72 | 91 | 180 | 99 | 204 | 117 | 225 | 0.77 (0.60-0.99) | 0.91 (0.71-1.16) | 0.98 (0.76-1.25) | 0.15 |
| | | | | | | | | Alcohol | | | | | | | | | | | | |
| GG | 218 | 389 | 207 | 407 | 201 | 341 | 1.00 (ref.) | 0.93 (0.77-1.13) | 1.09 (0.90-1.33) | | | | | | | | | | | |
| AG+AA | 113 | 192 | 92 | 227 | 102 | 190 | 1.00 (0.80-1.26) | 0.80 (0.63-1.03) | 1.03 (0.81-1.31) | 0.48 | | | | | | | | | | |

[a] Analysis adjusted for smoking status, alcohol intake, abstainers, HRT status (women only), BMI, use of NSAID, intake of red and processed meat, and dietary fibre.
[b] P-value for interaction between polymorphisms and dietary factors
Women: Tertiles of red and processed meat (<75.0270 g, 75.0270 g < and < 101.420 g, >101.420 g), fish (<26.9891 g, 26.9891 g < and < 42.7091 g, > 42.7091 g), dietary fibre (<16.9912 g, 16.9912 g < and < 22.0971 g, > 22.0971 g), cereals (<136.126 g, 136.126 g < and < 191.124 g, > 191.124 g), fruit (<141.858 g, 141.858 g < and < 263.602 g, > 263.602 g), vegetables (<122.013 g, 122.013 g < and < 213.125 g, > 213.125 g), alcohol (<4.48446 g, 4.48446 g < and < 13.0941 g, > 13.0941 g).
Men: Tertiles of red and processed meat (<116.935 g, 116.935 g < and < 159.387 g, >159.387 g), fish (<32.2760 g, 32.2760 g < and < 48.0483 g, > 48.0483 g), dietary fibre (<17.5329 g, 17.5329 g < and < 22.5676 g, > 22.5676 g), cereals (<166.378 g, 166.378 g < and < 233.488 g, > 233.488 g), fruit (<90.4576 g, 90.4576 g < and < 196.277 g, > 196.277 g), vegetables (<106.053 g, 106.053 g < and < 187.468 g, > 187.468 g), alcohol (<14.4960 g, 14.4960 g < and < 37.1134 g, > 37.1134 g).

**Table S4: IRR for CRC in relation to combinations of *ABCB1*/rs1045642 and *IL10*/rs3024505 genotypes and use of NSAID.**

| *Genotype combinations* | *NSAID use* $n_{cases}/n_{sub\text{-}cohort}$ | | *NSAID use* *IRR (95% CI)*[a] | | *NSAID use* *IRR (95% CI)*[b] | | *P-value*[c] |
|---|---|---|---|---|---|---|---|
| | *No* | *Yes* | *No* | *Yes* | *No* | *Yes* | |
| rs1045642-CC/rs3024505-CC | 68/154 | 55/67 | 1.00 (ref.) | 1.78 (1.27-2.50) | 1.00 (ref.) | 1.77 (1.27-1.48) | 0.007 |
| rs1045642-CC/rs3024505-CT+TT | 36/57 | 17/41 | 1.22 (0.84-1.78) | 1.00 (0.61-1.64) | 1.22 (0.83-1.77) | 1.02 (0.62-1.67) | |
| rs1045642-CT+TT/rs3024505-CC | 360/659 | 149/291 | 1.17 (0.93-1.48) | 1.12 (0.86-1.47) | 1.20 (0.95-1.52) | 1.13 (0.87-1.48) | |
| rs1045642-CT+TT/rs3024505-CT+TT | 174/321 | 64/142 | 1.13 (0.88-1.46) | 1.00 (0.73-1.38) | 1.17 (0.90-1.51) | 1.03 (0.75-1.41) | |

[a] Crude – adjusted for age and sex.
[b] In addition, adjusted for smoking status, alcohol intake, HRT status (women only), BMI, intake of red and processed meat, and dietary fibre.
[c] P-value for interaction for the adjusted risk estimates.

**Table S5: IRR for CRC in relation to combinations of *ABCG2*/rs2231137 and *IL10*/rs3024505 genotypes and use of NSAID.**

| *Genotype combination* | *NSAID use* $n_{cases}/n_{sub\text{-}cohort}$ | | *NSAID use* *IRR (95% CI)*[a] | | *NSAID use* *IRR (95% CI)*[b] | | *P-value*[c] |
|---|---|---|---|---|---|---|---|
| | *No* | *Yes* | *No* | *Yes* | *No* | *Yes* | |
| rs2231137-GG/rs3024505-CC | 388/768 | 199/340 | 1.00 (ref.) | 1.12 (0.95-1.33) | 1.00 (ref.) | 1.11 (0.94-1.32) | 0.02 |
| rs2231137-GG/rs3024505-CT+TT | 198/354 | 71/174 | 1.00 (0.85-1.18) | 0.81 (0.64-1.04) | 1.01 (0.86-1.19) | 0.82 (0.64-1.05) | |
| rs2231137-GA+AA/rs3024505-CC | 35/57 | 7/25 | 1.13 (0.79-1.62) | 0.53 (0.24-1.14) | 1.19 (0.84-1.70) | 0.52 (0.24-1.13) | |
| rs2231137-GA+AA/rs3024505-CT+TT | 11/27 | 10/13 | 0.92 (0.50-1.71) | 1.44 (0.79-2.61) | 0.90 (0.48-1.67) | 1.40 (0.78-2.51) | |

[a] Crude – adjusted for age and sex.
[b] In addition, adjusted for smoking status, alcohol intake, HRT status (women only), BMI, intake of red and processed meat, and dietary fibre.
[c] P-value for interaction for the adjusted risk estimates.

**Table S6: Interaction between smoking status and the studied polymorphisms in relation to risk of CRC.**

| *Gene* | *Genotype* | *Never smokers* | *Past smokers* | *Current smokers* | *Never smokers* | *Past smokers* | *Current smokers* | *Never smokers* | *Past smokers* | *Current smokers* | *P-value*[c] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $n_{cases}/n_{sub-cohort}$ | $n_{cases}/n_{sub-cohort}$ | $n_{cases}/n_{sub-cohort}$ | *IRR (95%CI)*[a] | *IRR (95%CI)*[a] | *IRR (95%CI)*[a] | *IRR (95%CI)*[b] | *IRR (95%CI)*[b] | *IRR (95%CI)*[b] | |
| *ABCB1* | rs1045642 | | | | | | | | | | |
| | TT | 102/190 | 93/161 | 105/212 | 1.00 (ref.) | 1.00 (0.76-1.31) | 0.91 (0.70-1.19) | 1.00 (ref.) | 0.97 (0.73-1.28) | 0.86 (0.65-1.13) | 0.12 |
| | TC+CC | 180/385 | 188/353 | 267/437 | 0.91 (0.71-1.15) | 0.93 (0.73-1.18) | 1.11 (0.89-1.39) | 0.89 (0.70-1.13) | 0.92 (0.72-1.17) | 1.06 (0.85-1.33) | |
| | | | | | | | | | | | |
| | TT | 102/190 | 93/161 | 105/212 | 1.00 (ref.) | 1.00 (0.76-1.31) | 0.91 (0.70-1.19) | 1.00 (ref.) | 0.96 (0.73-1.28) | 0.86 (0.65-1.13) | |
| | TC | 127/287 | 134/246 | 196/322 | 0.86 (0.66-1.11) | 0.93 (0.72-1.20) | 1.10 (0.87-1.40) | 0.85 (0.65-1.10) | 0.93 (0.72-1.20) | 1.06 (0.84-1.35) | 0.27 |
| | CC | 53/98 | 54/107 | 71/115 | 1.05 (0.75-1.46) | 0.93 (0.67-1.29) | 1.12 (0.83-1.50) | 1.00 (0.72-1.39) | 0.90 (0.65-1.25) | 1.05 (0.78-1.42) | |
| | | | | | | | | | | | |
| | CC | 53/98 | 54/107 | 71/115 | 1.00 (ref.) | 0.89 (0.61-1.29) | 1.07 (0.75-1.52) | 1.00 (ref.) | 0.90 (0.62-1.32) | 1.05 (0.74-1.49) | 0.84 |
| | TC+TT | 229/477 | 227/407 | 301/534 | 0.88 (0.65-1.18) | 0.91 (0.68-1.23) | 0.98 (0.73-1.31) | 0.91 (0.67-1.22) | 0.94 (0.70-1.27) | 0.98 (0.73-1.31) | |
| | rs1128503 | | | | | | | | | | |
| | CC | 89/178 | 97/181 | 114/214 | 1.00 (ref.) | 0.98 (0.74-1.30) | 1.03 (0.80-1.34) | 1.00 (ref.) | 1.00 (0.75-1.33) | 1.05 (0.79-1.38) | 0.93 |
| | CT+TT | 193/407 | 188/338 | 255/438 | 0.93 (0.72-1.20) | 0.98 (0.76-1.26) | 1.08 (0.85-1.37) | 0.95 (0.74-1.23) | 0.99 (0.77-1.29) | 1.06 (0.83-1.36) | |
| | rs3789243 | | | | | | | | | | |
| | CC | 75/165 | 77/132 | 87/185 | 1.00 (ref.) | 1.14 (0.83-1.56) | 0.98 (0.72-1.33) | 1.00 (ref.) | 1.14 (0.83-1.56) | 0.94 (0.69-1.28) | 0.16 |
| | CT+TT | 204/417 | 203/382 | 284/471 | 1.06 (0.82-1.38) | 1.05 (0.80-1.36) | 1.25 (0.98-1.61) | 1.06 (0.81-1.38) | 1.04 (0.80-1.36) | 1.22 (0.94-1.57) | |
| *ABCG2* | rs2231142 | | | | | | | | | | |
| | GG | 236/460 | 228/407 | 283/501 | 1.00 (ref.) | 0.99 (0.83-1.19) | 1.08 (0.91-1.29) | 1.00 (ref.) | 0.99 (0.82-1.19) | 1.05 (0.88-1.25) | 0.25 |
| | TG+TT | 42/126 | 55/114 | 90/157 | 0.73 (0.52-1.01) | 0.90 (0.67-1.21) | 1.07 (0.84-1.36) | 0.73 (0.53-1.02) | 0.92 (0.69-1.24) | 1.05 (0.83-1.33) | |
| | rs2231137 | | | | | | | | | | |
| | GG | 262/546 | 266/479 | 344/616 | 1.00 (ref.) | 1.04 (0.88-1.24) | 1.12 (0.95-1.32) | 1.00 (ref.) | 1.05 (0.88-1.25) | 1.09 (0.92-1.29) | 0.47 |
| | GA+AA | 19/43 | 17/41 | 27/39 | 1.01 (0.62-1.64) | 0.86 (0.52-1.42) | 1.27 (0.85-1.89) | 1.08 (0.67-1.74) | 0.84 (0.50-1.39) | 1.25 (0.84-1.86) | |
| | rs2622604 | | | | | | | | | | |
| | CC+TC | 257/530 | 270/487 | 351/611 | 1.00 (ref.) | 1.03 (0.86-1.22) | 1.12 (0.95-1.31) | 1.00 (ref.) | 1.03 (0.86-1.22) | 1.09 (0.92-1.28) | 0.77 |
| | TT | 20/54 | 14/30 | 19/41 | 0.75 (0.47-1.20) | 0.83 (0.48-1.44) | 0.99 (0.61-1.59) | 0.75 (0.47-1.21) | 0.83 (0.48-1.44) | 0.98 (0.61-1.58) | |
| *ABCC2* | rs2273697 | | | | | | | | | | |
| | GG+AG | 267/564 | 268/498 | 354/625 | 1.00 (ref.) | 1.05 (0.88-1.24) | 1.14 (0.97-1.34) | 1.00 (ref.) | 1.05 (0.88-1.24) | 1.11 (0.94-1.31) | 0.43 |
| | AA | 13/18 | 14/20 | 19/23 | 1.53 (0.88-2.65) | 1.05 (0.65-1.72) | 1.53 (1.00-2.32) | 1.48 (0.86-2.56) | 1.02 (0.62-1.69) | 1.49 (0.98-2.26) | |
| | rs717620 | | | | | | | | | | |
| | GG | 182/378 | 201/338 | 243/421 | 1.00 (ref.) | 1.09 (0.89-1.33) | 1.12 (0.93-1.36) | 1.00 (ref.) | 1.10 (0.90-1.35) | 1.09 (0.90-1.33) | 0.38 |
| | AG+AA | 96/201 | 83/180 | 128/228 | 0.99 (0.77-1.27) | 0.90 (0.69-1.17) | 1.14 (0.91-1.42) | 0.99 (0.77-1.27) | 0.89 (0.68-1.16) | 1.09 (0.87-1.38) | |

[a] Crude – adjusted for age and sex.
[b] In addition, adjusted for alcohol intake, HRT status (women only), BMI, use of NSAID, intake of red and processed meat, and dietary fibre.
[c] P-value for interaction for the adjusted estimates.

# Supplementary material for Paper V

**Figure S1. LD plot for *CYP19A1* illustrating two large haplotype blocks with the recombination spot where rs3751591 is located**

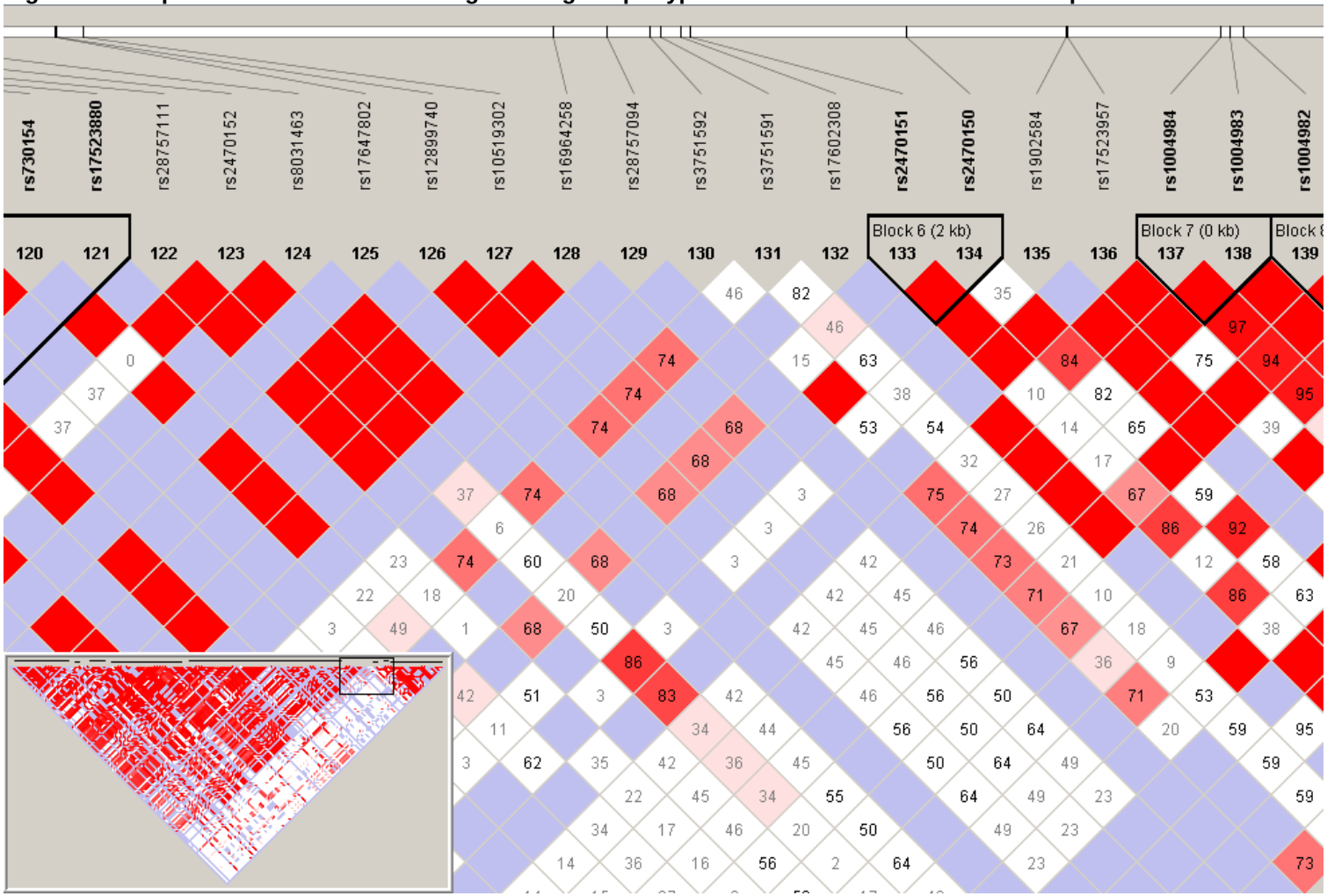

**Table S1: Plasma levels of estrone, estrone sulphate and SHBG among 335 never and past users of hormone replacement therapy as percentage change in hormonal measurements in relation to *CYP19A1* polymorphisms divided by usage of NSAID.**

| *Genotype* | *No* *n (%)* *n=217* | *Yes* *n (%)* *n=118* | *Estrone* | | *P-value* | *Estrone sulphate* | | *P-value* | *SHBG* | | *P-value* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *No* | *Yes* | | *No* | *Yes* | | *No* | *Yes* | |
| | | | *Δ (95% CI)[a]* | *Δ (95% CI)[a]* | | *Δ (95% CI)[a]* | *Δ (95% CI)[a]* | | *Δ (95% CI)[a]* | *Δ (95% CI)[a]* | |
| rs10519297 | | | | | | | | | | | |
| AA | 51 (24) | 28 (24) | 0 (ref.) | -14 (-30;6) | | 0 (ref.) | -11 (-25;6) | | 0 (ref.) | -3 (-20;17) | |
| AG+GG | 166 (76) | 90 (76) | 3 (-10;19) | -8 (-21;7) | 0.84 | 11 (-1;25) | -1 (-13;12) | 0.94 | -8 (-19;5) | 2 (-12;17) | 0.23 |
| rs749292 | | | | | | | | | | | |
| GG | 68 (31) | 33 (28) | 0 (ref.) | -14 (-28;4) | | 0 (ref.) | -18 (-30;-5) | | 0 (ref.) | 6 (-11;25) | |
| AG+AA | 149 (69) | 85 (72) | -5 (-16;8) | -16 (-27;-3) | 0.79 | -15 (-24;-6) | -22 (-30;-12) | 0.18 | 0 (-11;12) | 8 (-5;22) | 0.87 |
| rs1062033 | | | | | | | | | | | |
| CC | 58 (27) | 30 (25) | 0 (ref.) | -13 (-28;6) | | 0 (ref.) | -16 (-29;-1) | | 0 (ref.) | 13 (-5;35) | |
| CG+GG | 159 (73) | 88 (75) | -3 (-15;10) | -15 (-26;-1) | 0.92 | -14 (-23;-4) | -22 (-31;-11) | 0.43 | 3 (-9;17) | 8 (-5;24) | 0.47 |
| rs10046 | | | | | | | | | | | |
| AA | 54 (25) | 37 (31) | 0 (ref.) | -11 (-26;7) | | 0 (ref.) | -7 (-21;10) | | 0 (ref.) | 1 (-15;20) | |
| AG+GG | 163 (75) | 81 (69) | -1 (-14;14) | -13 (-25;1) | 0.89 | 7 (-5;21) | -6 (-18;7) | 0.50 | -3 (-15;10) | 6 (-8;22) | 0.45 |
| rs4646 | | | | | | | | | | | |
| CC | 119 (55) | 64 (54) | 0 (ref.) | -11 (-22;2) | | 0 (ref.) | -9 (-19;2) | | 0 (ref.) | 4 (-8;18) | |
| CA+AA | 98 (45) | 54 (46) | 1 (-10;14) | -12 (-24;1) | 0.83 | 7 (-3;19) | -7 (-18;5) | 0.57 | -6 (-15;5) | 4 (-9;18) | 0.57 |
| rs6493487 | | | | | | | | | | | |
| AA | 121 (56) | 80 (68) | 0 (ref.) | -14 (-24;-3) | | 0 (ref.) | -9 (-18;1) | | 0 (ref.) | -1 (-11;11) | |
| GA+GG | 96 (44) | 38 (32) | -9 (-19;2) | -19 (-31;-5) | 0.74 | 4 (-6;16) | -10 (-22;3) | 0.55 | -10 (-19;0) | 8 (-7;25) | 0.05 |
| rs2008691 | | | | | | | | | | | |
| AA | 148 (68) | 68 (58) | 0 (ref.) | -9 (-20;3) | | 0 (ref.) | -13 (-22;2) | | 0 (ref.) | 4 (-8;17) | |
| GA+GG | 69 (32) | 50 (42) | 11 (-2;26) | -9 (-21;5) | 0.31 | 2 (-8;14) | -8 (-18;4) | 0.73 | 1 (-10;14) | 12 (-1;28) | 0.49 |
| rs3751591 | | | | | | | | | | | |
| TT+TC | 213 (98) | 114 (97) | 0 (ref.) | -13 (-21;-3) | | 0 (ref.) | -13 (-20;-5) | | 0 (ref.) | 8 (-2;18) | |
| CC | 4 (2) | 4 (3) | 10 (-29;71) | 15 (-26;77) | 0.59 | -16 (-42;21) | 48 (3;114) | 0.008 | 71 (15;154) | 17 (-21;74) | 0.12 |
| rs2445762 | | | | | | | | | | | |
| TT | 118 (54) | 54 (46) | 0 (ref.) | -12 (-23;2) | | 0 (ref.) | -10 (-20;2) | | 0 (ref.) | 10 (-4;25) | |
| TC+CC | 99 (46) | 64 (54) | -3 (-14;9) | -14 (-25;-2) | 0.98 | -1 (-10;10) | -13 (-22;-2) | 0.75 | -4 (-14;7) | 2 (-10;15) | 0.69 |
| rs11070844 | | | | | | | | | | | |
| CC | 173 (80) | 92 (78) | 0 (ref.) | -15 (-24;-5) | | 0 (ref.) | -11 (-19;-2) | | 0 (ref.) | 8 (-3;19) | |
| TC+TT | 44 (20) | 26 (22) | 14 (-1;31) | 13 (-5;35) | 0.20 | 16 (3;32) | 2 (-12;19) | 0.93 | 8 (-6;23) | 11 (-6;31) | 0.69 |

Δ Percentage change in hormonal measurements compared to WT divided by usage of NSAID.
[a] Adjusted for age, smoking (never, past, current), abstainers, alcohol intake (increment of 10 g per day) and BMI ($kg/m^2$) at baseline.
[b] P-value for interaction.
SHBG: Sex-hormone binding globulin.

**Table S2: Risk estimates for different combinations of *CYP19A1* haplotypes in relation to risk of BC.**

| Haplotype[a] | **AAA** | G**A**G | GGG |
|---|---|---|---|
| **AAA** | 1.00 (ref.) (164/162) | 0.91 (0.68-1.22) (164/182) | 1.17 (0.86-1.59) (156/143) |
| G**A**G | | 1.25 (0.79-1.97) (48/43) | 1.56 (1.02-2.40) (74/56) |
| GGG | | | 0.99 (0.59-1.68) (34/35) |

IRR (95% CI) for BC for different combinations of haplotypes. The number of cases and controls in each cell is listed. Variant alleles are bold. Adjusted for parous/nulliparous, number of births, age at first birth, length of school education (low, medium, high), duration of HRT use (years), BMI ($kg/m^2$) and alcohol intake (10 g/day).
[a]Haplotype sequence: rs10046, rs6493487, rs10519297

**Table S3: IRR for BC in relation to *CYP19A1* polymorphisms per increment of 10 g alcohol per day.**

| *Genotype* | $n_{cases}$ *(%) (n=651)* | $n_{controls}$ *(%) (n=651)* | *IRR[a] (95% CI)* | *IRR[b] (95% CI)* | *P-value[c]* |
|---|---|---|---|---|---|
| rs10519297 | | | | | |
| AA | 159 (24) | 162 (25) | 1.13 (0.96-1.33) | 1.12 (0.94-1.32) | |
| AG+GG | 492 (76) | 489 (75) | 1.16 (1.06-1.27) | 1.14 (1.05-1.25) | 0.79 |
| rs749292 | | | | | |
| GG | 208 (32) | 193 (30) | 1.23 (1.07-1.42) | 1.23 (1.07-1.43) | |
| AG+AA | 443 (68) | 458 (70) | 1.12 (1.02-1.23) | 1.10 (1.00-1.21) | 0.18 |
| rs1062033 | | | | | |
| CC | 196 (30) | 177 (27) | 1.24 (1.07-1.43) | 1.23 (1.06-1.43) | |
| CG+GG | 455 (70) | 474 (73) | 1.13 (1.03-1.23) | 1.10 (1.01-1.21) | 0.22 |
| rs10046 | | | | | |
| AA | 171 (26) | 176 (27) | 1.16 (0.73-1.48) | 1.14 (0.96-1.34) | |
| AG+GG | 480 (74) | 475 (73) | 1.15 (1.06-1.26) | 1.14 (1.04-1.24) | 0.98 |
| rs4646 | | | | | |
| CC | 355 (55) | 349 (54) | 1.14 (1.03-1.27) | 1.18 (1.00-1.24) | |
| CA+AA | 296 (45) | 302 (46) | 1.17 (1.04-1.31) | 1.16 (1.04-1.30) | 0.62 |
| rs6493487 | | | | | |
| AA | 384 (59) | 405 (62) | 1.13 (1.02-1.25) | 1.12 (1.01-1.24) | |
| GA+GG | 267 (41) | 246 (38) | 1.18 (1.04-1.33) | 1.16 (1.03-1.31) | 0.64 |
| rs2008691 | | | | | |
| AA | 453 (70) | 443 (68) | 1.13 (1.03-1.23) | 1.11 (1.01-1.22) | |
| GA+GG | 198 (30) | 208 (32) | 1.22 (1.05-1.41) | 1.20 (1.04-1.38) | 0.37 |
| rs3751591 | | | | | |
| TT+TC | 626 (96) | 638 (98) | 1.15 (1.06-1.24) | 1.13 (1.04-1.22) | |
| CC | 25 (4) | 13 (2) | 1.66 (0.86-3.18) | 1.66 (0.88-3.14) | 0.24 |
| rs2445762 | | | | | |
| TT | 339 (52) | 347 (53) | 1.13 (1.02-1.25) | 1.11 (1.00-1.23) | |
| TC+CC | 312 (48) | 304 (47) | 1.18 (1.06-1.32) | 1.18 (1.05-1.32) | 0.43 |
| rs11070844 | | | | | |
| CC | 520 (80) | 526 (81) | 1.17 (1.07-1.27) | 1.18 (1.05-1.25) | |
| TC+TT | 131 (20) | 125 (19) | 1.11 (0.95-1.30) | 1.11 (0.95-1.29) | 0.69 |

[a]Crude.
[b]Adjusted for parity (parous/nulliparous, number of births, age at first birth), length of school education (low, medium, high), duration of HRT use (years) and body mass index (kg/m2) at baseline.
[c]P-value for interaction for adjusted risk estimates.

**Table S4. IRR for BC in relation to use of NSAID and *CYP19A1* polymorphisms.**

| *Genotype* | *No* $n_{cases}$/ $n_{controls}$ *(n=675)* | *Yes* $n_{cases}$/ $n_{controls}$ *(n=675)* | *No* *IRR (95% CI)*[a] | *Yes* *IRR (95% CI)*[a] | *No* *IRR (95% CI)*[b] | *Yes* *IRR (95% CI)*[b] | *P-value*[c] |
|---|---|---|---|---|---|---|---|
| rs10519297 | | | | | | | |
| AA | 87/111 | 81/59 | 1.00 (ref.) | 1.69 (1.08-2.63) | 1.00 (ref.) | 1.65 (1.05-2.59) | |
| AG+GG | 305/358 | 202/177 | 1.21 (0.87-1.68) | 1.45 (1.03-2.05) | 1.16 (0.82-1.63) | 1.44 (1.00-2.05) | 0.30 |
| rs749292 | | | | | | | |
| GG | 122/134 | 91/67 | 1.00 (ref.) | 1.44 (0.98-2.12) | 1.00 (ref.) | 1.53 (1.03-2.28) | |
| AG+AA | 270/305 | 192/169 | 0.96 (0.71-1.31) | 1.21 (0.88-1.67) | 1.00 (0.73-1.36) | 1.26 (0.90-1.75) | 0.42 |
| rs1062033 | | | | | | | |
| CC | 114/120 | 86/62 | 1.00 (ref.) | 1.40 (0.94-2.10) | 1.0 (ref.) | 1.48 (0.98-2.23) | |
| CG+GG | 278/319 | 197/174 | 0.89 (0.65-1.22) | 1.14 (0.82-1.59) | 0.92 (0.67-1.26) | 1.18 (0.84-1.65) | 0.58 |
| rs10046 | | | | | | | |
| AA | 93/120 | 86/63 | 1.00 (ref.) | 1.69 (1.11-2.58) | 1.00 (ref.) | 1.68 (1.09-2.59) | |
| AG+GG | 299/319 | 197/173 | 1.22 (0.89-1.68) | 1.46 (1.04-2.04) | 1.17 (0.84-1.61) | 1.43 (1.01-2.02) | 0.24 |
| rs4646 | | | | | | | |
| CC | 204/230 | 163/132 | 1.00 (ref.) | 1.32 (0.99-1.76) | 1.00 (ref.) | 1.33 (0.99-1.78) | |
| CA+AA | 188/209 | 120/104 | 1.01 (0.76-1.32) | 1.30 (0.94-1.80) | 0.97 (0.73-1.28) | 1.30 (0.94-1.82) | 0.95 |
| rs6493487 | | | | | | | |
| AA | 223/277 | 177/146 | 1.00 (ref.) | 1.46 (1.11-1.92) | 1.00 (ref.) | 1.49 (1.12-1.97) | |
| GA+GG | 169/162 | 106/90 | 1.31 (1.00-1.73) | 1.46 (1.05-2.03) | 1.30 (0.98-1.73) | 1.47 (1.05-2.08) | 0.26 |
| rs2008691 | | | | | | | |
| AA | 273/297 | 197/164 | 1.00 (ref.) | 1.26 (0.96-1.64) | 1.00 (ref.) | 1.27 (0.96-1.67) | |
| GA+GG | 119/142 | 86/72 | 0.89 (0.66-1.19) | 1.27 (0.90-1.79) | 0.88 (0.65-1.19) | 1.31 (0.92-1.87) | 0.51 |
| rs3751591 | | | | | | | |
| TT+TC | 374/429 | 275/233 | 1.00 (ref.) | 1.32 (1.06-1.65) | 1.00 (ref.) | 1.35 (1.08-1.69) | |
| CC | 18/10 | 8/3 | 2.14 (0.93-4.91) | 3.04 (0.80-11.60) | 2.09 (0.90-4.88) | 3.37 (0.85-13.36) | 0.83 |
| rs2445762 | | | | | | | |
| TT | 207/228 | 147/128 | 1.00 (ref.) | 1.24 (0.92-1.67) | 1.0 (ref.) | 1.24 (0.91-1.68) | |
| TC+CC | 185/211 | 136/108 | 0.96 (0.73-1.27) | 1.35 (0.98-1.84) | 0.97 (0.73-1.29) | 1.43 (1.03-1.97) | 0.48 |
| rs11070844 | | | | | | | |
| CC | 325/357 | 216/192 | 1.0 (ref.) | 1.21 (0.95-1.54) | 1.00 (ref.) | 1.26 (0.98-1.61) | |
| TC+TT | 67/82 | 67/44 | 0.91 (0.64-1.31) | 1.62 (1.09-2.41) | 0.94 (0.65-1.36) | 1.59 (1.05-2.39) | 0.30 |

[a]Crude.
[b]Adjusted for parity (parous/nulliparous, number of births, age at first birth), length of school education (low, medium, high), duration of HRT use (years), body mass index (kg/m$^2$), and alcohol intake (increment of 10 g per day) at baseline.
[c]*P*-value for interaction for adjusted risk estimates.

**Table S5: IRR for BC in relation to combinations of *PPARG* Pro$^{12}$Ala and *CYP19A1* genotypes**

| *Genotype* | *PPARG Pro$^{12}$Ala* | | *PPARG Pro$^{12}$Ala* | | *PPARG Pro$^{12}$Ala* | | *P-value*[c] |
|---|---|---|---|---|---|---|---|
| | *Pro/Pro $n_{cases}$/ $n_{controls}$ (n=685)* | *Ala-carriers $n_{case}$/ $n_{controls}$ (n=685)* | *Pro/Pro IRR (95% CI)*[a] | *Ala-carriers IRR (95% CI)*[a] | *Pro/Pro IRR (95% CI)*[b] | *Ala-carriers IRR (95% CI)*[b] | |
| rs10519297 | | | | | | | |
| AA | 132/121 | 37/51 | 1.0 (ref.) | 0.65 (0.39-1.08) | 1.0 (ref.) | 0.61 (0.36-1.03) | 0.38 |
| AG+GG | 396/367 | 120/146 | 0.99 (0.75-1.31) | 0.75 (0.52-1.08) | 0.95 (0.71-1.27) | 0.75 (0.52-1.09) | |
| rs749292 | | | | | | | |
| GG | 165/143 | 51/60 | 1.0 (ref.) | 0.73 (0.46-1.14) | 1.00 (ref.) | 0.75 (0.47-1.18) | 1.00 |
| AG+AA | 363/345 | 106/137 | 0.91 (0.68-1.21) | 0.67 (0.47-0.94) | 0.92 (0.68-1.23) | 0.68 (0.48-0.98) | |
| rs1062033 | | | | | | | |
| CC | 152/132 | 51/54 | 1.00 (ref.) | 0.81 (0.51-1.27) | 1.00 (ref.) | 0.84 (0.52-1.34) | 0.57 |
| CG+GG | 376/356 | 106/143 | 0.90 (0.67-1.21) | 0.63 (0.45-0.91) | 0.91 (0.68-1.23) | 0.65 (0.45-0.93) | |
| rs10046 | | | | | | | |
| AA | 137/131 | 44/55 | 1.00 (ref.) | 0.76 (0.47-1.21) | 1.00 (ref.) | 0.75 (0.47-1.22) | 0.97 |
| AG+GG | 391/357 | 113/142 | 1.04 (0.79-1.36) | 0.75 (0.53-1.08) | 1.00 (0.76-1.32) | 0.75 (0.51-1.08) | |
| rs4646 | | | | | | | |
| CC | 286/252 | 84/117 | 1.00 (ref.) | 0.62 (0.44-0.87) | 1.00 (ref.) | 0.63 (0.44-0.90) | 0.17 |
| CA+AA | 242/236 | 73/80 | 0.90 (0.71-1.14) | 0.80 (0.56-1.16) | 0.88 (0.69-1.13) | 0.80 (0.55-1.15) | |
| rs6493487 | | | | | | | |
| AA | 306/302 | 99/126 | 1.00 (ref.) | 0.78 (0.57-1.06) | 1.00 (ref.) | 0.78 (0.57-1.08) | 0.74 |
| GA+GG | 222/186 | 58/71 | 1.20 (0.93-1.54) | 0.82 (0.54-1.24) | 1.18 (0.91-1.53) | 0.84 (0.55-1.29) | |
| rs2008691 | | | | | | | |
| AA | 372/334 | 106/134 | 1.00 (ref.) | 0.70 (0.52-0.96) | 1.00 (ref.) | 0.71 (0.52-0.97) | 0.53 |
| GA+GG | 156/154 | 51/63 | 0.89 (0.69-1.17) | 0.71 (0.47-1.07) | 0.89 (0.67-1.17) | 0.74 (0.49-1.13) | |
| rs3751591 | | | | | | | |
| TT+TC | 508/478 | 151/194 | 1.00 (ref.) | 0.74 (0.57-0.95) | 1.00 (ref.) | 0.75 (0.57-0.97) | 0.37 |
| CC | 20/10 | 6/3 | 1.83 (0.82-4.10) | 2.11 (0.41-10.97) | 1.68 (0.74-3.81) | 2.88 (0.54-15.33) | |
| rs2445762 | | | | | | | |
| TT | 271/260 | 87/104 | 1.00 (ref.) | 0.79 (0.56-1.12) | 1.00 (ref.) | 0.83 (0.59-1.18) | 0.37 |
| TC+CC | 257/228 | 70/93 | 1.07 (0.84-1.36) | 0.71 (0.50-1.03) | 1.11 (0.87-1.43) | 0.74 (0.51-1.07) | |
| rs11070844 | | | | | | | |
| CC | 435/396 | 116/159 | 1.00 (ref.) | 0.66 (0.50-0.88) | 1.00 (ref.) | 0.68 (0.52-0.92) | 0.24 |
| TC+TT | 93/92 | 41/38 | 0.94 (0.69-1.29) | 0.99 (0.62-1.58) | 0.96 (0.70-1.33) | 0.96 (0.59-1.55) | |

[a]Crude.
[b]Adjusted for parity (parous/nulliparous, number of births, age at first birth), length of school education (low, medium, high), duration of HRT use (years), body mass index (kg/m2) and alcohol intake (increment of 10 g per day) at baseline.
[c]P-value for interaction for the adjusted risk estimates.

**Table S6: IRR for BC in relation to combinations of *PPARGC1A* Gly$^{482}$Ser and *CYP19A1* genotypes**

| *Genotype* | *PPARGC1A Gly$^{482}$Ser* | | *PPARGC1A Gly$^{482}$Ser* | | *PPARGC1A Gly$^{482}$Ser* | | *P-value$^{c}$* |
|---|---|---|---|---|---|---|---|
| | *Gly-carriers* $n_{cases}$/ $n_{controls}$ *(n=686)* | *Ser/Ser* $n_{case}$/ $n_{controls}$ *(n=686)* | *Gly-carriers IRR (95% CI)$^{a}$* | *Ser/Ser IRR (95% CI) $^{a}$* | *Gly-carriers IRR (95% CI)$^{b}$* | *Ser/Ser IRR (95% CI)$^{b}$* | |
| rs10519297 | | | | | | | |
| AA | 147/148 | 21/26 | 1.00 (ref.) | 0.83 (0.45-1.52) | 1.00 (ref.) | 0.78 (0.42-1.46) | 0.92 |
| AG+GG | 461/449 | 55/63 | 1.04 (0.80-1.36) | 0.89 (0.57-1.37) | 1.02 (0.77-1.34) | 0.83 (0.53-1.29) | |
| rs749292 | | | | | | | |
| GG | 192/181 | 24/22 | 1.00 (ref.) | 1.10 (0.57-2.12) | 1.00 (ref.) | 1.02 (0.52-2.00) | 0.41 |
| AG+AA | 418/416 | 52/67 | 0.94 (0.73-1.21) | 0.72 (0.47-1.11) | 0.95 (0.73-1.23) | 0.69 (0.44-1.08) | |
| rs1062033 | | | | | | | |
| CC | 179/166 | 24/20 | 1.00 (ref.) | 1.22 (0.62-2.39) | 1.00 (ref.) | 1.14 (0.57-2.26) | 0.24 |
| CG+GG | 431/431 | 52/69 | 0.91 (0.71-1.17) | 0.67 (0.43-1.04) | 0.91 (0.70-1.18) | 0.64 (0.41-1.01) | |
| rs10046 | | | | | | | |
| AA | 162/164 | 20/24 | 1.00 (ref.) | 0.86 (0.46-1.62) | 1.00 (ref.) | 0.84 (0.44-1.60) | 0.88 |
| AG+GG | 448/433 | 56/65 | 1.07 (0.83-1.37) | 0.89 (0.58-1.36) | 1.03 (0.80-1.34) | 0.81 (0.52-1.26) | |
| rs4646 | | | | | | | |
| CC | 326/326 | 45/45 | 1.00 (ref.) | 1.00 (0.64-1.55) | 1.00 (ref.) | 0.98 (0.62-1.54) | 0.21 |
| CA+AA | 284/271 | 31/44 | 1.06 (0.84-1.32) | 0.72 (0.44-1.16) | 1.04 (0.82-1.31) | 0.65 (0.40-1.07) | |
| rs6493487 | | | | | | | |
| AA | 364/369 | 43/60 | 1.00 (ref.) | 0.74 (0.49-1.13) | 1.00 (ref.) | 0.68 (0.44-1.05) | 0.19 |
| GA+GG | 246/228 | 33/29 | 1.12 (0.89-1.42) | 1.24 (0.71-2.18) | 1.10 (0.86-1.40) | 1.23 (0.69-2.19) | |
| rs2008691 | | | | | | | |
| AA | 427/406 | 51/63 | 1.00 (ref.) | 0.78 (0.52-1.16) | 1.00 (ref.) | 0.73 (0.48-1.11) | 0.43 |
| GA+GG | 183/191 | 25/26 | 0.91 (0.71-1.15) | 0.92 (0.52-1.62) | 0.91 (0.71-1.17) | 0.89 (0.50-1.60) | |
| rs3751591 | | | | | | | |
| TT+TC | 584/586 | 76/87 | 1.00 (ref.) | 0.91 (0.65-1.27) | 1.00 (ref.) | 0.87 (0.61-1.23) | 0.02 |
| CC | 25/9 | -/2 | 2.50 (1.15-5.42) | - | 2.52 (1.15-5.56) | - | |
| rs2445762 | | | | | | | |
| TT | 322/319 | 36/46 | 1.00 (ref.) | 0.79 (0.49-1.27) | 1.00 (ref.) | 0.76 (0.47-1.23) | 0.76 |
| TC+CC | 288/278 | 40/43 | 1.02 (0.81-1.29) | 0.91 (0.58-1.44) | 1.05 (0.83-1.34) | 0.89 (0.56-1.41) | |
| rs11070844 | | | | | | | |
| CC | 493/480 | 59/75 | 1.00 (ref.) | 0.77 (0.53-1.11) | 1.00 (ref.) | 0.74 (0.50-1.07) | 0.29 |
| TC+TT | 117/117 | 17/14 | 0.99 (0.75-1.31) | 1.26 (0.61-2.61) | 0.99 (0.74-1.32) | 1.16 (0.55-2.44) | |

[a]Crude.
[b]Adjusted for parity (parous/nulliparous, number of births, age at first birth), length of school education (low, medium, high), duration of HRT use (years), body mass index (kg/m2) and alcohol intake (increment of 10 g per day) at baseline.
[c]P-value for interaction for the adjusted risk estimates.

**Table S7: IRR for BC in relation to combinations of *PPARGC1A* Thr$^{612}$Met and *CYP19A1* genotypes**

| *Genotype* | *PPARGC1A Thr$^{612}$Met* | | *PPARGC1A Thr$^{612}$Met* | | *PPARGC1A Thr$^{612}$Met* | | *P-value$^{c}$* |
|---|---|---|---|---|---|---|---|
| | *Thr/Thr $n_{cases}$/ $n_{controls}$ (n=686)* | *Met-carriers $n_{case}$/ $n_{controls}$ (n=686)* | *Thr/Thr IRR (95% CI)$^{a}$* | *Met-carriers IRR (95% CI) $^{a}$* | *Thr/Thr IRR (95% CI)$^{b}$* | *Met-carriers IRR (95% CI)$^{b}$* | |
| rs10519297 | | | | | | | |
| AA | 152/164 | 18/10 | 1.00 (ref.) | 2.12 (0.89-5.05) | 1.00 (ref.) | 1.87 (0.77-4.55) | 0.16 |
| AG+GG | 465/460 | 51/52 | 1.10 (0.85-1.42) | 1.08 (0.69-1.68) | 1.07 (0.82-1.39) | 1.02 (0.65-1.62) | |
| rs749292 | | | | | | | |
| GG | 195/178 | 21/24 | 1.00 (ref.) | 0.77 (0.41-1.42) | 1.00 (ref.) | 0.77 (0.41-1.45) | 0.18 |
| AG+AA | 422/446 | 48/38 | 0.85 (0.66-1.09) | 1.20 (0.73-1.97) | 0.86 (0.67-1.12) | 1.13 (0.68-1.89) | |
| rs1062033 | | | | | | | |
| CC | 186/163 | 17/22 | 1.00 (ref.) | 0.70 (0.36-1.34) | 1.00 (ref.) | 0.71 (0.36-1.38) | 0.13 |
| CG+GG | 431/461 | 52/40 | 0.81 (0.63-1.05) | 1.16 (0.71-1.91) | 0.82 (0.63-1.07) | 1.09 (0.65-1.81) | |
| rs10046 | | | | | | | |
| AA | 163/177 | 19/11 | 1.00 (ref.) | 2.04 (0.89-4.67) | 1.00 (ref.) | 1.82 (0.78-4.26) | 0.63 |
| AG+GG | 454/447 | 50/51 | 1.12 (0.87-1.43) | 1.09 (0.70-1.70) | 1.07 (0.83-1.38) | 1.02 (0.65-1.61) | |
| rs4646 | | | | | | | |
| CC | 326/347 | 45/24 | 1.00 (ref.) | 2.17 (1.25-3.76) | 1.00 (ref.) | 2.06 (1.17-3.65) | 0.002 |
| CA+AA | 291/277 | 24/38 | 1.11 (0.89-1.39) | 0.66 (0.38-1.12) | 1.09 (0.87-1.36) | 0.62 (0.36-1.08) | |
| rs6493487 | | | | | | | |
| AA | 366/396 | 41/34 | 1.00 (ref.) | 1.29 (0.79-2.11) | 1.00 (ref.) | 1.20 (0.73-1.99) | 0.49 |
| GA+GG | 241/228 | 28/28 | 1.22 (0.97-1.54) | 1.16 (0.67-2.00) | 1.21 (0.95-1.53) | 1.12 (0.63-1.97) | |
| rs2008691 | | | | | | | |
| AA | 427/428 | 51/41 | 1.00 (ref.) | 1.30 (0.83-2.04) | 1.00 (ref.) | 1.23 (0.77-1.95) | 0.35 |
| GA+GG | 190/196 | 18/21 | 0.96 (0.76-1.22) | 0.82 (0.43-1.57) | 0.97 (0.76-1.23) | 0.80 (0.41-1.57) | |
| rs3751591 | | | | | | | |
| TT+TC | 592/614 | 68/59 | 1.00 (ref.) | 1.20 (0.82-1.76) | 1.00 (ref.) | 1.14 (0.77-1.69) | 0.06 |
| CC | 25/10 | 1/3 | 2.59 (1.20-5.59) | - | 2.49 (1.14-5.45) | - | |
| rs2445762 | | | | | | | |
| TT | 320/331 | 38/33 | 1.00 (ref.) | 1.22 (0.75-1.99) | 1.00 (ref.) | 1.17 (0.70-1.94) | 0.64 |
| TC+CC | 297/293 | 31/29 | 1.05 (0.84-1.31) | 1.09 (0.63-1.89) | 1.08 (0.86-1.36) | 1.06 (0.60-1.87) | |
| rs11070844 | | | | | | | |
| CC | 498/504 | 53/51 | 1.00 (ref.) | 1.06 (0.70-1.62) | 1.00 (ref.) | 1.02 (0.66-1.58) | 0.58 |
| TC+TT | 119/120 | 16/11 | 1.02 (0.77-1.35) | 1.47 (0.68-3.17) | 1.02 (0.76-1.36) | 1.36 (0.61-3.02) | |

[a]Crude.
[b]Adjusted for parity (parous/nulliparous, number of births, age at first birth), length of school education (low, medium, high), duration of HRT use (years), body mass index (kg/m2) and alcohol intake (increment of 10 g per day) at baseline.
[c]P-value for interaction for the adjusted risk estimates.

**Table S8: IRR for BC per 10 g alcohol/day for combinations of *PPARG* Pro[12]Ala and *CYP19A1* genotypes**

| *Genotype* | *PPARG Pro[12]Ala* | | *PPARG Pro[12]Ala* | | *PPARG Pro[12]Ala* | | *P-value*[c] |
|---|---|---|---|---|---|---|---|
| | *Pro/Pro* $n_{cases}$/ $n_{controls}$ *(n=650)* | *Ala-carriers* $n_{case}$/ $n_{controls}$ *(n=650)* | *Pro/Pro IRR (95% CI)*[a] | *Ala-carriers IRR (95% CI)*[a] | *Pro/Pro IRR (95% CI)*[b] | *Ala-carriers IRR (95% CI)*[b] | |
| rs10519297 | | | | | | | |
| AA | 128/115 | 31/46 | 1.17 (0.96-1.42) | 1.06 (0.77-1.46) | 1.15 (0.94-1.41) | 1.06 (0.77-1.47) | 0.08 |
| AG+GG | 375/350 | 116/139 | 1.24 (1.11-1.38) | 0.97 (0.82-1.14) | 1.23 (1.10-1.36) | 0.95 (0.81-1.12) | |
| rs749292 | | | | | | | |
| GG | 158/136 | 50/57 | 1.39 (1.15-1.67) | 0.98 (0.77-1.23) | 1.39 (1.15-1.68) | 0.98 (0.77-1.24) | 0.03 |
| AG+AA | 345/329 | 97/128 | 1.16 (1.04-1.29) | 0.99 (0.83-1.19) | 1.14 (1.02-1.27) | 0.97 (0.80-1.17) | |
| rs1062033 | | | | | | | |
| CC | 146/126 | 50/51 | 1.37 (1.13-1.66) | 1.01 (0.80-1.29) | 1.37 (1.13-1.66) | 1.01 (0.79-1.28) | 0.04 |
| CG+GG | 357/339 | 97/134 | 1.17 (1.05-1.30) | 0.96 (0.80-1.16) | 1.15 (1.03-1.29) | 0.95 (0.78-1.14) | |
| rs10046 | | | | | | | |
| AA | 133/125 | 38/50 | 1.18 (0.98-1.41) | 1.12 (0.79-1.58) | 1.15 (0.95-1.39) | 1.12 (0.79-1.58) | 0.07 |
| AG+GG | 370/340 | 109/135 | 1.24 (1.11-1.37) | 0.96 (0.81-1.12) | 1.22 (1.10-1.36) | 0.94 (0.80-1.11) | |
| rs4646 | | | | | | | |
| CC | 276/240 | 78/108 | 1.20 (1.06-1.35) | 0.95 (0.77-1.18) | 1.18 (1.04-1.33) | 0.93 (0.74-1.16) | 0.08 |
| CA+AA | 227/225 | 69/77 | 1.25 (1.09-1.43) | 1.00 (0.82-1.22) | 1.24 (1.08-1.43) | 1.00 (0.82-1.22) | |
| rs6493487 | | | | | | | |
| AA | 293/287 | 90/117 | 1.18 (1.04-1.34) | 1.02 (0.84-1.23) | 1.16 (1.03-1.32) | 1.01 (0.83-1.22) | 0.07 |
| GA+GG | 210/178 | 57/68 | 1.27 (1.10-1.48) | 0.94 (0.76-1.18) | 1.26 (1.08-1.46) | 0.93 (0.74-1.16) | |
| rs2008691 | | | | | | | |
| AA | 355/318 | 98/124 | 1.21 (1.08-1.34) | 0.90 (0.75-1.09) | 1.19 (1.07-1.33) | 0.89 (0.73-1.07) | 0.04 |
| GA+GG | 148/147 | 49/61 | 1.25 (1.05-1.50) | 1.12 (0.88-1.43) | 1.23 (1.03-1.47) | 1.11 (0.87-1.41) | |
| rs3751591 | | | | | | | |
| TT+TC | 483/455 | 142/182 | 1.21 (1.11-1.33) | 0.99 (0.85-1.14) | 1.20 (1.09-1.32) | 0.98 (0.84-1.13) | 0.07 |
| CC | 20/10 | 5/3 | 1.66 (0.84-3.29) | 2.24 (0.27-18.71) | 1.71 (0.88-3.34) | 2.18 (0.25-19.35) | |
| rs2445762 | | | | | | | |
| TT | 256/246 | 82/100 | 1.20 (1.06-1.36) | 0.97 (0.80-1.18) | 1.17 (1.03-1.33) | 0.96 (0.79-1.17) | 0.09 |
| TC+CC | 247/219 | 65/85 | 1.25 (1.09-1.43) | 1.01 (0.81-1.26) | 1.25 (1.09-1.43) | 0.99 (0.79-1.24) | |
| rs11070844 | | | | | | | |
| CC | 411/378 | 109/148 | 1.23 (1.11-1.36) | 0.99 (0.84-1.16) | 1.22 (1.09-1.35) | 0.97 (0.82-1.15) | 0.10 |
| TC+TT | 92/87 | 38/37 | 1.17 (0.96-1.42) | 0.96 (0.71-1.30) | 1.16 (0.95-1.41) | 0.97 (0.71-1.31) | |

[a]Crude.
[b]Adjusted for parity (parous/nulliparous, number of births, age at first birth), length of school education (low, medium, high), duration of HRT use (years) and body mass index (kg/m2).
[c]P-value for comparison of the adjusted risk estimates.

**Table S9: IRR for BC per 10 g alcohol/day for combinations *PPARGC1A* Gly$^{482}$Ser and *CYP19A1* genotypes**

| *Genotype* | *PPARGC1A Gly$^{482}$Ser* | | *PPARGC1A Gly$^{482}$Ser* | | *PPARGC1A Gly$^{482}$Ser* | | *P-value$^{c}$* |
|---|---|---|---|---|---|---|---|
| | *Gly-carriers $n_{cases}$/ $n_{controls}$ (n=650)* | *Ser/Ser $n_{case}$/ $n_{controls}$ (n=650)* | *Gly-carriers IRR (95% CI)$^{a}$* | *Ser/Ser IRR (95% CI)$^{a}$* | *Gly-carriers IRR (95% CI)$^{b}$* | *Ser/Ser IRR (95% CI)$^{b}$* | |
| rs10519297 | | | | | | | |
| AA | 139/136 | 20/26 | 1.16 (0.97-1.38) | 0.97 (0.59-1.59) | 1.13 (0.94-1.36) | 0.99 (0.59-1.63) | 0.92 |
| AG+GG | 437/427 | 54/61 | 1.17 (1.07-1.29) | 1.12 (0.89-1.42) | 1.16 (1.05-1.27) | 1.10 (0.87-1.40) | |
| rs749292 | | | | | | | |
| GG | 185/172 | 23/21 | 1.27 (1.09-1.49) | 1.05 (0.77-1.44) | 1.27 (1.08-1.49) | 1.08 (0.79-1.49) | 0.50 |
| AG+AA | 391/391 | 41/66 | 1.13 (1.03-1.25) | 1.11 (0.83-1.47) | 1.11 (1.00-1.22) | 1.06 (0.79-1.42) | |
| rs1062033 | | | | | | | |
| CC | 173/157 | 23/20 | 1.25 (1.06-1.47) | 1.16 (0.81-1.64) | 1.24 (1.05-1.46) | 1.19 (0.84-1.69) | 0.59 |
| CG+GG | 403/406 | 41/67 | 1.14 (1.04-1.26) | 1.04 (0.79-1.38) | 1.13 (1.02-1.24) | 1.00 (0.75-1.33) | |
| rs10046 | | | | | | | |
| AA | 152/152 | 19/24 | 1.18 (0.99-1.40) | 0.99 (0.60-1.64) | 1.15 (0.97-1.38) | 1.00 (0.60-1.67) | 0.94 |
| AG+GG | 424/411 | 55/63 | 1.17 (1.06-1.28) | 1.12 (0.88-1.41) | 1.15 (1.05-1.27) | 1.10 (0.87-1.39) | |
| rs4646 | | | | | | | |
| CC | 311/305 | 43/44 | 1.15 (1.03-1.28) | 1.06 (0.76-1.47) | 1.13 (1.01-1.26) | 1.01 (0.72-1.42) | 0.84 |
| CA+AA | 265/258 | 31/43 | 1.20 (1.05-1.35) | 1.12 (0.85-1.47) | 1.18 (1.04-1.34) | 1.14 (0.86-1.50) | |
| rs6493487 | | | | | | | |
| AA | 342/345 | 42/59 | 1.14 (1.02-1.28) | 1.11 (0.86-1.44) | 1.12 (1.00-1.26) | 1.12 (0.87-1.45) | 0.89 |
| GA+GG | 234/218 | 32/28 | 1.19 (1.05-1.36) | 1.08 (0.74-1.57) | 1.18 (1.04-1.34) | 1.03 (0.70-1.52) | |
| rs2008691 | | | | | | | |
| AA | 403/381 | 49/61 | 1.16 (1.05-1.28) | 0.97 (0.73-1.28) | 1.14 (1.04-1.26) | 0.94 (0.71-1.25) | 0.43 |
| GA+GG | 173/182 | 25/26 | 1.19 (1.02-1.40) | 1.42 (0.91-2.21) | 1.17 (1.01-1.37) | 1.40 (0.91-2.13) | |
| rs3751591 | | | | | | | |
| TT+TC | 551/552 | 74/85 | 1.16 (1.07-1.26) | 1.12 (0.90-1.39) | 1.14 (1.05-1.24) | 1.11 (0.89-1.38) | 0.37 |
| CC | 25/11 | -/2 | 3.85 (1.02-14.58) | - | 3.70 (0.99-13.85) | - | |
| rs2445762 | | | | | | | |
| TT | 303/301 | 39/46 | 1.13 (1.01-1.26) | 1.21 (0.91-1.60) | 1.10 (0.98-1.23) | 1.19 (0.90-1.58) | 0.42 |
| TC+CC | 273/262 | 35/41 | 1.22 (1.08-1.38) | 0.95 (0.68-1.33) | 1.21 (1.07-1.37) | 0.94 (0.67-1.32) | |
| rs11070844 | | | | | | | |
| CC | 463/451 | 57/74 | 1.17 (1.07-1.29) | 1.13 (0.86-1.49) | 1.16 (1.05-1.27) | 1.10 (0.83-1.46) | 0.83 |
| TC+TT | 113/113 | 17/13 | 1.15 (0.96-1.38) | 0.97 (0.69-1.36) | 1.14 (0.95-1.37) | 0.98 (0.70-1.38) | |

[a]Crude.
[b]Adjusted for parity (parous/nulliparous, number of births, age at first birth), length of school education (low, medium, high), duration of HRT use (years) and body mass index (kg/m2).
[c]P-value for comparison of the adjusted risk estimates.

**Table S10: IRR for BC per 10 g alcohol/day for combinations of *PPARGC1A* Thr$^{612}$Met and *CYP19A1* genotypes**

| *Genotype* | *PPARGC1A Thr$^{612}$Met* | | *PPARGC1A Thr$^{612}$Met* | | *PPARGC1A Thr$^{612}$Met* | | *P-value$^{c}$* |
|---|---|---|---|---|---|---|---|
| | *Thr/Thr $n_{cases}$/ $n_{controls}$ (n=650)* | *Met-carriers $n_{case}$/ $n_{controls}$ (n=650)* | *Thr/Thr IRR (95% CI)$^{a}$* | *Met-carriers IRR (95% CI) $^{a}$* | *Thr/Thr IRR (95% CI)$^{b}$* | *Met-carriers IRR (95% CI)$^{b}$* | |
| rs10519297 | | | | | | | |
| AA | 144/154 | 15/8 | 1.11 (0.92-1.33) | 1.24 (0.75-2.06) | 1.10 (0.91-1.32) | 1.22 (0.72-2.06) | 0.96 |
| AG+GG | 445/440 | 56/48 | 1.16 (1.06-1.28) | 1.15 (0.88-1.52) | 1.15 (1.05-1.26) | 1.11 (0.85-1.47) | |
| rs749292 | | | | | | | |
| GG | 190/174 | 18/22 | 1.23 (1.06-1.43) | 1.24 (0.73-2.11) | 1.23 (1.06-1.44) | 1.21 (0.71-2.06) | 0.63 |
| AG+AA | 399/424 | 43/34 | 1.12 (1.01-1.24) | 1.13 (0.86-1.48) | 1.10 (0.99-1.22) | 1.09 (0.83-1.43) | |
| rs1062033 | | | | | | | |
| CC | 182/156 | 14/20 | 1.22 (1.05-1.43) | 1.40 (0.79-2.47) | 1.22 (1.04-1.43) | 1.36 (0.77-2.40) | 0.64 |
| CG+GG | 407/438 | 47/36 | 1.13 (1.02-1.24) | 1.10 (0.85-1.43) | 1.11 (1.01-1.23) | 1.06 (0.81-1.38) | |
| rs10046 | | | | | | | |
| AA | 155/167 | 16/9 | 1.13 (0.95-1.35) | 1.31 (0.78-2.21) | 1.12 (0.93-1.33) | 1.28 (0.74-2.20) | 0.96 |
| AG+GG | 434/427 | 45/47 | 1.16 (1.05-1.27) | 1.14 (0.87-1.49) | 1.14 (1.04-1.26) | 1.10 (0.84-1.44) | |
| rs4646 | | | | | | | |
| CC | 315/328 | 39/21 | 1.11 (0.99-1.25) | 1.25 (0.87-1.79) | 1.09 (0.97-1.23) | 1.21 (0.84-1.73) | 0.49 |
| CA+AA | 274/266 | 22/35 | 1.19 (1.06-1.34) | 0.91 (0.58-1.42) | 1.19 (1.05-1.34) | 0.86 (0.55-1.36) | |
| rs6493487 | | | | | | | |
| AA | 348/375 | 36/30 | 1.13 (1.02-1.26) | 1.16 (0.85-1.58) | 1.12 (1.00-1.25) | 1.11 (0.80-1.52) | 0.95 |
| GA+GG | 241/219 | 25/26 | 1.18 (1.03-1.34) | 1.24 (0.87-1.77) | 1.16 (1.02-1.33) | 1.22 (0.85-1.73) | |
| rs2008691 | | | | | | | |
| AA | 406/405 | 46/37 | 1.11 (1.01-1.23) | 1.24 (0.94-1.64) | 1.10 (1.00-1.21) | 1.20 (0.91-1.59) | 0.46 |
| GA+GG | 183/189 | 15/19 | 1.24 (1.07-1.45) | 0.86 (0.45-1.64) | 1.22 (1.05-1.42) | 0.80 (0.41-1.58) | |
| rs3751591 | | | | | | | |
| TT+TC | 565/584 | 60/53 | 1.15 (1.06-1.24) | 1.17 (0.92-1.48) | 1.13 (1.04-1.23) | 1.13 (0.90-1.44) | 0.86 |
| CC | 24/10 | 1/3 | 1.46 (0.76-2.79) | - | 1.50 (0.79-2.85) | - | |
| rs2445762 | | | | | | | |
| TT | 305/317 | 33/29 | 1.13 (1.01-1.26) | 1.15 (0.87-1.53) | 1.11 (0.99-1.24) | 1.10 (0.83-1.47) | 0.90 |
| TC+CC | 284/277 | 28/27 | 1.18 (1.05-1.32) | 1.24 (0.81-1.91) | 1.17 (1.04-1.32) | 1.21 (0.78-1.86) | |
| rs11070844 | | | | | | | |
| CC | 473/478 | 46/47 | 1.18 (1.08-1.29) | 1.07 (0.82-1.41) | 1.16 (1.06-1.28) | 1.03 (0.78-1.36) | 0.55 |
| TC+TT | 116/116 | 15/9 | 1.07 (0.90-1.26) | 1.62 (0.84-3.10) | 1.07 (0.90-1.26) | 1.51 (0.80-2.84) | |

[a]Crude.
[b]Adjusted for parity (parous/nulliparous, number of births, age at first birth), length of school education (low, medium, high), duration of HRT use (years) and body mass index (kg/m2) at baseline.
[c]P-value for comparison of the adjusted risk estimates.

**Table S11. IRR for BC per 10 g alcohol/day in relation to combinations of NSAID use and *CYP19A1* polymorphisms**

| *Genotype* | *No* $n_{cases}$/ $n_{controls}$ *(n=640)* | *Yes* $n_{cases}$/ $n_{controls}$ *(n=640)* | *No* *IRR (95% CI)*[a] | *Yes* *IRR (95% CI)*[a] | *No* *IRR (95% CI)*[b] | *Yes* *IRR (95% CI)*[b] | *P-value*[c] |
|---|---|---|---|---|---|---|---|
| rs10519297 | | | | | | | |
| AA | 81/104 | 76/55 | 1.16 (0.92-1.45) | 1.14 (0.89-1.46) | 1.13 (0.89-1.43) | 1.14 (0.88-1.47) | 0.94 |
| AG+GG | 286/315 | 197/166 | 1.12 (1.00-1.26) | 1.18 (1.04-1.35) | 1.10 (0.98-1.24) | 1.17 (1.02-1.33) | |
| rs749292 | | | | | | | |
| GG | 119/128 | 86/63 | 1.14 (0.95-1.37) | 1.35 (1.06-1.69) | 1.14 (0.95-1.38) | 1.33 (1.06-1.68) | 0.49 |
| AG+AA | 248/291 | 187/158 | 1.13 (0.99-1.28) | 1.11 (0.97-1.28) | 1.10 (0.96-1.25) | 1.09 (0.95-1.26) | |
| rs1062033 | | | | | | | |
| CC | 111/115 | 82/58 | 1.16 (0.95-1.41) | 1.30 (1.03-1.64) | 1.17 (0.96-1.43) | 1.28 (1.02-1.61) | 0.67 |
| CG+GG | 256/304 | 191/163 | 1.12 (0.99-1.27) | 1.13 (0.99-1.29) | 1.09 (0.96-1.24) | 1.12 (0.97-1.28) | |
| rs10046 | | | | | | | |
| AA | 87/113 | 81/59 | 1.17 (0.94-1.47) | 1.18 (0.93-1.50) | 1.14 (0.90-1.44) | 1.17 (0.91-1.50) | 0.93 |
| AG+GG | 280/306 | 192/162 | 1.12 (0.99-1.25) | 1.17 (1.03-1.34) | 1.10 (0.97-1.24) | 1.16 (1.01-1.32) | |
| rs4646 | | | | | | | |
| CC | 193/219 | 157/122 | 1.12 (0.97-1.29) | 1.16 (0.99-1.36) | 1.09 (0.94-1.26) | 1.14 (0.98-1.34) | 0.91 |
| CA+AA | 174/200 | 116/99 | 1.14 (0.98-1.33) | 1.19 (1.00-1.43) | 1.13 (0.97-1.32) | 1.18 (0.99-1.41) | |
| rs6493487 | | | | | | | |
| AA | 209/263 | 169/136 | 1.14 (0.99-1.31) | 1.14 (0.97-1.33) | 1.12 (0.98-1.29) | 1.12 (0.96-1.31) | 0.83 |
| GA+GG | 158/156 | 104/87 | 1.11 (0.94-1.30) | 1.23 (1.02-1.48) | 1.08 (0.91-1.28) | 1.21 (1.01-1.46) | |
| rs2008691 | | | | | | | |
| AA | 253/282 | 192/153 | 1.11 (0.99-1.26) | 1.15 (1.00-1.31) | 1.08 (0.96-1.23) | 1.14 (0.99-1.31) | 0.81 |
| GA+GG | 114/137 | 81/68 | 1.18 (0.97-1.43) | 1.25 (1.00-1.56) | 1.17 (0.96-1.43) | 1.21 (0.97-1.50) | |
| rs3751591 | | | | | | | |
| TT+TC | 350/409 | 265/218 | 1.12 (1.00-1.24) | 1.17 (1.04-1.32) | 1.09 (0.97-1.21) | 1.16 (1.03-1.31) | 0.29 |
| CC | 17/10 | 8/3 | 1.47 (0.76-2.82) | 32 (0.42-2483) | 1.60 (0.83-3.08) | 26.0 (0.32-2191) | |
| rs2445762 | | | | | | | |
| TT | 192/217 | 142/122 | 1.16 (1.00-1.35) | 1.09 (0.95-1.26) | 1.13 (0.97-1.32) | 1.07 (0.93-1.23) | 0.35 |
| TC+CC | 175/192 | 131/99 | 1.10 (0.96-1.27) | 1.32 (1.09-1.61) | 1.09 (0.94-1.26) | 1.33 (1.09-1.62) | |
| rs11070844 | | | | | | | |
| CC | 303/340 | 207/180 | 1.18 (1.06-1.33) | 1.14 (0.99-1.30) | 1.16 (1.03-1.31) | 1.12 (0.98-1.28) | 0.20 |
| TC+TT | 64/79 | 66/41 | 0.91 (0.71-1.16) | 1.32 (1.00-1.75) | 0.89 (0.69-1.16) | 1.31 (1.00-1.72) | |

[a]Crude.
[b]Adjusted for parity (parous/nulliparous, number of births, age at first birth), length of school education (low, medium, high), duration of HRT use (years) and body mass index (kg/m$^2$) at baseline.
[c]*P*-value for comparison of the adjusted risk estimates.

**Table S12. Hormones, SHBG and ethanol measurements after acute ingestion of alcohol.**

| | ***PPARG* wild types (n=18)** | | ***PPARG* $^{12}$Ala carriers (n=7)** | |
|---|---|---|---|---|
| | *Alcohol+ placebo* | *Alcohol+ Ibuprofen* | *Alcohol+ placebo* | *Alcohol+ Ibuprofen* |
| **Estrone pmol/l** | | | | |
| 0 min | 26.25±12.94 | 27.50±16.53 | 14.60±7.31 | 31.50±2.50 |
| 30 min | 16.50±13.10 | 15.23±10.17 | 10.00±10.00 | 19.00±15.58 |
| 60 min | 15.36±10.41 | 11.60±9.19 | 9.83±8.90 | 8.00±8.04 |
| 90 min | 13.20±12.69 | 9.00±9.18 | 13.67±9.88 | 13.50±9.54 |
| 1200 min | 24.56±17.71 | 30.45±19.61 | 18.40±11.81 | 32.00±32.47 |
| **Estrone sulphate pmol/l** | | | | |
| 0 min | 797.45±493.44 | 731.39±473.95 | 492.29±162.22 | 714.79±422.03 |
| 30 min | 575.78±285.18 | 714.56±519.96 | 426.64±105.34 | 699.14±415.42 |
| 60 min | 578.29±473.07 | 627.67±452.80 | 413.36±110.07 | 640.21±395.21 |
| 90 min | 750.38±527.00 | 659.56±496.70 | 428.07±124.82 | 652.29±389.76 |
| 1200 min | 626.89±375.54 | 698.11±505.23 | 468.29±149.23 | 871.14±764.29 |
| **SHBG nmol/l** | | | | |
| 0 min | 64.96±26.99 | 65.01±22.27 | 69.23±16.00 | 66.58±13.90 |
| 30 min | 61.62±21.74 | 67.75±25.31 | 71.18±17.00 | 68.26±13.80 |
| 60 min | 62.41±23.93 | 61.38±18.45 | 65.18±12.54 | 66.32±12.08 |
| 90 min | 59.90±23.59 | 62.82±21.64 | 69.51±16.20 | 59.67±13.12 |
| 1200 min | 65.39±30.15 | 64.58±22.25 | 70.58±15.71 | 65.51±22.00 |
| **Ethanol g/l** | | | | |
| 0 min | <0.00 | <0.00 | <0.00 | <0.00 |
| 30 min | 0.19±0.12 | 0.20±0.11 | 0.11±0.076 | 0.19±0.086 |
| 60 min | 0.32±0.094 | 0.34±0.088 | 0.29±0.036 | 0.33±0.048 |
| 90 min | 0.30±0.070 | 0.31±0.062 | 0.28±0.028 | 0.32±0.047 |
| 1200 min | <0.00 | <0.00 | <0.00 | <0.00 |

Blood concentrations of estrone, estrone sulphate, SHBG and ethanol before and after 30, 60, 90 and 1200 minutes of drinking, respectively. Values are presented as means ± SD.

# Supplementary material for Paper VI

**Table S1. Result from Fig. 1-7 presented as values ±SD and %CV.**

| Transient transfection assays | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **Full-length PPARγ2** | | | | **Full-length PPARγ2 with 1 µM Rosiglitazone** | | | | |
| Conc. (%) | 0.1 | 0.3 | 1.0 | 3.0** | 0.0*** | 0.1 | 0.3 | 1.0 | 3.0** |
| | *DMSO* | | | | *DMSO* | | | | |
| Mean* (±SD) | 0.90 (±0.12) | 0.73 (±0.09) | 0.47 (±0.16) | 0.14 (±0.13) | 5.83 (±2.11) | 5.87 (±1.88) | 4.92 (±0.82) | 2.93 (±1.14) | 0.56 (±0.45) |
| %CV | 12.96 | 12.07 | 33.46 | 90.02 | 36.16 | 32.03 | 16.64 | 39.01 | 80.47 |
| | *Isopropanol* | | | | *Isopropanol* | | | | |
| Mean* (±SD) | 0.99 (±0.07) | 0.92 (±0.05) | 0.89 (±0.24) | 0.88 (±0.29) | 4.57 (±0.27) | 4.36 (±0.68) | 4.70 (±0.31) | 4.34 (±0.73) | 3.53 (±1.18) |
| %CV | 6.60 | 5.24 | 26.66 | 33.43 | 5.82 | 15.61 | 6.70 | 16.82 | 33.35 |
| | *Methanol* | | | | *Methanol* | | | | |
| Mean* (±SD) | 0.94 (±0.15) | 0.86 (±0.20) | 0.78 (±0.17) | 0.57 (±0.41) | 5.26 (±0.89) | 5.31 (±0.86) | 4.74 (±0.30) | 4.32 (±0.41) | 3.07 (±2.26) |
| %CV | 16.31 | 23.53 | 21.80 | 71.93 | 16.90 | 16.22 | 6.36 | 9.59 | 73.48 |
| | *Ethylene glycol* | | | | *Ethylene glycol* | | | | |
| Mean* (±SD) | 0.94 (±0.08) | 0.90 (±0.08) | 0.71 (±0.12) | 0.58 (±0.10) | 5.39 (±0.93) | 5.37 (±0.67) | 5.36 (±0.90) | 4.77 (±0.34) | 3.18 (±0.27) |
| %CV | 8.85 | 8.90 | 16.24 | 18.22 | 17.23 | 12.46 | 16.74 | 7.23 | 8.42 |
| | *1-Propanol* | | | | *1-Propanol* | | | | |
| Mean* (±SD) | 0.75 (±0.12) | 0.74 (±0.10) | 0.93 (±0.22) | | 4.66 (±0.85) | 4.20 (±0.35) | 4.13 (±0.71) | 4.02 (±0.24) | |
| %CV | 16.02 | 14.18 | 23.96 | | 18.18 | 8.26 | 17.12 | 5.85 | |
| | *Acetone* | | | | *Acetone* | | | | |
| Mean* (±SD) | 1.04 (±0.43) | 1.12 (±0.58) | 1.17 (±0.59) | | 5.53 (±0.75) | 4.80 (±0.41) | 4.46 (±0.58) | 4.92 (±1.16) | |
| %CV | 41.40 | 52.23 | 50.09 | | 13.62 | 8.51 | 12.94 | 23.56 | |
| | *Acetonitril* | | | | *Acetonitril* | | | | |
| Mean* (±SD) | 0.92 (±0.03) | 0.82 (±0.07) | 0.90 (±0.06) | | 5.06 (±0.22) | 5.20 (±0.63) | 4.75 (±0.24) | 5.09 (±0.88) | |
| %CV | 3.50 | 8.27 | 6.26 | | 4.37 | 12.18 | 5.12 | 17.30 | |
| | *N,N-Dimethylformamide* | | | | *N,N-Dimethylformamide* | | | | |
| Mean* (±SD) | 0.73 (±0.06) | 0.61 (±0.06) | 0.27 (±0.03) | | 5.05 (±1.09) | 4.17 (±0.48) | 3.10 (±0.55) | 1.23 (±0.19) | |
| %CV | 7.86 | 9.43 | 11.17 | | 21.66 | 11.39 | 17.78 | 15.78 | |
| | *Ethyl acetate* | | | | *Ethyl acetate* | | | | |
| Mean* (±SD) | 0.89 (±0.03) | 0.79 (±0.10) | 0.74 (±0.05) | 0.61 (±0.14) | 5.75 (±1.00) | 5.27 (±0.75) | 5.01 (±0.75) | 4.44 (±0.20) | 3.89 (±0.54) |
| %CV | 3.39 | 12.53 | 6.49 | 23.43 | 17.42 | 14.25 | 15.03 | 4.47 | 13.82 |
| | *1,4-Dioxane* | | | | *1,4-Dioxane* | | | | |
| Mean* (±SD) | 0.92 (±0.17) | 0.72 (±0.18) | 0.59 (±0.29) | | 6.49 (±0.78) | 6.46 (±1.32) | 5.11 (±0.81) | 3.25 (±1.32) | |
| %CV | 17.97 | 25.02 | 49.63 | | 12.01 | 20.37 | 15.79 | 40.54 | |
| | **pcDNA3hPPARγΔAB** | | | | **pcDNA3hPPARγΔAB with 1 µM Rosiglitazone** | | | | |
| Conc. (%) | 0.1 | 0.3 | 1.0 | 3.0** | 0.0*** | 0.1 | 0.3 | 1.0 | 3.0** |
| | *DMSO* | | | | *DMSO* | | | | |
| Mean* (±SD) | 0.89 (±0.14) | 0.76 (±0.17) | 0.56 (±0.16) | 0.23 (±0.07) | 5.89 (±1.02) | 5.25 (±0.25) | 5.62 (±1.08) | 4.70 (±0.82) | 1.12 (±0.29) |
| %CV | 15.73 | 21.90 | 29.59 | 29.02 | 17.30 | 4.86 | 19.26 | 17.52 | 25.87 |
| | *Ethylene glycol* | | | | *Ethylene glycol* | | | | |
| Mean* (±SD) | 0.95 (±0.14) | 0.90 (±0.16) | 0.92 (±0.28) | 0.58 (±0.19) | 8.79 (±0.53) | 8.96 (±0.65) | 9.26 (±0.78) | 8.09 (±1.64) | 5.39 (±1.41) |

| %CV | 14.50 | 17.36 | 30.32 | 32.63 | 6.05 | 7.28 | 8.39 | 20.27 | 26.22 |
|---|---|---|---|---|---|---|---|---|---|
| | *Ethyl acetate* | | | | *Ethyl acetate* | | | | |
| Mean* (±SD) | 1.29 (±0.54) | 1.14 (±0.25) | 1.02 (±0.27) | 0.86 (±0.20) | 7.50 (±1.05) | 7.73 (±0.65) | 7.84 (±0.23) | 5.52 (±1.13) | 2.41 (±0.38) |
| %CV | 41.70 | 22.11 | 26.58 | 23.53 | 14.03 | 8.46 | 2.98 | 20.50 | 15.71 |
| | **pM1-hPPARγLBD** | | | | **pM1-hPPARγLBD** | | | | |
| Conc. (%) | 0.1 | 0.3 | 1.0 | 3.0** | 0.0*** | 0.1 | 0.3 | 1.0 | 3.0** |
| | *DMSO* | | | | *DMSO* | | | | |
| Mean* (±SD) | 1.09 (±0.11) | 0.92 (±0.07) | 0.78 (±0.08) | 0.82 (±0.15) | 16.86 (±4.16) | 17.15 (±3.61) | 16.59 (±3.32) | 11.94 (±1.70) | 2.78 (±0.60) |
| %CV | 9.74 | 7.60 | 10.84 | 18.09 | 24.69 | 21.08 | 20.04 | 14.26 | 21.58 |
| | *Ethylene glycol* | | | | *Ethylene glycol* | | | | |
| Mean* (±SD) | 1.20 (±0.09) | 1.07 (±0.17) | 1.08 (±0.29) | 0.98 (±0.29) | 23.71 (±7.00) | 20.15 (±6.30) | 19.80 (±7.83) | 16.11 (±5.91) | 9.51 (±4.99) |
| %CV | 7.88 | 15.53 | 26.47 | 30.17 | 29.53 | 31.26 | 39.51 | 36.69 | 52.48 |
| | *Ethyl acetate* | | | | *Ethyl acetate* | | | | |
| Mean* (±SD) | 1.16 (±0.07) | 1.34 (±0.23) | 1.37 (±0.16) | 1.60 (±0.96) | 14.10 (±0.24) | 14.29 (±2.70) | 13.36 (±0.67) | 8.13 (±4.67) | 5.25 (±3.08) |
| %CV | 5.99 | 16.88 | 11.35 | 60.07 | 1.74 | 18.88 | 5.01 | 57.47 | 58.66 |
| **Hormone measurements** | | | | | | | | | |
| **Estradiol (pg/ml)** | | | | | **Testosterone (ng/ml)** | | | | |
| Control | 0.3% Etylene glycol | 0.3% DMSO | 3% Acetone | 3% Etyl acetate | Control | 0.3% Etylene glycol | 0.3% DMSO | 3% Acetone | 3% Etyl acetate |
| Mean (±SD) | | | | | | | | | |
| 242.90 (±20.28) | 288.83 (±7.01) | 248.68 (±13.06) | 260.13 (±5.97) | 230.25 (±28.75) | 1.21 (±0.07) | 1.12 (±0.07) | 1.29 (±0.08) | 1.07 (±0.02) | 1.04 (±0.04) |
| %CV | | | | | | | | | |
| 8.35 | 2.43 | 5.25 | 2.30 | 12.49 | 5.79 | 6.25 | 6.20 | 1.87 | 3.85 |

* Normalized activity values (PPARγ/pRL-CMV).
** Some of the solvents showed severe sign of cytotoxicity at 3% and the top dose was consequently reduced to 1%.
*** DMSO control

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ISBN: 978-87-93109-37-7