# Non-Steroidal Anti-Inflammatory Drugs, Chronic Inflammation and Colorectal Cancer Risk

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## Abstract

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Substantial experimental and epidemiological evidence shows that long-term use of aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) are protective against colorectal cancer (CRC). However, the underlying chemopreventive mechanisms of NSAIDs are not fully understood, and whether there are specific subgroups of the population for whom the benefits of NSAIDs clearly outweigh the risk remains unknown. Using information from 11,894 cases and 15,999 controls from the Genetics and Epidemiology of Colorectal Cancer Consortium (GECCO) and the Colon Cancer Family Registry (CCFR), we systematically evaluated the interactions between regular use of NSAIDs (aspirin and non-aspirin NSAIDs) and other lifestyle and dietary factors in relation to CRC risk (Aim 1). NSAIDs were also reported to reduce the concentration of C-reactive protein (CRP), a biomarker of low-grade chronic inflammation that

has been moderately associated with the risk of CRC. We used Mendelian randomization analysis to investigate whether the relationship between circulating CRP level and CRC risk were causal among 30,480 CRC cases and 22,844 controls from 33 observational studies (Aim 2). In addition to CRP-related pathways, several other inflammatory or carcinogenic pathways have been found to be involved in chemopreventive effect of aspirin. However, no trial has been carried out to systematically explore the biological mechanisms of aspirin in healthy humans. We assessed the difference in plasma protein levels after 60 days of regular dose aspirin (325 mg/day) compared to placebo in a randomized double-blinded crossover trial of 44 healthy non-smoking men and women, aged 21-45 years (Aim 3).

In Aim 1, we found that the association between aspirin and CRC risk statistically significantly differed by smoking status after adjusting for other risk factors (P-interaction=0.048). Regular aspirin use was associated with a 29% lower risk of CRC among non-smokers (OR=0.71; 95% CI: 0.64, 0.79), whereas it was associated with 19% and 17% lower risk of CRC among smokers of pack-years below median (OR=0.81; 95% CI: 0.71, 0.92) and above median (OR=0.83; 95% CI: 0.74, 0.94), respectively. There was a suggestive interaction between regular use of any NSAID and body mass index (BMI) (P-interaction=0.075), where the association of any NSAID on CRC risk was attenuated with increasing BMI (normal: OR=0.69; 95% CI: 0.63, 0.77; overweight: OR=0.76; 95% CI: 0.70, 0.83; obese: OR=0.85; 95% CI: 0.75, 0.96). We did not observe interactions between non-aspirin NSAIDs and other CRC risk factors.

In Aim 2, two of the 19 selected SNPs were significantly associated with CRC risk, where rs1260326 was significantly associated with higher risk of CRC ($p=7.5\times10^{-4}$), and rs6734238 was associated with lower CRC risk (p=0.003). Using all selected SNPs as instrumental variables, we found that a genetically predicted one-unit increase in the log-transformed CRP

(mg/L) level was associated with a non-significant 4% higher risk of CRC (OR=1.04; 95% CI: 0.97, 1.12; p=0.256). Genetically elevated CRP was also not associated with CRC risk among subgroups of the population stratified by other risk factors.

In Aim 3, among the 3,000 antibodies analyzed, statistically significant differences in plasma protein levels were observed for nine antibodies after adjusting for false discoveries (FDR adjusted p-value<0.1). The most significant protein was succinate dehydrogenase subunit C (SDHC), a key enzyme complex of the mitochondrial tricarboxylic acid (TCA) cycle. The other statistically significant proteins (NR2F1, MSI1, MYH1, FOXO1, KHDRBS3, NFKBIE, LYZ and IKZF1) are involved in multiple pathways, including DNA base-pair repair, inflammation and oncogenic pathways. However, none of the 258 KEGG and 1,139 GO pathways was found to be statistically significant after FDR adjustment.

Our results suggest that the association between regular use of NSAIDs, primarily driven by aspirin, and CRC risk may be modified by smoking status and BMI. The beneficial effect of aspirin on CRC risk appears to be attenuated, rather than enhanced, among those with greater CRC risk due to obesity and heavy smoking, making it unlikely that these groups would benefit from use of aspirin. In addition, we observed no association between genetically elevated CRP levels and CRC risk, suggesting that circulating CRP is unlikely to play a causal role in the development of CRC. However, several other proteins may be involved in the chemopreventive mechanisms of aspirin on CRC risk, but larger and confirmatory studies are needed.

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# DEDICATION

For my parents, Jie Wang and Qianwei Zhu, for their continual love and support.

# Chapter 1. INTRODUCTION: BACKGROUND, AIMS AND SIGNIFICANCE

## Colorectal Cancer

Colorectal cancer (CRC) is one of the most common and fatal cancers in the world. In the US, there are estimated 135,430 new cases and 50,260 deaths in 2017 (1). CRC is heterogeneous in nature. Approximately 20% of CRC cases have at least one first-degree relatives affected, among which 5-10% are hereditary, with two major types of familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC) (2). However, the majority of CRC cases are sporadic, and epidemiological studies suggest that a number of other risk factors are associated with increased risk of CRC, including obesity, high consumption of red and/or processed meat, physical inactivity, smoking, and moderate-to-heavy alcohol consumption (3). Conversely, high intake of dietary fiber from fruits and vegetables, and the use of non-steroidal anti-inflammatory drugs (NSAIDs) are associated with lower CRC risk (4). It is also known that most colorectal carcinomas develop slowly from adenoma via the adenoma-carcinoma sequence, which can occur over a period of 10 to 15 years (5). As modifiable risk factors represent an important factor in CRC risk, it is thought that 50-80% of sporadic CRC cases are potentially preventable either by changing lifestyle or through the use of chemopreventive agents (6).

In terms of primary and secondary prevention, screening for CRC using high-sensitivity fecal occult blood testing, sigmoidoscopy, or colonoscopy among high-risk individuals remains the mainstay (7). Whilst screening has a great advance in early detection of tumors and removing precancerous adenomas, screening strategies are expensive (8). Evidence from randomized controlled trials and cohort studies also suggest that sigmoidoscopy or colonoscopy might not be

as effective in identifying proximal colon cancer, compared to distal colon cancer and rectal cancer (9, 10).

**Chronic inflammation and colorectal cancer**

Chronic inflammation is characterized as a state of continuous, unresolved, low-grade inflammation in response to tissue damage (11), which increases the levels of growth factors, cytokines, and reactive oxygen and nitrogen species that may cause DNA damage (12). Chronic inflammation has also been established as a risk factor for CRC (13). Patients with chronic inflammatory bowel diseases, including ulcerative colitis and Crohn's disease, are found to have a higher risk of CRC than the general population (14, 15). The molecular mechanisms by which chronic inflammation plays a role in cancer development, however, are still being uncovered. Evidence has emerged from genetic, pharmacological and epidemiological data that immune cells, cytokines and other immune mediators play important roles in colon tumorigenesis, including initiation, promotion, progression and metastasis (16). Proinflammatory signaling by tumor necrosis factor (TNF)-α is shown to activate NF-κB/Akt pathways in colon tumor cells, and promote β-catenin signaling, which is essential for formation of adenoma (17, 18), and is linked with the promotion of colon cancer in T cells of patients with colon cancer (19). Higher level of inflammation also increases the expression of cyclooxygenase 2 (COX-2), the rate-limiting enzyme in prostaglandin biosynthesis, which plays a role in the adenoma-to-carcinoma transition. In addition, inflammatory cytokines can serve as tumor growth and survival factors, and can promote angiogenesis and suppress immune-mediated tumor elimination (20, 21). However, results from epidemiological studies on the association between inflammation biomarkers and CRC risk are inconsistent. A meta-analysis of prospective studies found moderate association between elevated CRP and increased risk of CRC (22), but no association

was found in a meta-analysis of two other cohort studies (23). The association between interleukin-6 (IL-6) and CRC risk was suggested by a cohort study among older individuals (24), but not confirmed in later prospective studies (23). A recent meta-analysis of 10 observational studies suggested that higher CRP levels were statistically significantly associated with higher risk of advanced colorectal adenoma, and the association may differ by smoking and use of aspirin or NSAIDs (25), but no statistically significant associations were found for TNF- α or IL-6. The mechanisms of inflammation and colorectal cancer need to be better understood in humans.

**Non-steroidal anti-inflammatory drugs and colorectal cancer**

Substantial experimental and epidemiological evidence suggests that long-term use of aspirin and other NSAIDs is associated with a 40% lower risk of CRC (26-29). A meta-analysis of five randomized trials with 20-years of follow-up found that long-term use of aspirin at doses greater than 75 mg daily significantly reduced the incidence of colon cancer by 24% (HR=0.76; 95% CI: 0.60-0.96) and mortality due to CRC by 35% (HR=0.65; 95% CI: 0.48-0.88), and the benefit increased with scheduled duration of treatment (27). The effect was reported to be greatest 10-14 years after randomization in patients who had had scheduled trial treatment of 5 years or more (HR=0.37; 95% CI: 0.20-0.70) (26). In addition, the benefit of NSAID use was found to be greatest for proximal colon cancers (HR=0.45; 95% CI: 0.28-0.74), which are not otherwise prevented effectively by screening with sigmoidoscopy or colonoscopy (27). In the meta-analysis of observational studies, a similar association between maximum use of aspirin or NSAIDs and CRC risk was also reported (OR=0.59; 95% CI: 0.52-0.68) (26).

## Potential Mechanisms of the NSAID-CRC association

Extensive effort has also been made to understand the chemopreventive mechanisms of NSAIDs on CRC risk, both in animal experiments and human studies.

### *Evidence from animal studies*

Experiments in mice and rats consistently have found significant induction of tumor apoptosis after aspirin treatment, and that aspirin inhibited tumor cell growth and proliferation (30-42). It is also suggested that administration of low doses of aspirin and other NSAIDs in combination inhibited colon carcinogenesis more effectively than when they are given individually at higher doses (42), suggesting potential ways to have added chemopreventive benefits with minimal side effects. In addition, the effect of aspirin was suggested to be stronger in tumor tissues, but not in normal tissues, suggesting that aspirin may be more effective in overexpressed oncogenic pathways.

Various mechanisms may contribute to the observed effects of suppressed tumor growth and induced apoptosis, several of which have been evaluated as potential chemopreventive mechanisms of aspirin in identified animal studies. In general, they are categorized as COX-dependent and independent pathways.

#### *COX-dependent pathways*

Animal studies support a key role of COX-2 in the initiation of CRC in which the treatment of $APC^{\Delta 716(+/-)}$ mice with a COX-2 inhibitor reduces the number of intestinal polyps (34, 43). In the nude mouse model with chemoresistant xenografts, Rahman and colleagues (34) observed higher COX-2 expression, which is inhibited significantly after chemotherapy in combination with celecoxib, but not aspirin, suggesting a major role of COX-2 in colon carcinogenesis.

Comparison between the efficacy of mofezolac, a COX-1 selective inhibitor, and nimesulide, a COX-2 selective inhibitor, also found that a higher dose of mofezolac (1200 p.p.m), is required in order to achieve the same effect as nimesulide (400 p.p.m); this supports the dominant role of COX-2 in intestinal tumorigenesis (44). Furthermore, long-term treatment with aspirin for 10 months was associated with a 50% reduction of prostaglandin $E_2$ ($PGE_2$) concentration in the rat colon (40). NO-aspirin (NO-ASA) have also been shown to inhibit not only COX-2 activity (52-75% inhibition), but also the formation of prostaglandins from arachidonic acid (53-77% inhibition) in Azoxymethane (AOM)-induced colon tumors (35). Parallel suppression in nitric oxide synthase 2 (NOS-2) catalytic activity also suggested that the interplay between NOS-2 and COX-2 signals may promote colon tumorigenesis (35).

In addition to COX-2, genetic disruption of either the prostaglandin endoperoxide synthase-1 (PTSG-1) or PTST-2 gene decreased the number of intestinal polyps in *Min* mice by ~80% (45), suggesting the involvement of COX-1 isoform, in addition to COX-2, in $PEG_2$ production and colon carcinogenesis. In addition, the expression of vascular endothelial growth factor (VEGF) was significantly inhibited in the NO-ASA injected mice, compared to non-injection and vehicle-injected mice, which possibly led to suppression of angiogenesis, followed by significantly higher necrosis in tumors, which may explain part of the antineoplastic effect of aspirin (33).

*COX-independent pathways*

Systematic review has found inconsistent interactions between COX expressions and NSAID use in relation to CRC risk (46), indicating that other pathways may also play a role. Several of these have been evaluated in animal models.

*Wnt/β-catenin:* Aspirin had significant antitumor effects in mice with dysfunction of the *APC* gene and therefore dysregulation of β-catenin activity, compared to wide-type mice (31, 32, 38). The role of β-catenin in colon carcinogenesis is well established as its overexpression leads to excessive colonocyte proliferation (47, 48). Several studies specifically evaluated the involvement of Wnt/β-catenin signaling pathway in colorectal carcinogenesis. NO-ASA via diet has been shown to suppress β-catenin expression in AOM-induced colon tumors in rats (35). In the β-catenin-/lox-villin-creERT2$^{21}$ mouse tissues, the deletion of β-catenin upregulates the prostaglandin transporter (PGT) protein (49), as well as intestinal epithelial 15-prostaglandin dehydrogenase (15-PGDH) (50). Both proteins are identified as colorectal tumor suppressors (51, 52), and are shown to be down-regulated at an early stage in colorectal tumorigenesis (53, 54). The ability of β-catenin to down-regulate both regulators of prostaglandin catabolism, suggests that the chemopreventive effect of aspirin may partially act through inhibition of β-catenin to decrease elevated prostaglandin levels during early colorectal neoplasia.

*PPARs:* Peroxisome proliferator-activated receptors (PPARs) are transcription factors that enable the cell to respond to extracellular stimuli through transcriptional regulation of gene expression (55, 56). They were also identified as a target of APC and related to the β-catenin pathway *in vitro* (57). Due to the *APC* gene knockout, significantly higher PPARδ expression was observed in *Min* mice, compared to wild-type mice, which was significantly inhibited by up to 55% after intrarectal administration of NO-ASA (100 mg/kg/day) for 3 weeks (32). The suppression of PPARδ expression levels was concomitant with 38-59% lower intestinal tumor incidence, indicating that the antitumor effect of aspirin may act, in part, through its inhibitory effect on PPARδ (32).

*NF-κB:* Aspirin was also shown to activate NF-κB signaling by inducing phosphorylation and degradation of IκBα, and to induce apoptosis in a time-dependent manner in xenograpfted HT-29 tumors and in adenomas from *Min* mice (37). Similarly, NO-ASA was associated with significantly decreased NF-κB activation in intestinal epithelial cells of *Min* mice by 38.4% ($p<0.01$), compared with the vehicle group (58). However, the nuclear levels of the two p50 and p65 NF-κB subunits were virtually unaffected, suggesting the inhibitory mechanism of aspirin may be different from directly suppression on subunit translocation into the nucleus. However, in *Min* mice, treatment of NO-ASA induced ANXA1, which inhibits the activation of NF-κB by binding to its p65 subunit, and administration of ANXA1-based oligopeptides in nude mice were shown to inhibit the growth of SW480 human colon xenografts by 58% compared to controls ($p<0.01$) (39). In addition, NO-ASA more than doubled the amount of tyrosine nitration in the HT-29 xenografts tissues in nude mice ($p=0.03$) (59). Tyrosine nitration of p65 rapidly inactivates NF-κB (60), which may further modulate cell signaling. These results, in combination, suggested that aspirin may not directly interact with NF-κB, but rather act through ANXA1 to inhibit the activity of NF-κB to inhibit colorectal tumors.

*Bcl-2/Bax:* The intrinsic apoptotic pathway is controlled by members of the Bcl-2 family, specifically the anti-apoptotic Bcl-2 protein and the pro-apoptotic Bax protein. The Bcl-2/Bax ratio is considered as a determinant factor for apoptosis in the way that the decrease in this ratio will favor mitochondria alterations and lead to apoptosis (61). It was found that aspirin treatment did not significantly modify the expression level of Bax which remained up-regulated in the mucosa of AOM rats, but rather caused a significant down-regulation of the Bcl-2 transcripts which were reduced to the level in saline control rats ($p<0.01$) (40). This finding suggested that

long-term treatment of low-dose aspirin may induce apoptosis through the activation of the intrinsic mitochondrial pathway by the decrease of the Bcl-2/Bax transcript ratio.

*Oxidative stress and redox signaling:* Oxidative stress represents an irreversible state in which the intracellular level of reactive oxygen and nitrogen species (RONS) is increased, which may contribute to the antineoplastic effect of NSAIDs (62). Levels of urinary F2-isoprostane, a marker of oxidative stress, were increased after the treatment of phosphor-ibuprofen in nude mice bearing SW480 xenografts (63). Supplementation with salicylic acid was also observed to help to alleviate the elevated plasma pyruvate kinase activity ($p<0.001$) and lipid peroxidation in the rat colon due to Vitamin E deficiency ($p<0.01$) (64). Reductions in oxidative stress and prostaglandin production on supplementation with salicylic acid were also associated with an increase in glutathione peroxidase activity, which are key antioxidant enzymes catalyzing the decomposition of potentially toxic lipid peroxides (65), and have been associated with protection against colon cancer (66, 67). In the same rat model with dietary supplementation of aspirin using a proteomic approach, a total of 35 proteins differed significantly between Vitamin E deficient diet supplemented with salicylic acid, and Vitamin E sufficient diet (68). Among the identified proteins, 7 proteins were involved in two major redox pathways of thioredoxin and glutathione, which are related to maintenance of the redox environment (68).

*Phase-II enzymes:* The balance between the phase I carcinogen-activating enzymes and the phase II detoxifying enzymes is considered to be critical to determining an individual's risk for cancer (69). Aspirin has been proposed to modulate metabolizing enzymes, particularly phase II enzymes, leading to facilitated elimination of carcinogens in cancer chemoprevention. The activities of phase II enzymes, including the NAD(P)H:quinone oxireductase-1 (NQO-1; $p<0.05$) and glutathione S-transferases (GSTP1-1 and GSTA1-1; $p<0.005$), were significantly increased

in the mouse liver and intestine, but not in kidney, after treatment of NO-ASA for three weeks, compared with untreated *Min* mice (70). However, the activity of phase I enzymes, including CYP1A1 and CYP2E1 did not differ between treated and untreated groups, indicating that *in vivo* NO-ASA is a monofunctional phase II inducer. Similar results were observed in studies specifically investigating the effect of NSAIDs and phase-II metabolizing enzymes. Dietary administration of NSAIDs have been shown to enhance GST class theta (GSTT) levels in rat colon, with a 2-fold increase in GSTT1-1 ($p<0.01$) and a 1.7-fold increase in GSTT2-2 ($p<0.05$) induced by aspirin (71). In addition, the enhancement of colonic GSTT1-1 levels seemed to be common across multiple NSAIDs treatment groups, whereas increased GSTT2-2 levels were only observed in aspirin-treated group. Similarly, oral administration of daily hydrogen sulfide-releasing aspirin (HS-ASA) at 100 mg/kg body weight for 4 weeks induced a statistically significant 1.5-fold increase in both hepatic GST and NQO1 enzyme activities of colon ($p<0.05$), whereas CYP1A1 protein levels were not altered among male Wistar rats (72). Moreover, UGT1A1 levels was significantly increased with a more than 3-fold change after aspirin treatment ($p<0.01$), but its involvement in colorectal carcinogenesis is so far unclear.

In summary, several chemopreventive mechanisms of aspirin have been suggested based on animal studies, including COX-dependent and independent pathways. However, the majority of animal studies identified used NO-aspirin, phosphor-aspirin, meta- or para- isomers of aspirin, instead of conventional aspirin. Nonconventional forms of aspirin have not been widely used in humans, and thus their efficacy and safety profiles from human studies are limited. Moreover, most animal studies administered aspirin after generation of colon tumors, and their treatment period was relatively short. It is also known that mechanisms of action in humans often differ

from those in animals. Therefore, we should be cautious on drawing optimal inferences on the long-term preventive effect of aspirin on CRC risk from these studies.

***Evidence from Human Studies***

In comparison to animal experiments, mechanistic evidence in human subjects has been mostly generated from observational studies and has used genetic variants as surrogates instead of direct measurement of enzymes.

Among the GWAS-identified CRC-related loci, the *SMAD7* gene, located on chromosome 8q21 is involved in inflammation-related pathways, and has been shown to modulate transforming growth factor-β (TNF-β) and *Wnt* signaling (73). Therefore, Slattery and colleagues (74) evaluated the interaction of three GWAS-identified SNPs within the *SMAD7* gene in a case-control study, and found that the CC genotype of rs4939827 had a larger association with lower risk of CRC among individuals reporting recent aspirin/NSAID use (OR=0.60; 95% CI: 0.43-0.85), whereas the association was not significant among non-users (OR=0.86; 95% CI: 0.68-1.09; p-heterogeneity=0.08). In addition, the TT genotype of rs1285371 had a larger association with higher risk of CRC among NSAID users (OR=1.69; 95% CI: 1.20-2.38), compared to the association among non-users (OR=1.22; 95% CI: 0.96-1.56; p-heterogeneity=0.10). SMAD7 has been reported to promote anti-inflammatory action of the TGF-β signaling pathway, which further activate NF-κB (75, 76), and has also been shown to degrade β-catenin signaling, altering the *Wnt* signaling pathway (77). Although the interaction was not statistically significant, the results suggested the interplay between aspirin and NF-κB and *Wnt*/β-catenin signaling pathways

in cancer development, which was consistent with previously discussed findings in vivo (35, 37, 39, 49, 50, 58, 59). However, other studies using identified CRC-related loci did not find statistically significant interactions between aspirin or NSAID use on CRC risk (78, 79).

A genome-wide investigation of aspirin and NSAID use and genetic variants on CRC risk in a large population found that the association between aspirin or other NSAIDs differed by genetic variation at 2 SNPs after adjustment for multiple comparisons (80). Aspirin or other NSAID use was associated with a lower risk of CRC among individuals with the TT genotype of the SNP rs2965667 (OR=0.66; 95% CI: 0.61-0.70), located chromosome 12p12.3 near the *MGST1* gene, but with a higher risk of CRC among those with TA or AA genotypes (OR=1.89; 95% CI: 1.27-2.81; p-interaction=$4.6\times10^{-9}$). MGST1 has high sequence homology to prostaglandin E synthase (MGST1L1) (81), both of which are up-regulated in CRC (82). The combined activity of MGST1L1 and COX-2 increased $PGE_2$ production, which promotes carcinogenesis through several mechanisms, including the *Wnt* signaling pathway (83, 84). The SNP rs2965667 is also located near phosphatidylinositol-4-phosphase 3-kinase, catalytic subunit type 2 gamma (PIK3C2G) gene, which has been suggested to enhance production of COX-2 and $PGE_2$ (85). Furthermore, in the case-only interaction analysis in that GWA-study (80), regular aspirin/NSAID use was associated with a lower risk of CRC among individuals with the AA genotype of the SNP rs16873225 (OR=0.66; 95% CI: 0.62-0.71), located on chromosome 15q25.2 near the IL16 gene, but had no association among those with AC or CC genotypes (OR=0.97; 95% CI: 0.78-1.20; p-interaction=$8.2\times10^{-9}$). Previous evidence suggested that IL16 may stimulate proinflammatory cytokines associated with tumorigenesis, including IL6 and TNF-α, induction of COX-2 expression, and activation of Wnt signaling (86, 87). Taken together, the SNPs identified in GWA-studies reinforce several mechanisms observed in animal

studies, including COX-2/$PGE_2$ expression and Wnt/β-catenin signaling pathways, indicating that they play central roles in the chemopreventive effect of aspirin against CRC.

Both animal studies and population-based GWA-studies have suggested the two major pathways, including COX-2/$PGE_2$ expression and Wnt/β-catenin signaling pathways; this may partially explain the protective effect of aspirin in colorectal carcinogenesis. Other pathways suggested from animal studies, did not show direct interaction with aspirin use on CRC risk in human populations. However, the GWA-studies were limited to the genetic variants, which mostly affect the expression level of enzymes involved in these pathways. It is possible that aspirin suppress the bioactivity of related enzymes, rather than their expression levels.

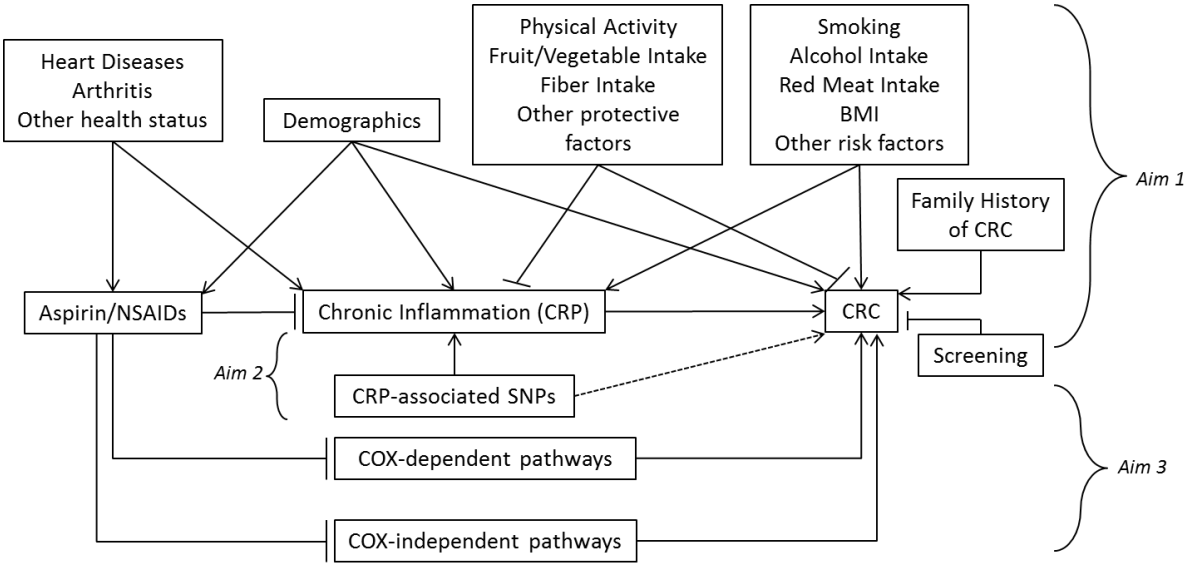
**Dissertation Aims**

In this project, we aimed to better understand the mechanisms of the chemoprevention effect of aspirin and other NSAIDs on CRC risk from three aspects (summarized in Figure 1.1). Using ~27,000 CRC cases and controls from GECCO and CCFR, we systematically evaluated whether the effect of NSAIDs on CRC risk was modified by other CRC risk factors (Aim 1). We also tested whether the relationship between circulating CRP levels and CRC risk is causal using CRP-related genetic variants as instrumental variables in a Mendelian randomization analysis, utilizing ~50,000 CRC cases and controls from GECCO, CCFR and CORECT (Aim 2). In addition, we explored other potential pathways of aspirin by systematically testing the biological effect of aspirin using microarray of 3,000 protein antibodies, in a randomized double-blinded cross-over trial among 44 healthy men and women (Aim 3).

**Dissertation Significance**

Despite the availability of screening tests, CRC remains one of the most common and fatal cancers in the US and worldwide. A majority of CRC cases is believed to be preventable based on the adenoma-carcinoma sequence in CRC development. Aspirin and other NSAIDs are promising chemopreventive candidates, but their biological mechanisms are not fully understood. Findings of our project will add to the knowledge of chemopreventive mechanisms of aspirin and NSAIDs and to the current understanding of carcinogenic pathways that are related to chronic inflammation, which can help to inform primary prevention strategies of CRC in the future.

**Figure 1.1 Overview of the relationships of aspirin/NSAID use, chronic inflammation and CRC risk**



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# Chapter 2. INTERACTIONS BETWEEN NONSTEROIDAL ANTI-INFLAMMATORY DRUGS AND OTHER FACTORS IN RELATION TO COLORECTAL CANCER RISK


## ABSTRACT

*Background:* Long-term use of nonsteroidal anti-inflammatory drugs (NSAIDs) has consistently been associated with lower risk of colorectal cancer (CRC). However, no national organizations have made recommendations to any subgroup of the population for whom the benefits of NSAID use clearly outweigh the risks. *Methods:* Using information from 11,894 cases and 15,999 controls from the Genetics and Epidemiology of Colorectal Cancer Consortium (GECCO) and the Colon Cancer Family Registry (CCFR), we performed multivariable logistic regression analysis to test for the interaction between regular use of NSAIDs (aspirin and non-aspirin NSAIDs) and other factors in relation to CRC risk, including lifestyle and dietary factors. Fixed-effects meta-analyses with inverse-variance weighting were used for stratified analyses across studies for each risk factor and to summarize the estimates from interactions. *Results:* Regular use of any NSAID, aspirin, or non-aspirin NSAIDs was statistically significantly associated with a lower risk of CRC within almost all subgroups stratified by other CRC risk factors. The association between aspirin and CRC risk statistically significantly differed by smoking status after adjusting for other risk factors (P-interaction=0.048). Regular aspirin use was associated with a 29% lower risk of CRC among non-smokers (OR=0.71; 95% CI: 0.64, 0.79), whereas it was associated with 19% and 17% lower risk of CRC among smokers of pack-years below median (OR=0.81; 95% CI: 0.71, 0.92) and above median (OR=0.83; 95% CI: 0.74, 0.94), respectively. There was a suggestive interaction between any NSAID and BMI (P-interaction=0.075), where the association was attenuated with increasing BMI. *Conclusions:* Our results suggest that smoking status and BMI may modify the association between NSAID use,

primarily aspirin, and CRC risk. The beneficial effect of aspirin on CRC risk appears to be attenuated, rather than enhanced, among those with greater CRC risk due to obesity and heavy smoking, making it unlikely that these groups would benefit from use of aspirin.

## INTRODUCTION

Colorectal cancer (CRC) is one of the most common and fatal cancers in the world. Non-steroidal anti-inflammatory drugs (NSAIDs), including aspirin and non-aspirin NSAIDs, are consistently observed to be protective against CRC (1, 2). Long-term use of aspirin at doses greater than 75 mg daily was found to significantly reduce the incidence of CRC by 24%, and the benefit increased with scheduled duration of treatment from 20-year follow up of five randomized trials(1). A similar association between higher use of aspirin or non-aspirin NSAIDs and CRC risk was also reported in a meta-analysis of observational studies (2). Despite its promising chemopreventive effects, aspirin is recommended only to prevent cardiovascular disease and CRC in those who are at high risk of cardiovascular disease; there is no broad recommendation from national organization in place due to concerns about gastrointestinal bleeding (3).

The main preventive mechanism of NSAIDs is the inhibition of cyclooxygenase-2 (COX-2) activity and subsequent formation of prostaglandin E2 ($PGE_2$) (4). Aspirin also inhibits the oncogenic Wnt/β-catenin pathway (5, 6) and the extracellular-signal-regulated kinase (ERK) signaling pathway (7). In addition, NSAIDs may function partially through NFκB-signaling pathway (8) and PI3K signaling pathway (9) in colorectal carcinogenesis. Other pathways related to transcription factors, cell proliferation and apoptosis have also been suggested (10).

It is suspected that the association of NSAID use and CRC risk may be modified by other risk factors that are also related to inflammation, but the results have been inconsistent. Sex-differences were reported in cohort studies where regular use of aspirin was associated with a larger decrease in CRC risk in men than in women (11, 12), but meta-analyses did not find this difference to be statistically significant (2, 13). Non-aspirin NSAID use was found to be

associated with a borderline statistically significant lower risk of CRC among individuals with body mass index (BMI) >25, but not with BMI ≤25, in a cohort study (14); interaction analyses on BMI and NSAID use from other cohort studies did not reach statistical significance (15-18). A case-control study found current use of NSAIDs was associated with larger reduction of CRC risk among individuals who had smoked for >40 years, compared to non-smokers (p-interaction=0.049) (19). However, cohort studies found no interaction between NSAID use and smoking on CRC risk (16, 17). In contrast, recent clinical trials among patients with colorectal adenomas found that aspirin was statistically significantly associated with lower risk of colorectal adenomas among non-smokers, but not among current smokers (20-22). A population-based case-control study found an interaction between NSAID use and post-menopausal hormone (PMH) use on colon cancer risk, with NSAID use associated with lower colon cancer risk among PMH non-users, but not among PMH users (p-interaction=0.06) (23). In addition, a randomized clinical trial reported synergistic effects of calcium and any NSAID use in lowering the risk of advanced colorectal neoplastic polyps (p-interaction=0.01) (24); but the interaction between NSAID use and calcium on CRC risk did not reach statistical significance in a cohort study (17).

To our knowledge, no subgroups of the population stratified by lifestyle or dietary risk factors have been consistently identified who have a clearly larger benefit from use of aspirin or non-aspirin NSAIDs. However, most studies did not have sufficient power to detect statistically significant differences in the effects of NSAIDs between population subgroups. Thus, we aimed to evaluate the potential effect modification of other CRC risk factors on the associations of regular use of any NSAID, aspirin, and non-aspirin NSAIDs with CRC risk using studies from a large, international consortium.

## METHODS

### *Study Participants*

Study participants were from the Genetics and Epidemiology of Colorectal Cancer Consortium (GECCO), an international collaboration that involves 12 case-control and cohort studies from North America, Australia and Europe (25). The studies included are listed in Table 1, and details have been described previously (9). In brief, we used data from 7 nested case-control studies in prospective US cohorts [Health Professionals Follow-up Study (HPFS); Multiethnic Cohort Study (MEC); Nurses' Health Study (NHS); Physician's Health Study (PHS); Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial (PLCO); VITamins And Lifestyle Study (VITAL); Women's Health Initiative (WHI)] and 5 case-control studies from the US, Canada and Europe [Assessment of Risk for Colorectal Tumors in Canada (ARCTIC); Hawai'i Colorectal Cancer Studies 2 & 3 (Colo2&3); Darmkrebs: Chancen der Verhutüng durch Screening (DACHS); Diet, Activity and Lifestyle Survey (DALS); Postmenopausal Hormone Study (PMH)]. Informed consent was given by all participants, and studies were approved by their respective Institutional Review Boards.

Each study identified incident, invasive CRC cases (International Classification of Disease for Oncology Code 18.0-18.9, 19.9 and 20.9), confirmed by medical record, pathology report, or death certificate. Age at diagnosis, cancer subsites and stages were obtained from medical records and registries. Controls were individuals without history of CRC at the time of selection, and were selected based on study-specific eligibility and matching criteria (mostly sex and age; as well as smoking status for PHS). For PLCO and WHI, additional controls selected from previous GWA-studies of prostate cancer and lung cancer (PLCO) or hip fracture (WHI) were also included.

Participants reported as members of racial/ethnic groups other than White were excluded, and European ancestry was confirmed using principal components analysis (26). Participants with missing information on both aspirin and non-aspirin NSAID use were excluded. A total of 11,894 colorectal cases and 15,999 controls were included in the analysis.

*Assessment of NSAID Use and Covariates*

Demographics and environmental exposures were self-reported at either in-person interview or via structured self-administered questionnaires, based on each participating study. A multistep, iterative data harmonization procedure was applied, reconciling each study's unique protocols and data collection instruments. Numerous quality-control checks were performed, and outlying values of variables were truncated to the minimum or maximum value of an established range for each variable. Variables were combined into a single dataset with common definition, standardized coding, and standardized permissible values.

For the main exposure variables (regular use of any NSAID, aspirin, and non-aspirin NSAIDs), a common definition of "regular" was not possible due to variability in the questions across studies. The study-specific definitions of regular use of aspirin and/or non-aspirin NSAIDs across studies are given in Table 1. Use of aspirin included both low-dose aspirin (81 mg), and regular or extra-strength aspirin (≥325 mg). Use of non-aspirin NSAIDs included ibuprofen, naproxen or other pain relievers, based on each study. Regular use of any NSAID was defined as regular use of either aspirin or non-aspirin NSAIDs. For participants in PHS, regular use of non-aspirin NSAIDs was not ascertained; therefore, participants from PHS were excluded from the analysis for non-aspirin NSAIDs.

An *a priori* list of potential confounders were also ascertained and harmonized, including study, age, sex, education, BMI ($kg/m^2$), smoking (non-smokers and pack-years), physical activity (hours/week), first-degree family history of CRC, history of endoscopy (colonoscopy or sigmoidoscopy), diabetes, and postmenopausal hormone (PMH) use in women. Age was defined as age at diagnosis for cases and age at selection for controls. Dietary covariates were ascertained using food frequency questionnaires (FFQ), including intakes of alcohol (non-drinkers, 1-28g/day, >28g/day), fruit, vegetables, dietary fiber, red meat, processed meat and total energy, plus total (diet plus supplemental) intakes of calcium and folate. Sex- and study-specific quartiles were created for smoking, physical activity, and all dietary variables except alcohol. For studies that collected dietary information in categories that did not allow conversion into quartiles, binary variables with the threshold between low and high consumption defined by sex-study-specific medians were used. The binary variable was coded as quartile 2 and 3 for these studies.

*Statistical Analyses*

Statistical analyses were conducted using individual-level data. For each study, logistic regression was used to estimate odds ratios (ORs) and corresponding 95% confidence intervals (CI) for each NSAID variable (any NSAID use, aspirin use, and non-aspirin NSAID use) by comparing regular users and non-regular users after adjusting for covariates (as specified in footnotes to tables). Indicators were used for missing covariates. Regular use of non-aspirin NSAIDs was also adjusted for in the analyses for aspirin, and vice versa. Study-specific estimates were combined, using a fixed-effects model, into summary ORs and corresponding 95% CIs. Heterogeneity across studies was tested using Cochran's Q test (27).

To assess factors that may modify the association between NSAID use and CRC risk, we computed stratum-specific estimates in each study, using logistic regression within each stratum

of each factor adjusting for all other covariates, which were then combined into summary stratum-specific ORs and corresponding 95% CIs. Interaction was tested as the significance of the cross product of the NSAID variable and the effect modifier in the multivariable model that also included the main effects of the NSAID variable and the potential effect modifier. Demographic characteristics and lifestyle factors were evaluated including age at diagnosis for cases or selection for controls (<70 and ≥70 years old), sex, BMI ($kg/m^2$; normal [18.5-24.9], overweight [25-29.9] and obese [≥30]), smoking (pack-years; non-smoker, ≤median and >median), moderate/vigorous physical activity (quartiles), first-degree family history of CRC, history of endoscopy, diabetes and PMH use in women, Dietary factors were also tested for potential effect modification, including alcohol intake (non-drinker, 1-28 g/day and >28 g/day), fruit intake (quartiles), vegetable intake (quartiles), red meat intake (quartiles), processed meat intake (quartiles), dietary fiber intake (quartiles), total calcium intake (quartiles) and total folate intake (quartiles). The potential effect modifiers with more than two categories were modeled as group linear (trend) in multiplicative interaction terms. The study-specific estimates for cross products were combined into summary estimates for a single p-value for interaction, using a fixed-effect meta-analysis. Most interaction analyses did not show statistically significant heterogeneity across studies, and we would not expect the mechanisms of interaction between NSAID use and other risk factors to differ across studies. Therefore, we did not use a random-effects meta-analysis. For each potential effect modifier, studies with constant values were excluded from corresponding interaction analyses: specifically, WHI, NHS, HPFS and PHS, each of which only included members of only one sex were excluded in the interaction analysis of NSAID use and sex; PHS was excluded in the interaction analysis of NSAID use and smoking; and HPFS and PHS were excluded in the interaction analysis of NSAID use and PMH

use. For statistically significant effect modifiers, we further tested whether the observed interaction differed by sex or study type (case-control and cohort).

Secondary analyses were also performed. Stratified analyses by cancer subsites (proximal colon, distal colon and rectal) and stages (local, regional and distant) were also performed for both the main effect of NSAID use (any NSAID use, aspirin use, and non-aspirin NSAID use) and interaction analyses of statistically significant effect modifiers. Site-specific or stage-specific cases were compared to the same control group in stratified analyses; logistic regression limited to cases was used to test for heterogeneity. Other site-specific interaction analyses were also performed for several risk factors that were previously related to subsites of CRC: specifically, the interaction analysis of NSAID use and physical activity, as well as PMH use in women, on colon cancer, and the interaction analysis of NSAID use and alcohol intake on rectal cancer. All analyses were performed in Stata v.14 (StataCorp).

## RESULTS

Descriptions of the study populations and the definitions of regular use of NSAIDs in each participating study are shown in Table 2.1. The main effects of NSAID use on CRC risk were examined for all studies (Figure 2.1). For each type of NSAID use (any NSAID, aspirin use, and non-aspirin NSAID use), regular NSAID use was statistically significantly associated with lower risk of CRC after adjusting for all the covariates, compared to non-regular users ($p<0.001$). Any NSAID use was associated with 25% lower risk of CRC, compared to non-regular NSAID use (OR=0.75, 95% CI: 0.71, 0.79; P<0.001; P-heterogeneity<0.001). The association was stronger among case-control studies.

Regular use of any NSAID, aspirin, or non-aspirin NSAIDs was statistically significantly associated with a lower risk of CRC across almost all subgroups, stratified by demographic and lifestyle factors (Table 2.2) and by dietary factors (Table 2.3). There was minimal heterogeneity by study in the test for interaction for all analyses, except for age and processed meat. The association between aspirin and CRC risk statistically significantly differed by smoking status after adjusting for other risk factors in the meta-analysis (P-interaction=0.048). Regular use of aspirin was associated with a 29% lower risk of CRC among non-smokers (OR=0.71; 95% CI: 0.64, 0.79), whereas it was associated with 19% and 17% lower risk of CRC among individuals with below the median of pack-years of smoking (OR=0.81; 95% CI: 0.71, 0.92) and above the median of pack-years (OR=0.83; 95% CI: 0.74, 0.94), respectively. There was a suggestion of interaction between regular use of any NSAID and BMI (P-interaction=0.075), where the association between any NSAID use and CRC risk appeared to attenuate with increasing BMI (normal: OR=0.69, 95% CI: 0.63, 0.77; overweight: OR=0.76, 95% CI: 0.70, 0.83; obese: OR=0.85, 95% CI: 0.75, 0.96). This possible interaction was primarily driven by aspirin (p-

interaction=0.074), and not by non-aspirin NSAIDs. The association of regular use of aspirin on CRC risk was stronger among individuals with normal BMI (OR=0.75; 95% CI: 0.67, 0.84) and overweight (OR=0.75; 95% CI: 0.68, 0.83), and statistically non-significant among the obese (OR=0.93; 95% CI: 0.80, 1.08). No other interactions between NSAIDs and other risk factors of CRC were observed in meta-analyses.

We examined the effect modification of smoking and BMI on the association between NSAID use and CRC, stratified by sex (Table 2.4). Results for interactions were stronger in men for interaction between aspirin use and BMI (p-interaction=0.024), and between use of aspirin and smoking (p-interaction=0.097). While the direction of effect modifications was similar in women as men, the tests for interaction were non-significant.

Because there were significant differences in the main effects of NSAID use on CRC risk between case-control and cohort studies (Figure 2.1), we evaluated whether the effect modification of smoking and BMI differed by study type. The interaction terms for smoking and aspirin were almost identical for case-control (Interaction OR=1.08; 95% CI: 0.97, 1.21) and cohort studies (Interaction OR=1.07; 95% CI: 0.98, 1.18; between-group p-heterogeneity=0.95). Similarly, the interaction terms for BMI and any NSAIDs were similar for case-control (Interaction OR=1.12; 95% CI: 0.94, 1.34) and cohort studies (Interaction OR=1.09; 95% CI: 0.95, 1.26; between-study p-heterogeneity=0.82). However, the interaction terms for BMI and aspirin use appeared to differ between case-control (Interaction OR=1.17; 95% CI: 1.03, 1.33) and cohort studies (Interaction OR=1.02; 95% CI: 0.92, 1.12; between-group p-heterogeneity=0.085).

No statistically significant differences in the associations between regular use of NSAIDs and CRC risk were observed between cancer subsites or stages (Supplemental Table 1). We also examined the interaction between NSAID use and smoking or BMI by cancer subsites and stages (data not shown). The interaction between regular use of any NSAID and smoking remained statistically significant for distal colon cancer (p-interaction=0.014), but not for proximal colon or rectal cancer (p-interaction=0.137 for proximal colon cancer; p-interaction=0.753 for rectal cancer). The interaction between regular use of aspirin and smoking was also statistically significant for distal colon (p-interaction=0.007) and proximal colon cancer (p-interaction=0.047), but not for rectal cancer. Similarly, the interaction between regular use of any NSAIDs and BMI was statistically significant for distal colon cancer only (p-interaction=0.013), and the interaction was mainly driven by aspirin (p-interaction=0.017). For CRC stages, the interaction between regular use of aspirin and smoking was statistically significant for local CRC only (p-interaction=0.032). The interaction between regular use of any NSAID and BMI was also statistically significant for local CRC only (p-interaction=0.043), and was mainly driven by aspirin (p-interaction=0.030). No interaction was observed for NSAID use and BMI/smoking in regional or distant CRC. No interaction was found for other site-specific analysis for alcohol, physical activity or PMH use in women.

## DISCUSSION

Consistent with previous evidence from randomized clinical trials and observational studies, regular use of aspirin and/or non-aspirin NSAIDs was statistically significantly associated with lower risk of CRC in this large consortium study. The association remained statistically significant among almost all the population subgroups stratified by other CRC risk factors.

In addition, we found a statistically significant interaction between regular use of aspirin and smoking, where regular use of aspirin was associated with a larger decrease in CRC risk among non-smokers, than among smokers. Similar to our findings, recent clinical trials among patients with colorectal adenomas also suggested that aspirin was statistically significantly associated with lower risk of colorectal adenomas among non-smokers, but not among current smokers (20-22). In a large randomized trial of low-dose aspirin in combination with the calcium supplements calcitriol and calcium carbonate among patients with colorectal adenomas, the treatment was suggested to be protective against adenoma recurrence among nonsmokers, but was associated with higher risk of recurrence among current smokers (p-interaction=0.046) (20). Similar interactions were observed in two small trials of colorectal adenomas in Asian populations such that the protective effect of low-dose aspirin was abrogated among current smokers (21, 22). A cross-sectional study of colonoscopy patients also found that daily NSAID use was associated with lower risk of colorectal polyps among non-smokers, but not among current smokers (p-interaction=0.04) (28). However, one cohort study reported no statistically significant interaction between NSAID use and smoking on CRC risk (16). In contrast, a case-control study found that current NSAID use was associated with larger decrease in CRC risk among individuals who smoked for >40 years than among non-smokers (p-interaction=0.049) (19).

The mechanisms by which smoking modifies the preventive effect of NSAIDs on CRC risk remain unclear. It was previously reported that smoking was strongly associated with increased risk of aspirin resistance (29), probably due to smoking-induced platelet hyper-reactivity (30). In addition, cigarette smoking was found to be more strongly associated with colorectal tumors that are microsatellite instability (MSI) positive (19). MSI is a hallmark of the serrated polyp pathway, an alternative to the adenomatous polyp pathway in CRC development (31). MSI-positive tumors arise more frequently found in proximal colon, than distal colon (32, 33). A randomized trial to prevent serrated polyps also found that aspirin use was only significantly associated with a lower risk of polyps in the right colon but not in the left colon (34). It is possible that the effect of aspirin differs by carcinogenesis pathways of colorectal tumors among smokers and non-smokers.

Although the association between NSAID use and CRC risk was similar in men and women, we found that the interaction between aspirin and smoking status was statistically significant among men only. No previous study has reported this sex-difference. Men had higher cumulative levels of smoking than women (means: 29.7 pack-years among men; 24.1 pack-years among women), which allowed a larger window for interactions between aspirin and smoking. In addition, there were approximately 20% women that were PMH users in our study, and NSAIDs were previously shown to be significantly associated with lower colon cancer risk among PMH non-users only, but not among PMH users (23), which was also suggested in our study (Table 2). Thus, the sex-difference of the interaction between aspirin and smoking may also be partially due to PMH use among women.

We also found a suggestion of interaction between NSAID use and BMI, by which regular use of any NSAID was associated with the lowest relative risk of CRC among individuals with normal

BMI, followed by overweight, and was highest among obese individuals. Consistently, a slightly more pronounced protection of regular use of NSAIDs on the prevalence of left-sided colorectal adenomas was observed among individuals with normal BMI than among those who were overweight or obese (p-interaction=0.09), in a multi-center cancer screening trial (35). However, several cohort studies observed no interaction between BMI and aspirin on colon cancer risk (14-16, 18). This could be due to the fact that previous studies combined the overweight and obese subgroups or had imprecise estimates for three BMI categories due to small sample sizes. It has been proposed that NSAIDs inhibit $PGE_2$ synthesis and chronic inflammation levels that are associated with higher BMI (36). High doses of salicylates were also shown to reverse insulin resistance in obese rodents (37), which could otherwise contribute to tumor development (38). However, our data suggested that the benefit of NSAIDs is attenuated, rather than enhanced as expected, among obese people. It is possible that larger dose, higher frequency, and longer duration of NSAID use are needed to reduce the elevated risk of colorectal neoplasia among individuals with higher BMI, who have higher chronic inflammation levels.

Our study suggested that only aspirin, rather than non-aspirin NSAIDs, interacted with BMI or smoking on CRC risk, which may be partially explained by unique mechanisms of actions of aspirin that are not shared by other NSAIDs. Low-dose aspirin has been shown to be associated with lower risk of CRC in randomized trials, suggesting the antiplatelet effect of aspirin may also play a role in the inhibition of colorectal tumor cells (1). In addition, aspirin can also acetylate COX-2 to synthesize anti-tumorigenic “aspirin-triggered lipoxin” (ATL), which is anti-inflammatory and inhibits carcinoma cell proliferation (39). The generation of ATL by aspirin was also observed at low, antiplatelet doses of aspirin in a small intervention study of healthy humans (40).

Our study has several strengths. First, we had a larger sample size and therefore greater statistical power for interaction analyses than prior studies. Secondly, we had detailed assessment for most of the CRC risk factors from all participating studies, a characteristic not seen in previous meta-analyses, which allowed us to perform systematic analyses on potential effect modification. In addition, we were able to adjust for potential confounders in all the analyses, whereas meta-analyses using published data have had limited control for confounding. Furthermore, we used an iterative harmonization process on all the environmental variables across all 12 cohort and case-control studies. Multiple quality-control checks were also performed to reduce the level of heterogeneity and the impact of outliers.

There are also some limitations. As all the environmental factors were assessed via questionnaires and varied across studies, there may be measurement errors in the main NSAID exposures and the covariates. For example, the definition of "regular use of NSAIDs" varied across studies, ranging from current use to ≥4 days/week for ≥1 year. However, despite these differences, there was no evidence of heterogeneity across studies in the interaction analysis of NSAIDs with almost all variables examined. Secondly, the main effects of NSAIDs on CRC differed between case-control studies and case-control studies nested in cohorts. This could be due to potentially longer duration between exposure assessment and time to diagnosis and therefore the associations between NSAID use and CRC risk were weaker among nested case-control studies, which resulted in smaller sizes of interaction. In addition, there might have been recall bias in case-control studies. Case-control studies may also be more susceptible to selection bias in that the response of participants may be jointly influenced by NSAID use, CRC status and effect modifier status. However, the odds ratios for our main results of interaction of smoking with aspirin use and of BMI with any NSAID use showed no evidence of heterogeneity between

study types. Furthermore, all participants in our study were of European ancestry; therefore, our results may not be applicable to other race/ethnicity groups. Lastly, we acknowledge that some of the observed interactions were of borderline statistical significance, and we did not adjust for multiple testing in the analysis.

To our knowledge, this is the largest study to systematically analyze the interactions between NSAID use and other risk factors in relation to CRC risk. Regular use of NSAIDs, including both aspirin and non-aspirin NSAIDs was statistically significantly protective against CRC risk in almost all subgroups stratified by other CRC risk factors. We observed stronger associations between aspirin and CRC risk among non-smokers than among smokers. We also found a suggestion of interaction between any NSAID use and BMI on CRC risk, primarily driven by aspirin. The beneficial effect of aspirin on CRC risk appears to be attenuated, rather than enhanced, among those with greater CRC risk due to obesity and heavy smoking, making it unlikely that these groups would benefit from use of NSAIDs, specifically aspirin, for the prevention of CRC.

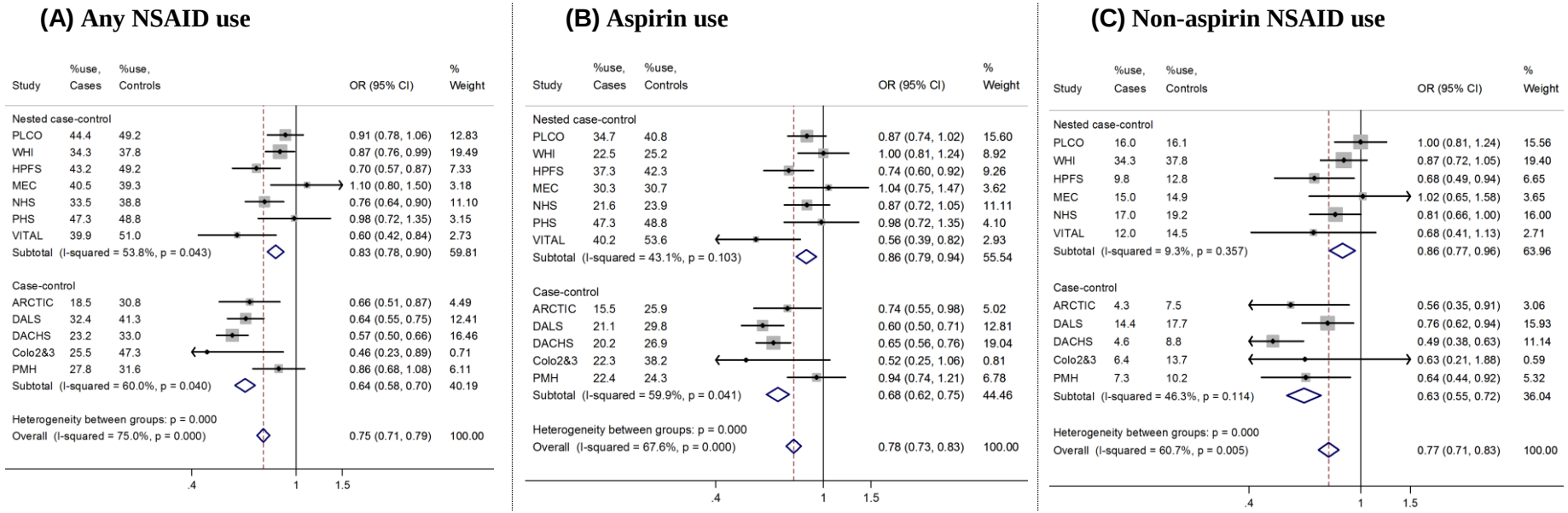
**Table 2.1 Definition of regular use of NSAIDs among participating studies**

| Study Design | Study | Country | Case N | Control N | Male, N (%) | Age, mean (sd), year | Definition of regular use of aspirin and/or non-aspirin NSAIDs[a] |
|---|---|---|---|---|---|---|---|
| **Cohort (nested case-control)** | PLCO | United States | 1,096 | 2,719 | 2,597 (68.1) | 69.0 (6.1) | ≥2 times/week in the last 12 months |
| | WHI | United States | 1,740 | 2,962 | 0 | 73.1 (7.3) | ≥1 time/week for at least the last 2 weeks |
| | HPFS | United States | 646 | 1,164 | 1,810 (100) | 69.6 (9.1) | Currently taking ≥2 times/week |
| | MEC | United States | 356 | 366 | 381 (52.8) | 70.0 (8.3) | ≥2 times/week for ≥1 month |
| | NHS | United States | 1,001 | 1,817 | 0 | 66.5 (8.2) | Currently using ≥15 days/month |
| | PHS | United States | 309 | 455 | 764 (100) | 69.2 (9.6) | Currently using ≥1 time/week |
| | VITAL | United States | 333 | 337 | 365 (54.3) | 70.5 (6.6) | ≥4 days/week for 1 year |
| **Case-control** | ARCTIC | Canada | 1,066 | 1,204 | 1,098 (48.4) | 62.1 (8.7) | ≥2 times/week for >1 month about 2 years ago |
| | DALS | United States | 1,451 | 1,474 | 1,644 (56.2) | 65.0 (9.9) | ≥3 times/week for ≥1 month within the last 2 years |
| | DACHS | Germany | 2,859 | 2,355 | 3,136 (60.2) | 68.6 (10.5) | Currently using for ≥2 time/week for ≥1 years |
| | Colo2&3 | United States | 94 | 131 | 128 (56.8) | 64.7 (11.4) | Currently using |
| | PMH | United States | 943 | 1,015 | 0 | 64.5 (7.2) | ≥twice/week for >1 month |
| **Overall** | | | **11,894** | **15,999** | **11,922 (42.7)** | **68.2 (9.1)** | |

Abbreviations: ARCTIC: Assessment of Risk for Colorectal Tumors in Canada; DALS: Diet, Activity and Lifestyle Study; PLCO: Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial; WHI: Women's Health Initiative; DACHS: Darmkrebs: Chancen der Verhutüng durch Screening Study; Colon 2&3: a case-control study from the University of Hawai'i; HPFS: Health Professionals Follow-up Study; MEC: Multiethnic Cohort; NHS: Nurses' Health Study; PHS: Physicians' Health Study; VITAL: Vitamins and Lifestyle Study; PMH: Postmenopausal Hormone Study – Colon Cancer Family Registry.

[a] Definition of regular use of aspirin and/or NSAIDs was assessed at corresponding referent period: in cohort studies, baseline; case-control studies, at the time of diagnosis for cases, and at analogue time for controls.

**Figure 2.1 Estimated associations between regular use of aspirin and/or NSAIDs and colorectal cancer risk**[a, b, c]




Abbreviations: OR: odds ratio; 95% CI: 95% confidence interval.

[a] The size of the data markers is proportional to the precision of the estimate, which is the inverse of the variance.

[b] Study-specific ORs and 95% CIs are estimated using logistic regression models, adjusting for age, sex, education (less than high school, high school graduate or GED, some college, college graduate, graduate degree), first-degree family history of colorectal cancer (yes/no), history of endoscopy (yes/no), postmenopausal hormone use among women (yes/no), history of diabetes(yes/no), body mass index (kg/m$^2$), moderate/vigorous activity (hours/week), smoking (non-smokers and quartiles of pack-years), alcohol intake (none, 1-28g/day, >28g/day), dietary intakes (quartiles) of fruit, vegetables, red meat, processed meat and fiber, total energy intake (quartiles), total (dietary and supplemental) intakes of calcium and folate (quartiles). Covariates in quartiles are adjusted as group linear variables in the model. For aspirin or non-aspirin NSAID use only, the other type was also adjusted for.

[c] Subtotal and overall ORs and 95% CIs are estimated using fixed-effect meta-analysis. The estimates using random-effect are: (A) Any aspirin or NSAID use: OR=0.75 (0.67, 0.85) (B) Aspirin use: OR=0.79 (0.70, 0.89) (C) Non-aspirin NSAID use: OR=0.74 (0.64, 0.86).

**Table 2.2 Interactions between regular use of NSAIDs and demographic and lifestyle factors in relation to colorectal cancer risk**

| | Any NSAID | | | | Aspirin | | | | Non-aspirin NSAIDs | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Cases | Controls | OR (95% CI)[a] | P value | Cases | Controls | OR (95% CI)[a] | P value | Cases | Controls | OR (95% CI)[a] | P value |
| **Age, years** | | | | | | | | | | | | |
| <70 | 6,518 | 8,213 | 0.74 (0.68, 0.80) | <0.001 | 6,467 | 8,181 | 0.79 (0.72, 0.87) | <0.001 | 6,337 | 7,910 | 0.75 (0.67, 0.84) | <0.001 |
| >=70 | 5,376 | 7,786 | 0.76 (0.70, 0.82) | <0.001 | 5,321 | 7,733 | 0.76 (0.69, 0.83) | <0.001 | 5,195 | 7,593 | 0.79 (0.70, 0.90) | <0.001 |
| *P value for interaction*[b] | | | | 0.672 | | | | 0.320 | | | | 0.954 |
| **Sex**[d] | | | | | | | | | | | | |
| Male | 3,993 | 5,355 | 0.68 (0.61, 0.75) | <0.001 | 3,943 | 5,314 | 0.72 (0.65, 0.80) | <0.001 | 3,970 | 5,337 | 0.70 (0.59, 0.83) | <0.001 |
| Female | 3,262 | 3,231 | 0.71 (0.63, 0.80) | <0.001 | 3,222 | 3,194 | 0.69 (0.60, 0.79) | <0.001 | 3,238 | 3,213 | 0.80 (0.68, 0.95) | 0.009 |
| *P value for interaction*[b] | | | | 0.963 | | | | 0.436 | | | | 0.309 |
| **BMI, kg/m$^2$** | | | | | | | | | | | | |
| Normal (18.5-24.9) | 4,113 | 6,311 | 0.69 (0.63, 0.77) | <0.001 | 4,080 | 6,286 | 0.75 (0.67, 0.84) | <0.001 | 3,944 | 6,028 | 0.72 (0.61, 0.84) | <0.001 |
| Overweight (25-29.9) | 4,827 | 6,322 | 0.76 (0.70, 0.83) | <0.001 | 4,783 | 6,284 | 0.75 (0.68, 0.83) | <0.001 | 4,663 | 6,139 | 0.80 (0.70, 0.91) | 0.001 |
| Obese (≥30) | 2,647 | 2,957 | 0.85 (0.75, 0.96) | 0.006 | 2,623 | 2,939 | 0.93 (0.80, 1.08) | 0.361 | 2,621 | 2,928 | 0.79 (0.67, 0.93) | 0.005 |
| *P value for interaction*[b] | | | | 0.075 | | | | 0.074 | | | | 0.967 |
| **Smoking, pack-years**[e] | | | | | | | | | | | | |
| Non-smoker | 4,902 | 6,930 | 0.71 (0.65, 0.77) | <0.001 | 4,854 | 6,889 | 0.71 (0.64, 0.79) | <0.001 | 4,882 | 6,911 | 0.74 (0.65, 0.84) | <0.001 |
| ≤ median | 2,934 | 4,211 | 0.79 (0.70, 0.88) | <0.001 | 2,913 | 4,192 | 0.81 (0.71, 0.92) | 0.002 | 2,915 | 4,204 | 0.79 (0.67, 0.93) | 0.005 |
| > median | 3,444 | 4,053 | 0.77 (0.69, 0.86) | <0.001 | 3,412 | 4,030 | 0.83 (0.74, 0.94) | 0.004 | 3,434 | 4,040 | 0.78 (0.66, 0.91) | 0.002 |
| *P value for interaction*[b] | | | | 0.167 | | | | 0.048 | | | | 0.459 |
| **Physical activity** | | | | | | | | | | | | |
| Quartile 1 | 2,092 | 2,574 | 0.63 (0.54, 0.72) | <0.001 | 2,047 | 2,538 | 0.65 (0.55, 0.76) | <0.001 | 1,999 | 2,455 | 0.70 (0.57, 0.86) | 0.001 |
| Quartile 2 | 1,724 | 2,289 | 0.74 (0.63, 0.86) | <0.001 | 1,721 | 2,286 | 0.73 (0.61, 0.86) | <0.001 | 1,556 | 2,016 | 0.84 (0.66, 1.07) | 0.154 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Quartile 3 | 1,484 | 2,258 | 0.77 (0.65, 0.90) | 0.001 | 1,474 | 2,246 | 0.75 (0.63, 0.91) | 0.003 | 1,471 | 2,256 | 0.80 (0.62, 1.03) | 0.084 |
| Quartile 4 | 1,399 | 1,681 | 0.73 (0.61, 0.88) | 0.001 | 1,382 | 1,668 | 0.78 (0.64, 0.96) | 0.018 | 1,340 | 1,610 | 0.66 (0.50, 0.87) | 0.003 |
| *P value for interaction*[b] | | | | 0.218 | | | | 0.263 | | | | 0.894 |
| **CRC family history** | | | | | | | | | | | | |
| Yes | 1,955 | 1,941 | 0.77 (0.66, 0.90) | 0.001 | 1,941 | 1,926 | 0.81 (0.67, 0.97) | 0.023 | 1,948 | 1,935 | 0.90 (0.71, 1.13) | 0.363 |
| No | 9,325 | 13,117 | 0.74 (0.69, 0.79) | <0.001 | 9,236 | 13,049 | 0.76 (0.70, 0.81) | <0.001 | 9,285 | 13,090 | 0.75 (0.69, 0.83) | <0.001 |
| *P value for interaction*[b] | | | | 0.659 | | | | 0.764 | | | | 0.143 |
| **History of endoscopy** | | | | | | | | | | | | |
| Yes | 4,595 | 6,100 | 0.75 (0.69, 0.82) | <0.001 | 4,544 | 6,049 | 0.78 (0.70, 0.86) | <0.001 | 4,560 | 6,084 | 0.75 (0.65, 0.86) | <0.001 |
| No | 6,166 | 8,321 | 0.73 (0.67, 0.79) | <0.001 | 6,117 | 8,290 | 0.76 (0.69, 0.83) | <0.001 | 6,160 | 8,305 | 0.79 (0.70, 0.88) | <0.001 |
| *P value for interaction*[b] | | | | 0.900 | | | | 0.886 | | | | 0.679 |
| **Diabetes** | | | | | | | | | | | | |
| Yes | 954 | 877 | 0.73 (0.59, 0.92) | 0.007 | 953 | 877 | 0.77 (0.60, 0.98) | 0.031 | 954 | 877 | 0.60 (0.42, 0.87) | 0.007 |
| No | 7,366 | 11,153 | 0.76 (0.71, 0.82) | <0.001 | 7,351 | 11,146 | 0.81 (0.75, 0.88) | <0.001 | 7,360 | 11,148 | 0.78 (0.71, 0.87) | <0.001 |
| *P value for interaction*[b] | | | | 0.442 | | | | 0.442 | | | | 0.674 |
| **PMH use in women**[g] | | | | | | | | | | | | |
| Yes | 2,002 | 3,362 | 0.87 (0.77, 0.99) | 0.035 | 1,985 | 3,342 | 0.92 (0.78, 1.09) | 0.304 | 2,000 | 3,354 | 0.89 (0.76, 1.05) | 0.178 |
| No | 4,259 | 4,909 | 0.75 (0.68, 0.83) | <0.001 | 4,224 | 4,889 | 0.75 (0.66, 0.85) | <0.001 | 4,241 | 4,896 | 0.75 (0.65, 0.87) | 0.001 |
| *P value for interaction*[b] | | | | 0.147 | | | | 0.178 | | | | 0.242 |

* CRC: colorectal cancer; PMH: postmenopausal hormone; BMI: body mass index

[a] Study-specific ORs and 95% CIs are estimated using logistic regression models, adjusting for age, sex, education (less than high school, high school graduate or GED, some college, college graduate, graduate degree), first-degree family history of colorectal cancer (yes/no), history of endoscopy (yes/no), postmenopausal hormone use among women (yes/no), history of diabetes(yes/no), body mass index (kg/m$^2$), moderate/vigorous activity (hours/week), smoking (non-smokers and quartiles of pack-years), alcohol intake (none, 1-28g/day, >28g/day), dietary intakes (quartiles) of fruit, vegetables, red meat, processed meat and fiber, total energy intake (quartiles), total (dietary and supplemental) intakes of calcium and folate (quartiles). Covariates in quartiles are adjusted as group linear variables in the model. For aspirin or non-aspirin NSAID use only, the other type was also adjusted for.

[b] P for interaction based on interaction of dichotomous NSAID variable and linear (trend) effect modifier variable, using fixed-effect meta-analysis. The p values for heterogeneity were all >0.05, except for age. More details are described in methods.

[c] Multivariable regression models also adjusted for interaction between age and participating studies.

[d] WHI, NHS, HPFS, PHS and PMH were excluded in subgroup and interaction analyses for sex since all participants have the same sex in each study.

[e] PHS was excluded in subgroup and interaction analyses for smoking since cases and controls were matched on smoking status in PHS.

[f] Multivariable regression models also adjusted for interaction between age and history of endoscopy.
[g] HPFS and PHS were excluded in subgroup and interaction analyses for PMH use in women since all participants were men.

**Table 2.3 Interactions between regular use of NSAIDs and dietary factors in relation to colorectal cancer risk**

| | Any NSAID | | | | Aspirin | | | | Non-aspirin NSAIDs | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Cases | Controls | OR (95% CI)[a] | P value | Cases | Controls | OR (95% CI)[a] | P value | Cases | Controls | OR (95% CI)[a] | P value |
| **Alcohol** | | | | | | | | | | | | |
| Non-drinker | 3,785 | 5,364 | 0.77 (0.70, 0.85) | <0.001 | 3,759 | 5,328 | 0.77 (0.69, 0.87) | <0.001 | 3,720 | 3,720 | 0.78 (0.68, 0.90) | <0.001 |
| 1-28g/day | 4,131 | 6,219 | 0.72 (0.66, 0.79) | <0.001 | 4,097 | 6,195 | 0.80 (0.72, 0.88) | <0.001 | 3,890 | 3,890 | 0.74 (0.64, 0.85) | <0.001 |
| >28g/day | 1,204 | 1,377 | 0.65 (0.53, 0.80) | <0.001 | 1,188 | 1,373 | 0.66 (0.53, 0.82) | <0.001 | 1,367 | 1,186 | 0.84 (0.61, 1.15) | 0.265 |
| *P value for interaction*[b] | | | | 0.572 | | | | 0.790 | | | | 0.540 |
| **Fruit intake** | | | | | | | | | | | | |
| Quartile 1 | 2,125 | 3,079 | 0.81 (0.71, 0.92) | 0.002 | 2,108 | 3,060 | 0.84 (0.71, 0.98) | 0.030 | 2,013 | 2,945 | 0.83 (0.69, 1.01) | 0.067 |
| Quartile 2 | 4,848 | 5,452 | 0.69 (0.63, 0.77) | <0.001 | 4,805 | 5,431 | 0.76 (0.68, 0.84) | <0.001 | 4,763 | 5,335 | 0.65 (0.56, 0.76) | <0.001 |
| Quartile 3 | 2,516 | 3,888 | 0.73 (0.64, 0.82) | <0.001 | 2,493 | 3,867 | 0.72 (0.63, 0.83) | <0.001 | 2,425 | 3,766 | 0.83 (0.70, 1.00) | 0.045 |
| Quartile 4 | 1,594 | 2,793 | 0.74 (0.64, 0.86) | <0.001 | 1,581 | 2,775 | 0.80 (0.67, 0.95) | 0.010 | 1,527 | 2,677 | 0.70 (0.56, 0.87) | 0.001 |
| *P value for interaction*[b] | | | | 0.428 | | | | 0.337 | | | | 0.120 |
| **Vegetable intake** | | | | | | | | | | | | |
| Quartile 1 | 1,889 | 2,785 | 0.73 (0.63, 0.84) | <0.001 | 1,868 | 2,770 | 0.81 (0.69, 0.96) | 0.014 | 1,787 | 2,668 | 0.74 (0.60, 0.90) | 0.003 |
| Quartile 2 | 5,122 | 5,817 | 0.73 (0.66, 0.80) | <0.001 | 5,086 | 5,786 | 0.78 (0.70, 0.87) | <0.001 | 5,017 | 5,686 | 0.69 (0.59, 0.80) | <0.001 |
| Quartile 3 | 2,501 | 3,869 | 0.72 (0.63, 0.81) | <0.001 | 2,476 | 3,853 | 0.73 (0.63, 0.84) | <0.001 | 2,408 | 3,741 | 0.74 (0.62, 0.89) | 0.001 |
| Quartile 4 | 1,623 | 2,771 | 0.83 (0.71, 0.95) | 0.009 | 1,607 | 2,754 | 0.78 (0.66, 0.93) | 0.006 | 1,567 | 2,658 | 0.91 (0.74, 1.11) | 0.354 |
| *P value for interaction*[b] | | | | 0.234 | | | | 0.881 | | | | 0.119 |
| **Fiber intake** | | | | | | | | | | | | |
| Quartile 1 | 1,516 | 2,356 | 0.69 (0.60, 0.81) | <0.001 | 1,500 | 2,342 | 0.71 (0.59, 0.86) | <0.001 | 1,509 | 2,353 | 0.81 (0.66, 1.00) | 0.048 |
| Quartile 2 | 1,532 | 2,411 | 0.82 (0.71, 0.95) | 0.009 | 1,516 | 2,395 | 0.82 (0.68, 0.98) | 0.027 | 1,529 | 2,407 | 0.87 (0.71, 1.07) | 0.173 |
| Quartile 3 | 1,337 | 2,407 | 0.79 (0.68, 0.92) | 0.002 | 1,323 | 2,390 | 0.84 (0.70, 1.01) | 0.063 | 1,334 | 2,404 | 0.78 (0.63, 0.97) | 0.027 |
| Quartile 4 | 1,405 | 2,415 | 0.83 | 0.015 | 1,390 | 2,403 | 0.76 | 0.003 | 1,403 | 2,413 | 0.90 | 0.328 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | (0.71, 0.96) | | | | (0.63, 0.91) | | | | (0.73, 1.11) | |
| *P value for interaction*[b] | | | | 0.142 | | | | 0.495 | | | | 0.559 |
| **Red meat intake** | | | | | | | | | | | | |
| Quartile 1 | 2,739 | 4,239 | 0.76 (0.67, 0.85) | <0.001 | 2,712 | 4,219 | 0.84 (0.73, 0.96) | 0.012 | 2,653 | 4,109 | 0.71 (0.58, 0.86) | <0.001 |
| Quartile 2 | 3,041 | 4,110 | 0.73 (0.65, 0.82) | <0.001 | 3,011 | 4,087 | 0.78 (0.68, 0.89) | <0.001 | 2,953 | 3,989 | 0.72 (0.61, 0.86) | <0.001 |
| Quartile 3 | 2,922 | 3,714 | 0.72 (0.64, 0.81) | <0.001 | 2,905 | 3,696 | 0.69 (0.60, 0.79) | <0.001 | 2,817 | 3,579 | 0.79 (0.67, 0.94) | 0.009 |
| Quartile 4 | 2,439 | 3,251 | 0.75 (0.66, 0.85) | <0.001 | 2,417 | 3,235 | 0.78 (0.67, 0.91) | 0.001 | 2,367 | 3,159 | 0.79 (0.66, 0.95) | 0.012 |
| *P value for interaction*[b] | | | | 0.484 | | | | 0.146 | | | | 0.876 |
| **Processed meat intake** | | | | | | | | | | | | |
| Quartile 1 | 1,795 | 2,693 | 0.74 (0.64, 0.86) | <0.001 | 1,779 | 2,675 | 0.81 (0.68, 0.96) | 0.014 | 1,719 | 2,576 | 0.74 (0.58, 0.93) | 0.011 |
| Quartile 2 | 3,325 | 5,045 | 0.74 (0.67, 0.82) | <0.001 | 3,301 | 5,032 | 0.79 (0.70, 0.89) | <0.001 | 3,210 | 4,884 | 0.74 (0.64, 0.86) | <0.001 |
| Quartile 3 | 2,019 | 2,877 | 0.76 (0.67, 0.87) | <0.001 | 2,006 | 2,865 | 0.77 (0.65, 0.90) | 0.001 | 1,956 | 2,799 | 0.81 (0.66, 0.98) | 0.028 |
| Quartile 4 | 1,993 | 2,392 | 0.71 (0.61, 0.82) | <0.001 | 1,974 | 2,375 | 0.68 (0.57, 0.81) | <0.001 | 1,929 | 2,293 | 0.81 (0.66, 1.00) | 0.051 |
| *P value for interaction*[b] | | | | 0.508 | | | | 0.181 | | | | 0.627 |
| **Total calcium intake** | | | | | | | | | | | | |
| Quartile 1 | 2,602 | 3,172 | 0.71 (0.63, 0.81) | <0.001 | 2,581 | 3,159 | 0.73 (0.63, 0.85) | <0.001 | 2,514 | 3,054 | 0.79 (0.65, 0.96) | 0.015 |
| Quartile 2 | 3,737 | 4,583 | 0.72 (0.65, 0.81) | <0.001 | 3,697 | 4,548 | 0.75 (0.66, 0.86) | <0.001 | 3,637 | 4,452 | 0.69 (0.58, 0.82) | <0.001 |
| Quartile 3 | 2,806 | 4,193 | 0.81 (0.72, 0.91) | <0.001 | 2,782 | 4,171 | 0.85 (0.74, 0.97) | 0.016 | 2,706 | 4,063 | 0.80 (0.67, 0.96) | 0.014 |
| Quartile 4 | 1,983 | 3,266 | 0.72 (0.63, 0.82) | <0.001 | 1,965 | 3,252 | 0.77 (0.66, 0.90) | 0.001 | 1,916 | 3,154 | 0.72 (0.60, 0.88) | 0.001 |
| *P value for interaction*[b] | | | | 0.726 | | | | 0.896 | | | | 0.644 |
| **Total folate intake** | | | | | | | | | | | | |
| Quartile 1 | 1,608 | 2,540 | 0.72 (0.62, 0.84) | <0.001 | 1,584 | 2,530 | 0.74 (0.62, 0.89) | 0.001 | 1,603 | 2,535 | 0.82 (0.67, 1.01) | 0.061 |
| Quartile 2 | 3,375 | 4,679 | 0.82 (0.73, 0.91) | <0.001 | 3,342 | 4,640 | 0.88 (0.77, 1.00) | 0.044 | 3,096 | 4,295 | 0.73 (0.61, 0.87) | <0.001 |
| Quartile 3 | 1,851 | 3,086 | 0.79 (0.69, 0.91) | 0.001 | 1,833 | 3,067 | 0.80 (0.68, 0.94) | 0.007 | 1,786 | 2,989 | 0.82 (0.67, 1.00) | 0.051 |
| Quartile 4 | 1,467 | 2,589 | 0.79 (0.68, 0.91) | 0.001 | 1,452 | 2,574 | 0.79 (0.67, 0.95) | 0.009 | 1,463 | 2,586 | 0.83 (0.68, 1.01) | 0.058 |
| *P value for interaction*[b] | | | | 0.679 | | | | 0.848 | | | | 0.703 |

[a] Study-specific ORs and 95% CIs are estimated using logistic regression models, adjusting for age, sex, education (less than high school, high school graduate or GED, some college, college graduate, graduate degree), first-degree family history of colorectal cancer (yes/no), history of endoscopy (yes/no), postmenopausal hormone use among women (yes/no), history of diabetes(yes/no), body mass index ($kg/m^2$), moderate/vigorous activity (hours/week), smoking (non-smokers and quartiles of pack-years), alcohol intake (none, 1-28g/day, >28g/day), dietary intakes (quartiles) of fruit, vegetables, red meat, processed meat and fiber, total energy intake (quartiles), total (dietary and supplemental) intakes of calcium and folate (quartiles). Covariates in quartiles are adjusted as group linear variables in the model. For aspirin or non-aspirin NSAID use only, the other type was also adjusted for.

[b] P for interaction based on interaction of dichotomous NSAID variable and linear (trend) effect modifier variable, using fixed-effect meta-analysis. The p values for heterogeneity were all >0.05, except for processed meat. More details are described in methods.

**Table 2.4 Interaction between regular use of NSAIDs and BMI/smoking in relation to colorectal cancer risk by sex**

| | Any NSAID | | | | Aspirin | | | | Non-aspirin NSAIDs | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Cases | Controls | OR (95% CI)[a] | P value | Cases | Controls | OR (95% CI)[a] | P value | Cases | Controls | OR (95% CI)[a] | P value |
| **Men** | | | | | | | | | | | | |
| **BMI, kg/m$^2$** | | | | | | | | | | | | |
| Normal | 1,403 | 2,413 | 0.65 (0.55, 0.77) | <0.001 | 1,390 | 2,412 | 0.68 (0.57, 0.81) | <0.001 | 1,245 | 2,150 | 0.73 (0.53, 1.01) | 0.061 |
| Overweight | 2,473 | 3,302 | 0.68 (0.60, 0.77) | <0.001 | 2,446 | 3,284 | 0.71 (0.62, 0.81) | <0.001 | 2,323 | 3,125 | 0.68 (0.55, 0.85) | 0.001 |
| Obese | 982 | 1,104 | 0.93 (0.74, 1.18) | 0.560 | 972 | 1,093 | 1.07 (0.84, 1.36) | 0.587 | 960 | 1,082 | 0.67 (0.48, 0.92) | 0.014 |
| *P value for interaction*[b] | | | | 0.058 | | | | 0.024 | | | | 0.546 |
| **Smoking, pack-years**[c] | | | | | | | | | | | | |
| Non-smoker | 1,587 | 2,512 | 0.62 (0.53, 0.73) | <0.001 | 1,572 | 2,495 | 0.67 (0.56, 0.79) | <0.001 | 1,452 | 2,312 | 0.50 (0.38, 0.67) | <0.001 |
| ≤ median | 1,443 | 2,064 | 0.79 (0.66, 0.93) | 0.005 | 1,429 | 2,052 | 0.79 (0.66, 0.94) | 0.007 | 1,420 | 2,044 | 0.87 (0.67, 1.15) | 0.372 |
| > median | 1,658 | 2,012 | 0.69 (0.58, 0.81) | <0.001 | 1,638 | 2,002 | 0.77 (0.66, 0.92) | 0.003 | 1,636 | 1,988 | 0.71 (0.54, 0.93) | 0.012 |
| *P value for interaction*[b] | | | | 0.143 | | | | 0.097 | | | | 0.075 |
| **Women** | | | | | | | | | | | | |
| **BMI, kg/m$^2$** | | | | | | | | | | | | |
| Normal | 2,710 | 3,888 | 0.73 (0.64, 0.82) | <0.001 | 2,690 | 3,874 | 0.82 (0.70, 0.95) | 0.010 | 2,699 | 3,878 | 0.72 (0.60, 0.87) | 0.001 |
| Overweight | 2,354 | 3,020 | 0.84 (0.74, 0.95) | 0.007 | 2,337 | 3,000 | 0.81 (0.69, 0.95) | 0.010 | 2,340 | 3,014 | 0.86 (0.72, 1.03) | 0.093 |
| Obese | 1,665 | 1,853 | 0.83 (0.71, 0.98) | 0.024 | 1,651 | 1,846 | 0.88 (0.72, 1.08) | 0.217 | 1,661 | 1,846 | 0.86 (0.71, 1.06) | 0.161 |
| *P value for interaction*[b] | | | | 0.458 | | | | 0.852 | | | | 0.631 |
| **Smoking, pack-years**[c] | | | | | | | | | | | | |
| Non-smoker | 3,443 | 4,612 | 0.76 (0.69, 0.85) | <0.001 | 3,410 | 4,588 | 0.76 (0.66, 0.87) | <0.001 | 3,430 | 4,599 | 0.82 (0.71, 0.95) | 0.010 |
| ≤ median | 1,504 | 2,165 | 0.79 (0.67, 0.93) | 0.004 | 1,497 | 2,158 | 0.85 (0.69, 1.04) | 0.119 | 1,495 | 2,160 | 0.75 (0.61, 0.93) | 0.008 |
| > median | 1,803 | 2,056 | 0.85 (0.73, 1.00) | 0.045 | 1,791 | 2,043 | 0.93 (0.77, 1.13) | 0.453 | 1,798 | 2,052 | 0.82 (0.66, 1.01) | 0.067 |
| *P value for interaction*[b] | | | | 0.628 | | | | 0.333 | | | | 0.898 |

[a] Study-specific ORs and 95% CIs are estimated using logistic regression models, adjusting for age, sex, education (less than high school, high school graduate or GED, some college, college graduate, graduate degree), first-degree family history of colorectal cancer (yes/no), history of endoscopy (yes/no), postmenopausal hormone use among women (yes/no), history of diabetes(yes/no), body mass index ($kg/m^2$), moderate/vigorous activity (hours/week), smoking (non-smokers and quartiles of pack-years), alcohol intake (none, 1-28g/day, >28g/day), dietary intakes (quartiles) of fruit, vegetables, red meat, processed meat and fiber, total energy intake (quartiles), total (dietary and supplemental) intakes of calcium and folate (quartiles). Covariates in quartiles are adjusted as group linear variables in the model. For aspirin or non-aspirin NSAID use only, the other type was also adjusted for.

[b] P for interaction based on interaction of dichotomous NSAID variable and linear (trend) effect modifier variable, using fixed-effect meta-analysis. All p values for heterogeneity were >0.05. More details are described in methods.

[c] PHS was excluded in subgroup and interaction analyses for smoking since cases and controls were matched on smoking status in PHS.

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# Chapter 3. MENDELIAN RANDOMIZATION OF C-REACTIVE PROTEIN ON COLORECTAL CANCER RISK


## ABSTRACT

*Background:* Several lines of evidence suggest that chronic inflammation is a risk factor for colorectal cancer (CRC). C-reactive protein (CRP), a biomarker of low-grade chronic inflammation has also been moderately associated with CRC risk in observational studies. However, observational studies are susceptible to unmeasured confounding or reverse causality. Using genetic risk variants as instrumental variables, Mendelian randomization analysis provides an alternative approach to assess the causal relationship. We investigated the association between genetically elevated CRP concentration and CRC risk using a Mendelian randomization approach. *Methods:* Epidemiological and genetic data from 30,480 colorectal cancer cases and 22,844 controls from 33 participating studies in three international colorectal cancer consortia were used: the Genetics and Epidemiology of Colorectal Cancer Consortium (GECCO), the Colorectal Transdisciplinary Study (CORECT) and the Colon Cancer Family Registry (CCFR). As instrumental variables, we included 19 SNPs that were associated with CRP concentration. The association between SNPs and CRC risk was estimated using a logistic regression model adjusted for age, sex, principal components and genotyping phases. An inverse-variance weighted method was then applied to estimate the causal effect of CRP on CRC risk. *Results:* Rs1260326 was significantly associated with higher risk of CRC ($p=7.5\times10^{-4}$), and rs6734238 was associated with lower CRC risk (p=0.003). A genetically predicted one-unit increase in the log-transformed CRP concentrations (mg/L) was associated with a 4% higher risk of CRC (OR=1.04; 95% CI: 0.97, 1.12; p=0.256). CRP was not associated with CRC risk among subgroups of the population stratified by other risk factors. *Conclusion:* Genetically elevated

CRP concentration was not associated with increased risk of CRC. Our findings suggested that circulating CRP is unlikely to be a causal factor in CRC development.

## INTRODUCTION

Chronic inflammation has been shown to play a role in the pathogenesis of colorectal cancer (CRC) (1), and studies have found a reduced risk of CRC associated with long-term use of aspirin or other nonsteroidal anti-inflammatory drugs (NSAIDs) (2-5). Circulating C-reactive protein (CRP) is the most commonly used biomarker of low-grade chronic inflammation, which is a sensitive, nonspecific marker produced in the liver in response to inflammation, infection and tissue injury (6). The heritability of CRP concentration was estimated to range between 25-40%, indicating that genetic variation influences elevated CRP concentrations (7). Although plasma CRP concentration is largely regulated under transcriptional control (6), assessment of the temporal reproducibility of CRP concentrations over a 5-year period suggests that it is comparatively stable over time within each individual (8). Lifestyle factors, such as older age (9), adiposity (10), tobacco smoking (11, 12), less physical activity (13), and lower use of NSAIDs (14) are also associated with increased circulating CRP concentrations.

Meta-analyses of observational studies have shown that a one unit (mg/L) increase in log-transformed high-sensitivity CRP was associated with 12% higher risk of colorectal cancer (15, 16), suggesting the involvement of inflammation pathways in CRC carcinogenesis. The association was stronger among men than among women. In addition, this association was primarily driven by the significant association in colon cancer, but not in rectal cancer. Although results from meta-analyses support a role of chronic inflammation and CRP in colorectal carcinogenesis, they were susceptible to potential bias by unmeasured confounding factors in the original studies. Furthermore, observational studies may be susceptible to reverse causality in which elevated CRP concentrations could be due to immune response and inflammation induced by premalignant or preclinical lesions during tumor growth (17, 18).

As an alternative study design, Mendelian randomization analysis is less susceptible to confounding or reverse causality by taking advantage of the random assortment of genetic alleles from parents to offspring during gamete formation (19). Since genetic variants are distributed randomly at conception, they are generally unrelated to potentially confounding socioeconomic or lifestyle factors, and temporally precede both lifestyle factors and the disease process. Studies of CRC used CRP-related genetic variants as a proxy of lifelong CRP concentrations and reported inconsistent findings. A nested case-control study found genetically determined two-fold higher CRP concentration (mg/L), based on seven SNPs in the *CRP* gene, was associated with higher CRC risk (20). Another case-control study found a tagSNP in the *CRP* gene to be associated with higher risk of colon cancer, and another SNP associated with lower risk of rectal cancer (21). However, other studies using various numbers of SNPs within the *CRP* gene did not find statistically significant associations between CRP and CRC risk (22-24). More recently, Prizment et al (25) found a statistically significant association between a weighted CRP genetic risk score and CRC risk in a prospective cohort, based on 20 SNPs identified to be significantly associated with CRP concentrations in a meta-analysis of GWAS studies (7), corroborating a causative role of chronic low-grade inflammation in colorectal carcinogenesis. However, their sample size was small (205 CRC cases among 7,603 participants) and had limited power for stratified analysis by subgroups of population. In addition, most previous studies assumed a homogeneous population without adjusting for population stratification, which could potentially bias their results. Other SNPs that also reached statistical significance in association with serum CRP concentration among European Americans in a large consortium were not included in previous analyses (26). Furthermore, the effect of genetically elevated CRP concentration on

CRC risk may differ by other risk factors, such as smoking and NSAID use (27), but the differences were not fully analyzed in previous studies.

Findings from previous human genetic studies were inconsistent and had insufficient power to assess a low or moderate causal relationship between CRP and CRC risk. In this study, we aimed to investigate whether CRP plays a causal role in CRC risk using genetic variants that were previously reported to be significantly associated with circulating CRP concentration as instrumental variables, based on the information from 50,437 individuals in 33 epidemiologic studies the largest sample size attempted for such analyses to date. We also aimed to further explore whether the association differs by other risk factors of CRC.

## METHODS

### *Study Participants*

We used epidemiological and genetic data from 30,480 CRC cases and 22,844 controls from 33 participating studies in three international CRC consortia: the Genetics and Epidemiology of Colorectal Cancer Consortium (GECCO), the Colorectal Transdisciplinary Study (CORECT) and the Colon Cancer Family Registry (CCFR). Full details have been published previously (28), and the demographic characteristics of study participants are summarized in Supplemental Table 2. In brief, 10,644 cases and 10,729 controls from GECCO were included from nested case-control studies in 8 cohorts and 6 case-control studies from the US, Canada and Europe. And 19,836 cases and 12,115 controls were included from CORECT from nested case-control studies in 7 cohorts, 9 case-control studies and 3 case-series studies. Nested case-control studies from different sites of CCFR participated as individual studies in GECCO and/or CORECT, and thus were analyzed as such. Several other studies contributed to both consortium, but there is no overlap of participants.

Participants reported as race/ethnicity groups other than European ancestry were excluded from analysis. Informed consent was given by all participants, and studies were approved by their respective Institutional Review Boards.

### *Assessment of Outcomes and Environmental Variables*

Invasive CRC cases (International Classification of Disease for Oncology Code 18.0-18.9, 19.9 and 20.9) were identified by medical record, pathology report, or death certificate in each study. Age at diagnosis, cancer subsites and stages were obtained from medical records and registries. Controls were selected based on study-specific eligibility and matching criteria, except for case-series studies which only contributed cases in this study.

Demographic factors and environmental exposures were self-reported at either in-person interview or via structured self-administered questionnaires, based on each participating study. A multistep, iterative data harmonization procedure was applied, and variables were combined into a single dataset with common definition, standardized coding, and standardized permissible values for all participating studies. Reference age was defined as age at CRC diagnosis of cases, or age at selection for controls. BMI was calculated based on height and weight ($kg/m^2$), and was categorized into three groups: normal (18.5-24.9), overweight (25-29.9), and obese (≥30). Smoking status was defined as never and ever smokers. Regular use of any non-steroidal anti-inflammatory drugs (NSAIDs), aspirin, or non-aspirin NSAIDs were defined as binary variables (yes/no) based on study-specific definitions. Family history of CRC was defined as CRC in any first degree relative. History of endoscopy included both sigmoidoscopy and colonoscopy.

*Genotyping*

Details on genotyping and imputation has been previously reported (29). In brief, DNA was mostly obtained from blood samples, with some from buccal swabs. Several different platforms (the Illumina HumanHap 300k, 240k, 550k and OncoArray 610k BeadChip Array system, or Affymetrix platform) were used for genotyping (30, 31). Quality control checks were implemented, and exclusion criteria included average sample call rate ≤97%, heterozygosity, unexpected duplicates or relative pairs, gender discrepancy and principle component analysis (PCA) outlier of HapMap2 CEU cluster. SNPs were also excluded if they were not consistent across platforms, call rate <98%, out of Hardy-Weinberg equilibrium (HWE) among controls ($p<0.0001$), or minor allele frequency (MAF) <0.05% (30). SNPs were imputed to 1000 Genome Project if they were not genotyped on each platform. Imputation accuracy as evaluated by $R^2$ is used as exclusion criteria for SNPs with low quality ($R^2>0.3$ for SNPs with MAF>1%).

*Instrumental Variables*

We selected SNPs from two resources as instrumental variables for CRP: 18 SNPs that have been previously used as instrumental variables (7) and 9 new SNPs based on the findings among participants of European ancestry from the PAGE study (26) (summarized in Table 3.1). Only SNPs that were significantly associated with CRP concentration at the threshold of $p<5\times10^{-8}$ were selected. In order to combine all the SNPs into a single set, we checked independence between the 27 selected SNPs using linkage disequilibrium (LD) analysis. If two SNPs were in LD ($r^2>0.2$), the SNP with the smaller p-value was included, and the other one excluded in the final SNP set. We also conducted a GWAS-catalog literature search for additional SNPs that were statistically significantly associated with CRP concentration ($p<5\times10^{-8}$) among participants of European ancestry, had estimated effect sizes and standard errors on per unit increase in CRP concent ration (mg/L), and were not in linkage disequilibrium (LD) with the 27 selected SNPs. No additional SNPs were identified via this search. In the end, 19 SNPs were included in the final set for the CRP instrumental variable.

There are several assumptions for a valid instrumental variable (IV) in the Mendelian randomization approach. The three basic assumptions for a single instrumental variable are that (i) the genetic marker is robustly associated with the exposure, (ii) the genetic marker is independent of the outcome, given the exposure and all confounders of the exposure-outcome association (i.e. the genetic marker has no pleiotropic effect, which means it only acts through the exposure and not through other pathways), and (iii) the genetic marker is independent of factors that confound the exposure-outcome relation (32). The first assumption was met since we only included SNPs that are significantly associated with CRP concentrations in GWA-studies. The second assumption could not be tested directly because CRP measures were not available

and we may not have all confounding variables measured in our study, but the Egger test and other sensitivity analyses were performed to indirectly test the pleiotropic effects. The third assumption was tested by evaluating the association between each set of SNPs and each potential confounder of the CRP-CRC association among controls, and no violation of this assumption was observed. If these three assumptions do hold, genetic variants can be used as instrumental variables for evaluating causal association. In addition, if multiple instrumental variables are combined into a single estimate by the inverse-variance weighted (IVW) method, a further assumption is made that the variants provide independent information. In other words, they should not be correlated with each other (i.e. linkage disequilibrium or gene-gene interaction) (33). Furthermore, even for a variable that satisfies the IV assumptions, the statistical association between the risk factor and the IV should be strong enough to provide unbiased and precise estimates in finite samples (34).

*Statistical Analysis*

Assuming all the prior assumptions are met, genetic variant $k, (k = 1 \ldots K)$ is associated with an observed $X_k$ mean change in the risk factor per additional variant allele with standard error $\sigma_{Xk}$ and an observed $Y_k$ log-odds change in the outcome per allele with standard error $\sigma_{Yk}$. An inverse-variance weighted (IVW) estimate of the causal effect combining the ratio estimates and standard errors of single SNPs can be computed using a fixed effect meta-analysis model (33):

$$\hat{\beta}_{IVW} = \frac{\sum_k X_k Y_k \, \sigma_{Yk}^{-2}}{\sum_k X_k^2 \, \sigma_{Yk}^{-2}}$$

and the approximate standard error will be $se(\hat{\beta}_{IVW}) = \sqrt{\frac{1}{\sum_k X_k^2 \sigma_{Yk}^{-2}}}$.

The effect sizes of genetic variants on CRP concentration ($X_k$ and $\sigma_{Xk}$) were obtained from prior studies (7, 26), and the effect size of genetic variants on CRC risk were estimated within our study populations. We used logistic regression models to estimate the association between each genetic variant and CRC risk in GECCO and CORECT separately, adjusting for age, sex, genotyping phase, and principle components (log-odds change in CRC risk per risk allele: $Y_k$ and $\sigma_{Yk}$). The Mendelian randomization estimates from GECCO and CORECT were then combined into a summary estimate using fixed-effect meta-analysis.

Stratified analysis were also carried out as exploratory analysis by an *a priori* list of CRC risk factors using the same regression models, including sex, BMI, smoking, NSAID use, aspirin use, family history of CRC and history of endoscopy. In addition, we also evaluated differences among cancer subsites and stages.

We also performed sensitivity analysis using other Mendelian randomization methods, including weighted median estimates (35) and Egger regression estimates (36).

*Power Calculation*

Based on methods described by Burgess (37), our sample size of 53,324 participants (30,480 CRC cases and 22,844 controls) has an estimated 99.4% power to detect the previously estimated causal effect size of CRP (OR=1.19) (25) at a significance level of 0.05, assuming the SNPs explains a total of 5% variance of CRP based on previous estimates (7). Alternatively, we have 82.5% power to detect a minimal odds ratio of 1.12 (15, 16) at a significance level of 0.05, given our sample size.

## RESULTS

The mean age among participants was 63.4 years (SD=10), and 50.7% are male (Supplemental Table 2). A total of 27 SNPs were identified and their associations with CRP concentration are summarized in Table 3.1. The imputation accuracy ($R^2$) ranges between 0.84 and 1.0. SNPs that were in LD ($R^2$>0.2) were identify. For each pair of correlated SNPs, the one with the strongest association with CRP concentration (smaller p-values) were included in the final set, and the others were excluded, leaving 19 SNPs for analysis. The estimated associations between these 19 SNPs and CRC risk are shown in Figure 3.1. In pooled analysis combining GECCO and CORECT estimates, rs1260326 was significantly associated with higher risk of CRC ($p=7.5\times10^{-4}$), and rs6734238 was associated with lower CRC risk (p=0.003). None of the other SNPs were statistically significantly associated with CRC.

Using the 19 SNPs as instrumental variables, we found that one unit increase in the log-transformed genetically elevated CRP concentration (mg/L) was associated with a non-significant 4% higher risk of CRC (OR=1.04; 95% CI: 0.97, 1.12; p=0.256; Table 3.2). Although the association was stronger in GECCO (OR=1.07; 95% CI: 0.96, 1.20; p=0.217) than that in CORECT (OR=1.02; 95% CI: 0.93, 1.12; p=0.654), there was no evidence of heterogeneity between the two consortia (p-heterogeneity=0.509).

In stratified analysis, genetically elevated CRP concentration was not associated with CRC risk in any of the subgroups by sex, BMI, smoking, NSAID use, family history of CRC or history of endoscopy (Table 3.3). The associations between genetically elevated CRP concentration and CRC risk were similar between men and women, NSAID/aspirin users and non-users, and never and ever smokers. None of the tests for interaction between genetically elevated CRP concentration and these risk factors on CRC risk reached statistical significance.

We also stratified by CRC subsites and stages. Genetically elevated CRP concentration was not associated with any subsite of CRC, although the association seemed to be stronger in proximal and distal colon cancer, compared to rectal cancer. However, there was a marginally significant association between genetically elevated CRP concentration and distant CRC (OR=1.19; 95% CI: 1.00, 1.42; p=0.049), but not for local or regional CRC.

Our results persisted using other Mendelian randomization methods in combined analysis (Supplemental Figure 1). We used inverse-variance weighting method, which seems to be more robust than median estimating, and more conservative than Egger regression. We used Egger regression to test for global pleiotropic effect of instrumental variables by regressing the associations between SNPs and CRC risk against the associations between SNPs and CRP (Figure 3.2). None of the p-values for the intercepts was statistically significant ($p>0.05$), suggesting no global violation of pleiotropic assumptions. We also used a newly developed method to test for pleiotropic effects of individual SNPs. By comparing observed p-values of estimated direct effects to expected p-values, no SNP had a significant pleiotropic effect after adjusting for false discovery rate (FDR) at 0.05. However, two of the SNPs had suggestive pleiotropic effects at FDR<0.2 (Supplemental Figure 2). The Mendelian randomization estimates were minimally changed after excluding these two SNPs (OR=1.03; 95% CI: 0.96, 1.11; p=0.365).

## DISCUSSION

In this large consortium study, we found a non-significant 4% increase in CRC risk per one unit increase in CRP concentration (mg/L) among participants of European ancestry. No association between genetically elevated CRP concentration and CRC risk was found in subgroups stratified by other CRC risk factors. Our results suggested that circulating CRP does not play a causal role in colorectal carcinogenesis.

Our estimate of the CRP-CRC association is smaller than the 12% found in meta-analyses of prospective observational studies that used measured CRP concentrations (15, 16), suggesting that the association between measured CRP concentrations and CRC risk may be partially due to confounding by other environmental factors. Our findings are also different from the only previous study that used GWAS identified SNPs as instrumental variables for assessing the relationship between CRP-related SNPs and CRC risk (25). Prizment et al found a statistically significant 19% higher risk in CRC with a one unit (mg/L) increment of the CRP instrumental variable created with 20 SNPs, while our analysis suggested a smaller effect size of 4% and the association was not statistically significant. The sample size in the previous study was small, with 105 CRC cases among 7,603 participants in the instrumental variable analysis.

Comparatively, we have a much larger sample size of 29,014 CRC cases and 21,423 controls for larger statistical power to test for the possibility of moderate causal association. In addition, the majority of SNPs (18 out of 19) used in our study are the same as those in that prospective cohort study (25). Prizment et al included two other SNPs that were not statistically significantly associated with CRP concentrations in the previous GWAS analysis (p-value > 5×10-8) (7). In comparison, we only included an additional SNP that were recently found to be statistically significantly associated with CRP concentration among individuals of European ancestry (p-

value < 5×10-8) (26) and were independent of the previous 18 SNPs. Our estimates were less likely to be biased or overestimated by including strong and independent instrumental variables. There is also possibility that the SNPs associated with CRP were also associated with other inflammation-related traits, and led to a spurious positive association between CRP and CRC in the previous analysis. We identified two SNPs, rs6734238 and rs1260326, that had suggestive pleiotropic effects at FDR<0.2 in our sensitivity analyses. They are located near the GCKR and IL1F10 genes, which were found to be associated with lipid or cholesterol concentrations (38, 39) and fibrinogen (40), respectively.

Our study is consistent with other prospective cohort studies that used multiple SNPs as instrumental variables and did not observe a statistically significant association between CRP and CRC risk (22-24). However, individual SNPs were found to be significantly associated with colon cancer or rectal cancer in population-based case-control studies (21). A nested case-control study found genetically two-fold higher CRP concentration (mg/L), based on seven SNPs in the *CRP* gene, was associated with higher CRC risk (20). However, the effect of genetically elevated CRP was not significantly attenuated after adjusting for measured CRP concentrations, indicating a potentially pleiotropic effect of selected SNPs which could lead to biased estimates of SNP-CRP relationship in the first stage.

Chronic inflammation has been established as a key predisposing factor in colorectal neoplasia (1), but the exact mechanisms of action are yet unknown. It was suggested that chronic inflammation has been found to create a microenvironment that promotes inflammatory cells to release reactive oxygen and nitrogen species which could potentially lead to DNA alteration (41), as well as to increase the production of inflammatory cytokines and proteins which promote tumor growth (42). As a proxy biomarker of low-grade chronic inflammation level, CRP has

been proposed to play a role in colorectal carcinogenesis. In addition, circulating CRP was found to be a major serum leptin-interacting protein that directly inhibited the binding of leptin to its receptors and its ability to signal *in vitro*, which resulted in leptin resistance and obesity *in vivo* (43). Lower concentrations of leptin and circulating adiponectin was also found in patients with colorectal cancer and adenomas than controls in a meta-analysis (44), suggesting the possibility of interaction between CRP and leptin on colorectal carcinogenesis. However, a previous case-control study reported no association between circulating CRP concentration and pathologic measures of colonic inflammation (45). Mendelian randomization analysis on CRP and coronary heart disease also found that CRP concentration itself was unlikely to be a causal factor in coronary heart disease (46), although persistent inflammation was found to contribute to coronary heart disease (47). Similar to coronary heart disease, it is possible that chronic inflammation promotes colorectal carcinogenesis through inflammatory mediators other than CRP.

Our study has several strengths. It is the largest study to investigate causality between CRP and CRC risk using genetic variants, and had adequate statistical power to detect a moderate association. We were also the first study to explore whether the effects differed among population subgroups by other CRC risk factors, and cancer subsites and stages, which was not feasible in previous studies. Since we have environmental factor measures in most of the participating studies, we were able to test the assumption that the genetic variants of interest were not associated with confounders of CRP and CRC. In addition, we used a comprehensive set of GWAS-identified genetic variants as instrumental variables in our analysis, which were strong instruments and provided robust estimates. Taking advantage of the random assortment of alleles during gamete formation, our results from Mendelian randomization should be less

susceptible to confounding and reverse causality compared to observational studies. Therefore, our results provide more evidence of non-causality than the observational studies on this topic. In addition, the association between genetically determined CRP concentration and CRC risk indicate lifelong exposure to higher CRP concentrations than CRP measures at a one or a few points in time, which could be influenced by other factors. Furthermore, although only participants of European ancestry was included in the analysis, we still accounted for population stratification by adjusting for principal components instead of assuming genetic homogeneity. The majority of previous studies of the CRP genotypes and CRC risk did not adjust for population stratification. Lastly, we were able to test for pleiotropic effects of individual SNPs which were not evaluated in previous studies, and we found suggestive pleiotropic effects of two SNPs that potentially biased previously reported associations between CRP and CRC risk.

There are also some limitations to this study. First, two of the three Mendelian randomization assumptions could not be fully tested in our study. Therefore, potential violations of the assumptions cannot be ruled out. Although we tested the assumption of no association between genetic variants and confounders, there are possible unmeasured confounders. We also performed several falsification tests, including Egger regression to test the assumption of no pleiotropic effect and showed no evidence for global violation of the assumptions. However, we found suggestive evidence that two individual genetic variants might violate this assumption and therefore bias the estimates. Second, we only investigated genetically elevated CRP in relation to CRC risk. Since CRP concentrations are also influenced by various environmental factors, there is still possibility that it can still serve as an inflammation mediator of environmental factors on CRC risk. In addition, we did not have sufficient statistical power in the stratified analysis, even

though we are the largest study to date. Lastly, since our analysis only included participants of European ancestry, our results may not be applicable to other race/ethnicity groups.

In summary, we found that genetically elevated CRP concentration was not statistically significantly associated with increased risk of CRC among participants of European ancestry. In addition, the associations did not differ by subgroups of population by other CRC risk factors. Our findings did not support a causal role of CRP in CRC risk.

**Table 3.1 Association of genome-wide significant loci with CRP concentrations in previous studies**

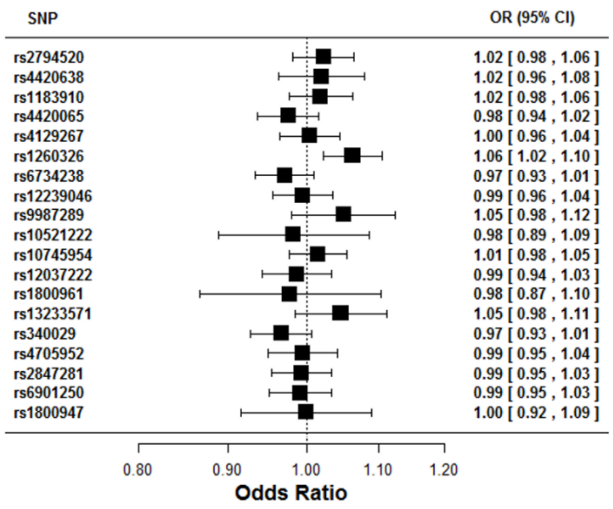
| SNP | Chr | Position | Gene | EA | BA | EAF | $R^2$ | β[a] | SE | p-value | Study | LD exclusion[b] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| rs2794520 | 1 | 159678816 | CRP | C | T | 0.67 | 1.01 | 0.160 | 0.006 | $2.0\times10^{-186}$ | | No |
| rs4420638 | 19 | 45422946 | APOC1 | A | G | 0.83 | 0.84 | 0.236 | 0.009 | $8.8\times10^{-139}$ | | No |
| rs1183910 | 12 | 121420807 | HNF1A | G | A | 0.68 | 1.00 | 0.149 | 0.006 | $2.1\times10^{-124}$ | | No |
| rs4420065 | 1 | 66161461 | LEPR | C | T | 0.62 | 0.98 | 0.090 | 0.005 | $3.5\times10^{-62}$ | | No |
| rs4129267 | 1 | 154426264 | IL6R | C | T | 0.60 | 0.99 | 0.079 | 0.005 | $2.1\times10^{-48}$ | | No |
| rs1260326 | 2 | 27730940 | GCKR | T | C | 0.42 | 1.01 | 0.072 | 0.005 | $4.6\times10^{-40}$ | | No |
| rs6734238 | 2 | 113841030 | IL1F10 | G | A | 0.41 | 1.00 | 0.050 | 0.006 | $1.8\times10^{-17}$ | | No |
| rs12239046 | 1 | 247601595 | NLRP3 | C | T | 0.62 | 0.99 | 0.047 | 0.006 | $1.2\times10^{-15}$ | | No |
| rs9987289 | 8 | 9183358 | PPP1R3B | A | G | 0.08 | 1.00 | 0.069 | 0.011 | $3.4\times10^{-13}$ | Dehghan et al (7) | No |
| rs10521222 | 16 | 51158710 | SALL1 | C | T | 0.95 | 0.97 | 0.104 | 0.015 | $8.5\times10^{-13}$ | | No |
| rs10745954 | 12 | 103483094 | ASCL1 | A | G | 0.52 | 0.99 | 0.039 | 0.006 | $1.6\times10^{-11}$ | | No |
| rs12037222 | 1 | 40064961 | PABPC4 | A | G | 0.23 | 0.99 | 0.045 | 0.007 | $6.4\times10^{-11}$ | | No |
| rs1800961 | 20 | 43042364 | HNF4A | C | T | 0.97 | 0.88 | 0.088 | 0.015 | $2.2\times10^{-9}$ | | No |
| rs13233571 | 7 | 72971231 | BCL7B | C | T | 0.89 | 1.00 | 0.054 | 0.009 | $3.6\times10^{-9}$ | | No |
| rs340029 | 15 | 60894965 | RORA | T | C | 0.62 | 0.97 | 0.032 | 0.006 | $4.1\times10^{-9}$ | | No |
| rs4705952 | 5 | 131839618 | IRF1 | G | A | 0.25 | 0.96 | 0.042 | 0.007 | $1.3\times10^{-8}$ | | No |
| rs2847281 | 18 | 12821593 | PTPN2 | A | G | 0.60 | 0.99 | 0.031 | 0.006 | $2.2\times10^{-8}$ | | No |
| rs6901250 | 6 | 117114025 | GPRC6A | A | G | 0.32 | 0.99 | 0.035 | 0.006 | $4.8\times10^{-8}$ | | No |
| rs2075650 | 19 | 45395619 | TOMM40 | A | G | 0.86 | 1.00 | 0.220 | 0.020 | $1.83\times10^{-38}$ | | Yes |
| rs1205 | 1 | 159682233 | CRP | C | T | 0.67 | 1.01 | 0.170 | 0.010 | $1.03\times10^{-31}$ | | Yes |
| rs1800947 | 1 | 159683438 | CRP | C | G | 0.94 | 0.85 | 0.300 | 0.030 | $3.1\times10^{-25}$ | | No |
| rs2650000 | 12 | 121388962 | HNF1A | C | A | 0.65 | 1.00 | 0.120 | 0.010 | $2.62\times10^{-23}$ | Kocarnik et al (26) | Yes |
| rs2228145 | 1 | 154426970 | IL6R | A | C | 0.60 | 0.99 | 0.100 | 0.010 | $1.47\times10^{-18}$ | | Yes |
| rs780094 | 2 | 27741237 | GCKR | T | C | 0.41 | 1.00 | 0.100 | 0.010 | $1.53\times10^{-16}$ | | Yes |
| rs7310409 | 12 | 121424861 | HNF1A | G | A | 0.60 | 1.00 | 0.180 | 0.030 | $1.57\times10^{-10}$ | | Yes |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| rs6857 | 19 | 45392254 | PVRL2 | C | T | 0.84 | 0.97 | 0.230 | 0.040 | $2.07\times10^{-10}$ | | Yes |
| rs429358 | 19 | 45411941 | APOE | T | C | 0.86 | 0.96 | 0.240 | 0.040 | $2.41\times10^{-10}$ | | Yes |

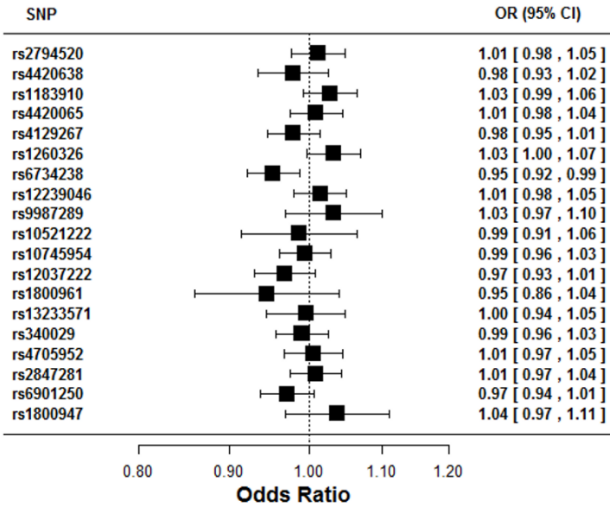
Chr: chromosome; EA: Effect allele; BA: baseline allele; EAF: Effect allele frequency;
[a] β coefficient represents 1-unit increase in the natural log-transformed CRP (mg/L) per copy increment in the effect allele; SE: standard error.
[b] SNPs that are in linkage disequilibrium (LD; $r^2>0.2$) with other SNPs are excluded in the analysis.

**Figure 3.1 Associations between 19 SNPs and colorectal cancer risk**

A) SNP-CRC association in GECCO




B) SNP-CRC association in CORECT




C) SNP-CRC association in GECCO & CORECT combined

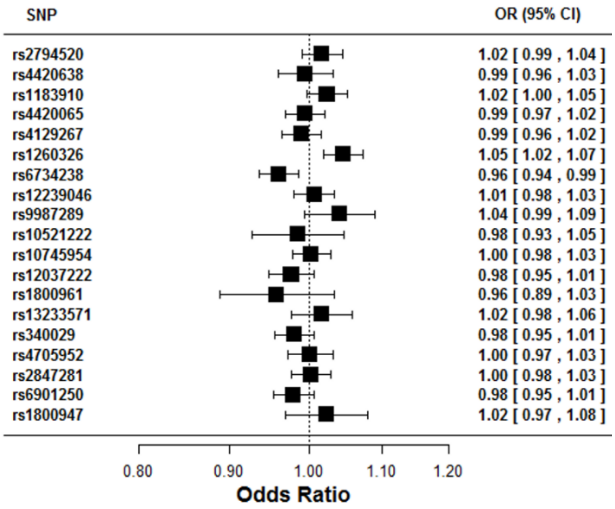


**Table 3.2 Mendelian randomization estimates of the causal effect of genetically elevated C-reactive protein and colorectal cancer risk**

| Study | N Cases | N Controls | OR (95% CI)[a,b] | p-value | p-het[c] |
|---|---|---|---|---|---|
| **GECCO** | 10,644 | 10,729 | 1.07 (0.96, 1.20) | 0.217 | |
| **CORECT** | 19,836 | 12,115 | 1.02 (0.93, 1.12) | 0.654 | |
| **Combined**[d] | 30,480 | 22,844 | 1.04 (0.97, 1.12) | 0.256 | 0.509 |

OR: Odds ratio; 95% CI: 95% confidence interval;

[a] Final set includes 19 SNPs identified from both studies, excluding the SNPs that are in linkage disequilibrium ($r^2$>0.8).

[b] Inverse-variance weighted method was used to estimate causal effect of genetically elevated CRP and CRC risk, and corresponding 95% CIs. Odds ratio represents the change in odds of colorectal cancer risk with one unit increase in the log-transformed genetically elevated CRP concentration (mg/L).

[c] P-het is p-value for heterogeneity of differences between GECCO and CORECT estimates.

[d] Fixed-effects meta-analysis was used to combine estimates from GECCO and CORECT.

**Table 3.3 Mendelian randomization estimates of the causal effect of genetically elevated CRP and CRC risk by subgroups**

| Subgroups | GECCO | | | CORECT | | | Combined[b] | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Cases/ Controls | OR[a] (95% CI) | p-value | Cases/ Controls | OR[a] (95% CI) | p-value | Cases/ Controls | OR[a] 95% CI | p-value | p-het[c] |
| **Sex** | | | | | | | | | | |
| Male | 5,027/4,940 | 1.12 (0.95, 1.32) | 0.172 | 10,854/6,181 | 1.03 (0.91, 1.17) | 0.653 | 15,881/11,121 | 1.06 (0.96, 1.18) | 0.229 | 0.431 |
| Female | 5,617/5,789 | 1.03 (0.88, 1.20) | 0.717 | 8,913/5,934 | 1.01 (0.89, 1.16) | 0.859 | 14,530/11,723 | 1.02 (0.92, 1.13) | 0.710 | 0.876 |
| **BMI** | | | | | | | | | | |
| Normal | 3,249/3,875 | 1.18 (0.98, 1.43) | 0.087 | 3,672/3,139 | 0.99 (0.82, 1.20) | 0.934 | 6,921/7,014 | 1.08 (0.95, 1.24) | 0.245 | 0.205 |
| Overweight | 3,959/3,935 | 0.99 (0.82, 1.18) | 0.872 | 4,637/3,503 | 0.97 (0.82, 1.16) | 0.779 | 8,596/7,438 | 0.98 (0.86, 1.11) | 0.754 | 0.936 |
| Obese | 2,216/1,757 | 1.01 (0.78, 1.31) | 0.921 | 2,772/1,748 | 1.19 (0.93, 1.51) | 0.171 | 4,988/3,505 | 1.10 (0.92, 1.31) | 0.286 | 0.388 |
| **Smoking** | | | | | | | | | | |
| Never | 4,612/5,107 | 1.12 (0.95, 1.33) | 0.169 | 3,031/2,983 | 1.02 (0.87, 1.19) | 0.836 | 10,139/9,279 | 1.07 (0.95, 1.20) | 0.269 | 0.399 |
| Ever | 5,855/5,654 | 1.03 (0.89, 1.20) | 0.670 | 7,025/4,515 | 1.08 (0.92, 1.28) | 0.342 | 11,425/9,789 | 1.06 (0.94, 1.18) | 0.338 | 0.681 |
| **NSAID use** | | | | | | | | | | |
| Yes | 2,847/3,722 | 1.04 (0.85, 1.27) | 0.732 | 3,031/2,983 | 1.05 (0.86, 1.29) | 0.635 | 5,878/6,705 | 1.04 (0.90, 1.20) | 0.563 | 0.926 |
| No | 5,855/5,924 | 1.03 (0.89, 1.19) | 0.693 | 7,025/4,515 | 1.02 (0.88, 1.19) | 0.790 | 13,478/10,439 | 1.03 (0.92, 1.14) | 0.639 | 0.936 |
| **Aspirin use** | | | | | | | | | | |
| Yes | 2,182/2,882 | 1.02 (0.81, 1.28) | 0.880 | 2,518/2,448 | 1.07 (0.86, 1.34) | 0.538 | 4,700/5,330 | 1.05 (0.89, 1.23) | 0.582 | 0.751 |
| No | 7,034/6,695 | 1.05 (0.92, 1.21) | 0.472 | 6,938/4,949 | 1.03 (0.89, 1.20) | 0.676 | 13,972/11,644 | 1.04 (0.94, 1.15) | 0.418 | 0.852 |
| **Non-aspirin NSAID use** | | | | | | | | | | |
| Yes | 1,192/1,539 | 0.97 (0.71, 1.34) | 0.864 | 680/766 | 1.05 (0.68, 1.62) | 0.831 | 1,872/2,305 | 1.00 (0.77, 1.29) | 0.990 | 0.784 |
| No | 7,681/7,682 | 1.03 (0.91, 1.18) | 0.635 | 9,226/6,623 | 1.00 (0.88, 1.14) | 0.972 | 16,907/14,305 | 1.02 (0.93, 1.11) | 0.721 | 0.752 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Family History of CRC** | | | | | | | | | | |
| Yes | 1,715/1,312 | 0.88 (0.64, 1.21) | 0.425 | 2,023/1,089 | 0.92 (0.68, 1.25) | 0.610 | 3,738/2,401 | 0.90 (0.72, 1.12) | 0.358 | 0.821 |
| No | 8,299/7,835 | 1.08 (0.95, 1.23) | 0.247 | 8,867/6,819 | 1.00 (0.88, 1.14) | 0.960 | 17,166/14,654 | 1.04 (0.95, 1.14) | 0.399 | 0.427 |
| **History of endoscopy** | | | | | | | | | | |
| Yes | 2,842/3,809 | 1.11 (0.90, 1.37) | 0.321 | 7,792/3,086 | 1.00 (0.84, 1.19) | 0.986 | 10,634/6,895 | 1.04 (0.91, 1.19) | 0.538 | 0.437 |
| No | 5,774/4,826 | 1.09 (0.93, 1.28) | 0.293 | 1,581/4,105 | 1.15 (0.90, 1.47) | 0.272 | 7,355/8,931 | 1.11 (0.97, 1.27) | 0.139 | 0.730 |

[a] Inverse-variance weighted method was used to estimate causal effect of genetically elevated CRP and CRC risk, and corresponding 95% CIs. Odds ratio represents the change in odds of colorectal cancer risk with one unit increase in the log-transformed genetically elevated CRP concentration.
[b] Fixed-effects meta-analysis was used to combine estimates from GECCO and CORECT.
[c] P-het is p-value for heterogeneity of differences between GECCO and CORECT estimates.

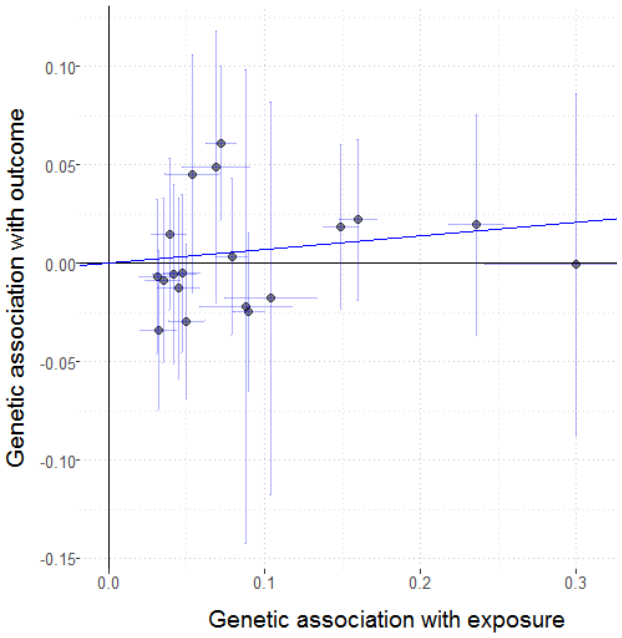
**Table 3.4 Mendelian randomization estimates of the causal effect of genetically elevated CRP and CRC risk by subgroups**

| Subgroups | GECCO | | | CORECT | | | Combined[b] | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Cases/ Controls | OR[a] (95% CI) | p-value | Cases/ Controls | OR[a] (95% CI) | p-value | Cases/ Controls | OR[a] (95% CI) | p-value | p-het[c] |
| **Subsite** | | | | | | | | | | |
| Colon | 7,662/10,729 | 1.13 (1.00, 1.27) | 0.062 | 11,964/12,115 | 1.02 (0.92, 1.13) | 0.748 | 19,626/22,844 | 1.06 (0.98, 1.15) | 0.131 | 0.199 |
| Proximal | 4,180/10,729 | 1.17 (0.98, 1.32) | 0.084 | 5,815/12,115 | 1.01 (0.89, 1.14) | 0.907 | 9,995/22,844 | 1.06 (0.96, 1.17) | 0.226 | 0.215 |
| Distal | 3,343/10,729 | 1.12 (0.96, 1.32) | 0.155 | 5,448/12,115 | 1.03 (0.90, 1.17) | 0.651 | 8,791/22,844 | 1.07 (0.96, 1.18) | 0.213 | 0.412 |
| Rectal | 2,780/10,729 | 0.92 (0.77, 1.11) | 0.385 | 6,617/12,115 | 1.06 (0.93, 1.20) | 0.410 | 9,397/22,844 | 1.01 (0.91, 1.12) | 0.867 | 0.237 |
| **Stage** | | | | | | | | | | |
| Local | 2,653/10,729 | 1.11 (0.93, 1.32) | 0.270 | 2,780/12,115 | 1.04 (0.88, 1.24) | 0.624 | 5,433/22,844 | 1.07 (0.95, 1.21) | 0.263 | 0.652 |
| Regional | 5,002/10,729 | 1.09 (0.94, 1.25) | 0.254 | 7,484/12,115 | 1.04 (0.91, 1.18) | 0.586 | 12,486/22,844 | 1.06 (0.96, 1.16) | 0.242 | 0.630 |
| Distant | 1,118/10,729 | 1.26 (0.98, 1.63) | 0.069 | 1,206/12,115 | 1.13 (0.89, 1.43) | 0.320 | 2,324/22,844 | 1.19 (1.00, 1.42) | 0.049 | 0.524 |

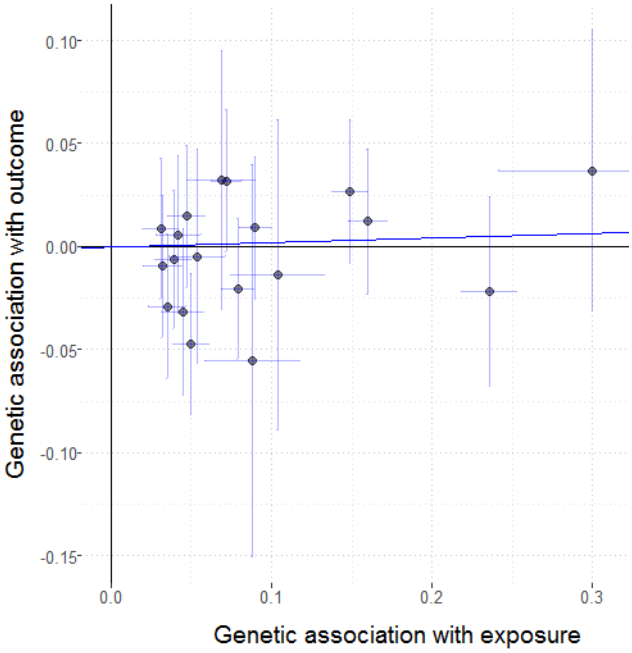
[a] Inverse-variance weighted method was used to estimate causal effect of genetically elevated CRP and CRC risk, and corresponding 95% CIs. Odds ratio represents the change in odds of colorectal cancer risk with one unit increase in the log-transformed genetically elevated CRP concentration (mg/L).
[b] Fixed-effects meta-analysis was used to combine estimates from GECCO and CORECT.
[c] P-het is p-value for heterogeneity of differences between GECCO and CORECT estimates.

**Figure 3.2 Scatter plots of SNP-CRP and SNP-CRC associations for 19 SNPs**

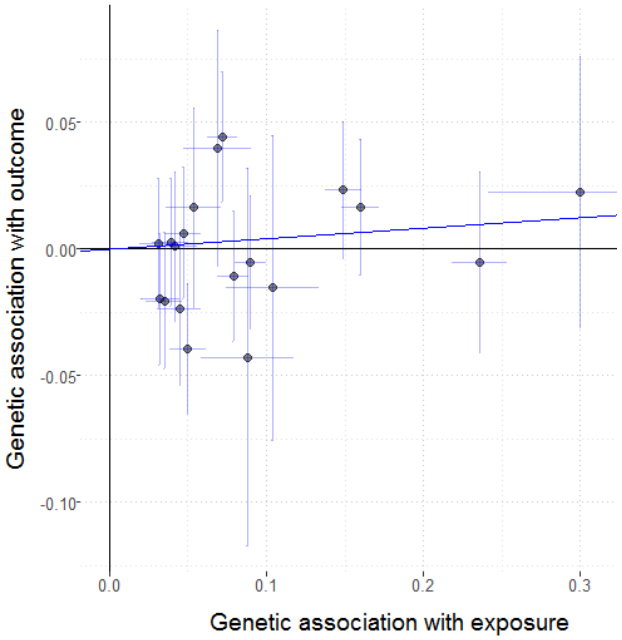
**A) GECCO**
(MR Egger intercept p-value = 0.442)




**B) CORECT**
(MR Egger intercept p-value = 0.224)




**C) GECCO & CORECT combined**
(MR Egger intercept p-value = 0.159)



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# Chapter 4. EXPLORATORY PLASMA PROTEOMIC ANALYSIS IN A RANDOMIZED CROSSOVER TRIAL OF ASPIRIN AMONG HEALTHY MEN AND WOMEN


## ABSTRACT

Long-term use of aspirin is associated with lower risk of colorectal cancer and other cancers; however, the mechanism of chemopreventive effect of aspirin is not fully understood. Animal studies suggest that COX-2, NFκB signaling and Wnt/β-catenin pathways may play a role, but no clinical trials have systematically evaluated the biological response to aspirin in healthy humans. Using a high-density antibody array, we assessed the difference in plasma protein levels after 60 days of regular dose aspirin (325 mg/day) compared to placebo in a randomized double-blinded crossover trial of 44 healthy non-smoking men and women, aged 21-45 years. The plasma proteome was analyzed on an antibody microarray with ~3,300 full-length antibodies, printed in triplicate. Moderated paired t-tests were performed on individual antibodies, and gene-set analyses were performed based on KEGG and GO pathways. Among the 3,000 antibodies analyzed, statistically significant differences in plasma protein levels were observed for nine antibodies after adjusting for false discoveries (FDR adjusted p-value<0.1). The most significant protein was succinate dehydrogenase subunit C (SDHC), a key enzyme complex of the mitochondrial tricarboxylic acid (TCA) cycle. The other statistically significant proteins (NR2F1, MSI1, MYH1, FOXO1, KHDRBS3, NFKBIE, LYZ and IKZF1) are involved in multiple pathways, including DNA base-pair repair, inflammation and oncogenic pathways. None of the 258 KEGG and 1,139 GO pathways was found to be statistically significant after FDR adjustment. This study suggests several chemopreventive mechanisms of aspirin in humans, which have previously been reported to play a role in anti- or pro-carcinogenesis in cell systems; however, larger, confirmatory studies are needed.


**KEYWORDS:** Aspirin; randomized trial; antibody microarray; proteomics

## INTRODUCTION

Low-dose and regular-strength aspirin use is consistently observed to be associated with reduced long-term risk of colorectal cancer (CRC) risk of adenomatous polyps, pre-cancerous lesions that increase risk of CRC (1, 2). Benefit increases with duration of aspirin use and is associated with 34% reduction in 20-year CRC risk (1, 3). Evidence consistently suggests that aspirin plays a role at an early stage or even before tumorigenesis (4). Therefore, studies of aspirin involving healthy individuals may help elucidate biological responses related to the chemopreventive effects of aspirin.

The presumed main mechanism by which aspirin lowers adenomatous polyps and CRC risk is by reducing inflammatory mediators through the inhibition of cyclooxygenase-2 (COX-2) activity (5, 6) and subsequent formation of prostaglandin E2 (PGE2) (7). Aspirin has also been shown to inhibit the oncogenic Wnt/β-catenin pathway (8) and the extracellular-signal-regulated kinase (ERK) signaling pathway (9) in colon cancer cell lines. Indirect support for these pathways from human studies comes from nested case-control studies which suggest that interactions between the use of non-steroidal anti-inflammatory drugs (NSAIDs) and polymorphisms in oncogenes in the Wnt/β-catenin signaling pathway (10) and NFκB-signaling pathway (11) modify CRC risk (12). Recently, a genome-wide investigation of gene-environment interactions reported that the association of NSAIDs with CRC risk differed according to genetic variation at 2 SNPs (13); these are related to genes involved in activation of the PI3K signaling pathway. Other pathways related to transcription factors, cell proliferation and apoptosis have also been suggested (14).

No human intervention trials have yet systematically explored proteomic profiling of aspirin use among human subjects. A randomized controlled trial of diclofenac among overweight individuals identified a group of inflammation-related modulators (15); another trial suggested a

variety of pathways, including cytokine activity pathways, in response to glucosamine and chondroitin supplementation (16). These studies support the utility of proteomic profiling in characterizing responses to drugs or supplements with pleiotropic effects. We created a high density antibody array containing >3,200 different antibodies to ~2,100 different proteins that we use to interrogate plasma or other biological samples for cellular activation status including proteins involved in apoptosis, proliferation, angiogenesis, immune cell activity/infiltration, and metabolism, etc. Many of the antibodies are to secreted proteins such as cytokines and growth factors including 21 proteins with insulin in their names. We have used these arrays to find biomarkers of ovarian (17, 18), breast (19), pancreas (20, 21) and colon (22) cancer and used the values derived to find pathways important in obesity (23), supplement usage (16), anti-apoptotic cell survival signaling pathways (22), and incisional hernia (24). The objective of this study was to explore potential mechanisms relevant to the effects of aspirin through proteomic analysis in healthy participants in a randomized trial of aspirin, with a focus on proteins that are related to cancer development.

## METHODS

### *Study design*

The Aspirin and the Biology of the Colon (ABC) study was a randomized, double-blinded, placebo-controlled, crossover trial (25). During each intervention period, participants took 325 mg aspirin or a visually identical placebo orally each day for 60 days, with a 3-month washout period between the treatment periods. Study activities, including participant interviews and blood draws, were conducted at the Fred Hutchinson Cancer Research Center (Fred Hutch) Prevention Center Research Clinic. The study procedures were approved by the Fred Hutch Institutional Review Board; informed, written consent was obtained from all participants prior to participation in the study.

### *Study participants*

Details of the study and the study population have been described previously (25, 26) and are summarized in Supplemental Figure 3. Briefly, healthy men and women, aged 21 to 45 years, were recruited from participants from the greater Seattle area who completed a cross-sectional study of diet and aspirin metabolism between June 2003 and March 2007 (27). Individuals were excluded if they had: a medical history of gastrointestinal, hepatic, or renal disorders; family history of familial adenomatous polyposis or Lynch syndrome; known intolerance to aspirin or other NSAIDs; weight change greater than 4.5 kg within the past year; current use of prescription medication (including oral contraceptives) or over-the-counter medications; alcohol intake >2 drinks/day; were pregnant or lactating; or were planning to move out of the greater Seattle area within the 12 months of the study period. Given that an aim of the parent study was to determine whether genetic variation in *UGT1A6* influenced response to aspirin (25), participants who met these criteria were further selected based on *UGT1A6* genotypes (rs2070959 and rs1105879) so

that all subjects with a **2/*2* genotype and sex-matched participants with a **1/*1* genotype were invited to participate. Additionally, two participants with a **2/*4* genotype were also included and randomized. Clinical measurements were also assessed and participants with abnormal laboratory values were excluded from participation. A total of 55 healthy men and women were recruited into the trial, randomly assigned, blocked on sex and *UGT1A6* genotype, to the order of receiving aspirin or placebo. Forty-four participants completed both intervention periods. The reasons participants dropped out were not related to either intervention or placebo period.

*Data Collection*

Demographics and medical history were obtained through questionnaires at the time of recruitment, including age, ethnicity, previous smoking habits, dietary supplement use, alcohol intake, history of weight change, and general health. Twelve-hour fasting morning blood samples were drawn on day -5 and day 55 of the first intervention period, and days 1 and 55 of the second intervention period. The pre-intervention blood samples were tested for liver and kidney function, and post-intervention blood samples were used for research purposes. Blood samples were collected in EDTA-containing vacutainer tubes; plasma was aliquoted into cryovials and stored at -80°C until analysis.

*Proteomics analyses*

Plasma samples were analyzed on a customized antibody array populated with ~3,300 full-length antibodies, printed in triplicate on a single microarray according to published methods (20-24, 28). Briefly, each sample (200 µg) was combined with the same amount of a Cy3 labeled "reference" pool (from 5 healthy men and 5 women) of albumin and IgG-depleted plasma, placed on the array and Cy3 and Cy5 signals determined. The log2-transformed Cy5/Cy3 ratio, noted as M value, determined the relative concentration of protein compared to reference. For

quality-control purposes, triplicate antibodies with coefficients of variation >10% were removed, and experimental variation was normalized using within-array print-tip loess normalization and between-array quartile normalization (29). The median for each antibody was taken from triplicates as the summary measure.

The two samples from the same person collected at the termination of the two periods (aspirin and placebo) were analyzed in the same batch with the order of treatment periods randomized. Sex and genotypes were randomly distributed across batches. Additional possible batch effects were checked by principal component analysis (data not shown).

Previous analyses showed that coefficients of variation, for triplicates, for >85% antibodies on the array were less than 10% (18, 28, 30, 31). Intra-class correlation ($R_1$) for triplicates was also used to evaluate the reliability of triplicates in this study (32). 92.5% of the antibodies had at least moderate correlation among triplicates ($R_1$>0.5), and 83.5% antibodies had strong correlations among triplicates ($R_1$>0.7), suggesting reliable measurements of plasma protein levels. Antibodies were also highly correlated between quality-control duplicate samples that were blinded to the lab analysist.

*Statistical analysis*

Antibodies with more than 30% missing values across the arrays were excluded from further analysis. Remaining missing data were imputed using the local least squares imputation method, which replaces a target protein that has missing values with a linear combination of 10 similar proteins, chosen by k-nearest neighbors based on Pearson correlation coefficients (33). Of ~3,300 antibodies on the array, 3,000 proteins were available for statistical analyses and complete data were available on all 44 participants after imputation. Moderated paired t-tests

(34) were performed for individual proteins to determine statistically significant differences between aspirin and placebo treatments. Adjustment for potential confounders, including batch effect, sample positions, and covariate effects of sex and *UGT1A6* genotype was carried out using a mixed linear regression model. The proteins were then ranked on the basis of p-values adjusted by Benjamini-Hochberg false discovery rate (FDR) correction, at a significance level of 0.1 (34). Both the moderated t-tests and FDR corrections were performed using the R LIMMA package (29).

Pathway analyses were also carried out (35), using gene sets in Kyoto Encyclopedia of Genes and Genomes (KEGG) and the Gene Ontology (GO). KEGG gene sets were obtained via REST server to KEGG, and GO gene sets were obtained from MSigDB (http://www.broadinstitute.org/gsea/msigdb/index.jsp). Simulation analyses were performed to compare several gene-set analytical tools (35-37). The Significance Analysis of Functional Categories (SAFE) framework (35) outperformed the other methods in relation to sensitivity and specificity, and was used in further analysis. Gene sets with fewer than 3 genes were excluded from the analysis. The enriched gene sets were ranked on the basis of adjusted p-values, with an FDR significance level of 0.1.

## RESULTS

The demographic characteristics of the 44 study participants are summarized in Table 4.1. The study population was predominantly Caucasian, and approximately half of the participants were overweight or obese (BMI≥25 kg/$m^2$).

Among the 3,000 proteins tested, nine were statistically different between the aspirin and placebo periods after FDR correction (adjusted p-value<0.1) (Table 4.2), including energy convertors, hormone receptors, transcriptional factors, and RNA- and DNA-binding proteins. Among these nine proteins, six (MYH1, FOXO1, KHDRBS3, NFKBIE, LYZ and IKZF1) had a higher expression level on aspirin than placebo, whereas the other three (SDHC, NR2F1 and MSI1) had a lower expression level on aspirin than placebo. The most significant protein was succinate dehydrogenase subunit C (SDHC), with an average 34% lower expression level on aspirin than placebo (p-value=4.47×10-05; FDR-adjusted p-value=0.06). The next most significant was myosin-1 (MYH1). The expression level of MYH1 on aspirin treatment was 62% higher on average than that on placebo (p-value=6.83×10-05; FDR-adjusted p-value=0.06). The largest decrease was observed in a nuclear hormone receptor NR2F1 with 60% lower expression level on aspirin than placebo. NF-κB inhibitor epsilon (NFKBIE) had one of the largest increases: the expression level was 63% higher on aspirin than placebo. Because the study was conducted as a randomized crossover study, each participant served as their own control; predictably, adjusting for batch effects, sample positions, order of aspirin and placebo period, and other covariates did not change test results.

Analyses of differences in individual protein expression levels were also undertaken stratified by sex (Supplemental Table 3). For the nine proteins that were statistically different in the overall analysis, the direction of effect (increased or decreased) was consistent across men and women;

however, none of the within-group changes in protein levels was statistically significant after FDR correction, probably due to reduced sample size in subgroup analyses.

In gene-set analyses using the SAFE framework, a total of 257 KEGG pathways and 1139 GO pathways were tested. Among them, 21 KEGG and 63 GO pathways were statistically significantly different on aspirin than placebo (p-value<0.05); however, none of these gene categories reached statistical significance after FDR correction at significance level of 0.1 (listed in Supplemental Table 4).

## DISCUSSION

In this placebo-controlled randomized crossover trial among healthy individuals, plasma levels of nine of the total of ~3,000 antibodies were significantly different between 60-day regular-dose aspirin and placebo, after adjustment for FDR at 0.1. Among these nine proteins, six had higher expression levels, and three were lower on aspirin than placebo. These proteins play important roles in various pathways, including the mitochondrial Kreb's cycle, DNA base-pair repair, and inflammation. However, when correcting for multiple comparisons, we did not identify overall pathways that were statistically significantly different between aspirin and placebo.

The protein with the most significant difference between treatments was SDHC; plasma levels were lower after aspirin treatment. SDHC is a subunit of succinate dehydrogenase (SDH), a key enzyme complex of the mitochondrial tricarboxylic acid (TCA) cycle, which oxidizes succinate to fumarate (38). As one part of SDH (also called complex II), it also facilitates transfer of electrons to coenzyme Q (ubiquinone) (39). Aspirin has been shown to interfere with mitochondrial function (40), as well as inhibit the activity of SDH in rats (41). Further, repeated mild inhibition of oxidative phosphorylation via inhibition of SDH protects against the decrease in ATP that usually accompanies severe hypoxia and thus can act as neuroprotection (42). Treatment with aspirin has also been shown *in vivo* to slow down the decline of intracellular ATP by this mechanism of inhibiting SDH (43) and therefore to protect against hypoxia, a common hallmark of tumors that promotes metabolic adaptations and angiogenesis (44). Furthermore, accumulation of succinate, due to reduced efficiency of SDHC, results in the stabilization of HIF1-α, the degradation of which is promoted by the oncometabolite (R)-2-hydroxyglutarate (45). Metabolomic analysis in the present study has shown that plasma concentrations of 2-hydroxyglutarate decreased after aspirin treatment in both men and women

(p=0.005) (26). It is relevant that plasma concentration of HIF1-α was lower after aspirin treatment among carriers of wild *UGT1A6*1/*1* genotype (Data not shown; $p = 6.2 \times 10^{-05}$; adjusted p-value=0.186), but not among carriers of *UGT1A6*2/*2* genotype, suggesting that the genotypes of *UGT1A6*, which encodes a UDP-glucuronosyltransferase that participates in glucuronidation of aspirin (46), may modulate the effect of aspirin on downstream metabolic functions. In summary, results from our proteomic analyses, as well as those from metabolomic analyses support a possible additional mechanism for aspirin in cancer prevention.

The expression level of MYH1 was also statistically significantly higher by 62% on aspirin than placebo. As a member of the human homologue of the base excision repair (BER) gene, MYH1 is a DNA glycosylase that removes adenine mispaired with 8-hydroxyguanine from DNA and protects against oxidative DNA damage (47, 48). Inherited variants in *MYH* that cause reduced enzyme function have been associated with significantly increased risk of familial and sporadic CRC in observational studies (49-52). In addition, the prevalence of low-frequency microsatellite instability (MSI) has been found to be higher among *MYH* mutation carriers (51, 53), suggesting possible interaction between the BER and MSI pathways. However, most previous observational studies assessed the association between germline mutations of *MYH* and CRC risk, whereas our study directly measured the plasma level of MYH1. Our findings suggest that environmental factors, such as aspirin, may also have an effect on enzyme levels, regardless of genetic background.

Similarly, NFKBIE was 63% higher on average on aspirin than placebo. NFKBIE is involved in the NF-κB signaling pathway. After cellular stimulation, NFKBIE is highly induced to bind the NF-κB dimer, and provides negative feedback regulation that inhibits NF-κB DNA-binding activity and prevents its nuclear accumulation (54). As the NF-κB pathway plays an important

role in chronic inflammation and tumor promotion, reduction of NF-κB activity is critical in inhibiting the production of pro-inflammatory cytokines (55, 56). In an observational study among 315 chronic lymphocytic leukemia (CLL) patients, targeted deep sequencing of 18 core complex genes within the NF-κB pathway found that the most frequently mutated genes was *NFKBIE*; further screening revealed that truncated NFKBIE predominated in patients with poor prognosis (57). Similarly, exome sequencing has also suggested that NFKBIE was highly mutated among melanoma patients (58). Aspirin has been found to inhibit IκB kinase (IKK) β, which inhibits NF-κB inhibitors by phosphorylation (59). In a nested case-control study, polymorphisms in IκBKβ were associated with lower CRC risk and the association was stronger among current NSAID users (11). Findings from our study are among the first in humans to suggest a biologic interaction between aspirin and NFKBIE, thus providing further support for the likelihood that the NF-κB signaling pathway is involved in one of the mechanisms of action of aspirin.

Among the other significant proteins, aspirin treatment was associated with 29% lower level of Musashi1 (MSI1), a neural RNA-binding protein. A previous study among colon cancer patients has shown that overexpression of MSI1 in colon cancer lesions, compared to paired normal colonic mucosa, was associated with poorer metastasis-free survival and poorer overall survival (60). A variety of other potential mechanisms have also been suggested by our findings, including those involving NR2F1 as a nuclear hormone receptor, Forkhead Box O1 (FOXO1) in Akt-mTOR signaling pathway, and KHDRBS3 in RNA-binding and cell-cycle control. Several of these have limited evidence for a relationship with aspirin in humans, probably due to the fact that most previous human studies have focused on candidate genes, proteins, or pathways. Randomized controlled trials of aspirin conducted among patients with cardiovascular disease

found statistically significant reductions in circulating concentrations of high-sensitivity C-reactive protein (CRP), tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and thromboxane B2 (TXB2) (61-63).

Compared to previous human studies of aspirin or other NSAIDs, our study has the strength that our antibody microarray has a high coverage of the proteome (2,100 proteins from 3,000 antibodies), which allowed for more complex proteomic profiling of the impact of aspirin. Most of the proteins that differed significantly between aspirin and placebo treatment are involved in various pathways associated with carcinogenesis, illustrating the potential range of biologic effects of aspirin *in vivo*. Secondly, most of the previous studies were focused on mechanisms inhibited by aspirin in tumor cells or in patients with a focus on tumor progression; ours is among the first to directly evaluate the effects of aspirin among healthy individuals. Therefore, we had the opportunity to identify, agnostically, proteins and mechanisms that are promoted by aspirin treatment; this, nonetheless, remains relevant to understanding how aspirin prevents tumor progression. In addition, the crossover design allowed participants to serve as their own control, which minimized unmeasured inter-individual variability. Because the samples from the same participant were randomly ordered and analyzed within the same batch, additional adjustment for batch effects and sample positions did not change the results. There was also a 3-month wash-out period between treatments (longer than the usual 60-day periods); this minimizes carry-over effects.

There are also some limitations. First, our analysis was primarily designed to examine the signaling effects of aspirin and has no specific hypothesis or protein to validate. Subsequent steps would involve testing on another set of subjects who took aspirin or placebo but these samples are not available to us at this time. Second, the duration of treatment was two months,

and this may not characterize the long-term effects of aspirin use in cancer prevention. Thirdly, our sample size was relatively small and lacked substantial as plasma protein levels were not the main outcome of the intervention. Effect sizes were also small which may reflect the fact that all the participants were generally healthy and therefore would have had relatively low expression of pro-inflammatory proteins; this, in turn would leave less room for change with aspirin intervention. Furthermore, the expression levels of proteins are context-specific: our findings using plasma may differ from those in other tissues, such as colon mucosa. Six of the nine proteins typically are found in the nucleus, so their presence in plasma might result from cellular leakage/damage or exosome formation. In support of the latter, aspirin has been reported to affect the content of platelet-derived exosomes (64). Lastly, we used a liberal significance level of 0.1 for FDR adjustment. Although a higher threshold was used to identify more possible candidates, it might also lead to more false positive results. Therefore, further investigations of these identified proteins are needed.

To our knowledge, this is the first randomized trial to systematically evaluate the effect of aspirin on plasma protein profiles in healthy men and women. We identified several proteins that differed significantly between aspirin and placebo, some of which have been previously reported as playing a role in inflammation and carcinogenesis. The involvement of various biologic pathways suggests that the chemopreventive mechanisms of aspirin in humans are complex. Larger, confirmatory studies with a longer period of aspirin exposure are needed.

**Table 4.1 Demographic characteristics of 44 participants**

| **Characteristics** | **N (%)** |
|---|---|
| Age, Mean(SD) | 30.43 (5.97) |
| Sex | |
| Male | 20 (45.5) |
| Female | 24 (54.5) |
| BMI, $kg/m^2$ | |
| Normal (<25) | 23 (52.3) |
| Overweight or obese (≥25) | 21 (47.7) |
| Race/Ethnicity | |
| Caucasian | 33 (75.0) |
| African-American | 1 (2.3) |
| Asian | 5 (11.4) |
| Other | 5 (11.4) |

**Table 4.2 Proteins that differed significantly between aspirin and placebo periods with false discovery rate (FDR) <0.10**

| Symbol | Function[a] | Missing %[b] | Average expression[c] | | Effect size[d] | Fold change[e] | p-value[f] | Adjusted p-value[g] |
|---|---|---|---|---|---|---|---|---|
| | | | aspirin | placebo | | | | |
| SDHC | Conservative effector of mitochondrial Krebs cycle and respiratory chain | 20.5 | -0.259 | -0.112 | -0.594 | 0.662 | $4.47\times10^{-05}$ | 0.058 |
| MYH1 | Energy convertor in base excision repair (BER) pathway | 18.2 | -0.262 | -0.454 | 0.698 | 1.622 | $6.83\times10^{-05}$ | 0.058 |
| NR2F1 | Nuclear hormone receptor and transcriptional regulator | 11.4 | -0.371 | -0.265 | -0.755 | 0.592 | $6.94\times10^{-05}$ | 0.058 |
| FOXO1 | Transcription factor in carbohydrates metabolism and Akt-mTOR signaling pathway | 17.0 | 1.003 | 0.845 | 0.520 | 1.434 | $7.72\times10^{-05}$ | 0.058 |
| KHDRBS3 | RNA-binding protein regulating pre-mRNA splicing, signaling and cell cycle control | 27.3 | 0.096 | -0.028 | 0.400 | 1.320 | $2.07\times10^{-04}$ | 0.087 |
| NFKBIE | NF-κB inhibitor epsilon | 23.9 | 0.530 | 0.374 | 0.709 | 1.634 | $2.15\times10^{-04}$ | 0.087 |
| LYZ | Lysozyme, antimicrobial enzyme | 26.1 | 0.004 | -0.147 | 0.689 | 1.612 | $2.29\times10^{-04}$ | 0.087 |
| MSI1 | RNA-binding protein, posttranscriptional regulator of proliferative activity | 26.1 | -0.351 | -0.176 | -0.487 | 0.714 | $2.32\times10^{-04}$ | 0.087 |
| IKZF1 | Transcription factor of zinc-finger DNA-binding and lymphocyte differentiation regulator | 4.5 | -0.310 | -0.426 | 0.542 | 1.456 | $2.67\times10^{-04}$ | 0.089 |

[a] Information pertaining to encoded protein function was derived from PubMed Gene unless otherwise noted.
[b] Missing% is the proportion of samples with missing values on this protein among all 88 samples.
[c] Average expression level was presented in median M values for each protein.
[d] Effect size was the mean difference of M values between two treatment periods standardized by standard deviation of average expression in placebo period .
[e] Fold-change was the standardized ratio between median M values of aspirin and placebo treatment. A fold change >1 indicated greater antibody expression after aspirin treatment compared to placebo; a fold change <1 indicated lower expression after aspirin treatment.
[f] P-values were obtained from mixed linear regression model, adjusted for batch effect, sample position, gender and genotype.
[h] P-values were adjusted for false discovery rate using Benjamini-Horchberg procedure.

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# Chapter 5. CONCLUSION

In this project, we aimed to better understand the relationships and underlying mechanisms of aspirin and NSAIDs, chronic inflammation and CRC risk. To achieve these aims, we conducted in-depth analyses of various aspects of these relationships, using multiple study design and statistical approaches.

To our knowledge, we were the largest study to systematically analyze the interactions between NSAID use and other risk factors in relation to CRC risk. We observed that regular use of NSAIDs, including both aspirin and non-aspirin NSAIDs, was statistically significantly protective against CRC risk in almost all subgroups stratified by other CRC risk factors. We found stronger associations between aspirin and CRC risk among non-smokers than among smokers. We also found a suggestion of interaction between any NSAID use and BMI on CRC risk, primarily driven by aspirin, with the protective effect of aspirin smaller as BMI increased. The beneficial effect of aspirin on CRC risk appears to be attenuated, rather than enhanced, among those with greater CRC risk due to obesity and heavy smoking, making it unlikely that these groups would benefit from use of NSAIDs, specifically aspirin, for the prevention of CRC.

One of the proposed chemopreventive mechanisms of aspirin and NSAIDs is via reduced chronic inflammation. As the most commonly used non-specific biomarker for low-grade chronic inflammation, CRP has been reported to be moderately associated with increased CRC risk. Using information from three international consortia, we further investigated the possibility of causal relationship between CRP and CRC risk. Using GWAS-identified genetic variants as instrumental variables for circulating CRP levels, we found that elevated CRP level was not statistically significantly associated with increased CRC risk among participants of European

ancestry. Our findings suggested that the previously reported association between CRP and CRC risk in observational studies might be due to confounding by environmental factors, or reversed causality from precancerous lesions or preclinical disease increasing the inflammation level in the tissue microenvironment leading to increases in CRP levels. It is also possible that CRP is not specific enough to CRC-related inflammation pathways, and therefore it is unlikely that CRP plays a causal role in colorectal carcinogenesis. Although we had greater statistical power to test for the relationship between CRP and CRC risk, compared to previous studies, we did not have direct CRP measures in our study and therefore we were not able to directly test for the exclusion restriction assumption in our data and we were not able to jointly estimate the effect of SNPs on CRC risk. In the future, we plan to obtain the CRP measures and other biomarkers in a subset of the study population, and can therefore further test the Mendelian randomization assumptions and replicate our findings from this study.

In addition to CRP-related pathways, we found several proteins that differed significantly between aspirin and placebo among healthy individuals in a randomized crossover trial. These proteins were previously reported to be associated with carcinogenesis and inflammation and could play important roles in various pathways, including the mitochondrial Kreb's cycle, DNA base-pair repair, and inflammation, but evidence was largely from cell line or animal studies, and limited from human studies. Our study was the first randomized trial to systematically evaluate the effect of aspirin on plasma protein profiles in healthy men and women. Our findings suggested the involvement of various biologic pathways in the chemopreventive mechanisms of aspirin in humans, although larger, confirmatory studies are needed.

As a next step, we plan to assess the interaction between aspirin/NSAIDs and genes using functional information as gene expression scores in relation to CRC risk. We would also like to

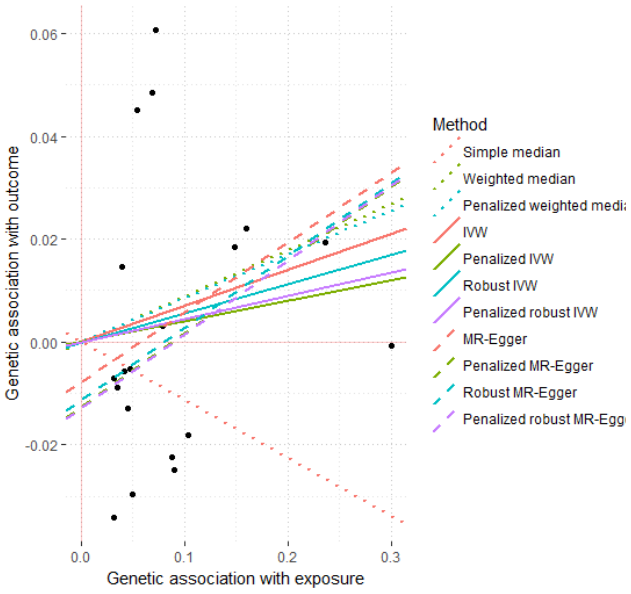
stratify the analysis by BMI and smoking status, since different subgroups of population may be susceptible to different biological pathways of cancer development.

Colorectal cancer is one of the most common and fatal cancers in the world, and aspirin and other NSAIDs are promising chemopreventive candidates. Findings of our project add to the knowledge of mechanisms of aspirin and other NSAIDs and to the current understanding of carcinogenic pathways that are related to chronic inflammation, and also may help inform future guidelines for primary prevention of CRC.

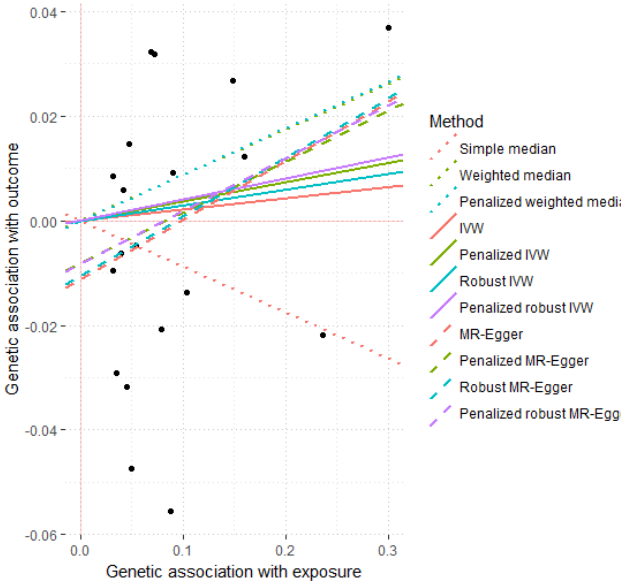
## APPENDIX A: SUPPLEMENTAL FIGURES

**Supplemental Figure 1 Sensitivity analyses of the final set using other Mendelian randomization methods (Chapter 3)**

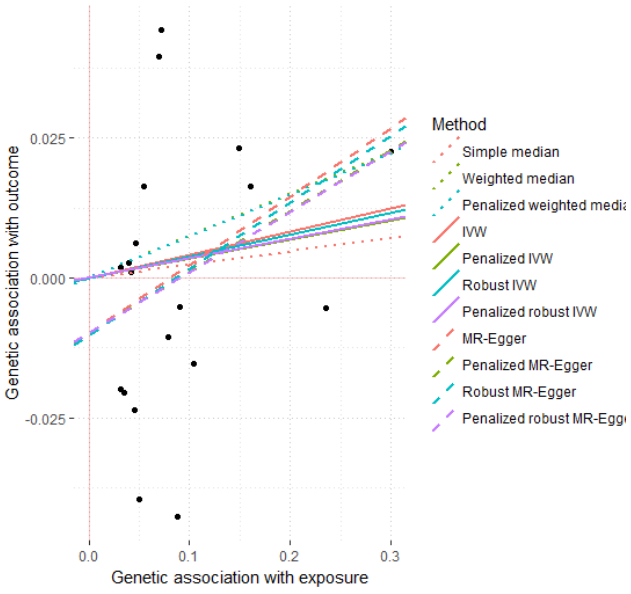
**A) GECCO**



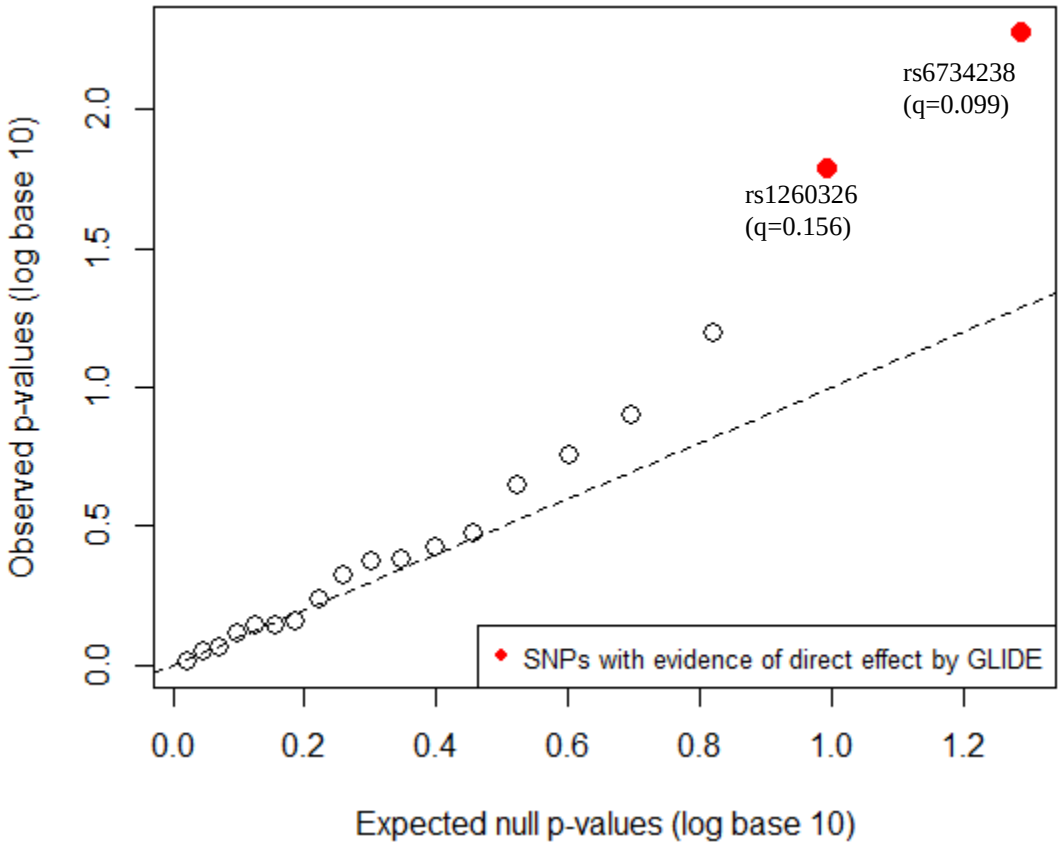

**B) CORECT**




**C) GECCO & CORECT combined**

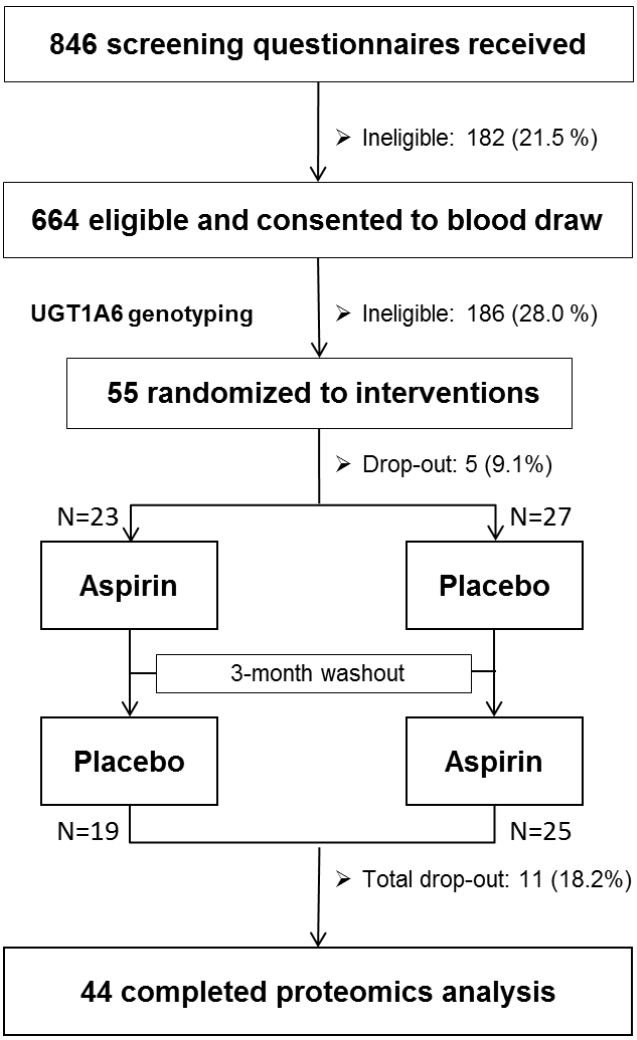


**Supplemental Figure 2. Diagnostic testing for pleiotropic effects of individual SNPs in combined data (Chapter 3)**




* No SNPs had q value <0.05 after FDR adjustment; SNP in solid dots had q value <0.2 after FDR adjustment.

**Supplemental Figure 3. Flow chart of participant enrollment and study design (Chapter 4)**



## APPENDIX B: SUPPLEMENTAL TABLES

### Supplemental Table 1. Estimated associations between regular use of NSAIDs and colorectal cancer risk by subsites and stages (Chapter 2)

| | Any NSAID | | | | Aspirin | | | | Non-aspirin NSAIDs | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Cases** | **Controls** | **OR (95% CI)[a]** | **P value** | **Cases** | **Controls** | **OR (95% CI)[a]** | **P value** | **Cases** | **Controls** | **OR (95% CI)[a]** | **P value** |
| **Subsite** | | | | | | | | | | | | |
| proximal | 4,750 | 15,999 | 0.73 (0.68, 0.79) | <0.001 | 4,698 | 15,914 | 0.78 (0.72, 0.85) | <0.001 | 4,621 | 15,503 | 0.82 (0.74, 0.90) | <0.001 |
| distal | 3,271 | 15,999 | 0.73 (0.67, 0.80) | <0.001 | 3,249 | 15,914 | 0.78 (0.71, 0.85) | <0.001 | 3,156 | 15,503 | 0.78 (0.69, 0.88) | <0.001 |
| rectal | 2,605 | 15,999 | 0.65 (0.59, 0.72) | <0.001 | 2,574 | 15,914 | 0.73 (0.65, 0.82) | <0.001 | 2,528 | 15,503 | 0.74 (0.64, 0.86) | <0.001 |
| *P value for heterogeneity*[b] | | | | | | | | | | | | |
| *Rectal vs colon* | | | | 0.146 | | | | 0.091 | | | | 0.695 |
| *Distal vs proximal* | | | | 0.433 | | | | 0.162 | | | | 0.831 |
| **Stage** | | | | | | | | | | | | |
| local | 3,092 | 15,999 | 0.74 (0.68, 0.81) | <0.001 | 3,069 | 15,914 | 0.79 (0.72, 0.87) | <0.001 | 3,018 | 15,503 | 0.82 (0.73, 0.92) | <0.001 |
| regional | 5,130 | 15,999 | 0.71 (0.66, 0.76) | <0.001 | 5,087 | 15,914 | 0.76 (0.70, 0.83) | <0.001 | 5,003 | 15,503 | 0.80 (0.72, 0.88) | <0.001 |
| distant | 1,150 | 15,999 | 0.76 (0.66, 0.87) | <0.001 | 1,139 | 15,914 | 0.80 (0.69, 0.93) | 0.003 | 1,126 | 15,503 | 0.84 (0.69, 1.00) | 0.056 |
| *P value for heterogeneity*[b] | | | | | | | | | | | | |
| *Regional vs local* | | | | 0.371 | | | | 0.350 | | | | 0.370 |
| *Distant vs local* | | | | 0.465 | | | | 0.843 | | | | 0.490 |

[a] Study-specific ORs and 95% CIs are estimated using logistic regression models, adjusting for age, sex, education (less than high school, high school graduate or GED, some college, college graduate, graduate degree), first-degree family history of colorectal cancer (yes/no), history of endoscopy (yes/no), postmenopausal hormone use among women (yes/no), history of diabetes(yes/no), body mass index ($kg/m^2$), moderate/vigorous activity (hours/week), smoking (non-smokers and quartiles of pack-years), alcohol intake (none, 1-28g/day, >28g/day), dietary intakes (quartiles) of fruit, vegetables, red meat, processed meat and fiber, total energy intake (quartiles), total (dietary and supplemental) intakes of calcium and folate (quartiles). Covariates in quartiles are adjusted as group linear variables in the model. For aspirin or non-aspirin NSAID use only, the other type was also adjusted for.

[b] P value for heterogeneity used to test for differences across cancer subsite and stage among cases only, using fixed-effect meta-analysis. More details are described in methods.

**Supplemental Table 2. Demographic characteristics of participating studies (Chapter 3)**

| | Study | Country | N Cases | N Controls | Male, N (%) | Age, mean (sd), years | Study Design |
|---|---|---|---|---|---|---|---|
| **GECCO** | ASTERISK | France | 892 | 947 | 1,076 (58.5) | 65.2 (10.6) | Hospital case-control |
| | CCFR | United States | 1,434 | 1,230 | 1,315 (49.4) | 53.4 (11.6) | Case-control |
| | Colo 2&3 | United States | 87 | 123 | 117 (55.5) | 65.3 (11.3) | Population case-control |
| | DACHS | Germany | 2,373 | 2,200 | 2,745 (60.0) | 68.7 (10.4) | Population case-control |
| | DALS | United States | 1,110 | 1,170 | 1,255 (55.0) | 63.8 (9.9) | Population case-control |
| | HPFS | United States | 173 | 229 | 402 (100) | 66.0 (8.8) | Cohort |
| | MEC | United States | 326 | 346 | 361 (53.7) | 63.0 (8.0) | Cohort |
| | NHS | United States | 298 | 774 | 0 (0) | 59.9 (6.6) | Cohort |
| | OFCCR | Canada | 598 | 522 | 540 (48.2) | 62.1 (7.9) | Population case-control |
| | PHS | United States | 375 | 389 | 764 (100) | 58.9 (9.0) | Cohort |
| | PLCO | United States | 979 | 861 | 1,093 (59.4) | 64.2 (5.2) | Cohort |
| | PMH | United States | 276 | 122 | 0 (0) | 62.7 (7.1) | Population case-control |
| | VITAL | United States | 282 | 287 | 299 (52.5) | 66.5 (6.2) | Cohort |
| | WHI | United States | 1,441 | 1,528 | 0 (0) | 66.4 (6.6) | Cohort |
| | **Subtotal** | | **10,644** | **10,729** | **9,967 (46.6)** | **63.7 (10.3)** | |
| **CORECT** | CCFR | United States, Canada, Australia | 2,776 | 1,197 | 2,127 (53.5) | 52.7 (12.1) | Case-control |
| | CPSII | United States | 540 | 536 | 548 (50.9) | 68.7 (5.5) | Cohort |
| | MECC | United States | 4,146 | 3,113 | 3,842 (52.9) | 70.2 (11.9) | Population case-control |
| | MCCS | Australia | 709 | 634 | 691 (51.5) | 59.7 (7.6) | Cohort |
| | NFCCR | Newfoundland | 184 | 456 | 376 (65.8) | 60.0 (9.0) | Population case-control |
| | Kentucky | United States | 1,035 | 1,132 | 1,070 (49.4) | 62.7 (9.4) | Population case-control |
| | Spain | Spain | 742 | 786 | 896 (58.6) | 66.0 (11.4) | Case-control |
| | SEARCH | United Kingdom | 4,139 | 115 | 2,442 (57.4) | 63.1 (7.9) | Case-control |
| | SWEDEN_Wolk | Sweden | 519 | 831 | 811 (60.1) | 63.6 (8.1) | Cohort |
| | ATBC | Finland | 147 | 30 | 177 (100) | 57.4 (4.8) | Cohort |
| | ColoCare_heidelberg | Germany | 187 | 36 | 141 (63.2) | 61.4 (12.1) | Case-series |
| | ColoCare_Seattle | United States | 169 | 0 | 96 (56.8) | 56.6 (13.2) | Case-series |
| | ESTHER_VERDI | Germany | 397 | 420 | 534 (65.4) | 65.1 (6.8) | Case-control |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | Kiel | Germany | 1,103 | 0 | 621 (56.3) | 63.8 (9.0) | Case-control |
| | MEC | United States | 63 | 81 | 71 (49.3) | 61.4 (8.9) | Cohort |
| | MSKCC | United States | 68 | 0 | 27 (39.7) | 60.5 (12.4) | Case-series |
| | NHS2 | United States | 87 | 80 | 0 (0) | 37.0 (4.3) | Cohort |
| | SWEDEN_Lindblom | Sweden | 2,504 | 2,281 | 2,565 (53.6) | 61.7 (0) | Cohort |
| | USC_HRT_CRC | United States | 321 | 387 | 0 (0) | 65.6 (5.3) | Population case-control |
| | **Subtotal** | | **19,836** | **12,115** | **17,035 (53.4)** | **63.3 (10.8)** | |
| | **Total** | | **30,480** | **22,844** | **27,002 (50.7)** | **63.4 (10.6)** | |

ASTERISK: Association Study Evaluating RISK for Sporadic Colorectal Cancer; CCFR: Colorectal Cancer Family Registry; Colo 2&3: a case-control study from the University of Hawai'I; DACHS: Darmkrebs: Chancen der Verhutüng durch Screening Study; DALS: Diet, Activity and Lifestyle Study; HPFS: Health Professionals Follow-up Study; MEC: Multiethnic Cohort; NHS: Nurses' Health Study; OFCCR: Ontario Familial Colorectal Cancer Registry; PHS: Physicians' Health Study; PLCO: Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial; PMH: Postmenopausal Hormone Study – Colon Cancer Family Registry; VITAL: Vitamins and Lifestyle Study; Women's Health Initiative; CPSII: Cancer Society Cancer Prevention Study II; MECC: Molecular Epidemiology of Colorectal Cancer Study; MCCS: Melbourne Case-Control Study (in Melbourne Collaborative Cohort); NFCCR: NewFoundland Case-Control Study; SEARCH: Studies of Epidemiology and Risk Factors in Cancer Heredity; SWEDEN_Wolk: The Swedish Mammography Cohort; ATBC: Alpha-Tocopherol, Beta-Carotene Cancer Prevention; ColoCare_heidelberg: The ColoCare study at the University Hospital Heidelberg; ColoCare_Seattle: The ColoCare study at the Fred Hutchinson Cancer Research Center; ESTHER_VERDI: ESTHER/VERDI study in Saarland, Germany; Kiel: Sample collected in PopGen Biobank; MSKCC: Memorial Sloan Kettering (MSK) cohort; NHS2: Nurses' Health Study II; SWEDEN_Lindblom: The Swedish Low-Risk Colorectal Cancer Study; USC_HRT_CRC: a case-control study for HRT use and CRC risk in Los Angeles county.

**Supplemental Table 3. Plasma level differences in the expression of the nine significant proteins, stratified by sex. (Chapter 4)**

| Gene name | Male (N=20) | | | | | | Female (N=24) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Average expression[a] | | Effect size[b] | Fold change[c] | p-value | Adjusted p-value[d] | Average expression[a] | | Effect size[b] | Fold change[c] | p-value | Adjusted p-value[d] |
| | aspirin | placebo | | | | | aspirin | placebo | | | | |
| SDHC | -0.189 | -0.076 | -0.432 | 0.741 | $7.19\times10^{-03}$ | 0.73 | -0.318 | -0.142 | -0.743 | 0.597 | $1.77\times10^{-03}$ | 0.36 |
| MYH1 | -0.295 | -0.387 | 0.421 | 1.339 | $9.21\times10^{-02}$ | 0.85 | -0.235 | -0.510 | 0.894 | 1.859 | $1.51\times10^{-04}$ | 0.23 |
| NR2F1 | -0.385 | -0.292 | -0.615 | 0.653 | $1.61\times10^{-02}$ | 0.73 | -0.360 | -0.242 | -0.901 | 0.536 | $2.38\times10^{-03}$ | 0.36 |
| FOXO1 | 0.986 | 0.821 | 0.660 | 1.580 | $2.94\times10^{-03}$ | 0.73 | 1.017 | 0.864 | 0.440 | 1.356 | $7.23\times10^{-03}$ | 0.45 |
| KHDRBS3 | 0.064 | -0.001 | 0.192 | 1.142 | $1.44\times10^{-01}$ | 0.85 | 0.121 | -0.050 | 0.604 | 1.520 | $3.23\times10^{-04}$ | 0.23 |
| NFKBIE | 0.471 | 0.365 | 0.439 | 1.355 | $1.21\times10^{-02}$ | 0.73 | 0.579 | 0.381 | 0.958 | 1.943 | $3.68\times10^{-03}$ | 0.37 |
| LYZ | 0.023 | -0.152 | 0.749 | 1.680 | $1.65\times10^{-02}$ | 0.73 | -0.013 | -0.143 | 0.620 | 1.537 | $2.16\times10^{-03}$ | 0.36 |
| MSI1 | -0.292 | -0.113 | -0.478 | 0.718 | $1.48\times10^{-02}$ | 0.73 | -0.400 | -0.229 | -0.497 | 0.709 | $4.69\times10^{-03}$ | 0.40 |
| IKZF1 | -0.289 | -0.438 | 0.656 | 1.575 | $1.43\times10^{-03}$ | 0.73 | -0.335 | -0.415 | 0.419 | 1.337 | $5.10\times10^{-02}$ | 0.65 |

[a] Average expression level was presented in median M values for each protein.
[b] Effect size was the mean difference of M values between two treatment periods standardized by standard deviation of average expression in placebo period .
[c] Fold-change was the standardized ratio between median M values of aspirin and placebo treatment. A fold-change >1 indicated greater antibody expression after aspirin treatment compared to placebo; a fold change <1 indicated lower expression after aspirin treatment.
[d] P-values were adjusted for false discovery rate using Benjamini-Horchberg procedure.

**Supplemental Table 4. List of gene sets with unadjusted p-values <0.05. (Chapter 4)**

| Database | Gene set name | Size[a] | p value | Adjusted p-value[b] |
|---|---|---|---|---|
| **KEGG** | Pertussis | 55 | 0.001 | 0.257 |
| | Retinol metabolism | 6 | 0.003 | 0.386 |
| | Salmonella infection | 43 | 0.007 | 0.551 |
| | Cytosolic DNA-sensing pathway | 21 | 0.013 | 0.551 |
| | Propanoate metabolism | 3 | 0.013 | 0.551 |
| | Rap1 signaling pathway | 150 | 0.014 | 0.551 |
| | Spliceosome | 12 | 0.015 | 0.551 |
| | Prion diseases | 35 | 0.019 | 0.570 |
| | Phagosome | 55 | 0.026 | 0.570 |
| | Glyoxylate and dicarboxylate metabolism | 7 | 0.026 | 0.570 |
| | NOD-like receptor signaling pathway | 35 | 0.030 | 0.570 |
| | Rheumatoid arthritis | 103 | 0.032 | 0.570 |
| | Bile secretion | 9 | 0.034 | 0.570 |
| | Amphetamine addiction | 13 | 0.036 | 0.570 |
| | Arachidonic acid metabolism | 8 | 0.037 | 0.570 |
| | RIG-I-like receptor signaling pathway | 24 | 0.040 | 0.570 |
| | Herpes simplex infection | 82 | 0.044 | 0.570 |
| | Vibrio cholerae infection | 9 | 0.046 | 0.570 |
| | Gastric acid secretion | 12 | 0.047 | 0.570 |
| | Cytokine-cytokine receptor interaction | 288 | 0.049 | 0.570 |
| | Glutathione metabolism | 9 | 0.049 | 0.570 |
| **GO** | Oxidoreductase activity | 5 | 0.003 | 0.616 |
| | Ion homeostasis | 89 | 0.005 | 0.616 |
| | Cellular cation homeostasis | 83 | 0.005 | 0.616 |
| | Cation homeostasis | 83 | 0.005 | 0.616 |
| | MAPKkk cascade | 41 | 0.005 | 0.616 |
| | Lipid binding | 37 | 0.005 | 0.616 |
| | Activation of MAPK activity | 16 | 0.005 | 0.616 |
| | Synaptic transmission | 22 | 0.006 | 0.616 |
| | Negative regulation of response to stimulus | 10 | 0.006 | 0.616 |
| | Cellular homeostasis | 96 | 0.007 | 0.616 |
| | Oxidoreductase activity | 53 | 0.007 | 0.616 |
| | Negative regulation of translation | 18 | 0.007 | 0.616 |
| | Apoptotic nuclear changes | 7 | 0.009 | 0.616 |
| | Nuclear organization and biogenesis | 7 | 0.009 | 0.616 |
| | Transmission of nerve impulse | 25 | 0.010 | 0.616 |
| | Isomerase activity | 8 | 0.010 | 0.616 |
| | Transcription cofactor activity | 48 | 0.011 | 0.616 |
| | Growth factor binding | 25 | 0.011 | 0.616 |

| | | | |
|---|---|---|---|
| Contractile fiber | 6 | 0.011 | 0.616 |
| Contractile fiber (part) | 6 | 0.011 | 0.616 |
| Response to chemical stimulus | 186 | 0.012 | 0.616 |
| Homeostatic process | 126 | 0.012 | 0.616 |
| Calcium ion binding | 38 | 0.012 | 0.616 |
| Regulation of cytokine biosynthetic process | 18 | 0.012 | 0.616 |
| Regulation of gene expression (epigenetic) | 4 | 0.013 | 0.641 |
| Chemical homeostasis | 102 | 0.015 | 0.655 |
| Brush border | 3 | 0.015 | 0.655 |
| Secondary metabolic process | 4 | 0.016 | 0.655 |
| Pigment biosynthetic process | 4 | 0.016 | 0.655 |
| Pigment metabolic process | 4 | 0.016 | 0.655 |
| Protease inhibitor activity | 23 | 0.017 | 0.655 |
| Tube morphogenesis | 8 | 0.017 | 0.655 |
| Cofactor biosynthetic process | 3 | 0.018 | 0.666 |
| Feeding behavior | 3 | 0.019 | 0.666 |
| Cysteine type endopeptidase activity | 15 | 0.020 | 0.666 |
| Anion transport | 5 | 0.020 | 0.666 |
| Late endosome | 5 | 0.020 | 0.666 |
| Cofactor metabolic process | 7 | 0.025 | 0.794 |
| External side of plasma membrane | 9 | 0.026 | 0.794 |
| Negative regulation of cellular biosynthetic process | 28 | 0.027 | 0.794 |
| Negative regulation of biosynthetic process | 28 | 0.027 | 0.794 |
| Transmembrane receptor activity | 121 | 0.029 | 0.794 |
| Hormone binding | 13 | 0.029 | 0.794 |
| Positive regulation of MAP kinase activity | 38 | 0.030 | 0.804 |
| Amino acid and derivative metabolic process | 13 | 0.032 | 0.806 |
| Behavior | 112 | 0.033 | 0.806 |
| Regulation of MAP kinase activity | 40 | 0.034 | 0.806 |
| Smooth muscle contraction | 5 | 0.034 | 0.806 |
| Structural constituent of muscle | 3 | 0.034 | 0.806 |
| Myofibril | 5 | 0.035 | 0.814 |
| Neuron projection | 5 | 0.037 | 0.828 |
| Transcription factor binding | 79 | 0.039 | 0.828 |
| Cellular component disassembly | 9 | 0.039 | 0.828 |
| Intramolecular oxidoreductase activity | 7 | 0.040 | 0.828 |
| Exopeptidase activity | 7 | 0.041 | 0.828 |
| Regulation of muscle contraction | 4 | 0.041 | 0.828 |
| Transmembrane receptor protein tyrosine kinase activity | 50 | 0.042 | 0.835 |
| Transmembrane receptor protein kinase activity | 51 | 0.044 | 0.860 |
| Cellular biosynthetic process | 92 | 0.046 | 0.865 |

| | | | |
|---|---|---|---|
| Negative regulation of cytokine biosynthetic process | 13 | 0.047 | 0.865 |
| Cortical actin cytoskeleton | 4 | 0.047 | 0.865 |
| Transcription corepressor activity | 24 | 0.048 | 0.865 |

[a] Size indicated the number of genes that had corresponding proteins tested in the analysis.
[b] P-values were adjusted for false discovery rate using Benjamini-Horchberg procedure

# VITA

Xiaoliang Wang was born and raised in Shanghai, China. In 2011, she earned a Bachelor of Science degree in Biological Sciences from Fudan University, China. In 2013, she earned a Master of Public Health degree in Epidemiology from Columbia University Mailman School of Public Health. In 2017, she earned a Doctor of Philosophy in Epidemiology from the University of Washington in Seattle.