Low Penetrance Alleles in Colorectal Cancer

the arachidonic acid pathway

Genetische aanleg voor dikke darmkanker

de arachidonzuur route

Proefschrift

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Voor Joost
Aan papa, mama
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List of abbreviations

AA	arachidonic acid
ALA	alpha-linolenic acid
APC	Adenomatous polyposis Coli
COX	cyclooxygenase
CRC	colorectal cancer
DHA	docosahexaenoic acid
DOM	diagnostisch onderzoek mammacarcinoom
EPA	eicosapentanoic acid
ESE	exonic splice enhancer
HNPCC	hereditary non-polyposis colorectal cancer
HODE	hydroxyoctadecadienoic acid
htSNP	haplotype tagging single nucleotide polymorphism
LA	linoleic acid
LD	linkage disequilibrium
LOX	lipoxygenase
MMR	mismatch repair
Mom	modifier of Min
NF-κB	nuclear factor κB
NSAID	nonsteroidal anti-inflammatory drug
OR	odds ratio
PCR	polymorease chain reaction
PG	prostaglandin
PLA2	phospholipase A2
PPAR	peroxisome prolifertor-activated receptor
PPHV	peilstation project hart en vaatziekten
PTGS	prostaglandin-endoperoxide synthase
PUFA	polyunsaturated fatty acid
RFLP	restriction fragment length polymorphism
RR	Relative risk
SD	standard deviation
SNP	single nucleotide polymorphism
TX	thromboxane
UTR	untranslated region

Chapter 1

Introduction

1.1 Colorectal cancer

1.1.1 Colorectal carcinogenesis

The distal tract of the human digestive apparatus consists of the colon and rectum, starting at the end of the small intestine and terminating at the anus. The surface of the large bowel comprises a single layer of epithelial cells. These cells arise from stem cells at the bottom of the crypts present in the colon. They multiply in the bottom third and differentiate in the upper two thirds of the crypt after which they migrate further into the luminal surface. In this fashion, intestinal cells are continuously renewed, with each proliferative cell within the crypt dividing twice a day, resulting in the production of about 300 cells per crypt per day.(1). This adds up to around 10^{11} cells that are shed into the lumen of the large bowel each day (2).
Colorectal tumors arise from defects in the delicate balance between stem cell renewal and differentiation resulting in clonal expansion of colonic epithelial cells. Colorectal cancer is a multistage process, in which the tumor develops through a series of distinct histopathological steps, each accompanied by specific genetic alterations. A model of this process, as proposed by Fearon and Vogelstein in 1990 (3), describes how the tumor proceeds from the earliest stage of aberrant crypt foci to various adenoma stages and eventually into malignant metastasizing carcinoma. This process is characterized by a series of genetic alterations in oncogenes, tumor suppressor genes and caretaker genes, as well as epigenetic events. Cells that have a selective advantage over others within the tumor through the acquisition of mutations enhancing their survival are selected, leading to clonal expansion. The genes involved in this process have been studied in detail and the key genes among many others include two genes disrupting the Wnt signaling pathway, the adenomatous polyposis coli (*APC*) gene and *β-catenin* gene, *K-RAS*, important in progression from small to larger adenomas and *TP53*, mainly in the later stages of tumor development. Other events include loss of heterozygosity of chromosome 18q, mutations in *PRL3* leading to metastasis, and microsatellite instability (4-6).

1.1.2 Epidemiology

1.1.2.1 Incidence

Colorectal cancer is the second leading cause of cancer deaths in the Western world. In the US it is estimated that 1 in 17 men and 1 in 18 women will develop colorectal cancer at some point in their life. This adds up to an estimated 145000 incident cases in 2005 in the US alone, with nearly 57000 estimated deaths due to colorectal cancer, accounting for 10% of all cancer deaths (7). In the Netherlands, the incidence and mortality rates reflect the rates of the US and other Western countries, with over 9500 new cases and nearly 4400 colorectal cancer deaths in the year 2002 (8). Interestingly, in western countries the incidence rates seem to be steadily declining, while in Japan colorectal cancer incidence is rapidly increasing (9).

1.1.2.2 Risk factors

There are several factors, both genetic and environmental, that influence the formation and development of colorectal cancer. Individuals can be at increased risk due to their genetic constitution. Genetic predisposition to colorectal cancer will be discussed in more detail in section 1.2.
Other high risk groups include individuals with adenomas, since approximately 5% of adenomas develop into carcinomas. Also inflammatory bowel diseases like ulcerative colitis and Crohn's disease can confer increased risk of colorectal cancer, indicating that inflammation also plays an important role in colon tumorigenesis. Although the mechanism by which inflammation influences tumorigenesis is not completely understood, there are several inflammation-induced changes observed in cells and tissues which might play a role in this process. These include changes in cytokine environment, prostaglandin formation,

glycosylation, apoptosis and angiogenesis (10,11). Moreover, inhibition of inflammatory mediators has been shown to inhibit tumor formation (12).
Epidemiological studies have indicated that a "Western lifestyle" is one of the main risk factors in colorectal cancer. This is illustrated by classical ecological studies involving Japanese immigrants to the US. Colorectal cancer incidence in Japan, where the traditional diet differs substantially from the Western diet, has always been much lower than in Western countries. This difference between populations is reduced when Japanese migrate to the US and adopt a more Western diet, subsequently increasing cancer incidence to reflect that of the adoptive country (13). Excess alcohol intake and diets high in red and processed meat are suggested to increase risk, while adequate levels of folate, calcium and vitamin D intake appear to be protective (14). The possible involvement of fatty acid composition will be discussed in more detail in section 1.3.2.
Other lifestyle and environmental factors that might influence the risk of colorectal cancer include low physical activity, excess body weight, smoking earlier in life and use of nonsteroidal anti-inflammatory drugs (NSAIDs) the latter of which will be discussed in section 1.3.3 (15).

1.2 Genetic predisposition to colorectal cancer

Colorectal cancer is traditionally divided in two distinct types, familial and sporadic cases, with familial cases being genetically predisposed to the disease on the account of inherited mutations in the germline, whereas in sporadic cases similar mutations accumulate in somatic cells, principally as the result of the involvement of endogenous and environmental risk factors. In a large study on nearly 45000 twin pairs it was estimated that 35% of colorectal cancer cases is due to heritable factors, indicating that our knowledge of the genetics of cancer is very limited (16). Moreover, a 2-fold increased risk in first-degree relatives of sporadic colorectal cancer patients has been observed, which suggest a mild predisposition and the involvement of low penetrance alleles. Taken together, this data suggests that familial and sporadic colorectal cancer are two very extremes with a large grey area in between. This grey area contains patients with no apparent or unknown family history as well as individuals with a weak family history. Only a few low penetrance alleles have been identified at present accounting for some of these cases, but many more are likely to be identified (17). Most, if not all of these low penetrance alleles will also require the involvement of environmental risk factors to result in the colorectal cancer phenotype. Quoting Judith Stern: "While genetics loads the gun, the environment pulls the trigger. Predisposition to colorectal cancer is therefore only in some exceptional cases just a straightforward matter of inheriting a high penetrance allele resulting in a familial syndrome and a very high chance of developing the disease, but in the majority of cases the presence of a combination of low penetrance alleles together with specific environmental factors (18).

1.2.1 High penetrance alleles

It is believed that high penetrance mutations play a role in up to 20% of colorectal cancer cases, whereas known high penetrance alleles giving rise to familial syndromes account for around 5% of cases (19). These syndromes are divided in two categories on the basis of presence or absence of polyposis. The polyposis syndromes consist of at least four members, familial adenomatous polyposis (FAP), Peutz-Jeghers syndrome, juvenile polyposis and MYH-polyposis, of which only FAP will be briefly described because of the involvement of the Wnt signaling pathway in sporadic colorectal cancer. FAP, accounting for less than 1% of colorectal cancer cases, is an autosomal dominant disease characterized by the formation of multiple adenomas in the colon during the second or third decade of life, leading to malignant tumors in near 100% of patients by the age of 40 to 50 years (19). The disease is initiated by a germline mutation in the adenomatous polyposis coli (APC) gene (first hit), resulting in disregulation of the Wnt signaling pathway and genetic instability (6).

For the formation of an adenoma a second hit, resulting in complete loss of functional APC, seems required. Similar loss of APC function is estimated to be essential in about 80% of sporadic colorectal cancer cases, although this involves other APC mutations compared to FAP and also includes epigenetic events. However, more recently contradictory reports on the absolute frequency of *APC* mutations in colorectal cancer have been published (20).
Hereditary non-polyposis colorectal cancer (HNPCC), also called Lynch syndrome, is the most common inherited colorectal cancer syndrome, accounting for approximately 3% of cases (21). This syndrome is characterized by early onset of disease (approximately 45 years of age) as compared to sporadic cancer, and a relatively large predisposition to other cancers as well. In this syndrome the progression from adenoma to carcinoma is accelerated to 2-3 years as opposed to 10-15 years in FAP or sporadic forms, so although adenomas form at a later age compared to FAP, the age of onset of adenocarcinomas is similar (22). HNPCC is caused by inactivating mutations in the family of mismatch repair (MMR) genes. The majority of mutations have been found in *MLH1* and *MSH2* (23-25), although rare mutations in other MMR genes exist. In sporadic colorectal cancer *MLH1* is also frequently inactivated by the epigenetic event of hypermethylation of its promoter region. Carriers with a germline mutation have a lifetime risk of developing the disease of 60-80%, indicating that environmental factors and modifier genes (see 1.2.2.1) may also play a role.
Other rare high penetrance alleles include mutations in *AXIN2*, *POLD*, *TGFβR2*, *LKB1*, *SMAD4*, *BMPR1* and *MYH* (18).

1.2.2 Susceptibility genes

To avoid confusion regularly observed in the literature concerning the terms modifier genes and low penetrance susceptibility alleles, the following distinction, as put forward by Balmain in his endnote, has been followed: Modifier genes modify the phenotype of tumors induced by a specific pathway, whereas low penetrance alleles confer weak susceptibility (26).

1.2.2.1 Low penetrance susceptibility alleles

The above mentioned alleles are rare as they only account for a small proportion of the familial clustering (3-5%) observed for colorectal cancer cases. This indicates that most genetic susceptibility to CRC will probably be the result of many genetic variants each with a modest individual effect on tumor susceptibility. Because these multiple variant alleles are generally low in penetrance and therefore only marginally contribute to tumor susceptibility, they are likely to have low estimates of relative risk (RR) when examined in association studies. However, if the frequency of a weak susceptibility allele is relatively high within a population, the proportion of cases attributable to this allele can be substantial, and account for a large proportion of cases in the previously described grey area. This is illustrated by a low penetrance variant in the *TGFβR1* gene. This gene, encoding for one of the two canonical receptors of the TGFβ signal transduction pathway, contains a polyalanine tract of variable length in the first exon. In the general population, approximately 14% of individuals carry an allele with six Ala repeats (TGFβR1*6Ala). This allele has been shown to confer a modest RR of 1.2 (27). However, due to the high carrier frequency, the population attributable risk of this allele is estimated at 3% of all colorectal cancer cases; a substantial proportion of cases.
Genetic heterogeneity within human study populations results in conflicting outcomes, which have led some researchers to investigate susceptibility alleles in genetically homogenous mice strains (26,28). Moreover, large sample sizes are required to be able to show moderate but statistically significantly RRs. These points make it difficult to identify low penetrance variations in humans, but some promising candidates are summarized in table 1.

Table 1: Candidate low penetrance alleles involved in predisposition to colorectal cancer

Gene	Polymorphism	Relative risk (95%CI)	Ref
APC	Ile1307Lys (Ashkenazi)	1.5-2.22 (1.21-2.07)	(29)
TGFβR1	6Ala repeat	1.20 (1.01-1.43)	(27)
HRAS1	Tandem repeat	2.50 (1.54-4.05)	(30)
MTHFR	C677T	0.76 (0.62-0.92)	(30)
BLM	2281delATCTGAinsTAGATTC	2.4 (1.5-3.7)	(31)
HFE	C282Y or H63D	1.40 (1.07-1.87)	(32)
CCND1	G870A	1.7 (1.0-2.66)	(33)

1.2.2.2 Modifier genes

A powerful alternative approach to the difficult search for low penetrance alleles is the use of mouse models to identify loci that alter the phenotype of that mouse model. Mapping these loci to identify the responsible modifier gene could help pinpoint the homologous gene in humans. An example of a locus that has been found to modify the phenotype of Apc^{Min} mice is the modifier of Min (Mom1) locus (34). The modifier gene on this locus encodes a phospholipase A_2 (Pla2g2a) enzyme, as discussed in more detail in section 1.2.3. Other recent examples include the identification of mouse *Ptprj*, encoding a receptor-type protein tyrosine phosphatase, as a candidate for the colon cancer susceptibility locus Scc1. The human homologue of this gene, *PTPRJ*, is frequently deleted in human cancers. Moreover, human tumors containing missense mutations in *PTPRJ* have been identified (35). Another recently identified modifier is the locus encompassing Aurora2 (*Stk6* in mouse and *STK15* in humans), a gene involved in mitotic chromosomal segregation. A functional polymorphism in the human gene has been identified and shown to interfere with mitosis which results in aneuploidy in colon tumors (36).

1.2.3 Genetic variation

1.2.3.1 Single Nucleotide Polymorphisms (SNPs)

The most abundant form of genetic variation in the human genome are single nucleotide polymorphisms (SNPs), positions at which more than one nucleotide can occur when genomes of different individuals are compared and that are present by definition in 1% or more in the population. One of the current hypotheses is that a large part of the low penetrance alleles yet to be identified will turn out to be SNP alleles. These SNPs are estimated to occur approximately at 1 out of 200-300 bases, with around 30% of minor alleles occurring at 5% or more in the population. Approximately 20% of SNPs are polymorphic in all 4 major ethnic groups (African, Asian, Caucasian and Hispanic-Latino).

The vast majority of SNPs is located in non-coding regions, either in introns within genes, in the non-coding regions between genes, or in 5' and 3' regulatory regions. It is not clear to which extent the first two types of SNPs have any functional relevance, although there is increasing evidence of regulatory sequences being present in introns. Even if it is unlikely that a SNP has any functional effect, due to its location, it could still be very useful for the identification of susceptibility allele in association studies, acting as a marker by linkage to another causal variant. However, SNPs in regulatory regions of a gene, for example the promoter, intron-exon boundaries or untranslated regions of exons, are potentially functionally relevant as they may exert an effect on several aspects of gene regulation, including expression levels, mRNA stability, splicing, transport and many others. SNPs in coding regions can either cause an amino acid substitution, the so-called non-synonymous SNP (nsSNP), or, despite the change in codon, encode the same amino acid, resulting in an unchanged protein, the so-called synonymous SNP (sSNPs). About half of all coding SNPs are synonymous (37). The functional relevance of synonymous SNPs has been subject to debate since the expressed protein is not affected. However, it has been shown more recently that there are consensus sequences within exons that facilitate splicing, the so-called exonic splice enhancers (ESE) (38). Changes within ESEs or other exon-specific

regulatory sequences can therefore interfere with the splice machinery, and may, amongst others, result in a change in the ratio of splice variants of the protein. Also, as with non-coding SNPs, sSNPs can act as markers of other variants.
nsSNPs are thought to have the most potential to contribute to disease phenotype since altered proteins are produced. An additional distinction is made between conservative and non-conservative amino acid substitutions, with the latter having a clear effect on protein function since the substituted amino acid is more likely to affect intrinsic properties of the wild-type protein.
The potential of SNPs to aid the mapping of disease genes and contribute to the understanding of complex diseases, has resulted in tremendous efforts to generate a SNP map of the human genome, with as major contributors the International SNP Map Working Group (39) and the SNP Consortium (40). Not all the SNP data that became available in the first few years could be directly applied to association and mapping studies since most of these SNPs were not validated and can only be considered as candidate SNPs. However, within the last two years hundreds of thousands of SNPs have undergone validation by population specific resequencing efforts and are publicly available on the internet in SNP databases of which dbSNP is the best known and most widely used (41).

1.2.3.2 Haplotypes
Every SNP is the result of a single mutation event and may be associated to other alleles present on that chromosome at the time of the mutation. The combination of a specific set of adjacent alleles that are formed this way on a part of a chromosome, is called a haplotype. Different haplotypes are formed when new mutations arise, or when recombination occurs in between two alleles within a haplotype, resulting in a new chromosome containing both maternal and paternal material components. When two SNP alleles are relatively close to each other and are consistently co-inherited, they are associated within a population, and are denoted to be in linkage disequilibrium (LD). The likelihood that recombination occurs between two alleles increases with distance, which means that the level of LD decreases with distance. A recent hypothesis relative to recombination is that it occurs at distinct hotspots with little or no recombination occurring between them, thus resulting in haplotype blocks that remain intact within a population (42). The number of haplotypes could in theory reach 2^n, where n is the number of independent SNPs; however in a chromosomal interval with little or no recombination the real number is closer to n+1. Several studies have investigated the nature and extent of these haplotype blocks and have showed great diversity in block structures, with some blocks extending only a few kb whereas others encompassing more than 100 kb (43,44).
The presence of strongly associated SNPs indicates that, although there can be many SNPs present within a chromosomal region, these are organized in only a few haplotypes. This means that for association studies, the selection of SNPs can be limited to those SNPs that are representative for a certain haplotype, the so-called haplotype tagging SNPs (htSNPs). It is estimated that out of the 15 million SNPs in the human genome over 1,000,000 can be considered as htSNPs (45). With this in mind, a large scale project has been set up called the International HapMap Project, aiming to create a genome wide map containing haplotype blocks from which htSNPs can be selected (46). The most informative SNPs for association studies, the so-called tagging SNPs, can also be selected by using the measure of LD across all SNPs within candidate genes (47).
An informative way of determining haplotypes is by using parental material from which the haplotype of the offspring can be deduced. In most cases however, this is not available and haplotypes can either be determined by physically separating the chromosomes (48), or more commonly, by estimation using algorithms designed for this purpose (49). A recent review on haplotype blocks and LD structure in the human genome is provided by Wall and Pritchard (50).

1.2.4 Arachidonic acid pathway genes as low penetrance candidate genes of CRC

1.2.4.1 Dietary polyunsaturated fatty acids

Several lines of evidence implicating arachidonic acid (AA) pathway genes in colon tumorigenesis have led to the hypothesis that functional polymorphisms in these genes may represent low penetrance susceptibility alleles. In the AA pathway shown in figure 1, dietary polyunsaturated fatty acids (PUFAs) are converted into prostanoids and leukotriens.

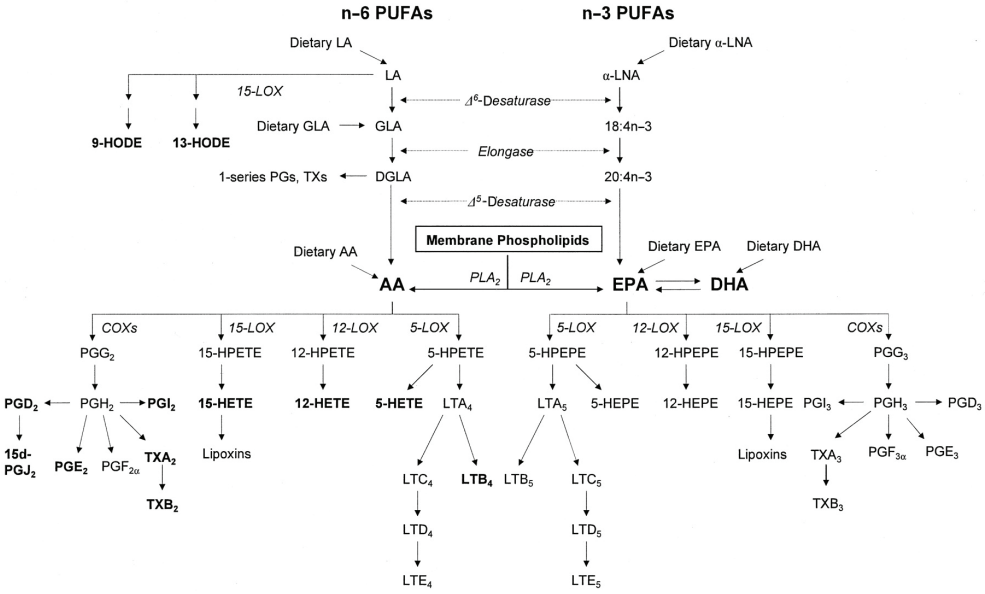

Figure 1: the AA pathway, from Larsson *et al*. 2004 (51).

The main PUFA in our diet is linoleic acid (LA; 18:2n-6), a member of the n-6 family of PUFAs which have their first double bond at the 6th carbon from the methyl terminus. The n-3 PUFA family, with the first double bond at the 3rd carbon atom, has as its parent compound α-linolenic acid (ALA; 18:3n-3). Both LA and ALA are converted in several steps by elongases and desaturases, including the rate limiting Δ-6 desaturase, into AA (20:4n-6) and eicosapentaenoic acid (EPA; 20:5n-3). The latter two can both be obtained from the diet as well. These eicosanoid precursors are incorporated and stored into cell membranes until they are released by different members of the phospholipase A_2 (PLA_2) family of enzymes.

1.2.4.2 Phospholipases

PLA_2 enzymes release AA and EPA by hydrolysis of the ester bond at the sn-2 position of phospholipids, thereby producing free fatty acids (52). Numerous lines of evidence implicate the PLA_2 family of enzymes in colon carcinogenesis. Several studies have suggested a tumor suppressing role for the gene encoding group 4A cytosolic PLA_2 (*PLA2G4A*). Upregulation of this enzyme results in release of free AA which can act as a signal for apoptosis via formation of ceramide (53), unless *PTGS2* (see section on cyclooxygenases) is simultaneously upregulated in which case the increased free AA is converted into prostanoids, and the apoptosis signal is lost, as shown in figure 2.

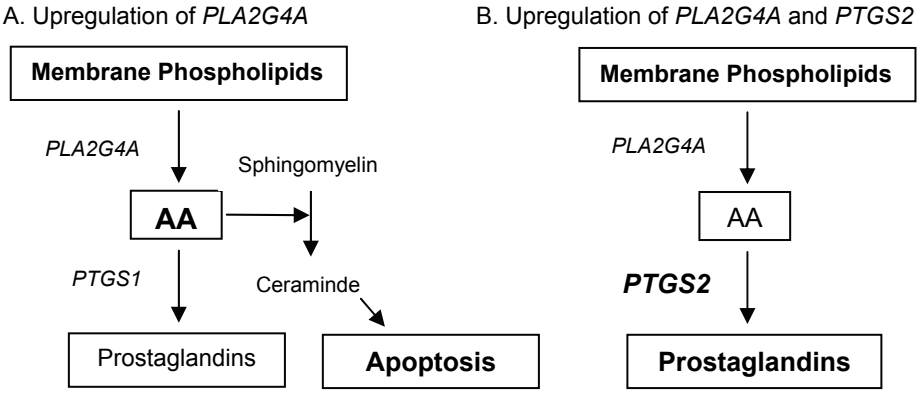

Figure 2: AA as a signal for apoptosis.

This hypothesis is reinforced by the inverse relationship found between *Pla2g4a* and *Ptgs2* in chemically induced mouse tumors (54). The importance of *Pla2g4a* is also demonstrated by a study in which an increase in tumor number in the colon was observed in mice with a deleted *Pla2g4a* gene despite a reduction in PGE_2 production (55). In addition, *Pla2g4a* heterozygous and null mice showed an increase in chemically induced colon tumors and a decrease in apoptosis within the epithelium (55,56). However, these effects seem to be specific for the colon, since mouse models for spontaneous intestinal cancer which predominantly develop polyps in the small intestine, show a protective effect on tumor multiplicity upon *Pla2g4a* deletion (57,58). This might be explained by differences in *Pla2g4a* expression between colon and small intestine, since, although *Pla2g4a* is expressed in the colon, its expression is barely detectable in the small intestine (55). Experiments on the secreted form of this enzyme (PLA2G2A) are not entirely consistent. *Pla2g2a* was first identified as the candidate gene for the modifier of Min (Mom1) locus in Apc^{Min} mice. An inactivating mutation in *Pla2g2a* negatively affects polyp multiplicity in Apc^{Min} mice (59). Efforts to confirm this effect in humans have been less convincing when using microsatellite markers around the *PLA2G2A* locus on chromosome 1 since these showed only weak associations (34,60). Since then, considerable effort has gone into elucidating the role of *PLA2G2A* in colon tumorigenesis and both overexpression and loss of the gene in human tumors has been found (61,62). However, numerous other studies have reported no association between alterations in the gene or gene expression levels and colorectal tumors (63-66), and also no functional polymorphisms or mutations have been found in patients with attenuated FAP as yet (67).

1.2.4.3 Cyclooxygenases

Free AA and EPA share the same enzymes for their conversion into 2-series prostanoids and 4-series leukotriens in the case of AA, and 3-series prostanoids and 5-series leukotriens in the case of EPA. The cyclooxygenase (COX) enzymes, also called prostaglandin-endoperoxide synthase (PTGS) catalyze the formation of prostanoids, including prostaglandins (PGs) and thromboxanes (TXs) via two distinct reactions, a cyclooxygenase reaction where two O_2 molecules are utilized to form the highly unstable intermediate PGG, and a peroxidase reaction where PGG is reduced into PGH (68). There are three PTGS isozymes, the constitutively expressed PTGS1, the inducible PTGS2, and the recently identified PTGS3 which is actually a splice variant of PTGS1 (69). Expression of PTGS2 is induced by numerous growth factors, cytokines and oncogenes, and regulated both transcriptionally and posttranscriptionally, through increased mRNA stability. Several pathways are involved in PTGS-modulation, some acting predominantly at the

transcriptional level (for example the Rho B pathway), whereas others predominantly regulate mRNA stability (for example the Akt/protein kinase B pathway) (70). Recently, it has been shown that the NF-κB pathway is also one of the key regulators of PTGS2 expression (71). Extensive research has gone into unraveling the mechanisms involved in the tumor promoting effect of PTGS2. Numerous studies have indicated PTGS2 as one of the key players in colon carcinogenesis, mainly based on the increased expression of the enzyme in colorectal tumor tissue (72-74). Further evidence for the relevance of PTGS2 in tumor formation was obtained through animal studies. Apc mutant mouse models showed a significant decrease in intestinal tumor multiplicities when the *Ptgs2* gene was knocked out (75) or inhibited (76,77). Also, treatment with PTGS inhibitors (nonsteroidal anti-inflammatory drugs, discussed in more detail in section 1.3.2) resulted in a decrease in adenoma number in FAP patients (78-80). There are several mechanisms by which elevated PTGS2 levels promote tumorigenesis, either PG dependent or PG independent. The NF-κB pathway is an example of a PG independent pathway, which will not be discussed further in this thesis. The PG dependent mechanisms will be discussed in more detail in section 1.2.3.1.

1.2.4.4 Lipoxygenases

The lipoxygenase (LOX) family of enzymes, including ALOX5, ALOX8, ALOX12 and ALOX15, catalyze the formation of leukotriens, hydroxy fatty acids and lipoxins. The ALOX15 enzyme has the unique ability to directly convert linoleic acid (LA) into a specific apoptosis inducer, namely 13-S-hydroxyoctadecadienoic acid (13-S-HODE). Two forms of ALOX15 have been identified as having anti-carcinogenic properties, and with respect to colorectal cancer, the main part of research has focused on the role of ALOX15-1 (81). It was found that ALOX15-1 expression and its metabolite 13-S-HODE, was decreased in colorectal tumors. In vitro studies revealed that 13-S-HODE induced cell cycle arrest, apoptosis and reduced cell proliferation (82,83). The mechanism by which 13-S-HODE can exert these effects have been further investigated and it is hypothesized that this eicosanoid can bind to the nuclear receptor peroxisome proliferator-activated receptor (PPAR) δ, thereby reducing its gene expression and activity, which in turn leads to apoptosis of cancer cells (84). The anti-carcinogenic effect of ALOX15 is further illustrated by a study on ALOX15-2, the other subtype of ALOX15 where its tumor suppressive effect was demonstrated for prostate cancer both in vitro and in vivo (85). At present the role of lipoxygenases in colorectal cancer is incompletely understood though a general working model is shown in figure 3, as adapted from Shureiqi and Lippman (81).

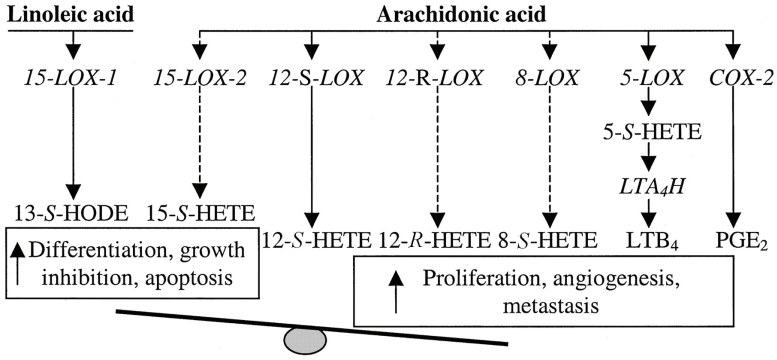

Figure 3: Lipoxygenase enzyme family and tumorigenesis. Adapted from Shureiqi and Lippman, 2001 (81)

1.2.4.5 Prostanoid signaling

The AA derived eicosanoids, which includes prostaglandins and leukotriens, represent potent lipid mediators. They are synthesized in cells which have been activated by mechanical trauma or numerous other stimuli including growth factors and cytokines. Leukotriens are predominantly produced in activated inflammatory cells, including leukocytes and macrophages. The eicosanoids derived from the precursor AA are in general pro-inflammatory, whereas the EPA derived eicosanoids are thought to contain anti-inflammatory properties (86). In addition to their pro-inflammatory action, several studies have implicated AA derived eicosanoids to play a role in tumorigenesis. PGH_2 is the precursor prostanoid formed by PTGS from AA after which, in a cell specific manner, other PGs and TXs are produced (87). It has been shown that the level of PGE_2 is increased in both human and mouse colorectal tumor cells when compared to normal mucosal cells (88,89). This has prompted researchers to investigate the role of this and other eicosanoids in tumorigenesis. Several in vitro studies have indicated a decrease in apoptosis after cells were treated with PGE_2 and PGD_2 (90,91). In human colorectal cancer cell lines HT-26 and SW1116, it was shown that most AA derived prostanoids, with the exception of prostacyclin (PGI_2), increased cell proliferation (92). PGE_2 has also been implicated to play a role in cell migration, adhesion, metastatic potential and growth of cancer cells (93-95) partly via activation of the Ras-MAPK cascade (96), and it has been shown to promote angiogenesis in murine tumors (97). Moreover, $Apc^{\Delta 716}$ mice with a homozygous deletion of the PGE_2 receptor EP2, showed a decrease in polyp size and multiplicities similar to *Ptgs2* knock-out mice, indicating that the PGE_2 tumor-promoting signal is mediated through the EP2 receptor (98). The mechanism by which PGE_2 induces apoptosis may be related to the increased expression of the proto-oncogene *Bcl-2* (99).

1.2.4.6 Interaction with Wnt signaling pathway

More clues that genes in the AA pathway might represent low penetrance alleles leading to increased susceptibility or resistance to colorectal cancer, have come from its interaction with the main pathway that is activated in most colorectal tumors, namely the Wnt signaling pathway. Wnt signaling activation by mutations in *APC* or *β-catenin*, leads to accumulation and nuclear translocation of β-catenin which in turn leads to transcription of oncogenic target genes (100). The putative link between the AA and Wnt pathways is among others suggested by interactions with peroxisome proliferator-activated receptors (PPARs). The PPARs are a family of ligand activated transcription factors that belong to the nuclear hormone receptor superfamily (101). Once activated by a ligand, they form a heterodimer with the RXR receptor after which the complex binds to the promoter of a target gene encompassing a peroxisome proliferator response element (PPRE). Depending on co-activators, this process induces or blocks transcription of the target genes. The PPARs are linked to the AA pathway since their ligands include PUFAs, in addition to specific eicosanoids produced via the AA pathway as well as nonsteroidal anti-inflammatory drugs (NSAIDs). For example, LA can act as a ligand for all three isotypes (α, β/δ and γ), whereas AA is mainly a PPARα activator. More selective eicosanoid ligands include PGI_2 for PPARδ, 13-S-HODE for PPARγ and LTB_4 for PPARα (102,103).

Both PPARδ and PPARγ have been suggested to interact with the Wnt signaling pathway. *PPARδ* has been identified as one of the downstream Wnt signaling targets, suggesting that the tumor promoting effects of mutant *APC* are at least in part due to changes in transcription caused by *PPARδ* expression. Moreover, decreased tumorigenesis was observed in mice inoculated with human colorectal cancer cells containing only one copy of the *PPARδ* gene (104). However, in a study investigating this cross-talk, the expected increase in *Pparδ* mRNA due to inactivation of *Apc* was not observed. Moreover, deletion of the *Pparδ* gene in Apc^{Min} mice did not result in decreased tumor number, but rather in increased tumor size (105). However, a decrease of tumor burden in Apc^{Min} mice was shown

upon Pparδ inhibition (106). Also, transactivation of Pparδ by PGE_2 through PI_3K/Akt signaling resulted in increased adenoma formation in this mouse model (107). Overall, removing or inactivating *PPARδ* has little effect but activation of *PPARδ* signaling plays an important role in stimulating intestinal polyp formation in vivo.
Several studies have investigated the relation between *PPARγ* and Wnt signaling. One study showed that loss of one copy of the *Pparγ* gene already resulted in an increase in tumor multiplicity and β-catenin accumulation in these tumors, but only in mice wild-type for *Apc* (108). This suggests a regulatory role for *PPARγ* in the Wnt signaling pathway, when this pathway has not been constitutively activated by mutations in *APC*. Although the latter results are rather controversial, even in cells carrying *APC* mutations, it has been shown that *PPARγ* activation causes β-catenin degradation (109). This is in line with findings that induction of *PPARγ* results in a decrease in β-catenin levels in adipocytes (110). Whether β-catenin is regulated by *PPARγ*, or *PPARγ* is a downstream target of Wnt signaling still remains to be elucidated suggested Jansson et al. as elevated PPARγ levels were observed upon induction of the Wnt signaling pathway in vitro (111).
PPARγ has also been implicated in colorectal cancer risk through association studies with SNPs. Both SNPs investigated in this thesis (*P12A* and *H477H*) have previously been shown to influence the risk of colorectal tumors. The *P12A* SNP has been associated with colorectal cancer by one study (112), which could not be replicated by another (113), and with colorectal adenomas (114). The *H477H* SNP has only been reported in one study on colorectal cancer, in which an association with colorectal cancer was found (113). These genetic associations provide further evidence that *PPARγ* plays an important role in colorectal tumorigenesis.

1.3 Environmental factors

In addition to genetic modifiers, environmental factors also play an important role in colorectal cancer susceptibility. Environmental factors that have been investigated in this thesis are discussed in more detail in the following sections.

1.3.1 n-3 polyunsaturated fatty acids

The hypothesis that n-3 PUFAs have a protective effect against colorectal cancer originates from studies with Greenland Eskimos a population characterized by a significantly lower incidence of colorectal cancer, and by a fish-enriched diet containing substantially more n-3 PUFAs compared to Western diets (115). A similar effect is also seen in Japanese populations consuming diets rich in fish. This protective effect is lost after migration to Western countries like the US, and adoption of the Western lifestyle (13). Epidemiological prospective studies however, have not been very consistent. For example, cancer incidence and fish consumption as a dietary proxy of n-3 PUFAs were found to be inversely associated in some studies (116-118), but not in others (119-121). In studies using nutrient calculations from food frequency questionnaires from which n-3 PUFA intake is deduced, similar inconsistencies were observed. One case-control study showed an inverse association between n-3 PUFA intake and colorectal cancer (122), whereas one case-control and one prospective study showed no association (123,124). Only recently, actual serum measurements of fatty acids have been used to investigate associations with colorectal cancer. Total n-3 PUFA, EPA and DHA content represent more reliable markers and have confirmed the inverse association with colorectal cancer incidence (125).
Animal and in vitro studies reinforce a potential protective effect of increased n-3 PUFA intake on colorectal cancer. For example, dietary supplementation with fish oil and/or EPA/DHA decreases tumor number in chemically-induced animal models of colorectal tumors (126) as well as in Apc^{Min} mice (127,128). N-3 PUFAs have also been shown to induce apoptosis and suppress cell growth in cell lines (129,130).

There are several proposed mechanisms by which n-3 PUFAs can exert their protective effect on tumor formation, as reviewed by Larsson et al. (51). One of the major mechanisms leads to the suppression of n-6 PUFA derived eicosanoids. Higher intake of n-3 PUFAs, compared to the n-6 variety, would result in a decrease in available AA for eicosanoid production through the incorporation of the n-3 PUFAs into membrane phospholipids (131). This effect is further enhanced by competition between n-3 and n-6 PUFAs for the elongases and desaturases that convert these PUFAs, since n-3 PUFAs have a higher affinity for these enzymes (87). N-3 PUFAs can also directly inhibit PTGS2 (132,133) and compete with n-6 PUFAs for PTGS2 to form prostanoids (134). Moreover, EPA is the preferred substrate for the LOX enzymes that utilize both AA and EPA, resulting in an increase in n-3 PUFA derived leukotriens (135).
Besides these direct effects on the AA pathway, dietary n-3 PUFAs or their metabolites can exert their effect by changing the ligand spectrum of PPARδ and PPARγ, and thereby modifying the signaling pathways of these two nuclear receptors (136,137). Other pathways might also play a role in the protective effect of n-3 PUFAs, for example the NF-κB signaling pathway (138) or decreased signaling by activated ras oncogene (139).

1.3.2 Nonsteroidal anti-inflammatory drugs (NSAIDs)

NSAIDs, of which aspirin is the best known example, are a class of drugs mainly used for analgesic purposes and to reduce inflammation, and are also used as anti-coagulants for individuals at increased risk of cardiovascular disease. From the effects on high risk individuals (Gardner syndrome), it became apparent that NSAIDs also played an active role in the protection against colorectal cancer. Numerous epidemiological studies have found associations between regular use of NSAIDs and decreased risk of colorectal cancer and adenomas, as reviewed by Thun et al. (140). Clinical trials with two NSAIDs, sulindac and celecoxib, have shown that treatment with these drugs can inhibit adenoma growth and cause regression of existing polyps in patients with FAP, one of the hereditary forms of colorectal cancer (78-80,141). Animal studies have also given clear indications about the protective effect of NSAIDs. In rat and mouse models of colorectal cancer, a significant reduction in tumor number was observed after treatment with sulindac and aspirin (142,143). The effect of aspirin was questioned however when one study found a reduction in tumor number in Apc^{Min} mice after treatment with indomethacin but not with aspirin (144). It seems that Apc^{Min} mice only have the beneficial effect of aspirin use when exposure to the drug has been life-long, and not just in adult life (145), although this has not been replicated by others (R. Smits, personal communication). How this translates to humans is not clear, but exposure at adult age seems to be effective in providing protection (146).
The mechanism of action of NSAIDs are generally divided into PTGS-dependent and PTGS-independent mechanisms. The ability of NSAIDs to inhibit the PTGS enzymes was originally thought to represent the main underlying mechanism of action, thereby reducing the production of prostanoids and increasing the pool of free AA, resulting in immune modulation, inhibition of tumor angiogenesis and promotion of apoptosis (147). Since inhibition of the constitutively expressed PTGS1 isoform is thought to be responsible for adverse gastro-intestinal effects, selective PTGS2 inhibitors may provide an alternative (148,149). Several animal studies have shown that treatment with selective PTGS2 inhibitors in mouse models for intestinal cancer resulted in reduced numbers of tumors (76,150). Moreover, genetic evidence suggests that both *Ptgs1* and *Ptgs2* are important in intestinal tumorigenesis as illustrated by the dose dependent decrease in polyp number in rats and mice after treatment with a selective Ptgs1 inhibitor (151). It has also been shown that genetic disruption of *Ptgs1,* as well as *Ptgs2*, results in a reduced tumorigenesis in Apc^{Min} mice (89).
One of the first clues that other enzymes might play a role in the mechanism of action of NSAIDs, the PTGS-independent mechanisms, came from studies on the sulindac metabolites sulfone, which has no anti-inflammatory activity, and sulindac sulfide, the active

metabolite and PTGS inhibitor. It was shown that although treatment of Apc^{Min} mice with sulindac sulfide resulted in a larger reduction in tumor burden, there was a trend toward a decrease in tumor number for mice treated with sulindac sulfone, which might be enhanced by higher dosages (152). Moreover, although treatment with sulindac caused a decrease in tumor number of Apc^{Min} mice, this did not coincide with the expected changes in PG or LT levels (153), although later results were not consistent with this finding (154). Other enzymes within the pathway have therefore been considered as targets for NSAIDs. Several in vitro studies have implicated ALOX15 as a target of NSAID action. Cancer cells treated with sulindac showed increased expression of ALOX15-1 and 13-S-HODE levels, as well as reduced growth and increased apoptosis. Inhibition of the enzyme blocked this effect, which was restored by addition of 13-S-HODE, indicating that in this cell line the effect of NSAIDs is caused in part by activation of ALOX15-1 (155). This same group demonstrated that the apoptotic effect of NSAIDs is independent of PTGS2 status within the cells (156), and that it is not limited to colorectal cancer (157). In gastric cancer cells the effect of NSAIDs on ALOX15-1 was even found for a selective PTGS2 inhibitor (158). Some first in vitro experiments have shown that inhibition of PLA2G4A might also be one of the mechanisms by which aspirin exerts one of its PTGS-independent protective effect (159).
Two subtypes of the PPAR family, which are activated by the products of the AA pathway, can also act as direct targets of NSAIDs. Sulindac can bind to PPARδ after which its activity and protein expression is down-regulated, inducing apoptosis (106). NSAIDs can also act as ligands for another receptor subtype, PPARγ (160). In contrast to PPARδ, this subtype has been shown to be activated by sulindac, which resulted in growth inhibition and apoptosis of cancer cells (161,162).
Besides their effect on the AA pathway, other pathways like the NF-κB signaling pathway might play a major role in the mechanism by which NSAIDs exert their effect on colorectal cancer (163,164). Also, the Wnt signaling pathway has recently been implicated as a target of NSAID action, as it was shown that after treatment with sulindac the tumors of FAP patients and colorectal cancer cell lines showed reduced β-catenin expression and hence a decreased expression of target genes (165).

1.4 Gene-gene and gene-environment interactions

There have been a large number of studies that have attempted the identification of low-penetrance alleles in candidate modifier genes of sporadic colorectal cancer (17). However, in the case of complex common diseases like cancer, numerous genetic and environmental factors interact in complex fashions and contribute to the development of the disease. It has been possible to detect associations between single SNPs or haplotypes in one gene and disease given large sample sizes and standardized experimental set up. Yet, it is generally assumed that most common diseases are due to multiple weak interacting loci (26). Therefore the effect of one SNP or haplotype might be dependent on the presence of another, and the association only becomes apparent when combinations of SNPs or haplotypes in different genes are investigated. This might explain part of the inconsistencies observed between the genetic association studies published to date in the literature.
Demographic differences in disease incidence indicate that apart from the above mentioned genetic modifiers also environmental factors play a very important role in disease development and progression, and are likely to interact with specific genetic determinants. This might partly explain the inconsistent results observed in genetic association studies. One of the problems with testing for gene-environment interactions is the fact that environmental factors are often “self-reported” (e.g. obtained through questionnaires filled in by individual cases and controls), and therefore prone to error (166). An example of an environmental factor for which it is difficult to obtain reliable data is diet. Despite the large improvements in the way food frequency questionnaires are set up, retrospectively recording

of dietary habits remains a challenge. A solution to this problem might be the use of measurable intermediates not influenced by the disease that could act as a proxy for the environmental factor under investigation (166), as exemplified by n-3 PUFA levels in adipose tissue or serum for fish consumption. However, intermediates are not always very obvious, and even when a clear correlation exists between a given environmental factor and the intermediate, they might not interact with the genetic factors in the same way, as demonstrated in this thesis in Chapters 3 and 5. By studying gene-environment interactions in association studies, a better estimate of the attributable risk of both separate factors to the disease is obtained. Moreover, associations between environmental factors and disease are strengthened when they are studied in genetically susceptible or resistant individuals (167). Ultimately, an individual tailored advise on disease prevention or treatment will become available in the foreseeable future.

1.5 Outline of this thesis

As discussed, it is likely that low penetrance alleles contribute to a large proportion of all colorectal cancer cases. These low penetrance alleles are likely to have a marginally detectable effect when analyzed individually but can interact with other variants or with environmental factors. We hypothesized that low penetrance alleles in the form of SNPs in genes involved in the AA pathway could influence colorectal cancer risk. To test this hypothesis we first attempted to generate a map of the common variation and accompanying haplotypes of the seven candidate genes, *PTGS1*, *PTGS2*, *ALOX15*, *PPARδ*, *PPARγ*, *PLA2G2A* and *PLA2G4A*, as described in Chapter 2. The associations between SNPs in our candidate genes and colorectal adenomas and the possible interaction between these SNPs and fish consumption in relation to colorectal adenomas is described in Chapter 3. Whether these associations are the same for colorectal cancer is investigated in Chapter 4. As the interactions shown between fish consumption and SNPs for colorectal adenomas is based on the hypothesis that fish consumption acts as a proxy for n-3 PUFAs, we investigated whether the SNPs interacted in the same way with serum n-3 PUFA levels, which is described in Chapter 5. In Chapter 6 we tested whether fish consumption as a proxy of n-3 PUFAs, and fatty acid tissue levels from fat biopsies were associated with colorectal adenomas. In Chapter 7 we tested the interaction of another protective factor, regular use of NSAIDs, with SNPs in relation to colorectal adenomas. The last Chapter of this thesis discusses the findings, puts them into context, and contains concluding remarks and thoughts for future research.

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Chapter 2

Genetic variation and haplotype block structure in arachidonic acid pathway genes in the Dutch population

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Abstract

Epidemiological and experimental evidence indicates that lipid metabolism, in particular the arachidonic acid (AA) pathway, plays a critical role in colorectal tumor development. Polymorphisms in key genes of this pathway may therefore contribute to susceptibility to colon cancer in the general population. In this study, the genetic variation of seven candidate genes from the AA pathway, *PTGS1*, *PTGS2*, *ALOX15*, *PPARδ, PPARγ, PLA2G2A* and *PLA2G4A*, has been determined in the Dutch population by direct sequencing of coding and regulatory regions. The fifty-eight single nucleotide polymorphisms (SNPs) identified, including thirteen newly described SNPs, were evaluated for their use in association studies.

Possible functional effects of the SNPs were evaluated by in silico tools. Thirteen SNPs were predicted to affect either protein function or splicing. Two software programs were used to compare estimated haplotypes and haplotype block structures, and some differences between publicly available haplotypes and our estimations were found. For example, although the block structure of *PPARγ* was consistent with both programs, the publicly available haplotypes for *PPARδ* were derived from insufficient SNP data to resolve the two blocks in the 3'UTR identified in our data.

These data may help to determine which SNPs are likely to be functionally important either at the protein or mRNA level and might therefore be candidates for association studies. Our study underlines the importance of re-sequencing of a number of subjects from the study population, especially in those genes where publicly available SNP and haplotype data is limited.

Introduction

Among others, diet and in particular lipid metabolism is thought to play a critical role in colon cancer. The fatty acids from our diet are in part converted into prostaglandins and leukotriens via the arachidonic acid (AA) pathway. There has been evidence from epidemiological studies and animal experiments that the enzymes and receptors in the AA pathway play an important role in colon tumorigenesis. Non-steroidal anti-inflammatory drugs (NSAIDs) for example, cause a considerable reduction in adenoma numbers in familial adenomatous polyposis patients(1) and in mouse models of intestinal cancer(2). It has been demonstrated that they exert their effect on the colon epithelium in part through the inhibition of the enzyme cyclooxygenase-2 also known as prostaglandin synthase-2 (PTGS2)(3). More recently it has also been shown that NSAIDs can have modulating effects on other players in the AA pathway as well, which might all contribute to the effect seen on colon cancer(4-6).

Variation in genes involved in the AA pathway in the form of Single Nucleotide Polymorphisms (SNPs) may modify the risk associated with colorectal cancer. The recent developments in SNP discovery have resulted in the availability of a multitude of SNP data. Publicly available databases (eg dbSNP), give access to lists of identified SNPs within any gene of interest. Besides the location of the SNP, data on validation status, population specificity and allele frequencies are often available. However, in order to obtain an overview of the genetic variation within a gene and population of interest, it is recommended to re-sequence a sample population(7).

Besides the use of SNPs individually as markers or functional variants in an association study, it is now commonly accepted that more specific information is gained from the combination of several SNP alleles on a chromosome, the so-called haplotypes. Our genetic material contains regions where little recombination occurs, resulting in a so-called block formation of highly variable sizes. SNPs present within these blocks are in linkage disequilibrium (LD) with each other which makes it feasible to estimate haplotypes(8). The SNPs within a block that are representative for the most common haplotypes are therefore

useful for association studies and are termed haplotype tagging SNPs (htSNPs)(9). The most informative SNPs for association studies can also be selected by using the measure of LD across all SNPs within candidate genes(10). The International HapMap project has as its main goal to determine common patterns of DNA sequence variation in the human genome. In particular, it aims at the estimation of the distribution of haplotypes across all chromosomes in four populations to identify those SNPs that are representative for all common haplotypes(11). Although the latter is very useful for SNP selection of whole genome association studies, for a candidate gene approach the SNPs evaluated by this project might, at present, be sparse within the genes of interest.
Another factor often considered in selecting candidate SNPs for association studies is the functional consequences of the specific polymorphisms. Possible biological explanations for specific associations are more plausible when it has been shown that a given SNP allele has a functional effect, for example by causing a change in protein function splicing or gene expression. In a recent review, the importance of other cis-acting elements, besides the consensus sequences at splice sites, to regulate splicing is underlined(12). These elements are termed exonic splice enhancers (ESE) for their location within exons. These sequences are thought to play an important role in splicing, especially when the consensus splice sequence at the exon-intron boundary is affected.
The aim of this study on colorectal cancer is to evaluate the genetic variation in seven candidate genes in the Dutch population, for use in further association studies. The candidate genes were selected for being involved in the AA pathway. Furthermore, each gene in this study, *PTGS1* and *PTGS2*(13), 15-lipoxygenase (*ALOX15*)(14), secreted phospholipase A2 (*PLA2G2A*)(15) and cytosolic phospholipase A2 (*PLA2G4A*)(16), peroxisome proliferated activated receptor δ (*PPAR*δ)(17) and γ (*PPAR*γ)(18), has been implicated individually to play a role in colon cancer progression. Five of the seven candidate genes have been screened for SNPs by direct sequencing of all coding and regulatory regions. Some exons of the remaining two genes have been sequenced to validate SNP data from other laboratories. Possible functional effects of the identified SNPs have been evaluated in silico, and common haplotypes and haplotype block structure were estimated and compared to publicly available data.

Materials and Methods

DNA samples

To detect genetic variants with a frequency > 1%, blood samples from 100 unrelated randomly selected healthy individuals from the Dutch population, incorporated in the so called REGENBOOG study were obtained(19). In this large Dutch health examination study, a random sample of the Dutch population was interviewed and 30% of those individuals participated in an additional health examination at a municipal health center. There were no major differences, with respect to many background and health related variables between participants at home and those who underwent physical examination. DNA was extracted from buffy coats by digestion with proteinase K, followed by salting out with potassium acetate and chloroform/isoamyl alcohol extraction(20).

Identification of polymorphisms

Off all genes involved, with the exception of *PTGS1* and *PTGS2* the complete coding sequences as well as the regulatory regions like promoter and 3'-UTR were sequenced. For *PTGS1* and *PTGS2* partial resequencing was performed based on published information(21) as well as on personal communication (Frederico Canzian, IARC), see also table 1. PCR Primers were selected using the Primer 3.0 program (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). Annotations of the genes involved and the sequences of the primers used are shown in table 1.

Table 1: PCR primers for sequencing genes

Gene (accession number)	Exon		Sequence	Gene	Exon		Sequence
PTGS1	7	For	gcgtatgtcatcgacagtgg	*PLA2G4A*	8	For	cccggccttattttcttctt
NM 000962		Rev	ctttgccagggaagaccat			Rev	tgtggcatcaaactggaaag
	9	For	cttggctgaccctatttcca		9	For	tgtgccttctttctttggaga
		Rev	caccaacatggaggtgttga			Rev	ggggagaatgggctcagta
	10	For	gaccactgctgtgcttctctc		10	For	tgaatagcattctttctgtgtctg
		Rev	atccaggaaacagctgctca			Rev	gggaaactgcaattcagagg
	11	For	tgcttgtcattccagagtgc		11	For	ccatgccatagcattttcct
		Rev	ttctggcagaccaaatgaga			Rev	actgccccagacacctaaaa
PTGS2	Prom	For	tcacatgctcctccctgag		12	For	aacccctttgcatttcttaatgg
NM 000963		Rev	ctccctgatgcgtggattat			Rev	tgagttttataacctcagtgtcaaa
	1	For	ccctcagacagcaaagccta		13	For	cactctgtggttttgctcaga
		Rev	ctgggatagacccaggaggt			Rev	tcctttcctcatggatctgg
	3	For	ggctccctccctttcttcgtcttcttg		14	For	cagcccaaagatccctttttt
		Rev	aggctaaaaaccttagaaag			Rev	tcctgcattagctcccacta
	10	For	aagaacgaaagtaaagatgtttgaa		15	For	aagcctgagggcctaatcat
		Rev	tcaaattattgtttcattgctgattt			Rev	ggcaagcaagagaatgtggt
ALOX15	Prom1	For	tgaggcagagaattgcttga		16	For	ggtcagaccaactgaatcaatg
NM 001140		Rev	cagtggcatttgaggaaggt			Rev	tgtcagtttcgtcccctactt
	Prom2	For	caaaaggaatgagtcccagtg		17	For	ggactcccactgctgtcttt
		Rev	gttgttggaaccggcataga			Rev	ggcatattttgggtggtgtc
	1	For	agaccaggaacacctccctct		18a	For	tgtgtatgcatgactcgtagattt
		Rev	gtcctccccggtatttgact			Rev	tcaacatcatttgtcaacctaactt
	2	For	cccgatacgtctcctcctct		18b	For	tgatcatgagagactggctga
		Rev	gcttctcacacagcccaga			Rev	ttttgcgtttgaaagcagtg
	3+4	For	gggtgctgaggctctcct	*PPARγ*	Prom	For	ttttgggcttcacaaatcag
		Rev	atcctgggccagtccaat	NM 015869		Rev	catggaataggggtttgctg
	5	For	gcctgtggtcgtcatgttag		B	For	tcaagcccagtcctttctgt
		Rev	cagagggacccaagtctctg			Rev	caaacacaacctggaagacaaa
	6	For	accccagtatgtctcccaac		1	For	tctgaaactctgtgagattgctg
		Rev	ctccgcagctactctcgtgt			Rev	ggttactgagagatgaggtccaa
	7	For	tggaggaatccgagtaggaa		2	For	aattatcctctcacatgtctcca
		Rev	tgataaggggctgagctttc			Rev	ggggttctgctgaaatgaaa
	8	For	gaacaggtttggaggacgaa		3	For	taggttgctgcttccatgtg
		Rev	cctggggcagagatagtgg			Rev	tccactggtctggcagctat
	9+10	For	ctcctctgactctgcccttc		4	For	tcatcctgtcattcctcttcc
		Rev	atgtcttctccacccccact			Rev	ccccaatgaagacagcagaa
	11	For	aggaggtctgcgtctggtt		5	For	acctgggatggcattcact
		Rev	tcttgggctttgtgtctgag			Rev	accatcatcccaccctcttt
	12+13	For	ctctgagcgtctgccctact		6	For	ctgaacccccctgttgtgttt
		Rev	cacagctgcctccttcctta			Rev	gaagggaaatgttggcagtg
	14	For	catttcaagactagaatagggggga	*PPARδ*	Prom1	For	aatcactcatccagatagctag
		Rev	catcaagcatatgaacccagaa	NM 006238		Rev	aacctcctcatcttccagtga
PLA2G2A	Prom+1	For	tgctggagagcaatgaaaaa		Prom2	For	tgatctcctccagtggacct
NM 000300		Rev	gattgctcccttctgctcaa			Rev	cagaattctcccgctcctg
	2	For	gagcggctgaatgaatgagt		1	For	gcggagaggcagaattagg
		Rev	tctgctcttgacaggacatca			Rev	ctacatggcaacgcacgac
	3	For	caccctggacttaggttgga		2	For	cctggcacctgtcagctaaa
		Rev	caagagtgcttccctttctgg			Rev	cctgtgtcagtggtggtcag
	4	For	tagcagagaggggcagagag		3	For	gattcgctctccaccacatt
		Rev	cctgggccagagtctaggag			Rev	tcagaggagcaactggcata
	5	For	tggagctgtgggacaagag		4	For	aggtccatctgcgttcagac
		Rev	ccccagcactgtctaaacaaa			Rev	gtcccctgccagtcctct
	6	For	cccacaagaagccactgaat		5	For	cagctgttaagtggctgagg
		Rev	gtgcatagggcaatgcatac			Rev	gaaagctttgtggagggtca
PLA2G4A	1	For	ggagaccagcccacatttta		6	For	acctcctggtggcctttc
NM 024420		Rev	aagctaaggagggtgggaga			Rev	accttggtttcaggctgtgt
	2	For	gaactgcatccaagaggaag		7	For	taaagggatggggatgtcag
		Rev	agacccccctccaagaaagaa			Rev	gacccagagcccaggatg
	3	For	aaatgaggttctatgttggaagc		8	For	cccgaggcctgatctctaa
		Rev	cctccgatttgtcaaggaac			Rev	ccaggacgactgggtgtg
	4	For	cagattcattgagaataaactgacaa		9	For	cacctcttggggtggaagt
		Rev	ctctcaacatgaatcacaacca			Rev	ctggccccattggagtct

Gene (accession number)	Exon		Sequence	Gene	Exon		Sequence
	5	For	ccatgggaaattttatttttgtg		10	For	ctccctgtgccctctctc
		Rev	acccaacttcccagaatcaa			Rev	ggaaccctgcctacttcca
	6	For	gggtcttcatggagctgtgt		11a	For	tccccctgctcctttctcta
		Rev	tggatcactcacaatatttctca			Rev	gcttgaaccccagttctcct
	7	For	gctacctggatggaaaacca		11b	For	gggcatgccatgtctgag
		Rev	tggggaaattgtccaagcta			Rev	tgtaagccccaggaaaaaca

Each PCR contained 5μl 2X hotstar master mix (Qiagen), 1μM of each primer, in some cases 2μl Q solution provided with the hotstar master mix, and 10 ng genomic DNA, in a total volume of 10μl. PCR reactions were carried out in a Perkin-Elmer 9600 thermal cycler under the following conditions: 95ºC for 15 min, 35 cycles of 94ºC for 45 s, 57ºC for 45 s, 72ºC for 1 min, followed by 72ºC for 10 min.
DNA Sequencing. Each PCR product was directly sequenced on an Applied Biosystems 3700 capillary sequencer, after purification with QIAquick multiwell PCR purification plates. The sequence reaction contained 1μl BigDye Terminator Reaction Kit, 200mM Tris, 5mM $MgCl_2$, 0.16 μM primer and 3-20ng PCR product in a total volume of 20μl. PCR reactions were carried out in a Perkin-Elmer 96 thermal cycler under the following conditions: 25 cycles of 96ºC for 10 s, 50ºC for 5 s, and 60ºC for 4 min.
Sequence analysis. Trace files of sequencing reactions were imported into the Seqman part of the Lasergene software program (DNAstar Inc). Polymorphisms were identified as conflicts in the sequence by the software.

In silico functional analysis
SIFT. Sorting intolerant from tolerant (SIFT)(22) is a sequence homology based software program. It is based on the premise that functionally important amino acids in a protein will be conserved within the protein family. It can thus predict the probability, by considering the type of amino acid change and the extent of conservation, whether an amino acid substitution is allowed at that position. A low probability ($p<0.05$) predicts an impact on protein function. Data for SIFT prediction was submitted at http://blocks.fhcrc.org/~pauline/SIFT_seq_submit2.html.
PolyPhen. Several parameters are used by PolyPhen to predict the possible outcome of an amino acid substitution on protein function. The prediction is based on analysis of the sequence, polygenetic and structural information of the protein. Results are classed as benign, possibly damaging or probably damaging. Data for PolyPhen prediction was submitted at http://www.bork.embl-heidelberg.de/PolyPhen/(23).
RESQUE-ESE. This program identifies hexanucleotide sequences as candidate ESEs on the basis that they have both significantly higher frequency of occurrence in exons than in introns and also significantly higher frequency in exons with weak (non-consensus) splice sites than in exons with strong (consensus) splice sites(24). Sequences for ESE identification were submitted at http://genes.mit.edu/burgelab/rescue-ese/.

Haplotype analysis
For each gene, haplotypes were estimated using PHASE v2.1 on the basis of individual genotype scores using default settings(25,26). The PHASE output file with the estimated haplotype scores for each individual was subsequently used to screen for haplotype block structure within each gene using HaploBlockFinder V0.7, also using default settings(27). PHASE can be found at: HTTP://www.stat.washington.edu/stephens/software.html. HaploBlockFinder V0.7 can be found at: HTTP://cgi.uc.edu/cgi-bin/kzhang/haploBlockFinder.cgi/. PTGS1 and PTGS2 were excluded from haplotype analysis, since we did not sequence the whole gene and therefore had no genotype data of all SNPs present in the gene. The haplotype blocks identified by HaploBlockFinder were

compared to block estimates obtained from Haploview with genotypes form the HapMap database, found at http://www.hapmap.org/index.html.en.

Results

Identification of polymorphisms

All polymorphisms identified in the seven selected candidate genes in our random selection of 100 healthy Dutch individuals are shown in table 2.

Screening our genes of interest for variants has resulted in the identification of fifty-eight SNPs. Of these, 78% (n=45) were already described in databases, thus showing that 22% are newly described SNPs. Out of the fifty-eight SNPs, seventeen were located within coding regions. Of these, only nine resulted in an amino acid substitution, the so-called non-synonymous SNP (nsSNP) (15.5% of total), illustrating the relatively low frequency at which these SNPs are present in the genome. Of the remaining forty-one SNPs, eighteen were located in intronic regions, usually in the proximities of exons, whereas twenty-one were situated in regulatory regions, either in the promoter, 5'UTR or 3'UTR. In two cases, the SNPs were situated just outside the gene. The minor allele frequencies of the SNPs ranged from 0.005 (0.5%) to 0.450 (45%). Most of the nsSNPs, eight out of nine, occurred at frequencies lower than 0.05, generally considered too low for association studies. Only one SNP in *PPAR*γ revealed a higher frequency (0.093). The low frequencies illustrate the selective pressure on nsSNPs, as they may negatively affect protein function.

Table 2: Overview of identified polymorphisms

Gene	SNP name		Location	Sequence	Minor allele frequency	Database[b]
PTGS1	*L237M*		exon 7	TGGAGACAAT[C/A]TGGAGCGTCA	0,033	5789
	c.762+15delA		intron 7	GTGCTGGGCC[A/-]GGGGGTAGGG	0,094	3215925
	V348E		exon 9	GAGGAGTACG[T/A]GCAGCAGCTG	0,006	
	S439S		exon 10	CCTACACCTC[C/T]TTCCAGGAGC	0,006	
	V444I		exon 10	CCAGGAGCTC[G/A]TAGGTGAGCA	0,012	5794
PTGS2	*c.-1329A>G*		promoter	TGAAATTCCA[A/G]CTGTCAAAAT	0,218	689466
	V102V		exon 3	TGAGTTATGT[G/C]TTGACATGTA	0,250	5277
	c.2242T>C		3'UTR	TACTTTTGGT[T/C]ATTTTTCTGT	0,296	5275
ALOX15	*c.-272G>T*		Promoter	GCGTGTTTTC[G/T]GTCCAAATCC	0,200	7220870
	c.-217G>C		Promoter	TCCACTCCCT[G/C]CTCTCGCCAG	0,200	2664592
	c.-185G>C		Promoter	TTTGGACTGG[G/C]GCTGAATTCT	0,200	2664593
	c.136-37G>A	x	Intron 1	CCTCCCGCCA[G/A]GCCCCACCGG	0,106	11568141
	D90H	x	exon 2	CGGAGCCGGG[G/C]ACGAGGTCAG	0,005	11568142
	c.337+8C>T	x	Intron 2	CCGGTAAGCG[C/T]GGGGCTGAGG	0,308	11078528
	c.542+150C>T	x	Intron 4	ACCCTTTGCC[C/T]CATCCTGCCC	0,016	
	F253F		exon 6	GCCTAGTGTT[C/T]CCTCCAGGCA	0,295	11568103
	G324V	x	exon 8	CCCCGCACAG[G/T]ATCCCCACCA	0,005	
	T485T	x	exon 11	ACTATAAGAC[A/G]GACGTGGCTG	0,062	743646
	c.1641+22C>G		Intron 12	AGATGGGCAG[C/G]TGGGAATTTG	0,030	
	c.1641+63G>A		Intron 12	GTCTGTGTGC[G/A]GGTGGACCAC	0,456	6502804
	c.1641+91C>T		Intron 12	GCGTGCTTGG[C/T]AGGCACTGAC	0,456	2619112
	T560M		exon 13	GCACCCTGCA[C/T]GATGCGGCTG	0,030	
PLA2G2A	*c.-218+94G>A*	x	Intron 1	ATATGAGACA[G/A]GGGTGGAGGA	0,006	
	c.-217-240C>T	x	Intron 1	GTCTCTGCCC[C/T]CCTTAGAGGC	0,027	11573155
	c.-217-96A>C	x	Intron 1	CCAATCCTCA[A/C]CTCTGTCCTG	0,007	1796923
	c.-180C>G	x	5'UTR	CCAGAGGGAG[C/G]AGCTATTTAA	0,289	11573156
	T32T	x	exon 4	TCAAGTTGAC[G/C]ACAGGAAAGG	0,145	2236771
	Y44Y	x	exon 4	ATGGCTTCTA[C/T]GGCTGCCACT	0,274	4744
	c.185+16C>T	x	intron 4	GCCACCTATC[C/T]CTCCCTACCC	0,274	2307246
	R143H	x	exon 6	AGCACCCCTC[G/A]TTGCTGAGTC	0,011	
	c.665C>T	x	3'UTR	AGCTGTACTC[C/T]GGGGGGTCTC	0,155	11677
	c.700+2T>A	x	Outside	TAGCAAATCA[T/A]GTATATGTGT	0,126	876018

Gene	SNP name[a]		Location	Sequence	Minor allele frequency	Database[b]
PLA2G4A	*c.-109A>C*	x	5'UTR	AAGGATCCTG[A/C]CTGAAAGCTA	0,006	12720485
	c.559-43G>A		Intron 7	TGTATTTTAT[G/A]ATTTTTACTT	0,031	12720586
	c.918+23C>T	x	Intron 9	TCAATCTACA[C/T]TGCTTTTATA	0,195	2307200
	c.918+31A>G	x	Intron 9	CACTGCTTTT[A/G]TAACAAGTAG	0,012	12720597
	c.919-22T>C	x	Intron 9	TAAAGTCATC[T/C]TTTTCTTTCT	0,021	2307195
	c.1264-57T>A	x	Intron 12	TTATATTAAA[T/A]TTAATTGTTA	0,067	4650709
	c.1336+3G>A	x	Intron 13	ACCCAAAGGT[G/A]AGTGAGCCGG	0,062	6661772
	R651K	x	exon 16	AGAAAGTACA[A/G]GGCTCCAGGT	0,025	2307198
	c.2605G>A	x	3'UTR	ATAAGACCTC[G/A]CATTATGTAT	0,094	12720707
	c.2721+17T>A		Outside	GATAACTGAG[T/A]TTTTTGTTAA	0.035	12720709
PPARγ	*P12A*	x	exon B	TCCTATTGAC[C/G]CAGAAAGCGA	0,088	1801282
	H477H	x	exon 6	TCACGGAACA[C/T]GTGCAGCTAC	0,114	3856806
PPARδ	*c.-1180C>T*	x	Promoter	AAGGTGTGGC[C/T]GAAGGAGAGG	0,006	
	c.-1175G>T	x	Promoter	GTGGCCGAAG[G/T]AGAGGGGAGG	0,006	
	c.-789C>T	x	Promoter	TCCAGTGGAC[C/T]TAGCACTGGG	0,051	
	c.-126A>G	x	5'UTR	CCGTGGAGCA[A/G]TGATCTCTAC	0,010	1883323
	c.285+11G>C	x	Intron 5	GTACGGACTG[G/C]GGGGAGCGGT	0,005	
	N163N	x	exon 7	TGACTGCAAA[T/C]GAGGGGAGCC	0,215	2076167
	c.1443G>A	x	3'UTR	CTCTTTCTCA[G/A]TTCCTCTTTC	0,022	9658167
	c.1867G>A	x	3'UTR	CAGGCTGAGA[G/A]CCAGATGCCT	0,017	9658170
	c.1973G>A	x	3'UTR	GACACCCAGT[G/A]TCCTTCCATC	0,011	9658172
	c.2021T>C	x	3'UTR	TGAAGCTGCC[T/C]CTCCAGCACA	0,189	3734254
	c.2246G>C	x	3'UTR	TGTTCATTCT[G/C]ATGTCCATTT	0,006	
	c.2589G>A	x	3'UTR	GGAGCCATTCT[G/A]TGTGTGACTC	0,052	1053046
	c.2629T>C	x	3'UTR	CCTGCCCCTA[T/C]GGGCGCTGCA	0,266	1053049
	c.2806C>G	x	3'UTR	TCCCTGCCCC[C/G]ACCCGGGTCT	0,188	9794

[a]Due to missing data, only SNPs with x were used for haplotype analysis.
[b]rs number in dbSNP. Missing number means SNP is previously undescribed.

In silico functional analysis

SIFT/PolyPhen. All nsSNPs were screened by the software programs SIFT and PolyPhen, to determine putative effects on protein function. Of the nine nsSNPs identified in this study, three were predicted by SIFT to affect protein function, namely *PTGS1 V348E*, *ALOX15 T560M* and *PLA2G2A R143H*. PolyPhen predicted probably damaging effects for two of these SNPs, but not for *PLA2G2A R143H*. Also, a possibly damaging effect was predicted for *PPARγ P12A*. Results are shown in table 3.

Table 3: Prediction of impact on protein function by amino acid substitutions by SIFT and Polyphen analysis

Gene	SNP	Predicted impact on protein function (SIFT)	Predicted impact on protein function (Polyphen)
PTGS1	*L237M*	Tolerated	Benign
	V348E	Affects protein function	Probably damaging - disruption of ligand binding site
	V481I	Tolerated	Benign
ALOX15	*D90H*	Tolerated	Benign
	G324V	Tolerated	Benign
	T560M	Affects protein function	Probably damaging – hydrophobicity change at buried site
PLA2G2A	*R143H*	Affects protein function	Benign
PLA2G4A	*R651K*	Tolerated	Benign
PPARγ	*P12A*	Tolerated	Possibly damaging

RESCUE-ESE. All exonic SNPs (including 5' and 3' UTR exons) newly identified or validated in our study were screened for the presence of ESE sequences. Out of thirty-one SNPs screened, ten were shown to affect an ESE sequence. It is believed that ESE sequences play an especially important role in splicing of exons characterized by weak consensus splice-sites. Of the ten SNPs interfering with ESE sequences, three were in exons with weak splice-sites. Results are shown in table 4.

Table 4: Identification of exonic polymorphisms likely to effect splicing by RESCUE-ESE analysis

Gene	SNP	Impact of minor allele on ESE-site	Weak splice-sites
PTGS1	*L237M*	New site is created	
ALOX15	*T485T*	Site is lost	Weak 5' splice site
	T560M	New site is created	
PLA2G2A	*T32T*	Site is lost	
PLA2G4A	*c.-109A>C*	Site changes in other consensus sequences	
	R651K	Site is lost	
PPARγ	*P12A*	New site is created	Weak 5' splice site
	H477H	New site is created	Weak 3' splice site
PPARδ	*N163N*	New site is created	
	c.1867G>A	New site is created	

Haplotype determination

The haplotype block structure for each gene is shown in figure 1. The haplotypes present within each block are shown in figure 2. The SNPs that can be selected for genotyping to represent all common haplotypes (htSNPs) according to haplotype block finder are shown in table 5.

We were unable to use all SNPs in the *ALOX15* gene for haplotype estimation due to missing data; therefore, haplotypes have been estimated using six of the fourteen identified SNPs. All six SNPs seem to lie within the same haplotype block, with three of them identified as htSNPs. The *PLA2G2A* SNPs are divided over three haplotype blocks. The first and last blocks contain only two common haplotypes each, which can be distinguished by a htSNP in each block. In the larger middle block, three htSNPs are identified, representing the four common haplotypes. There are two haplotype blocks present in *PLA2G4A*, the first encompassing four common haplotypes, with three and one htSNPs in the first and second block respectively. There are only two SNPs in *PPARγ* which are not in a haplotype block, therefore both representing htSNPs. The fourteen SNPs of *PPARδ* are divided into three haplotype blocks. The four common haplotypes of block 1 can be identified by three htSNPs, which is also the case for block 2, although the latter block only encompasses four SNPs. The last SNP of *PPARδ* is located in a separate block and is therefore by definition a htSNP.

Figure 1

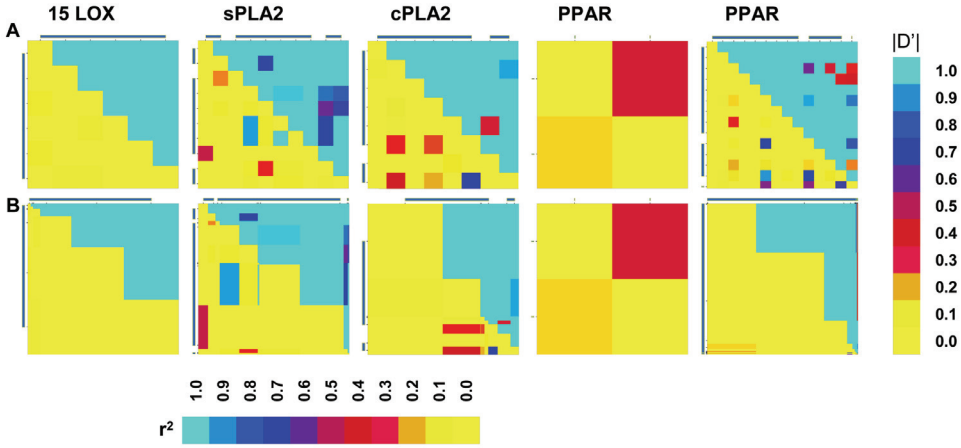

Fig 1: Pairwise linkage disequilibrium (LD) estimates (|D'| at the right and r^2 at the bottom) and haplotype block indications of each gene as given by HaploBlockFinder V0.7. Panel A shows the results along a scale where the distance between each SNP was set constant. Panel B shows the same results but now according to physical distance between each SNP. |D'| is 1.0 when no recombination has occurred between two SNPs, hence they are in complete LD and exist on one haplotype block. The measure for r^2 is then either 1.0, when the minor alleles of these two SNPs lie on the same haplotype allele, or 0.0 when the minor alleles lie on separate haplotypes alleles.

Figure 2

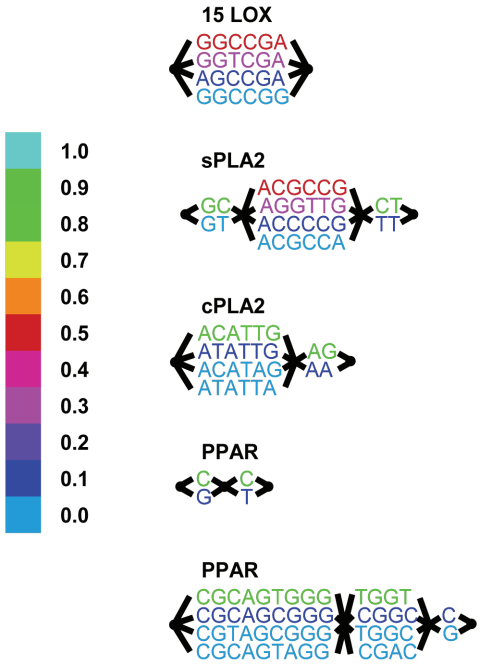

Fig 2: Estimated frequencies of the four most common haplotypes per haplotype block within each gene as given by HaploBlockFinder V0.7. Frequencies are approximated by different colors corresponding to the scale on the left. The lines separating the blocks indicate recombination occurring at these sites.

Figure 3

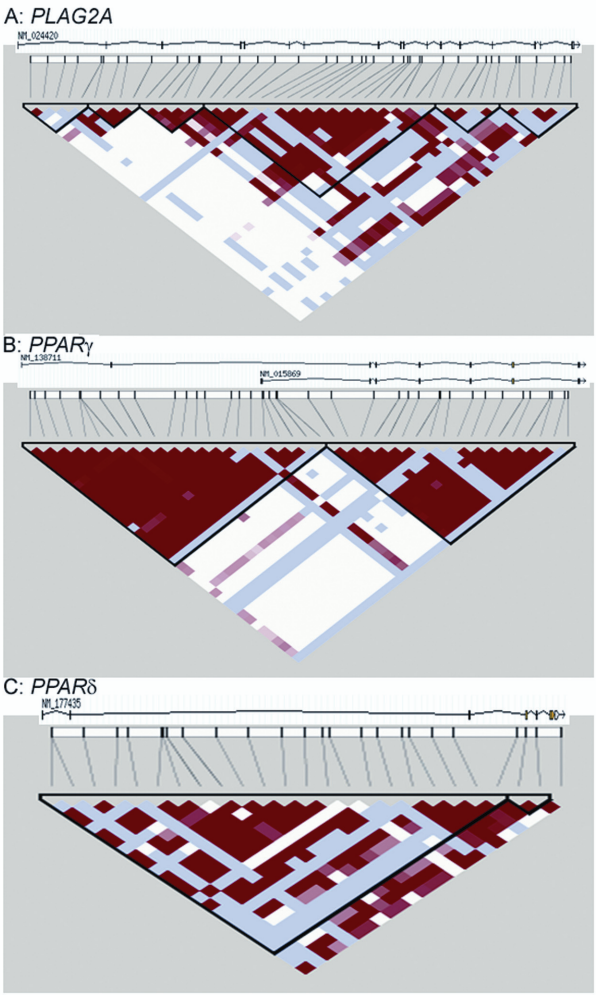

Fig 3: Pairwise LD plots of |D'| for *PLA2G4A* (A), *PPARγ* (B) and *PPARδ* (C), according to Haploview 3.0 with data form the HapMap project found at http://www.hapmap.org/index.html.en. Red indicates high |D'|, lighter colors indicate lower values of |D'|. Grey indicates high |D'| but low LOD scores. Haplotype blocks are indicated by black lines.

Table 5: Identification of htSNPs, representative for all major haplotypes

Gene	htSNP	Haplotype block
ALOX15	*c.136-37G>A*	Block 1
	c.337+8C>T	Block 1
	T485T	Block 1
PLA2G2A	*c.-217-240C>T*	Block 1
	c.-180C>G	Block 2
	T32T	Block 2
	R143H	Block 2
	c.665C>T	Block 3
PLA2G4A	*c.918+23C>T*	Block 1
	c.1264-57T>A	Block 1
	c.1336+3G>A	Block 1
	c.2605G>A	Block 2
PPARγ	*P12A*	Block 1
	H477H	Block 2
PPARδ	*c.-789C>T*	Block 1
	N163N	Block 1
	c.1443G>A	Block 1
	c.2021T>C	Block 2
	c.2589G>A	Block 2
	c.2629T>C	Block 2
	c.2806C>G	Block 3

The haplotype blocks obtained from HaploBlockFinder were compared to block estimates from Haploview with genotypes from the HapMap project. *ALOX15* and *PLA2G2A* did not contain enough genotype data for LD analysis in HapMap, therefore comparisons were restricted to *PLA2G4A*, *PPARγ* and *PPARδ*. LD plots with block structures from Haploview for these three genes are shown in figure 3. The LD analysis for *PLA2G4A* reveals a total of six blocks. The plots for *PPARγ* and *PPARδ* show one point of recombination each, resulting in two haplotype blocks.

Discussion

In this study we have investigated the genetic variation of a number of candidate genes and evaluated which polymorphisms might be useful in future association studies. We found that, despite the growing amount of information currently available on genetic variation in the human genome, it was still useful to conduct a re-sequencing study to identify all potentially interesting SNPs and maximize genetic variability.

As it holds for the whole genome, there are great differences in sequence variation among the selected candidate genes, due to different degrees of selective pressure or to their location in more or less recombinogenic chromosomal regions. It is therefore difficult to estimate the frequency with which SNPs occur in the human genome, which is also apparent by the different estimates found in the literature(28,29). The variation in *PPARδ* for example is much greater than in another nuclear receptor, *PPARγ*, with fourteen polymorphisms in 5366 bp for *PPARδ* compared to two polymorphisms in 3068 bp for *PPARγ*. With respect to variations in functional elements encoded in the human genome our still limited knowledge is largely restricted to data collected in databases like TRANSFAC, although recently comparative genomic approaches have greatly expanded the number of these functional elements, including the number of miRNAs(30).

Direct approach for selecting SNPs

According to the SIFT software program, three of the nine non-synonymous SNPs found were likely to affect protein function, and are therefore candidates to use in an association study. Unfortunately, these three SNPs have a minor allele frequency lower than 5%,

generally thought to be the minimum frequency required to guarantee sufficient statistical power, unless very large cohorts are employed. The other program able to predict impact on protein function, PolyPhen, was not consistent with SIFT only in the case of the *R143H* SNP in *PLA2G2A*. SIFT predicted this SNP to have an effect on protein function whereas PolyPhen predicted the substitution to be benign. The reason for this might be the difference in prediction criteria used by the two programs. SIFT only uses sequence homology whereas PolyPhen uses sequence, polygenetic and structural parameters to derive at its predictions. PolyPhen might therefore be the more accurate of the two programs. A SNP with a tolerant score from SIFT, *P12A* in *PPARγ*, was predicted to be possibly damaging by PolyPhen. However, as the score from SIFT was only borderline tolerant (0.07 as compared to the cut-off value 0.05), and as PolyPhen predicted only a possible damaging effect, these results may considered in agreement rather than contradictory. In general, as with many in silico tools, results may differ slightly between software programs due to different rules and assumptions for each program. Combining tools however, is likely to lead to more robust results.
One of the four SNPs, *ALOX15 T560M*, is also predicted by the RESCUE-ESE program to interfere with an ESE consensus sequence, and might therefore have an additional effect on the overall protein function. A further nine SNPs were identified by the software as interfering with ESE consensus sites, making them possible candidates for association studies.

Indirect approach for selecting SNPs
An alternative way of selecting SNPs for association studies, when no functional data are present, is by estimating haplotypes and haplotype blocks, and thereby identifying SNPs that can distinguish between all the common haplotypes present within that block. Within a block, little or no recombination occurs, therefore limiting the number of possible haplotypes. Between two blocks, hotspots for recombination occur(8,31). Sebastiani et al.(9) found that of the total number of SNPs within a gene, an estimated 10% could be considered as htSNPs. In our study this percentage is much higher (52%, twenty-one out of forty SNPs), because only SNPs in coding and regulatory regions were available for analysis, whereas Sebastiani et al. also employed intronic SNPs for their analysis.
We have found a clear haplotype block structure in most of the investigated genes, with the exception of *ALOX15* where all of the investigated SNPs lie within the same block. The maximum number of common haplotypes in each block was four, and the maximum number of htSNPs per block was three. This method is useful to aid in the selection of SNPs for an association study, but there are some limitations as pointed out by Fullerton et al.(32). This group analyzed the haplotype structure of the *APOA1/C3/A4/A5* gene cluster, by using all SNPs identified by re-sequencing, and comparing the results to the structure revealed using htSNPs. They found that many gene- and population-specific features of this gene cluster could not be identified using the htSNP approach. In particular, the differences in haplotype distribution between populations caused the inability to identify and correctly classify the estimated haplotypes, when the same set of htSNPs was used for each population. There was therefore considerable heterozygosity within haplotype classes. This illustrates a disadvantage of using a general set of htSNPs to be applied to all populations. Other disadvantages of this approach, as outlined by Jawaid et.al.(7), is that pooling of DNA samples, a method to substantially reduce the number of genotype assays needed, is not possible for the estimation of haplotypes. Moreover, haplotype maps are often based on incomplete SNP data, which might mean that unidentified SNPs could break up the estimated block structure. It is therefore recommended that a subset of the population under study is re-sequenced, to identify all SNPs present in the genes of interest and select htSNPs from this data to use in the association study. In a simulation study, it was suggested that as little as 25 individuals are sufficient to identify all common polymorphisms

and therefore all common haplotypes(33). However, in practice numbers ranging from 25 to 60 are more commonly used.
The need for re-sequencing is illustrated by the haplotype block structure according to Haploview analysis for *PPARδ*, where the relatively small blocks in the 3'UTR could not be identified by Haploview, due to the low SNP density in this region. While the Haploview results show a separate block towards the end of the gene, our results show a block structure which points to two recombination hotspots in the 3'UTR of the gene. Since so far only a few SNPs from this region have been analyzed in the HapMap project, no block structures have been identified; on the contrary in our study this region has been analyzed in more detail with several 3'UTR SNPs, making the haplotype blocks become apparent.
The results obtained for *PPARγ* in our study are in line with LD estimated with Haploview, although different SNPs were used in the analyses. Our two exonic SNPs seem to lie on different haplotype blocks, likely due to a recombination event occurred in between these two SNP locations. The Haploview results also show two distinct haplotype blocks, with a point of recombination distal to the first exon of the most common splice variant of PPARγ, in agreement with our block structure.
The results for *PLA2G4A* from Haploview however are not consistent with our results. The Haploview results show six regions of LD representing haplotype blocks, whereas our study only shows two blocks with the first haplotype block extending beyond exon 13. The reason for this inconsistency might reside in the number of SNPs used for the analysis in Haploview, which includes many more intronic SNPs. This illustrates the need to include as many SNPs as possible in the analysis of haplotype blocks and that intronic SNPs can refine the block structure. This same point was recently stressed by a large re-sequencing study, where it was shown that using all SNPs within a gene to infer the common haplotypes, as compared to coding SNPs only, resulted in most genes in the identification of more common haplotypes. These extra haplotypes would have been missed using coding SNPs only(34). However, although intronic SNPs add to the haplotype diversity, SNPs in coding regions are more likely to have a functional effect on the protein. It remains important to determine the relevant htSNPs for the population under study, which is difficult to compare between studies as each one employs different SNPs sets.
In conclusion, our study underlines the importance of re-sequencing of a number of subjects from the study population, especially in those genes where a limited number of SNPs has been validated and analyzed by the HapMap project. It was recently demonstrated(35) that irrespective of the method used to define haplotype blocks it is the density of the SNP coverage defining the ultimate reliability of any ht tagging strategy. The more saturated, the more different haplotype blocks will be found. As a consequence, more tagging SNPs should be used to cover the region of interest, which will render the tagging approach less cost effective. Ultimately, a common sense approach not only based on selecting htSNPs but also determining possible functional effects of the SNPs, either at the protein or mRNA level would result in an optimal htSNP selection.

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Chapter 3

Colorectal adenoma risk is modified by the interplay between polymorphisms in arachidonic acid pathway genes en fish consumption

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Abstract

Associations between polymorphisms in genes (SNPs) involved in the arachidonic acid (AA) pathway, and colorectal adenomas were investigated in a Dutch case control study including 384 cases and 403 polyp-free controls. Twenty-one polymorphisms in seven candidate genes were studied, and a potential modifying effect of fish consumption was considered.
A protective effect on colorectal adenomas was found for the CT genotype of SNP *H477H* in *PPARγ* and the GC genotype of SNP *V102V* in *PTGS2* (OR, 0.63; 95%CI, 0.45-0.89 and OR, 0.65; 95%CI, 0.46-0.92 respectively) compared to the homozygote major genotypes. An increase in adenoma risk was observed for the TC genotype of SNP *c.2242T>C* in *PTGS2* (OR, 1.47; 95%CI, 1.07-2.00) compared to the TT genotype. Analysis with estimated haplotypes confirmed these associations and revealed three additional associations with *PTGS2*, *PLA2G2A* and *ALOX15* haplotypes. Fish consumption modified the associations with *PTGS2* and *PPARδ* genotypes. For SNP *c.-789C>T* in *PPARδ,* the major genotype showed a decrease in adenoma risk for those in the highest tertile of fish consumption (T3), as compared to the lowest tertile (T1) (OR, 0.65; 95%CI, 0.41-1.02). Protective effects were also observed for SNPs *V102V* and *c.2242T>C* in PTGS2 and high fish intake. The interaction between fish consumption and *c.2242T>C* was statistically significant, with an OR for the TT genotype and high fish consumption of 0.52 (95%CI, 0.27-1.01) as compared to low fish intake.
These results indicate that SNPs in genes involved in the AA-pathway are associated with colorectal adenoma risk. Part of these associations is modified by fish consumption

Introduction

Epidemiological and experimental evidence indicates that lipid metabolism, in particular the arachidonic acid (AA) pathway, plays a critical role in colorectal tumor development, as reviewed by Jones *et al.* (1).
Prostaglandin endoperoxide synthases 1 and 2 (*PTGS1* and *PTGS2*), also known as Cyclooxygenase-1 and cyclooxygenase–2, are two key genes in the AA pathway, encoding enzymes that initiate the synthesis of biologically important prostanoids (PGs) and eicosanoids (2). Both genes have been demonstrated to be involved in intestinal tumorigenesis, by promoting tumor growth, angiogenesis and metastasis. Major evidence comes from the study of non-steroidal anti inflammatory drugs (NSAIDs), acting amongst others as COX inhibitors, which play an important role in cancer prevention (3). Inactivation of these genes in mouse models of intestinal cancer decreases the number of polyps in these mice (4,5). Phospholipase A_2 (PLA_2) enzymes are a family that, besides other conversions, catalyze the generation of free fatty acids, such as AA, from membrane bound phospholipids. Most important in the AA-pathway are cytosolic PLA_2 (PLA2G4A) and secretory PLA_2 (PLA2G2A), which have both been demonstrated in mouse models to be involved in tumorigenesis (6,7). Levels of *PLA2G4A* mRNA are reduced in mouse and human tumors, possibly indicating a tumor protective effect of this enzyme, which is further substantiated by the reduction in apoptosis *in vitro* after inhibiting PLA2G4A (8). A similar protective effect is found for the secretory form of the enzyme, PLA2G2A. This gene, present on the Mom1 locus, causes an increase in number of polyps when inactiviated in the Apc^{Min} mouse model for intestinal cancer (7). Lipoxygenase (LOX), is an enzyme for which AA is one of the substrates. Activation of the enzyme 15-LOX might inhibit carcinogenesis via the conversion of linoleic acid into 13-S-hydroxyoctadecadienoic (13-S-HODE) acid, which in turn downregulates PPARδ thereby restoring apoptosis (9). Peroxisome proliferator-activated receptors (PPARs) play an important regulatory role in lipid metabolism and cancer, and PPARs can be activated by a variety of eicosanoids (10,11). There are three distinct types of PPARs, α, δ and γ. Both PPARδ and PPARγ have been implicated to play a role in colorectal tumorigenesis, by transcriptionally controlling pathways involved in cell

proliferation, differentiation and survival. Activation of PPARδ was shown to increase the number and size of intestinal polyps in Apc^{Min} mice (12), whereas inactivation causes inhibition of tumor growth in a colorectal cancer xenograft model (13,14). Moreover, recently its role as a focal point of cross-talk between prostaglandin and Wnt pathways has been suggested (15). Most data of PPARγ suggests a tumor suppressive role, however, there is still some controversy about the increase of intestinal polyps in Apc^{Min} mice by ligands of PPARγ (16,17).

Genetic variants represented by Single Nucleotide Polymorphisms (SNPs) in genes encoding these key players of the AA-pathway may contribute to variation in susceptibility to colorectal cancer. Recently, the focus of attention has shifted from the use of single (putative causal) genetic variants in association studies (the 'direct' approach) to using sets of genetic markers without a priori functional effects, the so called indirect approach (18). By using information about the common SNPs in a particular population, combinations of SNP alleles called haplotypes can be estimated, after which differences in frequencies between cases and controls can be evaluated (19,20).

Colorectal adenomatous polyps, later referred to as colorectal adenomas, are presumed to be the pre-cancerous state of colon cancer (21). Studying colorectal adenomas instead of colon cancer may give information about the risk factors in the earlier stages of carcinogenesis.

The fatty acids utilized by the AA pathway include n-3 poly-unsaturated fatty acids (PUFAs) from fish. Some, but not all animal experimental studies and epidemiological studies have shown that fish consumption may decrease the risk of colorectal tumors (22,23). A high ratio of fish fatty acids (Fas) to arachidonic acid (AA) in adipose tissue, as a marker for fatty acid intake, has been associated with a lower risk of colorectal adenomas (24). High fish consumption can affect the AA pathway by causing a shift in substrates from n-6 to n-3 PUFAs. SNPs in AA pathway genes may interact with fish consumption by influencing the conversion of these PUFAs into eicosanoids.

In this association study, twenty-one SNPs in genes encoding five enzymes and two nuclear receptors have been used. The objective of this study was to assess the association between inherited SNPs and haplotypes involved in the AA-pathway, and the occurrence of colorectal adenomas. Moreover, the potential modifying effect of fish consumption was considered.

Methods and materials

Study population

A retrospective case-control study was conducted in the Netherlands, between 1997 and 2001. The study design has been described in detail elsewhere (25,26). In brief, both cases and controls were undergoing an endoscopy in one of eight hospitals. They were asked to participate without knowing whether they had colorectal adenomas or not. Cases and controls were Dutch-speaking persons, of European origin, and aged between 18 and 75 years at the time of endoscopy. They did not suffer from inflammatory bowel diseases, and did not have a history of colorectal cancer or (partial) bowel resection. Cases were defined as those subjects diagnosed with at least one histologically confirmed colorectal adenomatous polyp ever in their life. Controls were defined as those subjects without a history of any type of polyp, including hyperplastic and metaplastic polyps. Suspected cases of hereditary colorectal cancer syndromes were excluded (27,28).

Major indications for endoscopy among cases were complaints (47.7%), including bowel complaints, rectal bleeding and defecation problems, and screening (39.5%). For controls these numbers were 76.7% for complaints and 1.7% for screening.

Pathological anatomy reports provided information about polyp characteristics. Medical records were used for additional information on polyp recurrence and general health status of the participants.
The total study population included 925 subjects. The Medical Ethical Committees of all participating hospitals and of Wageningen University approved the study and all participants have provided written informed consent.

Genetic analysis
Twenty of the twenty-one SNPs have been selected on the basis of an inventory of the genetic variation in the Dutch population of the selected genes as described elsewhere, in which fifty-eight polymorphisms were identified (C.Siezen, in preparation). The SNP selection was based on allele frequency (with some exceptions only those SNPs with a minor allele frequency of 5% or higher were considered), position in the gene (when possible divided over the length of the gene), possible impact on protein function (amino acid changes), and linkage between the SNPs in one gene (of two or more tightly linked SNPs only one was selected). One SNP was selected on the basis of another population study on PTGS1 variants (29).
Genotypes of the 21 SNPs were determined using a technique known as Pyrosequencing^{TM}. Each PCR contained 5μl 2x Hotstar master mix (Qiagen), 1μM of one primer, 0.1μM of the second primer containing a so-called universal tail of 23 nucleotides, 0.9μM of a third primer with the same sequence as the tail and labeled with biotin, and 10 ng genomic DNA, in a total volume of 10μl. PCR reactions were carried out in a Perkin-Elmer 9600 thermal cycler under the following conditions: 95ºC for 15 min, 40 cycles of 94ºC for 45 s, 57ºC for 45 s, 72ºC for 1 min, followed by 72ºC for 10 min. From the biotinylated PCR products single stranded DNA's were prepared and subsequently genotyped using the PSQ 96MA system (Pyrosequencing AB) (http://www.pyrosequencing.com) and SNP reagent kit (Pyrosequencing AB), as previously described (30). Primers for each SNP are described in table 1 in the appendix. DNA was available from 808 participants. SNPs could not be assessed in one of these samples.

Fish consumption and other lifestyle factors
Information about dietary habits was obtained using a validated food-frequency questionnaire (31,32). Participants were requested to recall their dietary and lifestyle habits of the year previous to their last endoscopy. Data about fish consumption was collected as number of times consumed per day, week, month or year. A distinction was made between high fat fish, low fat fish, and shellfish.
Information on demographic and lifestyle factors, like smoking habits, physical activity level (33) and family history, was obtained from a self-administered questionnaire.
The intake of total energy and of various nutrients from this questionnaire was calculated with the computerized Dutch food composition table.

Statistical analysis
Subjects with incomplete dietary data (n=20) were excluded. The data-analysis thus included data of 787 participants: 384 cases and 403 controls.
Logistic regression analysis was performed to calculate odds ratios (Ors) and 95% confidence intervals (95%CI) of separate genotypes when possible. If the numbers were too small, analyses were performed using pooled heterozygote and homozygote minor genotypes. The only potential confounding variables included in the model were age and gender.
Haplotypes were estimated and Ors calculated using the Hplus program, available online at http://qge.fhcrc.org/hplus. Hplus is a SNP analysis tool for performing haplotype estimations, according to the distribution of genotypes in a population. It is able to handle datasets that include case-control status as well as covariates and SNP location variables (34).

Multiple logistic regression analysis was performed to evaluate the modifying effect of fish consumption. Fish consumption, in grams per day, was divided in tertiles according to the distribution of intake among controls. The lowest fish consumption tertile in combination with a homozygote major allele for the SNP of interest was considered as reference group.
The variables age, BMI, family history of colorectal cancer, gender, indication of endoscopy, insulin use, physical activity, education level, smoking, aspirin use, daily energy intake and intake of alcohol, calcium, fiber, fruit, red meat, poultry, processed meat, vegetables, β-carotene, vitamin C and vitamin E were considered as potential confounding factors. Besides the variables age and gender, also indication for endoscopy and alcohol consumption were included in the model, since these factors changed the β-estimates by more than 10%.
To test for linear trend, we modeled the tertile of fish consumption as a continuous variable in the logistic regression model, in which each tertile was assigned its median value. To test whether the combination of genotypes and fish consumption deviated from multiplicativity, we calculated p-values for interaction by inclusion of a numerical term for genotype, multiplied by fish consumption as a continuous variable into our multivariate models. To exclude the influence of previous adenomas among cases, and of undetected proximal polyps among controls, we repeated our analysis excluding these cases and controls.
The analyses were conducted using Statistical Analysis Software (SAS) for Windows, version 8.

Results

Cases and controls were similar with respect to family history of colorectal cancer, with 22.5% of cases and 19.2% of controls having a family history of cancer. The variables age and gender differed among cases and controls. The median age for cases was 59.9 and for controls 52.2, and 53.3% of cases was male versus 38.4% of controls. However, no effect modification was observed after stratification for age group and gender. A more detailed description of the study population characteristics was published previously (35,36). The genotypes of all SNPs studied were in Hardy Weinberg equilibrium.

Genotypes

Table I shows the associations between genotypes and colorectal adenomas. A statistically significant inverse association was observed for the CT genotype of SNP *H477H* (OR, 0.63; 95%CI, 0.45-0.89) in the *PPARγ* gene as compared to the CC genotype. The OR of the TT genotype did not reach significance, but the TC and TT genotypes together also showed a statistically significant inverse association (OR, 0.64; 95%CI, 0.46-0.90) (data not shown) as compared to the CC genotype. For the *PTGS2* gene two opposite associations were observed; an inverse association for the GC genotype of SNP *V102V* (OR, 0.65; 95%CI, 0.46-0.92), and a positive association for the TC genotype of SNP *c.2242T>C* (OR, 1.47; 95%CI, 1.07-2.00) as compared to the homozygote major genotypes. In the case of both SNPs, the homozygote minor genotypes did not show a statistically significant OR, but combining the heterozygotes and homozygotes minor genotypes resulted in a statistically significant association (OR, 0.68; 95%CI, 0.49-0.95 and OR, 1.40; 95%CI, 1.04-1.89 respectively) (data not shown), as compared to the homozygote major genotypes.
No statistically significant associations were observed for any of the SNPs in *PPARδ, PTGS1, PLA2G4A, PLA2G2A* or *ALOX15.*

Table 1: AA-pathway related genetic variants and colorectal adenomas

Genotype	Cases (n=384)	Controls (n=403)	OR (95%CI) [a]	Genotype	Cases (n=384)	Controls (n=403)	OR (95%CI) [a]
PPARδ				***PLA2G4A***			
SNP A:				SNP M:			
c.-789C>T				*c.918+23C>T*			
CC	333	351	1.00 (ref)	CC	255	272	1.00 (ref)
CT	31	32	1.04 (0.60-1.79)	CT	114	123	0.93 (0.67-1.28)
TT	1	1	0.64 (0.04-10.65)	TT	11	8	1.26 (0.47-3.37)
SNP B:				SNP N:			
N163N				*c.1336+3G>A*			
TT	240	245	1.00 (ref)	GG	344	348	1.00 (ref)
TC	121	143	0.92 (0.67-1.23)	GA+AA	40	53	0.71 (0.45-1.13)
CC	22	15	1.31 (0.64-2.68)	SNP O:			
SNP C:				*R651K*			
c.2021T>C				AA	327	353	1.00 (ref)
TT	249	256	1.00 (ref)	AG+GG	14	18	0.92 (0.43-1.95)
TC	118	133	0.98 (0.71-1.35)	SNP P:			
CC	17	12	1.41 (0.63-3.19)	*c.2605G>A*			
SNP D:				GG	269	298	1.00 (ref)
c.2589G>A				GA	41	54	0.74 (0.47-1.18)
GG	347	361	1.00 (ref)	AA	3	5	0.66 (0.15-2.95)
GA+AA	37	42	0.91 (0.56-1.50)	***PLA2G2A***			
SNP E:				SNP R:			
c.2806C>G				*c.-180C>G*			
CC	272	293	1.00 (ref)	CC	219	217	1.00 (ref)
CG	92	97	1.08 (0.76-1.53)	CG	137	162	0.87 (0.63-1.18)
GG	12	8	1.44 (0.55-3.79)	GG	20	19	1.13 (0.56-2.25)
PPARγ				SNP S:			
SNP F:				*T32T*			
P12A				GG	320	327	1.00 (ref)
CC	281	297	1.00 (ref)	GC	61	73	0.81 (0.54-1.20)
CG	92	97	0.92 (0.65-1.31)	CC	1	3	0.38 (0.04-4.26)
GG	6	9	0.72 (0.24-2.20)	SNP T:			
SNP G:				*c.665C>T*			
H477H				CC	297	319	1.00 (ref)
CC	276	263	1.00 (ref)	CT	75	77	1.14 (0.78-1.66)
CT	84	115	0.63 (0.45-0.89)	TT	6	3	2.47 (0.55-11.06)
TT	7	10	0.68 (0.24-1.93)	***ALOX15***			
PTGS1				SNP U:			
SNP H:				*c.-217G>C*			
W8R				GG	258	260	1.00 (ref)
CC	334	339	1.00 (ref)	GC	87	94	0.98 (0.68-1.40)
CT	38	51	0.95 (0.59-1.54)	CC	3	9	0.30 (0.08-1.20)
TT	1	2	0.42 (0.04-5.02)	SNP V:			
SNP I:				*T485T*			
L237M				AA	301	293	1.00 (ref)
CC	300	332	1.00 (ref)	AG	57	73	0.80 (0.53-1.19)
CA+AA	14	12	1.29 (0.56-2.94)	GG	5	10	0.50 (0.16-1.58)
PTGS2							
SNP J:							
c.-1329A>G							
AA	218	355	1.00 (ref)				
AG	131	122	1.23 (0.89-1.69)				
GG	22	16	1.62 (0.80-3.30)				
SNP K:							
V102V							
GG	284	267	1.00 (ref)				
GC	88	121	0.65 (0.46-0.92)				
CC	12	15	0.96 (0.41-2.24)				
SNP L:							
c.2242T>C							
TT	159	196	1.00 (ref)				
TC	185	162	1.47 (1.07-2.00)				
CC	35	38	1.16 (0.68-1.99)				

[a] Univariate adjustment for age and gender

Haplotypes

Table II shows the associations between the most common haplotypes estimated and colorectal adenoma risk. The haplotypes are represented as a series of 0 and 1, indicating for each SNP in that gene whether the major (0) or minor (1) allele is present in that haplotype. Five statistically significant associations between a specific haplotype and adenoma risk have been found.

Table 2: AA-pathway related haplotypes and colorectal adenomas

Haplotype[a]	Frequency among cases	Frequency among controls	OR (95% CI)[b]
PPARδ			
SNP order[c]: ABCDE			
00000	0.780	0.779	1.00 (ref)
01101	0.146	0.141	1.08 (0.80-1.46)
01110	0.018	0.028	0.63 (0.32-1.22)
11110	0.029	0.025	1.22 (0.63-2.35)
11000	0.016	0.020	0.75 (0.35-1.59)
PPARγ			
SNP order[c]: FG			
00	0.825	0.787	1.00 (ref)
11	0.095	0.104	0.84 (0.59-1.21)
01	0.038	0.070	0.47 (0.28-0.78)
10	0.042	0.040	0.98 (0.58-1.66)
PTGS1			
SNP order[c]: HI			
00	0.924	0.912	1.00 (ref)
10	0.053	0.070	0.91 (0.60-1.39)
01	0.022	0.017	1.28 (0.55-2.97)
PTGS2			
SNP order[c]: JKL			
000	0.282	0.318	1.00 (ref)
001	0.336	0.298	1.34 (1.02-1.76)
100	0.236	0.198	1.37 (1.01-1.86)
010	0.146	0.186	0.93 (0.67-1.28)
PLA2G4A			
SNP order[c]: MNOP			
0000	0.793	0.793	1.00 (ref)
1000	0.109	0.089	1.19 (0.82-1.74)
1101	0.050	0.059	0.72 (0.42-1.23)
0010	0.019	0.023	0.91 (0.43-1.90)
1001	0.014	0.018	0.81 (0.29-2.24)
0001	0.013	0.010	1.58 (0.63-3.95)
PLA2G2A			
SNP order[c]: RST			
000	0.640	0.623	1.00 (ref)
100	0.229	0.246	0.93 (0.72-1.22)
011	0.066	0.069	0.88 (0.55-1.40)
010	0.017	0.028	0.58 (0.28-1.21)
001	0.042	0.028	2.10 (1.11-3.95)
ALOX15			
SNP order[c]: UV			
00	0.819	0.795	1.00 (ref)
10	0.089	0.082	1.20 (0.77-1.86)
11	0.044	0.073	0.51 (0.30-0.87)
01	0.048	0.050	1.17 (0.70-1.97)

[a] 0 represents major allele, 1 represents minor allele;
[b] Adjusted for age and gender
[c] SNP order according to table I

A reduction in the risk for colorectal adenomas was observed for haplotype 01 in *PPARγ* (C allele for *P12A* and T allele for *H477H*), and 11 in *ALOX15* (C allele for *c.-217G>C*, and G allele for *T485T*). The Ors showed about a 50% reduction of colorectal adenoma risk (OR = 0.47 and 0.51 respectively), as compared to haplotype 00 (major alleles for both SNPs). The remaining 3 haplotypes showed a positive association. Haplotypes 100 and 001 in *PTGS2* (G alleles at position *c.-1329* and amino acid 102 and the T allele for *c.2242T>C*; and A allele at position *c.-1329,* G allele at amino acid 102 and C allele for *c.2242T>C*) showed similar Ors of 1.37 (95%CI, 1.01-1.86) and 1.34 (95%CI, 1.02-1.76), as compared to haplotypes 000. An even greater effect was observed for haplotype 001 in *PLA2G2A* (C allele for *c.-180C>G*, G allele for *T32T* and T allele for *c.665C>T*), with an OR of 2.10 (95%CI, 1.11-3.95), as compared to haplotype 000.

Gene-diet interaction

The associations between genotypes and colorectal adenomas stratified for fish consumption are shown in Table III. There was no statistically significant association between fish consumption and colorectal adenoma risk (data not shown). A statistically significant interaction was observed between fish consumption and SNP *c.-789C>T* in *PPARδ,* in adenoma risk. An inverse association was observed for those with the CC genotype and highest tertile (T3) of fish consumption as compared to those with lowest tertile (T1) of fish consumption (OR, 0.65; 95%CI, 0.41-1.02). However, for those with the CT or TT genotype, fish consumption increased risk (OR T3 versus T1, CC genotype: 2.22; 95%CI, 0.78-6.36).

For *PTGS2* the AG and GG genotypes of SNP *c.-1329A>G*, located in the promoter in *PTGS2*, showed a positive trend towards a reduced risk of adenomas with increasing fish consumption (p=0.01 and 0.03 respectively), as compared to those with the AA genotype and low fish consumption. Fish consumption strengthened the protective effect of SNP *V102V* in *PTGS2,* to a statistically significant OR of 0.42 (95%CI, 0.20-0.90) in the highest tertile of intake for individuals with the GC genotype, as compared to those with the GG genotype and low fish consumption. A statistically significant interaction was observed between fish consumption and SNP *c.2242T>C*, located in 3'UTR in *PTGS2* (p=0.01). The homozygote major T allele was inversely associated with colorectal adenomas in the highest fish consumption tertile. Although not statistically significant, the OR of 0.52 (95%CI, 0.27-1.01) reflects a reduction of almost 50% in the occurrence of colorectal adenomas, as compared to low fish consumption.

The AA genotype of SNP *R651K*, an A to G substitution in *PLA2G4A* was inversely associated with colorectal adenomas for individuals in the highest fish consumption tertile (OR, 0.64; 95%CI 0.41-0.96), as compared to low fish consumption. The risk of colorectal adenomas decreased also for the GG genotype of SNP *c.2605G>A* in *PLA2G4A* with high consumption of fish (OR, 0.59; 95%CI, 0.37-0.92), as compared to low consumption of fish.

After stratification for fish consumption, no statistically significant associations were observed between genotype and adenomas for either SNPs in *PPARγ,* or for the SNPs in *PTGS1*. There was also no effect on the associations between the genotypes of SNPs in *PLA2G2A* and *ALOX15*, and colorectal adenomas.

A summary of the major findings of this study is provided in Table 4.

All analyses were repeated for incident cases only (i.e. those with a first polyp at index scopy). No differences were observed in comparison to the analyses with the prevalent and incident cases together (data not shown). The same analyses were carried out for the cases with villous polyps. No marked differences were observed in comparison to the analyses with all cases, including cases with tubular and tubulovillous polyps, but the results were stronger for the cases with villous polyps (data not shown). Also, similar results were found with analyses including only subjects who underwent a full endoscopy (data not shown).

Table 3: AA-pathway related genotypes and colorectal adenomas, stratified by fish consumption.

Genotype	T_1 fish consumption [a]		T_2 fish consumption [a]		T_3 fish consumption [a]		p for trend [c]
	Cases/Controls	OR (95% CI) [b]	Cases/Controls	OR (95% CI) [b]	Cases/Controls	OR (95% CI) [b]	
PPARδ							
c.-789C>T							
CC	109/111	1.00 (ref)	128/117	1.18 (0.78-1.80)	96/123	0.65 (0.41-1.02)	0.06
CT+TT	9/20	0.35 (0.12-1.05)	8/6	1.43 (0.41-4.97)	15/7	2.22 (0.78-6.36)	0.02
p for interaction							0.01
N163N							
TT	80/82	1.00 (ref)	95/81	1.26 (0.76-2.08)	65/82	0.65 (0.38-1.12)	0.11
TC	42/53	0.65 (0.34-1.25)	39/41	0.85 (0.44-1.63)	40/49	0.66 (0.35-1.25)	0.74
CC	3/7	0.13 (0.01-1.17)	6/4	1.33 (0.32-5.53)	13/4	2.09 (0.56-7.83)	0.06
p for interaction							0.03
c.2021T>C							
TT	80/92	1.00 (ref)	99/81	1.49 (0.91-2.45)	70/83	0.83 (0.49-1.40)	0.41
TC	43/44	0.90 (0.47-1.73)	37/42	0.91 (0.47-1.75)	38/47	0.64 (0.33-1.25)	0.24
CC	2/5	0.20 (0.01-3.17)	5/3	1.55 (0.31-7.71)	10/4	2.19 (0.57-8.40)	0.24
P for interaction							0.12
c.2589G>A							
GG	112/128	1.00 (ref)	133/113	1.47 (0.96-2.26)	102/120	0.82 (0.52-1.29)	0.34
GA+AA	13/14	1.09 (0.39-2.99)	8/13	0.59 (0.19-1.80)	16/15	1.10 (0.46-2.63)	0.89
p for interaction							0.14
c.2806C>G							
CC	90/103	1.00 (ref)	102/93	1.28 (0.81-2.04)	80/97	0.80 (0.50-1.30)	0.36
CG	32/35	0.77 (0.37-1.59)	31/30	1.04 (0.52-2.09)	29/32	0.60 (0.28-1.27)	0.49
GG	1/3	0.12 (0.01-2.56)	5/1	3.60 (0.35-36.37)	6/4	1.39 (0.31-6.14)	0.51
p for interaction							0.23
PPARγ							
P12A							
CC	92/107	1.00 (ref)	107/90	1.64 (1.01-2.64)	82/100	0.83 (0.50-1.37)	0.39
CG	30/31	1.30 (0.65-2.62)	31/35	0.97 (0.50-1.90)	31/31	1.14 (0.58-2.25)	0.77
GG	1/4	0.68 (0.06-7.63)	3/1	2.22 (0.19-26.50)	2/4	0.52 (0.06-4.76)	0.99
p for interaction							0.19
H477H							
CC	90/92	1.00 (ref)	105/81	1.51 (0.94-2.43)	81/90	0.79 (0.48-1.31)	0.20
CT	28/40	0.75 (0.38-1.46)	29/36	0.68 (0.34-1.34)	27/39	0.58 (0.29-1.16)	0.64
TT	1/4	0.41 (0.04-4.34)	3/3	0.94 (0.14-6.18)	3/3	0.87 (0.10-7.96)	0.86
p for interaction							0.14

Genotype	T_1 fish consumption [a]		T_2 fish consumption [a]		T_3 fish consumption [a]		p for trend [c]
	Cases/Controls	OR (95% CI) [b]	Cases/Controls	OR (95% CI) [b]	Cases/Controls	OR (95% CI) [b]	
PTGS1							
W8R							
CC	111/119	1.00 (ref)	122/99	1.44 (0.93-2.23)	101/121	0.81 (0.52-1.26)	0.26
CT+TT	9/16	1.06 (0.39-2.89)	16/25	0.91 (0.41-2.03)	14/12	1.18 (0.43-3.29)	0.64
p for interaction							0.16
L237M							
CC	101/112	1.00 (ref)	106/105	1.07 (0.71-1.60)	93/115	0.68 (0.45-1.04)	0.20
CA+AA	3/6	0.42 (0.06-2.98)	5/3	1.38 (0.26-7.36)	6/3	1.22 (0.24-6.14)	0.29
p for interaction							0.08
PTGS2							
c.-1329A>G							
AA	70/96	1.00 (ref)	82/83	1.32 (0.78-2.23)	66/76	1.29 (0.75-2.23)	0.45
AG	44/36	2.47 (1.28-4.75)	45/36	2.85 (1.54-5.29)	42/50	0.88 (0.47-1.65)	0.01
GG	8/5	2.43 (0.58-10.25)	9/4	5.14 (1.32-19.91)	5/7	0.62 (0.14-2.88)	0.03
p for interaction							0.88
V102V							
GG	91/96	1.00 (ref)	98/80	1.40 (0.86-2.29)	95/91	0.89 (0.54-1.46)	0.32
GC	30/40	0.60 (0.29-1.24)	37/40	0.88 (0.47-1.66)	21/41	0.42 (0.20-0.90)	0.31
CC	4/6	0.85 (0.15-4.71)	6/6	0.90 (0.20-3.96)	2/3	0.61 (0.06-6.61)	0.82
p for interaction							0.58
c.2242T>C							
TT	52/66	1.00 (ref)	70/62	1.57 (0.87-2.83)	37/68	0.52 (0.27-1.01)	0.07
TC	59/61	1.14 (0.62-2.12)	58/44	1.74 (0.93-3.27)	68/57	1.30 (0.71-2.40)	0.79
CC	12/12	1.30 (0.42-4.00)	12/17	0.62 (0.21-1.84)	10/9	1.44 (0.46-4.47)	0.55
p interaction							0.01
PLA2G4A							
c.918+23C>T							
CC	82/101	1.00 (ref)	94/79	1.40 (0.85-2.31)	79/92	0.92 (0.55-1.53)	0.79
CT	39/39	1.05 (0.54-2.04)	40/44	1.06 (0.58-1.95)	35/40	0.67 (0.34-1.30)	0.13
TT	3/2	1.05 (0.09-11.72)	5/3	3.46 (0.72-16.54)	3/3	0.67 (0.11-4.28)	0.59
p for interaction							0.40
c.1336+3G>A							
GG	116/122	1.00 (ref)	124/107	1.34 (0.86-2.08)	104/119	0.79 (0.50-1.25)	0.18
GA+AA	9/19	0.49 (0.17-1.44)	17/19	1.01 (0.45-2.26)	14/15	0.88 (0.36-2.14)	0.45
p for interaction							0.09

Genotype	T_1 fish consumption [a]		T_2 fish consumption [a]		T_3 fish consumption [a]		p for trend [c]
	Cases/Controls	OR (95% CI) [b]	Cases/Controls	OR (95% CI) [b]	Cases/Controls	OR (95% CI) [b]	
R651K							
AA	108/120	1.00 (ref)	119/113	1.12 (0.75-1.68)	100/120	0.63 (0.41-0.96)	0.16
AG+GG	2/5	1.01 (0.16-6.28)	8/5	1.47 (0.39-5.57)	4/8	0.62 (0.16-2.36)	0.48
p for interaction							0.52
c.2605G>A							
GG	87/107	1.00 (ref)	99/88	1.11 (0.72-1.71)	83/103	0.59 (0.37-0.92)	0.26
GA+AA	14/20	0.57 (0.22-1.45)	18/20	1.03 (0.49-2.20)	12/19	0.53 (0.22-1.27)	0.47
p for interaction							0.49
PLA2G2A							
c.-180C>G							
CC	70/68	1.00 (ref)	82/70	1.15 (0.67-1.99)	67/79	0.71 (0.41-1.25)	0.27
CG	45/67	0.68 (0.37-1.27)	52/48	1.24 (0.68-2.26)	40/47	0.77 (0.41-1.45)	0.87
GG	8/6	1.60 (0.44-5.83)	6/7	1.23 (0.34-4.37)	6/6	0.35 (0.06-2.02)	0.42
p for interaction							0.30
T32T							
GG	98/110	1.00 (ref)	117/105	1.38 (0.87-2.18)	105/112	0.93 (0.58-1.48)	0.56
GC+CC	26/32	0.96 (0.46-2.00)	24/21	1.25 (0.59-2.66)	12/23	0.42 (0.16-1.08)	0.73
p for interaction							0.50
c.665C>T							
CC	92/109	1.00 (ref)	109/98	1.43 (0.90-2.27)	96/112	0.85 (0.53-1.37)	0.31
CT+TT	31/33	1.21 (0.61-2.43)	31/27	1.34 (0.67-2.69)	19/20	0.92 (0.38-2.19)	0.88
p for interaction							0.61
ALOX15							
c.-217G>C							
GG	86/91	1.00 (ref)	94/78	1.47 (0.93-2.32)	78/91	0.79 (0.49-1.28)	0.48
GC	29/32	1.12 (0.56-2.24)	33/32	1.17 (0.60-2.28)	25/30	0.83 (0.42-1.66)	0.51
CC	1/2	1.58 (0.13-19.28)	1/3	0.09 (0.00-3.77)	1/4	0.31 (0.03-2.98)	0.32
p for interaction							0.38
T485T							
AA	98/105	1.00 (ref)	110/96	1.47 (0.95-2.28)	93/92	0.98 (0.61-1.57)	0.60
AG	19/24	1.20 (0.54-2.66)	22/19	1.07 (0.46-2.47)	16/30	0.69 (0.33-1.46)	0.14
GG	1/2	0.53 (0.04-6.83)	2/5	0.56 (0.07-4.69)	2/3	0.83 (0.13-5.51)	0.67
p for interaction							0.43

[a] Tertiles of fish consumption, per day: 0.0-4.3g, 4.3-14.5g, and 14.5-84.1g.
[b] Multivariate adjustment for age, gender, indication for endoscopy and alcohol consumption.
[c] Trend test for fish consumption per genotype.

Table 4: Summary of statistical significant results

		Analysis					
Gene	SNP	Genotype	Risk	Haplotype	Risk	Genotype	Effect of fish
PPARδ	*c.-789C>T*					CT+TT	Trend[a]
							Interaction[b]
	N163N						Interaction[b]
PPARγ	*H477H*	CT	↓	01	↓↓		
PTGS2	*c.-1329A>G*			100	↑	AG+GG	Trend[c]
	V102V	GC	↓				
	c.2242T>C	TC	↑	001	↑		Interaction[b]
PLA2G	*R651K*					AA	T3/↓[d]
4A	*c.2605G>A*					GG	T3/↓[d]
PLA2G 2A				001	↑		
ALOX15				11	↓		

[a] Increased adenoma risk with increasing fish consumption
[b] Deviation from multiplicativity
[c] Decreased adenoma risk with increasing fish consumption
[d] Decreased adenoma risk with high fish consumption

Discussion

All AA-pathway genes investigated in this study have been associated with the etiology of colorectal tumors in previous in-vitro, animal model and human studies. We investigated whether polymorphisms in these genes alone or in combination with fish consumption contributed to adenoma risk, and found a number of statistically significant associations. The SNPs were selected with great care, however, there are SNPs with lower minor allele frequencies that could also influence colorectal adenoma risk, but because of insufficient numbers, it is not feasible to evaluate these effects in this study.

The associations found between SNPs in *PTGS2* and colorectal polyps are supported by a similar finding by Campa *et al*. (37), who found a positive association between the minor allele of SNP *c.2242T>C* in *PTGS2* and non-small cell lung cancer. This is not in line however, with a previously published study in which affected siblings were tested for linkage of *PTGS2* variants with colon neoplasia (38). The authors suggested that genetic variation in *PTGS2* is unlikely to contribute to colorectal cancer risk, but as pointed out by Ulrich and Potter (39) the population used in this study might not be representative of the general population and markers some distance away from the *PTGS2* locus were used. Moreover, the importance of the 3'UTR region of *PTGS2* is illustrated by a strong positive association between another SNP in this region and colorectal cancer observed in a study investigating a number of polymorphisms in *PTGS2* (40). This part of the 3'UTR was previously thought to lie outside the gene, hence this SNP was not included in our study. In line with published data on the importance of the 3'UTR in posttranscriptional regulation of *PTGS2* expression (41) it is hypothesized that SNPs in this region interfere with this process.

We found no associations between the two SNPs in *PTGS1* and colorectal adenomas. This is in line with two previous studies investigating one of the SNPs in *PTGS1* (*L237M*) neither of which showed a significant association (42,43). However, due to the low minor allele frequency of this SNP false negative results cannot be ruled out.

As far as we know *H477H* in *PPARγ* has not been tested before for colorectal tumor risk, whereas for the other variant in *PPARγ*, *P12A*, a modest protective effect of the minor allele has been found (44). This finding could not be replicated by our study, which may be explained in part by the different study populations; colorectal cancer vs adenoma patients. The involvement of this SNP in colorectal cancer development was also implicated by a study on colorectal cancer patients where the minor allele was frequently found in tumors

without a K-ras mutation (45), an observation that could not be reproduced by us (data not shown). Analyzing the haplotype containing the major allele of *P12A* and the minor allele of *H477H* strengthened the association we found between *H477H* in *PPARγ* and adenomas. Moreover, the importance of specific haplotypes containing these SNPs was also illustrated by two other studies, on body weight and type 2 diabetes. However, the statistically significant association in the second study concerned the haplotype containing the minor allele of *P12A* and the major allele of *H477H* (also known as *C1431T*) (46,47). This does indicate however, that there is likely a functional effect resulting from the combination of these two SNPs, either on protein function or on the amount of protein present.

We found a positive association between haplotype 001 (C allele for *c.-180C>G*, G allele for *T32T* and T allele for *c.665C>T*) of *PLA2G2A* and adenomas. When the SNPs were analyzed separately no associations were apparent, illustrating again the importance of specific haplotypes. This positive association is particularly striking since the role of PLA2G2A in human colorectal carcinogenesis following the discovery of the gene on the Mom-1 locus (7), has been the subject of much debate. Although one study found a germline PLA2G2A mutation in a sporadic colorectal cancer patient (48), there have been several other results questioning the tumor-suppressing role of human PLA2G2A. No somatic mutations in tumors have been found (49) neither do the differing disease phenotypes in FAP patients correlate with *PLA2G2A* mutations (50). Gene expression analysis revealed no change in expression levels of *PLA2G2A* between normal mucosa and tumors (51), and even an apparent lack of expression of *PLA2G2A* in colorectal cancer cell lines was observed (52). In line with this, a study investigating the effect of single SNPs, corresponding to two of our selected SNPs (*c.-180C>G* and *T32T*), on the phenotype of FAP patients and on sporadic colorectal cancer, found no associations (53). However, the effect of the third SNP (*c.665C>T*) and the corresponding haplotypes, was not considered in that study. Our results reinforce the notion that *PLA2G2A* is not only an important tumor suppressor in mice, but may also play a role in human tumors.

No previous studies have been reported evaluating associations between SNPs or haplotypes in the anticarcinogenic *ALOX15* gene and colorectal tumors. However, associations with a specific haplotype in another subtype of the lipoxygenase enzyme, the procarcinogenic 5LOX gene, have been reported recently (42), indicating again the importance of this family of enzymes in colorectal tumorigenesis.

Fish consumption can affect lipid metabolism through different mechanisms. An increase in n-3 PUFAs can lead to an increase in eicosapentaenoic acid (EPA) tissue content, and a decrease in n-6 PUFA derived AA. This will lead to an increase in eicosanoid synthesis from EPA, resulting in a shift in production of 2-series to 3-series prostaglandins (54,55). Since prostaglandins act as ligands for PPARδ (56), fish consumption may interact with PPARδ by modifying the spectrum of PPAR ligands. This can be influenced by SNPs, explaining the interactions founds between SNPs in *PPARδ* and fish consumption. Besides changing the substrates and therefore the products of the AA-pathway, n-3 PUFA can have a direct effect on the genes in the pathway as well. A high concentration of n-3 PUFAs has been shown to inhibit PTGS2 directly causing a decrease in the overall production of prostaglandins (57). SNPs in *PTGS2* might interfere with this process. Inhibition of Δ6-desaturase, the rate-limiting enzyme in the conversion of LA to AA, by n-3 PUFAs has also been demonstrated (58). These interactions between fish consumption, as a proxy for n-3 PUFAs, and genes in the AA-pathway make it plausible that the association between SNPs in these genes and colorectal adenomas is modulated by fish consumption, and therefore that some associations became apparent and others were strengthened, although the exact mechanisms remain far from clear.

Consideration must be given to the potential limitations of the present study in particular the possibility of chance findings due to evaluating a large number of genes and gene-diet interactions simultaneously. Although the genes examined have been previously shown to

be involved in colorectal carcinogenesis, the results of this study need to be confirmed by others.
In conclusion, this study has shown that polymorphisms in genes involved in the AA-pathway may be associated with colorectal adenomas. We have shown for the first time that these associations could be modified by fish consumption, but further research to understand the mechanisms involved is needed. For example, functional studies of the SNPs implicated in this study might provide a plausible basis for the associations observed, and to minimize information bias and to assess whether n-3 Fas are the active agents associated with colorectal tumor risk, measurements of n-3 Fas in plasma samples are recommended and presently ongoing.

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Appendix

Table 1: PCR and sequencing primer sequences for each SNP

SNP		Sequence (5'-3')	SNP		Sequence (5'-3')
PPARδ	Forward	[a]aatcactcatccagatagctag	***cPLA$_2$***	Forward	[a]gctctggacaacctgtcacc
c.-789C>T	Reverse	aacctcctcatcttccagtga	*c.918+23C>T*	Reverse	ggggagaatgggctcagta
	Sequence	gactcttaacccagtgcta		Sequence	tgtctacttgttataaaagc
N163N	Forward	[a]taaagggatggggatgtcag	*c.1336+3G>A*	Forward	[a]tgatagctcggacagtgatga
	Reverse	gacccagagcccaggatg		Reverse	catgcaacatgcaatcctct
	Sequence	tgtactggctcccctc		Sequence	agtttccggctcact
c.2021T>C	Forward	[a]tcccctgctcctttctcta	*R651K*	Forward	[a]ttatgtgtttgatcgggaagg
	Reverse	ggagcctgcaggtaaagtga		Reverse	tgtcagtttcgtcccctactt
	Sequence	atgtgtgtgtgctgga		Sequence	cctacttacctggagcc
c.2589G>A	Forward	[a]ggctggagtctcagagcaca	*c.2605G>A*	Forward	tgaacttcctgatacaaatgtaggg
	Reverse	atcctgccagcagagagtga		Reverse	[b]aaaaatagtgttgtctcatggtatgaa
	Sequence	ccacccagagtcacac		Sequence	ttcaatctcaataagacctc
c.2806C>G	Forward	tctctgctggcaggattctt	***sPLA$_2$***	Forward	[a]gctccgttggttggtaggta
	Reverse	[b]tggggctcctagcaaaaata	*c.-180C>G*	Reverse	tttcacagccacgtc
	Sequence	cgcgtccctgccc		Sequence	cctgctccccttaaat
PPARγ	Forward	[a]caaacccctattccatgctg	*T32T*	Forward	[a]caaatttccttccaggcctac
P12A	Reverse	caaacacaacctggaagacaaa		Reverse	cataactgagtgcggcttcc
	Sequence	aggaatcgctttctg		Sequence	cggcttcctttcctg
H477H	Forward	[a]aaccaccctgagtcctcaca	*c. 665C>T*	Forward	[a]catccctcacccatcctaga
	Reverse	gaagggaaatgttggcagtg		Reverse	catgatttgctaattgctttattca
	Sequence	cctgcagtagctgcac		Sequence	ttattcagaagagacccc
PTGS1	Forward	agcccctcatctctctcctc	***15LOX***	Forward	ctgtaccaggcgttgattcc
W8R	Reverse	[b]aggaggggttgaaaccag	*c.-217G>C*	Reverse	[b]agagcttcaaggggcagaat
	Sequence	tctgcagggagtctctt		Sequence	gatagtggtttccactcc
L237M	Forward	[a]caggtagacctcggccacat	*T485T*	Forward	[a]gctccgttggttggtaggta
	Reverse	ctttgccagggaagaccat		Reverse	tcttgggctttgtgtctgag
	Sequence	ttgatactgacgctcca		Sequence	tttcacagccacgtc
PTGS2	Forward	[a]atgctcctccctgagcacta			
c.-1329A>G	Reverse	tttcagttgcctgggcttat			
	Sequence	tggaagggagattttg			
V102V	Forward	cattcccttccttcgaaatg			
	Reverse	[b]gatggaaggcaaacttaaaagc			
	Sequence	aaatgcaattatgagttat			
c.2242T>C	Forward	tgtttccaatgcatcttcca			
	Reverse	[b]gcactgatacctgtttttgtttg			
	Sequence	aattttaaagtacttttggt			

[a]Forward tail and forward universal biotin labeled primer: gacgggacaccgctgatcgttta
[b]Reverse tail and reverse universal biotin labeled primer: agcgctgctccggttcatagatt

Chapter 4

Polymorphisms in arachidonic acid pathway genes, fish consumption and colorectal cancer risk

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Abstract
The objective of this study on colorectal cancer was to investigate the associations between SNPs in arachidonic acid (AA) pathway genes, their haplotypes and colorectal cancer. Moreover, interactions between SNPs and fish consumption were considered. In this study a total of 508 cases and 772 controls were included, originating from two prospective cohorts, the Monitoring Project on Cardiovascular Disease Risk Factors (PPHV) and Diagnostisch Onderzoek Mammacarcinoom (DOM). Genotypes of twenty-three SNPs in seven candidate genes were determined and the modifying effect of fish consumption was considered. A protective effect of the minor allele of SNP *V102V* in *PTGS2* was observed (OR, 0.37; 95%CI, 0.16-0.87). The haplotype representing this allele showed a weaker inverse association, indicating that two alleles are necessary to obtain this protective effect. Fish consumption data was available for 209 cases and 418 controls. Increased fish consumption was inversely associated with cancer, although not statistically significant (OR, 0.83; 95%CI, 0.57-1.20). Despite the substantial reductions in cancer risk for some genotypes in combination with high fish intake, no statistically significant interactions between any SNP studied and fish consumption were observed. We have previously described an association between colorectal adenomas and SNP *V102V* in *PTGS2* and have now confirmed this association for colorectal adenocarcinomas. Fish consumption of once a week or more might protect against colorectal cancer, but no statistically significant interactions with SNPs in AA-pathway genes could be detected within the study.

Introduction
Colorectal cancer is one of the leading causes of death through cancer in the developed world, with over 9500 new patients in The Netherlands in the year 2002 alone (1). Epidemiological and experimental evidence indicates that both genetic and environmental factors are involved. Lipid metabolism, in particular the arachidonic acid (AA) pathway, appears to play a critical role in colorectal tumor development, as reviewed by Jones *et al.* (2).

The evidence implicating several major genes within this pathway in colon tumorigenesis has been discussed previously (3), but is recapitulated in short. Cyclooxygenase-1 and cyclooxygenase–2, also known as prostaglandin endoperoxide synthases (*PTGSs*), are two key genes in the AA pathway, encoding enzymes that initiate the synthesis of biologically important prostanoids (PGs) and eicosanoids (4). Both genes have been demonstrated to be involved in intestinal tumorigenesis and proposed mechanisms include, promoting tumor growth, cell proliferation, angiogenesis and inhibiting apoptosis. Phospholipase A_2 (PLA_2) enzymes are a family that, besides other conversions, catalyze the generation of free fatty acids, such as AA, from membrane bound phospholipids. Most important in the AA-pathway are cytosolic PLA_2 (PLA2G4A) and secretory PLA_2 (PLA2G2A), which have both been demonstrated in mouse models to be involved in tumorigenesis (5,6). Lipoxygenase (ALOX), is an enzyme for which AA is one of the substrates. Activation of the enzyme ALOX15 might inhibit carcinogenesis via the conversion of linoleic acid into 13-S-hydroxyoctadecadienoic (13-S-HODE) acid, which in turn downregulates PPARδ thereby restoring apoptosis (7). Peroxisome □roliferators-activated receptors (PPARs) play an important regulatory role in lipid metabolism and cancer, and PPARs can be activated by a variety of eicosanoids (8,9). There are three distinct types of PPARs, α, δ and γ. Both PPARδ and PPARγ have been implicated to play a role in colorectal tumorigenesis, by transcriptionally controlling pathways involved in cell proliferation, differentiation and survival (8). Moreover, recently the role of both PPARδ and PPARγ as focal points of cross-talk between prostaglandin and Wnt signaling pathways has been suggested (10,11). Most data of PPARγ suggests a tumor suppressive role, however, there is still some controversy about the increase of intestinal polyps in Apc^{Min} mice by ligands of PPARγ (12,13).

Genetic variants represented by Single Nucleotide Polymorphisms (SNPs) in genes encoding these key players of the AA-pathway may contribute to variation in susceptibility to colorectal cancer. Recently, the focus of attention has shifted from the use of single genetic variants in association studies, to using haplotypes (14). By using information about the common SNPs in a particular population, combinations of SNP alleles called haplotypes can be estimated, after which differences in frequencies between cases and controls can be evaluated (15,16). Haplotypes can also be used to select those SNPs that are representative for a certain haplotype, the so-called tagging SNPs (15).
Among environmental factors, diet appears to play a major role in the risk of developing colorectal cancer. Several dietary components have been identified as risk factors, including red meat and alcohol, whereas others have been shown to reduce cancer risk, for example fruit, vegetables and calcium (17). Additionally, some, but not all animal experimental studies and epidemiological studies have shown that fish consumption may decrease the risk of colorectal tumors (18-20). The mechanism by which fish may exert its protective effect on colorectal tumors might include modulation of the AA pathway, by increasing the n-3 polyunsaturated fatty acids (PUFAs) content in cellular membranes. The AA pathway utilizes both dietary n-3 and n-6 PUFAs to produce eicosanoids. High fish consumption might therefore cause a shift in substrates from n-6 to n-3 PUFAs, resulting in a different class of eicosanoids. A high ratio of fish fatty acids (Fas) to arachidonic acid (AA) in adipose tissue, as a marker for fatty acid intake, has been associated with a lower risk of colorectal adenomas (21). SNPs in AA pathway genes may interact with fish consumption by influencing the conversion of these PUFAs into eicosanoids.
In this association study, twenty-three SNPs in genes encoding five enzymes and two nuclear receptors have been used. The objective of this study on colorectal cancer was to investigate whether SNPs previously found to play a role in adenoma formation, might be important in the progression of adenoma to carcinoma, and to evaluate the role of fish consumption in this process (3). In addition, a previously reported association between a SNP in the 3' UTR of *PTGS2* and colorectal cancer is investigated (22).

Materials and Methods

Study population

We conducted a nested case-control study using two Dutch studies. The first prospective study called Monitoring Project on Cardiovascular Disease Risk Factors (PPHV), conducted in three Dutch towns between 1987 and 1991 included over 36000 participants. A detailed description of the study was published previously (23). Follow-up for incident cancer for the period 1987 to mid 2003 was achieved via computerized record linkage with the Netherlands Cancer Registry and with the three regional cancer registries. In total 209 colorectal cancer cases (46 prevalent and 163 incident) could be identified. A random sample of controls was drawn from the same cohort, frequency matched on age (5 year intervals), gender and center, to include 418 subjects.
The second so-called 'DOM' (Diagnostisch Onderzoek Mammacarcinoom) project was conducted between 1976 and 1978, where all women born between 1911 and 1925 living in the city of Utrecht were invited to participate in a population-based screening program for the early detection of breast cancer (24). Each participant provided an overnight urine sample which was stored at -20°C. Follow-up for incident cancer from 1976 through linkage to the regional cancer registry resulted in a total of 355 colorectal cancer cases. A random selection of controls was drawn frequency matched on age at intake (5 year intervals) and all were of postmenopausal status, to include 426 subjects.

Fish consumption and other lifestyle factors

For the PPHV cohort, information about fish consumption and other lifestyle factors was obtained using a self-administered questionnaire. Dietary habits were estimated using a

validated semi-quantitative food-frequency method (25). Frequency of fish consumption was assessed in six categories: never, less than once a month, one to three times monthly, once per week, two to four times weekly, and more than four times a week.
The intake of total energy from this questionnaire was calculated with the computerized Dutch food composition table (26).

Genetic analysis
For participants of the PPHV cohort, genomic DNA was extracted from buffy coats by digestion with proteinase K, followed by salting out with potassium acetate and chloroform/isoamyl alcohol extraction (27). For participants of the DOM cohort, DNA was isolated from 100 ml frozen urine by alcohol precipitation as described earlier (28).
Twenty-one of the twenty-three SNPs have been selected on the basis of an inventory of the genetic variation in the Dutch population of the selected genes as described elsewhere, in which fifty-eight polymorphisms were identified (Siezen *et al*., submitted). The SNP selection was based on allele frequency (with some exceptions only those SNPs with a minor allele frequency of 5% or higher were considered), position in the gene (when possible evenly distributed across the gene), possible impact on protein function (amino acid changes), and linkage between the SNPs in one gene (of two or more tightly linked SNPs only one was selected). One SNP was selected on the basis of a population study on *PTGS1* variants (29), and one on a previous association between a SNP in *PTGS2* and colorectal cancer risk (22).
PCR and genotyping of twenty-one SNPs has been described previously (3). In short, all samples were genotyped using a technique known as Pyrosequencing™ (30), using a biotinylated single stranded PCR product. Two additional SNPs were genotyped using the restriction fragment length polymorphism (RFLP) method. SNP *c.136-37G>A* in *ALOX15* was genotyped using 2 units of HaeIII (New England Biolabs) and buffer provided, per 10μl PCR product. SNP *c.3618A>G* in *PTGS2* was genotyped using 1 unit of Alu and buffer provided, per 10μl PCR product. Both mixtures were incubated at 37°C for 2 hours. Each PCR plate contained ninety-two DNA samples, one negative (TE) control, and three positive controls, one for each genotype if available.
Primers for twenty-one SNP are described previously (3) and primers for two additional SNPs are in table 1 in the appendix. All SNPs were genotyped in the PPHV cohort from which DNA was available for 603 participants (204 cases and 399 controls). Based on i) these PPHV results, on ii) previously found associations (3), and iii) limited available DNA from the DOM cohort, the SNPs in *PPARγ* and *PTGS2* were selected for additional genotyping in the DOM cohort. Due to insufficient DNA or failed genotyping, which is not unusual for DNA extracted from urine (28), genotypes were determined for 677 participants from de DOM cohort (304 cases and 373 controls).

Data analysis
Analyses were performed on all genotyped participants. Logistic regression analysis was performed to calculate odds ratios (Ors) and 95% confidence intervals (95%CI) of separate genotypes when possible. If the numbers were too small, analyses were performed using pooled heterozygote and homozygote minor genotypes. The matching variables age, gender and cohort or centre were considered as potential confounding factors. Only the covariate cohort changed the β-estimate by more than 10%, therefore for the genotype analysis with pooled cohorts, this was included into the model as a covariate. All other factors were assumed to be unrelated to genotype.
Haplotypes were estimated and Ors calculated using the Hplus program, available online at http://qge.fhcrc.org/hplus. Hplus is a SNP analysis tool for performing haplotype estimations, according to the distribution of unphased genotypes in a population. It is able to handle datasets that include case-control status as well as covariates and SNP location variables (31).

Multiple logistic regression analysis was performed to evaluate the modifying effect of fish consumption in the PPHV cohort. Fish consumption, in 6 categories, was divided in low fish consumption (less than once a week) and high fish consumption (once a week or more frequent). The low fish consumption group in combination with a homozygote major allele for the SNP of interest was considered as reference group. Analysis was performed on incident cases only, since prevalent cases could have changed their eating habits after first diagnosis. This resulted in 161 cases for analysis.
The variables age, gender, smoking, aspirin use (ever/never), physical activity at work, physical activity in leisure time, education (high/low), insulin use (yes/no), fruit, vegetables, meat (all quartiles), total energy intake, alcohol, calcium, β-carotene, vitamin C, vitamin E, fiber, total cholesterol, HDL cholesterol and BMI (all continuous) were considered as potential confounding factors. No variables were included into the model since none of these factors changed the β-estimates by more than 10%.
To test whether the combination of genotypes and fish consumption deviated from multiplicativity, we calculated p-values for interaction in an exploratory way by inclusion of a numerical term for genotype, multiplied by fish consumption in 2 categories, low or high fish consumption, into our multivariate models.
The analyses were conducted using Statistical Analysis Software (SAS) for Windows, version 8.
Power calculations were conducted using an online software program provided by the UCLA department of statistics (32).

Results

Table 1 shows the characteristics of both study populations. All genotypes of the SNPs studied were in Hardy-Weinberg equilibrium, in each cohort separately as well as in the combined set.

Table 1: Characteristics of study population

	PPHV*		DOM†	
	Cases (n=204)	Controls (n=399)	Cases (n=304)	Controls (n=373)
Demographic				
Female (%)	44.1	44.9	100	100
Age at baseline, mean (SD)	51.2 (7.53)	52.6 (6.81)	57.3 (4.13)	56.2 (4.14)‡
Height, cm, mean (SD)	171.5 (9.54)	170.2 (9.30)	162.7 (5.89)	162.1 (10.5)
Weight, kg, mean (SD)	77.5 (12.4)	75.0 (12.5)	69.9 (10.7)	68.5 (10.4)
BMI, kg/m^2, mean (SD)	25.9 (3.68)	25.5 (3.61)	26.5 (3.92)	25.9 (3.53)
Total cholesterol, mmol/l (SD)	5.97 (1.23)	6.03 (1.12)		
HDL cholesterol, mmol/l (SD)	1.22 (0.35)	1.24 (0.33)		
Total/HDL cholesterol ratio (SD)	5.28 (1.87)	5.24 (1.76)		
Lifestyle				
Diet, mean (SD)				
Fish, frequency per month	2.49 (2.96)	2.89 (3.12)		
Meat, frequency per day	0.67 (0.29)	0.69 (0.34)		
Fruit, frequency per day	0.96 (0.66)	1.07 (0.66)		
Vegetables, frequency per day	1.12 (0.62)	1.13 (0.57)		
Total energy, kJ/day	6999 (1877)	6918 (2135)		
Alcohol, g/day	10.1 (13.1)	10.4 (13.8)		
Calcium, mg/day	969 (387)	957 (405)		
β-caroteen, mg/day	1.10 (0.56)	1.11 (0.60)		
Vitamin C, mg/day	55.9 (27.7)	57.4 (25.4)		
Vitamin E, mg/day	12.2 (5.22)	11.9 (5.18)		
Fiber, mg/day	17.2 (5.58)	16.4 (5.67)		
Other lifestyle, %				
Smoking, ever	69.3	71.2	26.8	26.4
Education, low	56.9	53.8		
Regular physical activity leisure time	63.4	71.4‡		
Regular physical activity work	23.7	32.7‡		
Use of insulin	2.45	1.00		
Use of aspirin	33.3	26.8		

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† Diagnostisch Onderzoek Mammacarcinoom

‡ p≤0.05 Wilcoxon rank test (continuous variables) or Fisher's exact test (categorical variables)

Tables 2 and 3 show the Ors for each genotype, or pooled genotypes and colorectal cancer. Subjects with the CC genotype of SNP *V102V* in *PTGS2* have a 63% reduction in colorectal cancer risk as compared to those with the GG genotype (OR, 0.37; 95%CI, 0.16-0.87). None of the other risk estimates were statistically significant, although the pooled minor genotypes of two SNPs in *PLA2G4A* (*c.1336+3G>A* and *c.2605G>A*) showed Ors of over 1.50. Analysis with only incident cases did not change the results.

Table 2: PPARγ and *PTGS2* variants and colorectal cancer in the PPHV and DOM cohorts

	PPHV cohort		DOM cohort			
Genotype (rs number) (33)	Cases (n=304)	Controls (n=373)	Cases (n=304)	Controls (n=373)	OR (95%CI) *	Power
PPARγ						
P12A (rs1801282)						
CC	160	325	387	596	1.00 (ref)	
CG	40	71	92	146	0.96 (0.72-1.28)	0.05
GG	1	2	8	8	1.34 (0.50-3.62)	0.08
H477H (rs3856806)						
CC	155	307	380	555	1.00 (ref)	
CT	42	79	92	162	0.82 (0.61-1.10)	0.28
TT	4	4	7	9	1.14 (0.42-3.11)	0.04
PTGS2						
c.-1329A>G (rs689466)						
AA	127	243	283	422	1.00 (ref)	
AG	59	128	132	226	0.87 (0.67-1.13)	0.20
GG	10	20	19	41	0.68 (0.39-1.20)	0.31
V102V (rs5277)						
GG	142	287	339	521	1.00 (ref)	
GC	56	100	133	195	1.04 (0.80-1.35)	0.05
CC	5	11	7	28	0.37 (0.16-0.87)	0.78
c.2242T>C (rs5275)						
TT	97	190	216	339	1.00 (ref)	
TC	83	163	171	281	0.97 (0.75-1.25)	0.05
CC	20	35	55	73	1.14 (0.77-1.69)	0.11
c.3618A>G (rs4648298)						
AA	194	368	461	699	1.00 (ref)	
AG+GG	5	21	13	36	0.56 (0.29-1.06)	0.49

49. Univariate adjustment for cohort, analysis of the two cohorts added up

Table 3: *PPARδ*, *PTGS1*, *PLA2G4A*, *PLA2G2A* and *ALOX15* variants and colorectal cancer in the PPHV cohort

Genotype (rs number)	Cases (n=204)	Controls (n=399)	OR (95%CI)	Power
PPARδ				
c.-789C>T				
CC	179	338	1.00 (ref)	
CT+TT	22	39	1.07 (0.62-1.86)	0.04
N163N (rs2076167)				
TT	123	250	1.00 (ref)	
TC	68	122	1.14 (0.79-1.65)	0.11
CC	8	24	0.68 (0.30-1.57)	0.16
c.2021T>C (rs3734254)				
TT	129	261	1.00 (ref)	
TC	65	114	1.16 (0.80-1.69)	0.12
CC	10	20	0.89 (0.41-1.92)	0.05
c.2589G>A (rs1053046)				
GG	178	347	1.00 (ref)	
GA+AA	22	50	0.86 (0.51-1.47)	0.08
c.2806C>G (rs9794)				
CC	145	279	1.00 (ref)	
CG+GG	49	91	1.04 (0.70-1.56)	0.04
PTGS1				
W8R (rs1236913)				
CC	169	335	1.00 (ref)	
CT+TT	33	55	1.18 (0.74-1.88)	0.10
L237M (rs5789)				
CC	183	348	1.00 (ref)	
CA+AA	8	31	0.49 (0.22-1.10)	0.49
PLA2G4A				
c.918+23C>T (rs2307200)				
CC	137	279	1.00 (ref)	
CT	57	99	1.15 (0.78-1.70)	0.11
TT	9	16	1.15 (0.49-2.66)	0.05
c.1336+3G>A (rs6661772)				
GG	178	364	1.00 (ref)	
GA+AA	25	34	1.51 (0.88-2.61)	0.32
R651K (rs2307198)				
AA	196	378	1.00 (ref)	
AG+GG	8	20	0.78 (0.34-1.79)	0.09
c.2605G>A (rs12720707)				
GG	163	347	1.00 (ref)	
GA+AA	38	50	1.58 (0.99-2.51)	0.49
PLA2G2A				
c.-180C>G (rs11573156)				
CC	128	239	1.00 (ref)	
CG	55	133	0.76 (0.52-1.11)	0.31
GG	18	18	1.87 (0.94-3.71)	0.45
T32T (rs2236771)				
GG	167	330	1.00 (ref)	
GC+CC	36	68	1.05 (0.68-1.64)	0.04
c.665C>T (rs11677)				
CC	164	314	1.00 (ref)	
CT+TT	39	84	0.89 (0.59-1.37)	0.08
ALOX15				
c.-217G>C (rs2664592)				
GG	126	257	1.00 (ref)	
GC	64	112	1.18 (0.81-1.71)	0.14
CC	9	14	1.32 (0.56-3.14)	0.10
c.136-37G>A (rs11568141)				
GG	157	319	1.00 (ref)	
GA+AA	45	77	1.20 (0.79-1.81)	0.13
T485T (rs743646)				
AA	146	300	1.00 (ref)	
AG+GG	45	78	1.19 (0.79-1.81)	0.13

The Ors for all major haplotypes and colorectal cancer risk are shown in tables 4 and 5. The haplotype representing the minor alleles of *c.1336+3G>A* and *c.2605G>A* in *PLA2G4A* (haplotype 1101, also containing the minor allele of *c.918+23C>T* and the major allele of *R651K*), showed an OR of over 1.50, in line with the pooled genotypes of the separate SNPs (OR, 1.54; 95%CI, 0.87-2.71). No statistically significant Ors for any of the haplotypes were observed.

Table 4: Haplotypes of *PPARγ* and *PTGS2* and colorectal cancer in the PPHV and DOM cohort

Haplotype*	Frequency cases	Frequency controls	OR (95%CI) †	Power
PPARγ				
00	0.849	0.847	1.00 (ref)	
11	0.071	0.079	0.88 (0.64-1.21)	0.13
01	0.038	0.044	0.89 (0.57-1.38)	0.08
10	0.041	0.030	1.33 (0.85-2.08)	0.25
PTGS2				
0000	0.333	0.308	1.00 (ref)	
0010	0.297	0.276	0.97 (0.79-1.21)	0.05
1000	0.195	0.222	0.81 (0.63-1.03)	0.55
0100	0.153	0.163	0.85 (0.67-1.08)	0.30
0011	0.014	0.024	0.51 (0.23-1.13)	0.51
1010	0.005	0.004	0.96 (0.17-5.53)	0.03

* 0 represents major allele, 1 represents minor allele. SNP order according to table 2
† Adjusted for cohort

Table 5: Haplotypes of *PPARδ*, *PTGS1*, *PLA2G4A*, *PLA2G2A* and *ALOX15* and colorectal cancer risk in the PPHV cohort

Haplotype*	Frequency cases	Frequency controls	OR (95%CI)	Power
PPARδ				
00000	0.776	0.777	1.00 (ref)	
01101	0.137	0.131	1.05 (0.75-1.48)	0.05
11110	0.040	0.041	0.97 (0.53-1.77)	0.03
01110	0.023	0.027	0.84 (0.38-1.85)	0.07
11000	0.010	0.014	0.72 (0.23-2.30)	0.07
PLA2G4A				
0000	0.791	0.798	1.00 (ref)	
1000	0.091	0.102	0.92 (0.62-1.37)	0.06
1101	0.059	0.039	1.54 (0.87-2.71)	0.34
0010	0.014	0.026	0.79 (0.36-1.73)	0.09
1001	0.025	0.020	1.37 (0.61-3.09)	0.12
0001	0.011	0.009	1.22 (0.38-3.93)	0.05
PLA2G2A				
000	0.639	0.647	1.00 (ref)	
100	0.224	0.213	1.06 (0.78-1.43)	0.06
011	0.064	0.065	1.00 (0.61-1.64)	0.03
001	0.042	0.045	0.91 (0.48-1.74)	0.05
010	0.031	0.025	1.24 (0.60-2.56)	0.08
ALOX15				
000	0.684	0.690	1.00 (ref)	
100	0.115	0.101	1.14 (0.03-8.77)	0.10
010	0.076	0.094	0.85 (0.03-28.7)	0.11
101	0.060	0.078	0.78 (0.02-33.6)	0.18
001	0.029	0.033	0.89 (0.00-52.4)	0.05
111	0.035	0.005	8.29 (0.00-112.8)	0.99
PTGS1				
00	0.894	0.879	1.00 (ref)	
10	0.085	0.079	1.05 (0.69-1.58)	0.04
01	0.021	0.042	0.50 (0.23-1.07)	0.50

* 0 represents major allele, 1 represents minor allele. SNP order according to table 3

There was an inverse association between fish consumption of once a week or more and colorectal cancer, as compared to less than once a week, although not statistically significant (OR, 0.83; 95%CI, 0.57-1.20). The distribution of fish consumption in six categories among incident cases and controls is shown in table 6.

Table 6: Distribution of fish consumption among incident cases and controls

Fish consumption category	Cases (n=160)*	Controls (n=397)*
Never	13	44
< once a month	41	82
1 to 3 times monthly	46	104
Once a week	54	144
2-4 times weekly	6	19
> 4 times weekly	0	4

*Three cases and two controls had missing data on fish consumption

In an exploratory analysis of the interaction between genotypes and fish, divided into low and high consumption, no statistically significant interactions were observed. The homozygous minor genotypes of two SNPs in *PPARδ* (*N163N* and *c.2021T>C*) however, showed a decrease of 80% in colorectal cancer risk for those with high fish intake only, as compared to the major genotypes and low fish intake, although not statistically significant. A similar reduction in risk was observed for the pooled CA+AA genotypes of *L237M* in *PTGS1* and high fish consumption, as compared to the CC genotype and low fish consumption (OR, 0.19; 95%CI, 0.03-1.45). Nearly 75% risk reduction was seen for the AG+GG genotypes of *c.3618A>G* in *PTGS2* only for those with high fish intake, as compared to the AA genotype and low fish intake (OR, 0.26; 95%CI, 0.03-2.06) (data not shown).

Discussion

A number of associations between a variety of SNPs in *PTGS2* and colorectal tumors has been reported (3,22,34-36). Together with the results from this study, this underlines the importance of this gene and the pro-inflammatory AA-pathway in the development of both colorectal adenomas and carcinomas. We have previously shown that the heterozygote genotype of SNP *V102V* in exon 3 of *PTGS2* has a protective effect on colorectal adenomas (3). Even though in the present study the protective effect is only evident when two C alleles are present, this result can still be considered as an indication of the importance of this SNP in colorectal tumor risk. Just one C allele appears to be sufficient for a reduction in risk of colorectal adenomas of 35%, whereas for the later stages of tumor development two alleles are needed to confer a risk reduction of 63%. These two inverse associations make it likely that there is either some functional effect of this SNP, for example on splicing, or that this SNP is in LD with another functional variant. We have so far not been able to demonstrate any functionality of this SNP either *in silico* or experimentally. Our results however, are not in line with another study on *PTGS2* variants in which this SNP in exon 3 was also tested and no association with colorectal cancer was evident (22). This study was performed in a Spanish population however who may have a slightly different genetic background compared to our Dutch cohorts, which may be one of the factors accounting for the different results. When we analyzed the haplotype representing the minor allele of this SNP (0100), a slight reduction in risk was apparent (OR, 0.85; 95%CI, 0.67-1.08). This illustrates that two copies of this allele are necessary to obtain the strong protective effect seen for the homozygous individuals. However, it must be pointed out that these haplotypes are estimated and not measured through parent data, which results in less reliable information.

Although in this study the association between *PTGS2* and colorectal cancer was only evident for a SNP in the coding region of the gene and one other study has shown an association between a coding SNP (*V511A*) and colorectal cancer (34), most other studies on associations between SNPs in *PTGS2* and disease observed an effect for SNPs in

regulatory regions of the gene, indicating that the effect of *PTGS2* on the development of colorectal tumors is due to changes in expression levels. This is in line with recent findings that the protective effect of NSAIDs might be due to inhibition of NF-κB, which is associated with down-regulation of *PTGS2* expression (37). A SNP in the 3'UTR of *PTGS2* (*c.2242T>G*) has previously been associated with colorectal adenomas (3), and lung cancer (38,39). The involvement of this SNP in colorectal cancer however, could not be demonstrated in this study. A possible explanation could be that this SNP is only important in the early stages of tumor formation but does not play a role in the development from polyps to malignant colorectal tumors. This is in line with another study in which no association was found between this 3'UTR SNP and colorectal cancer (22). In this same study, a rare SNP in the 3'UTR (*c.3618A>G*) did show a positive association between the minor allele and colorectal cancer risk. This association was not observed in this study. Our results even suggest an inverse relation between the minor allele and cancer risk (OR, 0.56; 95%CI, 0.29-1.06). However, the low minor allele frequency of this SNP (0.02 in controls) and therefore the small number of subjects with the minor genotypes might result in chance findings. Several studies have investigated the role of SNPs in the promoter of *PTGS2* in disease risk and several associations have been found (35,36,40-43), indicating direct involvement of changes in gene expression on disease etiology. Some null results however, have also been reported (22,44).

We did not find associations between any other SNP and colorectal cancer. The effects we have previously shown for genotypes and haplotypes of SNPs in *PPARγ*, *PLA2G2A* and *ALOX15* on adenomas (3) could not be demonstrated in this study on adenocarcinomas. This could be due to the difference in study populations, adenoma versus colorectal cancer patients, indicating that the SNPs and these genes may only affect the first stages of tumor development. Since approximately 5% of adenomas develop further into malignant tumors, the association found for adenoma risk might not be evident when the endpoint is adenocarcinomas. This is in line with an inverse association found between SNP *P12A* in *PPARγ* and colorectal adenomas (45). However, the association was stronger for more advanced adenomas, and moreover, an inverse association was observed in a colorectal cancer population for the minor allele of this SNP (46), although this is not confirmed by our data. No clear data is available about the exact stage of tumor development in which *PLA2G2A* and *ALOX15* play a role, but our data suggests that they may be most important in the early stages.

We hypothesized that increased fish consumption, as a proxy of n-3 fatty acid intake, can influence the risk of colorectal cancer by modulation of the AA-pathway. We found an inverse association, although not statistically significant, between fish consumption and colorectal cancer (OR, 0.83; 95%CI, 0.57-1.20) for subjects with fish consumption of once a week or more frequent as compared to less than once a week. This is in line with a strong protective effect of increased fish consumption shown recently in a large European study (20). This association between fish consumption and colorectal cancer might be modified by polymorphisms in genes involved in the conversion of free fatty acids into prostaglandins. We have previously demonstrated the association between SNPs in *PPARδ* and *PTGS2* and fish consumption in relation to colorectal adenomas (3). In this study however (using only the PPHV cohort), we have not been able to show any statistically significant interactions with any SNP and fish consumption, even though some substantial reductions in cancer risk were observed. This might be the cause of insufficient statistical power due to the relatively small population size. There are also some other possible explanations for the lack of significant interactions. First, the consumption of fish might be too low in our population to be able to measure an effect. We considered fish consumption of once a week or more as high fish intake. A large study on the effect of fish consumption on prostate cancer detected an inverse association for four or more servings of fish a week, which was statically significant (47). Second, the associations were calculated for total fish intake, because no distinction was made between the different types of fish in the food frequency

questionnaire. If n-3 fatty acids are considered the bioactive agents in fish, it is likely that fish high in n-3 fatty acids, for example salmon, mackerel and herring, are more strongly associated with cancer than lean fish like cod and haddock. Also, other sources of n-3 fatty acids that have not been considered in the analysis since no data was available, can influence the results. It has also been suggested that a low ratio n-6/n-3 fatty acids is more important than the total amount of n-3 fatty acids (21,48), confirmation of which is awaiting ongoing FA analysis.

Our PPHV cohort included both incident and prevalent cases. We repeated the analysis for genotypes with only incident cases, but this did not change our results. However, prevalent cases might have changed their dietary habits after the first time they developed cancer, therefore these cases where excluded in the gene-diet interaction analysis.

Consideration must be given to some potential limitations of the study. First, the use of self-administered questionnaires can give rise to uncertainties, especially since the answer categories were limited to six options. The dietary intake data is therefore less accurate than when a more open questionnaire is used. Measurements of specific fatty acid content in plasma samples might be more accurate, and is currently ongoing. Second, for the gene-diet interactions several potential confounders were tested. None of the variables tested effected the association, but it cannot be ruled that other factors for which no data was collected could cause confounding. Third, the lack of interaction between the SNPs tested and fish consumption does not rule out any interaction between these genes and fish consumption. It is possible there are other SNPs present in the genes, that have low minor allele frequencies and have therefore not been tested in this study, that modify the association. Fourth, we were not able to investigate gene-gene interactions due to small sample size, therefore associations between a combination of SNPs within two genes and colorectal cancer would not have been detected by our study if the effect of the single SNPs were negligible. Fifth, due to the small sample size, which is especially the case for the fish consumption data, there is a substantial lack of power to detect interactions. This is also the case for some main effects when allele or haplotype frequencies are low. And last, chance findings can never be excluded, especially when multiple testing is considered. However, we opted not to correct for multiple testing, since at this stage and with this sample size Bonferroni corrections were considered punitively conservative (49).

In conclusion, we have confirmed a previously found inverse association between SNP *V102V* in *PTGS2* and colorectal adenomas, in this study on colorectal cancer. Although the association between increased fish consumption and colorectal cancer was inverse, there were no statistically significant interactions between the SNPs investigated and fish consumption.

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Appendix

Table 1: Primer sequences of two additional SNPs

Gene/SNP	rs number	Strand	Sequence (5'-3')
ALOX15 c.136-37G>A	rs11568141	Forward	cccgatacgtctcctcctct
		Reverse	tccggtacttccaccttgag
PTGS2 c.3618A>G	rs4648298	Forward	ttcttttccacatctcattgtca
		Reverse	tcagagaggtaaccccaaagaa

Chapter 5

Serum n-3 and n-6 fatty acid levels, SNPs in fatty acid metabolizing genes and the risk of colorectal adenomas

Submitted

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Abstract

Dietary polyunsaturated fatty acids (PUFAs) may influence the etiology of colorectal tumors. It is believed that n-3 PUFAs, derived mainly from fish, are anti-carcinogenic and are associated with a decreased risk of colorectal cancer, whereas n-6 PUFAs are not. Single nucleotide polymorphisms (SNPs) in genes involved in fatty acid metabolism, including cyclooxygenases (*COX*) and peroxisome proliferator-activated receptors (*PPAR*) may modify these associations. Therefore we evaluated fatty acid-SNP associations in colorectal adenoma risk in a case-control study with 344 cases and 397 endoscopy controls.

Linoleic acid (LA) as well as total n-6 PUFAs was positively associated with colorectal adenomas. The odds ratio (OR) of the third tertile (T3) was 1.69 (95% confidence interval (CI), 1.09-2.61) for total n-6 PUFAs and 1.78 (95%CI, 1.15-2.76) for LA as compared to the first tertile (T1), with a statistically significant trend (0.02 and 0.008 respectively). Total n-3 PUFAs were inversely, although not statistically significantly associated with colorectal adenomas, (OR and 95% CI T3 versus T1: 0.71; 0.46-1.10). Results for EPA and DHA separately were similar. The ratio of total n-6/n-3 PUFAs was not associated with colorectal adenoma risk. None of the SNPs tested modified these associations.

In conclusion, our results suggest that increased serum LA levels increase colorectal adenoma risk. Increased EPA, DHA and total n-3 PUFA content may decrease adenoma risk. Interactions of other pathways with the arachidonic acid pathway may explain these observations.

Introduction

Colorectal cancer is the second leading cause of death due to cancer in the Western world (1). Differences in geographic incidence rates suggest that environmental influences play an important role. Besides a high consumption of red or processed meat, saturated fat was previously hypothesized to increase colorectal cancer risk (2,3). More recently, it was suggested that the dietary intake of polyunsaturated fatty acids (PUFAs) may be more important (4). Long chain PUFAs, in particular n-3 PUFAs, can inhibit carcinogenesis and reduce the risk of colorectal cancer (5). N-3 PUFAs are obtained mainly through the intake of fish in the form of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), but they are also present in some vegetable oils including flaxseed and soybean oil mainly as α-linolenic acid (ALA). N-6 PUFAs are present in eggs and meat, predominantly in the form of linoleic acid (LA), although they also contain arachidonic acid (AA) (6,7).

Dietary PUFAs are converted by elongases and desaturases in a pathway known as the arachidonic acid (AA) pathway, into eicosanoid precursors, the n-6 AA and the n-3 EPA. These are in turn converted into prostanoids (PG) and leukotriens (LT) utilizing cyclooxygenase (COX) and lipoxygenase (LOX) enzymes. The n-6 PUFA derived eicosanoids are in general pro-inflammatory and are associated with tumor progression, whereas the n-3 derived eicosanoids are thought to contain anti-inflammatory properties (8). There are several proposed mechanisms by which n-3 PUFAs can exert their protective effect on tumor formation, as reviewed by Larsson *et al*. (5). One of the major mechanisms leads to the suppression of n-6 PUFA derived eicosanoids. Higher intake of n-3 PUFAs compared to the n-6 variety would mean a decrease in available AA for eicosanoid production through the incorporation of the n-3 PUFAs into membrane phospholipids (9). This effect is further enhanced by competition between n-3 and n-6 PUFAs for the elongases and desaturases that convert these PUFAs, since n-3 PUFAs have a higher affinity for these enzymes (8). N-3 PUFAs can also directly inhibit COX2 (also known as prostaglandin-endoperoxide synthase-2 PTGS2), one of the key enzymes in the AA pathway (10,11) and compete with n-6 PUFAs for COX2 to form prostanoids (12).

We have previously shown that, although fish consumption did not significantly decrease colorectal adenoma risk, it did interact with a number of SNPs in genes involved in the AA-

pathway, thereby modulating the risk (13). We therefore used the previously genotyped twenty-one SNPs in seven genes involved in the AA pathway in a case-control study using patients with colorectal adenomas (13), and considered the modifying effect of these genotypes on the association between serum cholesterylester n-3 fatty acid composition as a proxy for fish consumption as well as serum cholesteryl n-6 fatty acids and colorectal adenomas.

Materials and Methods

Study population

A retrospective case-control study was conducted in the Netherlands, between 1997 and 2001. The study design has been previously described (14,15). In short, this case-control study was designed to examine the association between various dietary and other lifestyle factors, genetic susceptibility and colorectal adenomas. All participants underwent endoscopy in one of ten participating hospitals because of routine screening for colorectal adenomas or gastrointestinal complaints. Cases had one or more adenomas at index endoscopy or in their past. Controls had no adenomas at index endoscopy, nor ever in their past.

The total study population included 1,477 subjects (16). The study was approved by the Medical Ethical Committees of all participating hospitals and all participants provided written informed consent.

Data collection

Information about dietary habits was obtained using a validated food-frequency questionnaire (17,18). Participants were requested to recall their dietary and lifestyle habits of the year previous to their last endoscopy. Information on demographic and lifestyle factors, like smoking habits, physical activity level (19) and family history, was obtained from a self-administered questionnaire.

The intake of total energy and of various nutrients from this questionnaire was calculated with a computerized Dutch food composition table.

Serumcholesteryl fatty acid analysis

Non-fasting venous blood samples were obtained for measurement of serum cholesteryl esters. Serum was obtained by low-speed centrifugation for 10 minutes. Serum samples were stored within 2 h at -80°C until analysis.

Serum cholesterylester fatty acids were analyzed largely as described previously (20). Three ml of isopropanol and 1.0 ml of distilled water were added to 0.65 ml serum. After mixing, 3 ml of n-octane was added. After centrifugation for 5 min at 1580*g* the (upper) octane layer was removed and evaporated to dryness in a stream of nitrogen. The residue was redissolved in hexane/diethyl ether (39:1 v/v%) and cholesterylesters were selectively eluted from a 500 mg silicacolumn using hexane/diethylether (39/1 v/v%) as eluens. The cholesteryl esters were redissolved in 0.5 ml chloroform and methylated with 4 ml 4% H_2SO_4 in methanol for 1h at 90°C. One ml distilled water and 0.5 ml hexane, containing 0.25 mg butylated hydroxytoluene per ml, were added and the mixture was shaken for 2 min. After centrifugation for 3 min at 1580*g* the hexane layer was removed for analysis. The fatty acid methylesters were separated by gas chromatography (Hewlett Packard 5890), using a 25 m WCOT fused silica column (inside diameter 0.25 mm) coated with 0.2 µm CP Wax 58 (Chrompack, Middelburg, The Netherlands), using splitless injection. The injection volume was 0.5 µl. Hydrogen was used as carrier gas at a flow of 1.5 ml/min. The oven temperature was programmed to rise first from 60 °C to 180°C at a rate of 30°C/min, followed by a rise at a rate of 1.2°C/min to 215°C, thereafter at a rate of 40°C/min to 245°C. Total run time was 45 minutes. The temperature of the injector was 275°C and that of the hydrogen flame ionisation detector was 265°C. Peak areas were measured with GC Chemstation software

(Rev. A.09.03, Agilent Technologies, Palo Alto, CA, USA). Fatty acids were identified by comparison with known standards (Chrompack, Middelburg, the Netherlands). Ten % of all samples were analyzed in duplicate. To monitor analytical performance over time, a serum control sample was analyzed in duplicate in each run. Within-run and between-run coefficients of variation were respectively 2.4% and 2.4% for LA, 6.2% and <1% for ALA, 3.0% and 2.2% for AA, 3.8% and 3.7% for EPA, and 5.1% and 2.9% for DHA.

Genetic analysis

SNP selection and genotyping of twenty-one SNPs in *PLA2G2A*, *PLA2G4A*, *PTGS1*, *PTGS2*, *ALOX15*, *PPARδ* and *PPARγ* have been described previously (13). In short, genotyping of the SNPs was carried out using a technique known as Pyrosequencing™ (21) with a biotinylated single stranded PCR product. Primers for each SNP are described previously (13) Sequence primers for the pyrosequence reaction were designed using software available online (21). SNPs were determined using DNA from 808 randomly selected participants from the case-control study. Genotyping failed in one of these DNA samples.

Statistical analysis

Serum of 66 participants was not available for fatty acid analysis, therefore data analysis included 741 participants, 344 cases and 397 controls.

From all fatty acids analyzed, the median of the percentage in cholesterol esters and interquartile range was calculated for cases and controls separately. Differences between cases and controls were determined with a T-test.

Odds ratios (Ors) for the presence of colorectal adenomas and 95% confidence intervals (95% Cis) were estimated for total n-6 PUFAs, total n-3 PUFAs, n-6/n-3 ratio, LA, ALA, AA, EPA and DHA by using the lowest tertile of the fatty acid in question as a reference group. For this the values were divided into tertiles according to the distribution among controls.

The variables age, BMI, family history of colorectal cancer, gender, indication of endoscopy, insulin use, physical activity, education level, smoking, NSAID use, daily energy intake and intake of alcohol, calcium, fiber, fruit, red meat, poultry, processed meat, vegetables, β-carotene, vitamin C and vitamin E were considered as potential confounding factors. Besides the variables age and gender, also indication for endoscopy and alcohol consumption were included in the model, since only these factors changed the β-estimates by more than 10%.

To test for linear trend, we modeled the tertile of fatty acid content as a continuous variable in the logistic regression model, in which each tertile was assigned its median value.

Risk estimates stratified for genotypes, were calculated with separate genotypes where possible, with the lowest tertile in combination with the homozygote major allele of the SNP of interest considered as reference group. If the numbers were insufficient heterozygote and homozygote minor genotypes were pooled. To test whether the combination of genotypes and fatty acid content deviated from multiplicativity, we calculated p-values for interaction by inclusion of a numerical term for genotype, multiplied by total or specific fatty acid content as a continuous variable into our multivariate models.

The analyses were conducted using Statistical Analysis Software (SAS) for Windows, version 8.2 (SAS institute, Cary, NC).

Results

Characteristics of cases and controls are shown in table 1. Cases and controls were similar with respect to family history of colorectal cancer, total energy intake and fish consumption. The variables age and gender differed among cases and controls. The median age for cases was 59.1 and for controls 50.9, and 53.2% of cases was female versus 37.6% of controls. With regard to the fatty acid analysis, significant differences between cases and controls were seen for the rare fatty acids C16:2n-4, C18:3n-6 (γ-linolenic acid) and C22:5n-3 (docosapentaenoic acid). A more detailed description of the study population characteristics was published previously (14,15).

Table 1: Characteristics of study population

Characteristic	Common name	Cases (n=344)	Controls (n=397)
Female (%)		46.8[a]	62.4
Age (mean ± SD)		59.1[a] ± 10.5	50.9 ± 14.0
Family history of CRC[b] (%)		23.8	18.6
Dietary intake (mean ± SD)			
Energy (KJ/day)		8779 ± 2431	8681 ± 2600
Fish (g/day)		10.82 ± 9.90	11.31 ± 11.55
PUFAs[c] (median ± SD)			
n-3 PUFAs		1.98 ± 0.74	1.94 ± 0.74
C18:3n-3	α-Linolenic acid	0.52 ± 0.18	0.51 ± 0.18
C20:5n-3	Eicosapentaenoic acid	0.85 ± 0.54	0.81 ± 0.55
C22:5n-3	Docosapentaenoic acid	0.00 ± 0.03[d]	0.00 ± 0.03
C22:6n-3	Docosahexaenoic acid	0.57 ± 0.19	0.56 ± 0.20
n-6 PUFAs		62.28 ± 4.62	61.85 ± 4.31
C18:2n-6	Linoleic acid	53.13 ± 5.17	52.92 ± 4.99
C18:3n-6	γ-Linolenic acid	0.98 ± 0.37[d]	0.92 ± 0.36
C20:3n-6	Dihomo-γ-Linolenic acid	0.77 ± 0.17	0.79 ± 0.18
C20:4n-6	Arachidonic acid	6.99 ± 1.53	7.04 ± 1.61
n-6/n-3 ratio		31.28 ± 11.72	31.48 ± 12.35
Other Fas[c] (median ± SD)			
C15		0.17 ± 0.05	0.16 ± 0.05
C16	Palmitic acid	11.09 ± 0.87	11.04 ± 0.84
C16:1	Palmitoleic acid	3.00 ± 1.43	2.98 ± 1.51
C16:2n-4		0.00 ± 0.03[d]	0.00 ± 0.04
C16:3n-4		0.26 ± 0.15	0.25 ± 0.16
C17	Margaric acid	0.10 ± 0.05	0.10 ± 0.06
C18	Stearic acid	0.81 ± 0.17	0.79 ± 0.18
C18:1n-9	Oleic acid	16.53 ± 2.29	16.84 ± 2.17
C18:1n-7	Vaccenic acid	1.00 ± 0.25	1.03 ± 0.25
C20:1n-9	Gadoleic acid	0.15 ± 0.06	0.16 ± 0.06

[a] p<0.001, chi square for gender, t-test for age
[b] Colorectal cancer
[c] % of total cholesterol esters
[d] p<0.05 t-test

The association between total and specific fatty acid composition and colorectal adenomas is shown in table 2. There was an increase in adenoma risk with increasing serumcholesteryl LA content. The OR reached 1.78 (95%CI, 1.15-2.76) in the third tertile (T3) of LA content as compared to the first tertile (T1), with a statistically significant trend towards increased risk. In accordance with this, total n-6 PUFA content showed a positive association with adenomas, with the OR in T3 reaching 1.69 (95%CI, 1.09-2.61), and a

statistically significant trend. AA content in cholesterol ester fractions was not associated with colorectal adenoma risk.

Total n-3 PUFAs were inversely, although not statistically significantly associated with colorectal adenomas, the OR for those in T3 was 0.71 (95% CI 0.46-1.10) as compared to T1. There was no statistically significant trend towards decreased risk. Both EPA and DHA content were inversely associated with adenoma risk, although not statistically significant. There was a reduced risk of colorectal adenomas of about 25 to 30% for those in the second and third tertile of EPA content, as compared to the first tertile. The third tertile of DHA content reached 0.67 (95%CI, 0.43-1.04), as compared to T1. The association between serum n-3 PUFAs and colorectal adenomas was seen for both sexes, although it was somewhat stronger in women than in men (data not shown). ALA content was not statistically significantly associated with adenoma risk, although a moderate increase in risk with increasing ALA content was observed (OR T3 vs T1, 1.35; 95%CI, 0.88-2.07. A similar result is shown for the n-6/n-3 ratio (OR T3 vs T1, 1.36; 95%CI, 0.88-2.11). Our study population included both incident and prevalent cases. Since prevalent cases might have changed their diet after first diagnosis of cancer, we repeated the analysis with incident cases only, but this did not change the results (data not shown).

Table 2: Serum fatty acids and colorectal adenoma risk

	Fatty acid tertile						
	T1		T2		T3		
Fatty acid	Cases/ controls	OR (95%CI)[a]	Cases/ controls	OR (95%CI)[a]	Cases/ controls	OR (95%CI)[a]	p for trend
LA	111/133	1.00 (ref)	106/131	1.08 (0.68-1.70)	127/133	1.78 (1.15-2.76)	0.008
AA	114/132	1.00 (ref)	110/133	0.97 (0.63-1.50)	120/132	0.99 (0.65-1.52)	0.98
n-6 PUFAs	105/133	1.00 (ref)	108/131	1.22 (0.78-1.91)	131/133	1.69 (1.09-2.61)	0.02
ALA	98/133	1.00 (ref)	126/129	1.14 (0.73-1.76)	120/135	1.35 (0.88-2.07)	0.17
EPA	100/133	1.00 (ref)	109/132	0.73 (0.47-1.13)	135/132	0.69 (0.44-1.08)	0.12
DHA	108/133	1.00 (ref)	123/131	0.88 (0.57-1.35)	113/133	0.67 (0.43-1.04)	0.07
n-3 PUFAs	107/132	1.00 (ref)	105/132	0.62 (0.40-0.98)	132/133	0.71 (0.46-1.10)	0.16
n-6/n-3 ratio	134/132	1.00 (ref)	95/132	0.83 (0.53-1.30)	115/133	1.36 (0.88-2.11)	0.11

[a] Multivariate adjustment for age, gender, indication of endoscopy and alcohol consumption.

The association between total n-3 PUFAs, total n-6 PUFAs, and colorectal adenomas, stratified for genotype of SNPs in genes previously shown to interact with fish consumption, is shown in tables 3 and 4 respectively. There were no statistically significant interactions between any of the SNPs genotyped and any of the fatty acids analyzed.

Table 3: Serum n-3 level and colorectal adenoma risk stratified for genotypes of SNPs in *PPARδ* and *PTGS2*

	Total n-3 serum level[a]						
	T1		T2		T3		
Genotype	Cases/ controls	OR (95%CI)[b]	Cases/ controls	OR (95%CI)[b]	Cases/ controls	OR (95%CI)[b]	p for trend
PPARδ							
c.-789C>T							
CC	97/111	1.00 (ref)	93/117	0.59 (0.37-0.94)	110/118	0.68 (0.43-1.06)	0.11
CT+TT	7/10	0.81 (0.22-2.97)	8/9	0.76 (0.24-2.42)	13/13	0.71 (0.27-1.90)	0.35
p for interaction							0.97
N163N							
TT	73/77	1.00 (ref)	66/82	0.54 (0.31-0.93)	74/84	0.57 (0.33-0.98)	0.08
TC	29/48	0.53 (0.26-1.05)	35/46	0.41 (0.20-0.83)	45/45	0.54 (0.28-1.03)	0.60
CC	7/8	0.82 (0.24-2.86)	5/4	0.52 (0.09-2.96)	9/3	1.29 (0.28-6.05)	0.85
p for interaction							0.42
c.2021T>C							
TT	72/80	1.00 (ref)	71/84	0.60 (0.35-1.03)	79/89	0.61 (0.36-1.05)	0.14
TC+CC	37/52	0.66 (0.35-1.26)	35/47	0.38 (0.19-0.78)	50/43	0.59 (0.31-1.15)	0.57
p for interaction							0.44
c.2589G>A							
GG	98/122	1.00 (ref)	98/117	0.65 (0.41-1.04)	117/116	0.73 (0.46-1.15)	0.25
GA+AA	11/11	1.05 (0.35-3.11)	8/15	0.29 (0.09-0.96)	12/16	0.54 (0.21-1.43)	0.11
p for interaction							0.81
c.2806C>G							
CC	80/92	1.00 (ref)	74/95	0.58 (0.35-0.96)	85/103	0.59 (0.36-0.97)	0.08
CG+GG	27/41	0.63 (0.31-1.25)	29/34	0.46 (0.21-0.99)	41/27	0.74 (0.36-1.52)	0.85
p for interaction							0.29
PTGS2							
c.-1329A>G							
AA	67/83	1.00 (ref)	64/85	0.55 (0.32-0.97)	65/83	0.57 (0.33-0.99)	0.03
AG	33/41	1.16 (0.60-2.24)	33/39	0.78 (0.40-1.50)	50/40	1.06 (0.57-1.94)	0.92
GG	7/6	1.45 (0.36-5.87)	4/4	0.66 (0.12-3.76)	9/6	1.28 (0.35-4.69)	0.33
p for interaction							0.74
V102V							
GG	79/95	1.00 (ref)	73/79	0.77 (0.45-1.31)	95/91	0.82 (0.49-1.35)	0.28
GC	24/33	1.07 (0.52-2.19)	32/46	0.46 (0.23-0.91)	29/38	0.49 (0.24-1.00)	0.30
CC	6/5	1.92 (0.36-9.15)	1/7	0.11 (0.01-2.26)	5/3	1.20 (0.22-6.49)	0.34
p for interaction							0.64
c.2242T>C							
TT	42/59	1.00 (ref)	43/66	0.49 (0.25-0.94)	54/67	0.56 (0.29-1.05)	0.20
TC	58/58	1.02 (0.55-1.90)	53/52	0.83 (0.44-1.59)	61/50	0.92 (0.49-1.73)	0.74
CC	8/12	1.01 (0.33-3.13)	9/13	0.42 (0.12-1.52)	11/13	0.51 (0.16-1.65)	0.11
p for interaction							0.84

[a] Tertiles of total n-3 level as percentage of total cholesterol esters: 0.62-1.71, 1.71-2.14 and 2.14-7.63.
b Multivariate adjustment for age, gender, indication of endoscopy and alcohol consumption.

Table 4: Serum n-6 level and colorectal adenoma risk stratified for genotypes of SNPs in *PPARδ* and *PTGS2*

	Total n-6 serum level[a]						
	T1		T2		T3		
Genotype	Cases/ controls	OR (95%CI)[b]	Cases/ controls	OR (95%CI)[b]	Cases/ controls	OR (95%CI)[b]	p for trend
PPARδ							
c.-789C>T							
CC	94/113	1.00 (ref)	89/120	1.13 (0.71-1.80)	117/113	1.68 (1.08-2.63)	0.02
CT+TT	6/13	0.41 (0.11-1.61)	15/7	2.68 (0.98-7.37)	7/12	1.18 (0.34-4.08)	0.03
p for interaction							0.72
N163N							
TT	64/81	1.00 (ref)	64/83	1.11 (0.63-1.94)	85/79	1.66 (0.97-2.84)	0.07
TC	32/46	0.53 (0.25-1.12)	38/44	1.13 (0.58-2.18)	39/49	1.14 (0.59-2.22)	0.08
CC	8/6	1.43 (0.35-5.90)	6/4	2.21 (0.55-8.90)	7/5	1.28 (0.28-5.74)	0.95
p for interaction							0.64
c.2021T>C							
TT	66/83	1.00 (ref)	70/89	1.17 (0.68-2.01)	86/81	1.68 (0.99-2.85)	0.07
TC+CC	39/50	0.61 (0.30-1.22)	38/42	1.16 (0.60-2.24)	45/50	1.20 (0.63-2.27)	0.10
p for interaction							0.16
c.2589G>A							
GG	99/116	1.00 (ref)	95/119	1.23 (0.77-1.98)	119/120	1.59 (1.01-2.52)	0.05
GA+AA	6/17	0.32 (0.09-1.21)	13/12	1.26 (0.49-3.23)	12/13	1.32 (0.45-3.84)	0.01
p for interaction							0.68
c.2806C>G							
CC	71/98	1.00 (ref)	77/96	1.24 (0.75-2.05)	91/96	1.60 (0.97-2.62)	0.04
CG+GG	34/32	0.94 (0.45-1.97)	29/33	1.20 (0.58-2.48)	34/37	1.14 (0.57-2.30)	0.72
p for interaction							0.51
PTGS2							
c.-1329A>G							
AA	55/84	1.00 (ref)	62/86	1.44 (0.81-2.55)	79/81	2.07 (1.18-3.63)	0.01
AG	42/39	2.05 (1.07-3.94)	36/41	1.92 (0.98-3.76)	38/40	2.18 (1.11-4.31)	0.96
GG	4/5	0.96 (0.18-5.11)	7/3	3.74 (0.69-20.2)	9/8	3.11 (0.92-10.5)	0.13
p for interaction							0.47
V102V							
GG	75/89	1.00 (ref)	83/80	1.54 (0.91-2.62)	89/96	1.47 (0.87-2.47)	0.18
GC	24/39	0.61 (0.29-1.31)	24/44	0.61 (0.28-1.33)	37/34	1.73 (0.89-3.38)	0.03
CC	6/5	1.62 (0.34-7.70)	1/7	0.96 (0.17-5.42)	5/3	1.59 (0.15-17.4)	0.88
p for interaction							0.92
c.2242T>C							
TT	47/67	1.00 (ref)	38/64	0.88 (0.45-1.72)	54/61	1.75 (0.94-3.26)	0.07
TC	47/50	1.22 (0.63-2.34)	57/59	1.72 (0.94-3.17)	68/51	2.10 (1.13-3.90)	0.14
CC	9/15	0.52 (0.14-1.96)	12/6	2.90 (0.86-9.82)	7/17	0.92 (0.30-2.81)	0.29
p for interaction							0.86

[a] Tertiles of total n-6 level as percentage of total cholesterol esters: 45.9-60.1, 60.1-63.4 and 63.4-79.6.
b Multivariate adjustment for age, gender, indication of endoscopy and alcohol consumption.

Discussion

In this case-control study, LA and total n-6 PUFAs were positively associated with colorectal adenomas. Total n-3 PUFAs and EPA and DHA separately were inversely associated with colorectal adenomas, although not statistically significant. None of the SNPs tested modified these associations.

We found that individuals with an increased LA and total n-6 PUFA content, but not increased AA content, have an increased risk of colorectal adenomas. Previous findings on the relation between n-6 PUFAs such as LA and AA and colorectal carcinogenesis have been inconsistent. While our results are in line with most studies showing an increased risk of colorectal adenomas for those with a high n-6 PUFA dietary intake (22), other studies have reported no effect or even a tumor inhibiting effect of increased dietary LA (23), and n-6 PUFA levels in serum (24), and in *in vitro* studies a pro-apoptotic effect of AA has been shown (25). Our results show that increased AA content does neither reduce nor increase the risk of colorectal adenomas, but rather that its precursor LA might have a stimulating

effect on tumor formation. This suggests that additional pathways besides the AA pathway may be involved in the pro-carcinogenic effect of LA. This is in line with findings that LA intake of over 2.5% of total energy, which is below the typical average of 5-6% of energy, does not result in increased conversion to AA and PGs due to saturation of Δ-6 desaturase, the rate limiting enzyme in this pathway (26). Therefore the level of AA would not change much as a consequence of changes in LA intake. What other biochemical pathway might involve LA in tumor formation remains to be elucidated, but several other pathways have been indicated, including pathways involved in cellular proliferation, apoptosis, angiogenesis and metastasis (5).

Our results indicate that increased n-3 PUFA content, in particular EPA and DHA content decreases the risk of colorectal adenomas. As with n-6 PUFAs, studies with n-3 PUFAs have been inconsistent. Some epidemiological studies that have investigated the association between fish consumption as a proxy for n-3 PUFA intake and cancer found inverse associations (27-30), while others have not (31-33). In studies using nutrient calculations from food frequency questionnaires from which n-3 PUFA intake is deduced, the same inconsistency is observed. One case-control study showed an inverse association between n-3 PUFA intake and colorectal cancer (34), whereas two studies showed no association (35,36). Only one recent prospective study has used actual serum measurements of fatty acids to investigate associations with colorectal cancer, and found an inverse association between total n-3 PUFA, EPA and DHA content and colorectal cancer, although only in women (24). This selective effect on women was also seen in a study where n-3 PUFA intake was calculated from a food frequency questionnaire (34).
Several animal and *in vitro* studies indicate more consistently that n-3 PUFAs suppress the development of cancer, as reviewed by Roynette et.al (6). It has been shown that supplementation of the diet with fish oil decreases tumor number in animals with chemically induced colorectal tumors (37) as well as in mouse models of intestinal cancer (Apc^{Min} and $Apc^{\Delta 716}$) (38,39). N-3 PUFAs have also been shown in cancer cell lines to induce apoptosis and suppress cell growth (40,41). In human subjects, supplementation with fish oils containing n-3 PUFAs in patients with sporadic adenomatous polyps resulted in reduced intestinal hyperproliferation (42,43). These data taken together suggest a protective role of n-3 PUFAs against colorectal tumorigenesis, which is in line with our results. Our study did not show a significant effect of n-6/n-3 ratio on colorectal adenoma risk. Although a protective effect of a high ratio of n-3 fatty acids to AA in adipose tissue on colorectal adenomas has been shown previously(44), our result is in line with a large study in which fatty acid intake was calculated from a food frequency questionnaire and no protective effect of a high n-3/n-6 ratio was demonstrated (45).
It is hypothesized that n-3 and n-6 PUFAs have an effect on colorectal tumor formation via modulation of the AA pathway, by changing the substrates and products or by a direct effect on the genes involved in this pathway. We tested whether any of the fatty acids analyzed interacted with one or more of the twenty-one SNPs in genes involved in the AA pathway, and found that there were no statistically significant interactions at all (data not shown). This might point to interaction with other pathways that utilize n-3 PUFAs in addition to the AA pathway, for example activation of PPARγ (46), inhibition of NF-κB activation (47) or lowering of *ras* oncogene activation (48). However, although none of the SNPs analyzed interacted with n-3 PUFAs in modulating colorectal adenoma risk, there might be rare SNPs in the same genes with detrimental effects that could interact with the fatty acids (49). Also decreased statistical power due to stratification by genotypes might be a reason for not reaching statistical significance, although no trend was observed.
We have previously shown that SNPs in genes in the AA pathway interact with fish consumption modulating cancer risk. We found that the estimates of fish consumption based on the food frequency questionnaires were properly reflected by the n-3 fatty acid composition of analyzed sera. Although the Pearson correlation coefficients were relatively

low, they were statistically significant (data not shown). We hypothesized that n-3 PUFAs in fish were the bioactive compounds in this interaction. Our result with n-3 PUFAs however, do not confirm this and indicate that there may be other bioactive compounds in fish that interacts with AA pathway genes. A candidate for this could be vitamin D which is also abundant in fatty fish, and for which compelling epidemiological and experimental evidence is accumulating regarding its cancer protective effects (50,51). In its active form, $1,25(OH)_2D_3$, it restrains cell proliferation and induces apoptosis and cell differentiation, the latter by interfering with β-catenin signaling (52).
In conclusion, our results indicate that increased LA but not AA content might increase colorectal adenoma risk. Increased EPA, DHA and total n-3 PUFA content might decrease adenoma risk. Although n-3 PUFA content can act as a proxy for fish consumption, n-3 PUFAs do not interact with SNPs in AA pathway genes in the same way as fish consumption does, indicating the involvement of other pathways and possibly the involvement of another active agent in fish.

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Chapter 6

Tissue levels of fatty acids and risk of colorectal adenomas: a case control study

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Abstract
Epidemiological and animal studies have suggested that a high ratio of n-3 fish fatty acids to Arachidonic acid (AA), might protect against colorectal carcinogenesis. Competition of n-3 and n-6 fatty acids, especially AA, for the enzyme cyclooxygenase-2 may be responsible for this effect.
To examine the relation between fish intake and colorectal adenomas, data from a Dutch case-control study were analysed. All 52 cases and 57 controls filled out a food questionnaire, underwent a full colonic examination and have had a fat biopsy from the buttock.
Intake of fish and fish fatty acids was inversely associated with colorectal adenomas although not statistically significant. For the ratio of fish fatty acids to AA, the Ors in the second and third tertile were 1.2 and 0.8 (p-trend = 0.78).
Tissue levels of fish fatty acids were inversely associated and tissue levels of AA were positively associated with adenomas, although not statistically significant. However, the OR for the ratio of fish fatty acids to AA was 0.2 in the second and third tertile (p-trend = 0.002)).
In line with the hypothesis, a high ratio of fish fatty acids to AA in adipose tissue was associated with a lower risk of colorectal adenomas.

Introduction
Both epidemiological and experimental studies suggest that consumption of fish and fish oil correlates with a reduced risk of colorectal cancer. Ecological studies show that Alaskan and Greenland Eskimos have a lower incidence of colorectal cancer than other North Americans (1). Furthermore some coastal populations have a lower incidence of colorectal cancer compared to urban dwellers (2), and a report from 24 European countries shows an inverse relation between fish consumption and colorectal cancer risk (3). All these ecological observations might be attributed to high consumption of marine food rich in n-3 polyunsaturated fatty acids.
In case-control and cohort studies evidence that fish reduces the risk of colon cancer is less clear, probably due to the low range in fish consumption and inaccurate measurement of fish consumption (4-10). Nevertheless, a few case-control studies show protective effects of fish consumption against colorectal cancer (11-14). Based on a comprehensive review of epidemiological studies a recent panel report concluded that fish consumption possibly protects against cancer of the colon (15).
Intervention studies in humans indicate a protective effect of n-3 PUFA. For example, Anti et al. showed that supplementation with fish oil significantly reduced the mucosal cell proliferation in high-risk patients (16); a similar finding has been reported for healthy subjects (17).
Also a number of animal experiments show that fish oil consumption reduces the risk of colorectal cancer (18-22). Moreover these experiments suggest that the mechanism by which n-3 PUFA exert their protective effect might be through the cyclooxygenase 2 (COX-2) pathway. The COX-2 enzyme is involved in the formation of prostanoids from free PUFAs (23-25). It converts n-6 PUFAs such as arachidonic acid (AA) into the 2 series prostanoids and n-3 PUFAs into the 3 series prostanoids. The 2 series prostanoids promotes cell proliferation and decreases apoptosis and immune response, while the 3 series prostanoids inhibit tumorgenesis (26).
Several mechanisms have been proposed to explain how n-3 PUFAs, especially fish fatty acids (eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)), could protect against adenoma formation. One mechanism is the competition between n-3 fish fatty acids and n-6 PUFAs for COX-2 activity (27), leading to a decreased production of the 2-series prostanoids and an increase in 3-series products, inhibiting tumorgenesis (28;29). In addition to competition for COX-2, the n-3 and n-6 PUFAs are also competing for the

desaturases and elongases that convert the precursors Linoleic acid (LA) into AA and α-linoleic acid (ALA) into EPA (30). Therefore a high ratio of n-3 to n-6 PUFAs may lead to a decreased production of AA and 2-series prostanoids. This mechanism can also account for the antitumorgenic effect of a high ratio of fish fatty acids (EPA and DHA) to AA in animal models (31).
With regard to n-6 fatty acids alone, Zock and Katan concluded in a review article that animal studies show some evidence that n-6 fatty acids promote the growth of colorectal tumors, but the data are inconsistent (32). In addition case-control, prospective cohort studies and comparison of populations showed no consistent association between n-6 fatty acids and colorectal cancer risk (32).
We hypothesize that a high intake of fish fatty acids and a low intake of AA, i.e. a higher ratio of fish fatty acids to AA, might protect against colorectal adenomas. We investigated this hypothesis in a small case-control study in the Netherlands. In most epidemiological studies food questionnaires are used to assess intake of fish and n-3 and n-6 fatty acids. In this study the fatty acid composition in adipose tissue was used as a marker of fatty acid intake. Given the postulated mechanism this measure of fatty acid exposure is more directly of biological relevance as compared to estimated intake. Moreover, the fatty acids are determined specifically and problems of validity and reproducibility of food questionnaires and food tables are avoided.

Materials en Methods

Study population

For the present analysis, data from a case-control study are used. The study was conducted between 1995 and 1998 in the Netherlands to investigate the role of dietary factors and genetic susceptibility in sporadic and hereditary colorectal adenomas (33). The study was approved by the medical ethical committees of Wageningen University and the Nijmegen University Hospital. All participants were Dutch-speaking and of Western European origin, diagnosed before the age of 75, without a history of colorectal carcinomas, colon resection, polyposis coli, or inflammatory bowel disease. All underwent a full colonic examination (colonoscopy or sigmoidoscopy combined with barium enema) to assess presence of adenomas. The study population included sporadic subjects i.e., without a clear familial background of colon cancer as well as subjects with a familial background of HNPCC according the Amsterdam criteria. In both groups cases are subjects who had an adenoma removed during the colonic examination.
Sporadic subjects were recruited by the Department of Gastroenterology of the Nijmegen University Hospital. Sporadic cases were excluded if they had previous adenomas more than 3 years before entrance to the study. Sporadic controls were examined at the hospital for various reasons, for example abdominal pain, rectal blood loss or constipation.
HNPCC subjects were persons who came to the hospital for screening purposes. They were recruited in a similar fashion, either by the Nijmegen University Hospital or by the Netherlands Foundation for the Detection of Hereditary Tumors which keeps a registry of HNPCC families in The Netherlands.
This resulted in 137 eligible subjects. A total of 13 subjects decided not to participate in the study. So 124 subjects received a set of questionnaires at endoscopy and have had a fat biopsy from the buttock, by needle aspiration during endoscopy (34). Of the 124 subjects 12 did not return the questionnaires. Additionally, laboratory analyses of three biopsies was not successfully completed. This resulted in 109 subjects with complete data, i.e. 52 cases and 57 controls.

Fatty acid analysis

Gas-liquid chromatography was used to determine the fatty acid content of samples after saponification, petroleumether-extraction, and methylation of fatty acids. Chromatographic

analysis used split-injection, temperature-programmed runs with a CP-WAX-58 column with an inside diameter of 0.25 mm and 25 m length, with hydrogen serving as the carrier gas (35).

Food questionnaire

To quantify energy and nutrient intake a validated semi-quantitiative food frequency questionnaire was used, which was developed for the Dutch cohorts of the EPIC study (European Prospective Investigation into Cancer and Nutrition) (36;37). Frequency of consumption of food groups was based on the habitual consumption of 178 food items during the year before endoscopy. Nutrient intake was quantified for each individual using an extended version of the 1993 computerised Dutch food composition table. In the present analyses this EPIC questionnaire was used for information on nutrient intake and fish consumption.

With respect to the study hypothesis, the questionnaire asks for the frequency of fish consumption and type of fish consumed (subdivided in lean fish, fatty fish and other seafood). Comparing the questionnaire with the 24 hour recalls as gold standard it appears that the validity and reproducibility of total fat intake was generally good (36), but was not specified according to specific fatty acids (e.g. fish fatty acids and Arachidonic acid). However, validity was poor for fish consumption in men ($r = 0.32$) and woman ($r = 0.37$). Also reproducibility was low in men ($r = 0.49$), but slightly better in women ($r = 0.61$).

Data analysis

Means of demographic parameters (age, sex, height and weight), medical parameters (complaints and familial background), lifestyle characteristics (aspirin use and smoking habits), energy and fat intake were computed for cases and controls separately. Odds Ratios and corresponding 95% confidence intervals were computed for fish intake, fatty acid intake and fatty acids in the fat biopsy.

To identify possible confounders, the relation between exposure of n-3 fish fatty acids and the above mentioned variables was computed. Also other variables such as aspirine use and dietary variables (e.g. fibre, fat, fruit, vegetable and nutrient intake) were evaluated. When a variable differs between cases and controls and there was a relation between that variable and exposure to fish fatty acids we verified in further analyses whether substantial confounding was present.

Ors and corresponding 95% confidence intervals were computed using logistic regression models with fatty acids classified in tertiles, based on the distribution among controls. Because of the study design, sex and familial background (sporadic or HNPCC) were included as covariates in all analysis. Furthermore we adjusted for energy intake to account for differences in nutrient intake related to the differences in energy intake.

In addition, logistic regression was used to adjust for confounding, with fatty acid intake included as a continuous variable. For these continuous variables the exposure contrast represents the difference between the middle of the third and first tertile. When the regression coefficient obtained from a model with the potential confounder differed by more than 10% from the coefficient obtained from the model without that confounder we adjusted for that variable in all analyses. This resulted in adjustment for age and energy intake (as continuous variable) and for sex and familial background (as indicator variable).

The test for trend was conducted by scoring the subsequent categories of intake from 1 to 3 and entering this score as a continuous variable in the logistic regression model.

Finally, we verified whether other nutritional risk factors of colorectal carcinogenesis (fibre, proteins, alcohol, calcium, vitamin E, aspirin intake and saturated fatty acids intake) might have confounded our results. To decide whether substantial confounding by these factors was present we used the above-mentioned criteria, applied to logistic regression models with vs. without this additional variable. Using this procedure it appeared that dietary fibre needed to be added, together with the above-mentioned confounders for exposure to the

fatty acid AA. Ors and corresponding 95% confidence intervals for fish intake were adjusted for the same confounders as Ors for fatty acid intake.
All analysis were conducted using the statistical analysis system (SAS version 6.12, SAS institute).

Results

General characteristics of cases and controls were shown in Table 1. Weight, height and the percentage of subjects with bowel complaints were similar for cases and controls. Among cases age, energy intake and percentage of man was higher than in controls. The percentage of HNPCC subjects was higher in controls. Fat intake was slightly higher among cases. After adjustment for energy intake, nutrient intake did not differ between cases and controls (data not shown), except for intake of fibre and animal protein, which was slightly higher among cases.

Table 1: General characteristics of adenoma cases and controls.

Variables	Cases (n=52)	Controls (n=57)
	Mean ± SD	Mean ± SD
Demographic		
Age (year)	53 ± 12	42 ± 13
Weight (kg)	77 ± 12	75 ± 12
Height (cm)	173 ± 9	173 ± 10
Gender (%man)	52	37
Medical		
Complaints (%yes)	57	55
Familial background (%HNPCC)	17	40
Lifestyle		
Aspirin use (%yes)	72	70
Current smoking (%yes)	67	67
Diet		
Energy intake (MJ)	9.6 ± 2.5	9.3 ± 2.8
Total fat intake (g/day) [a]	94.7 ± 31.2	90.7 ± 33.3
Saturated fat (g/day)	36.0 ± 11.5	34.6 ± 12.3
Monounsaturated fat (g/day)	34.2 ± 11.4	32.5 ± 12.6
Polyunsaturated fat (g/day)	20.4 ± 9.3	19.3 ± 8.5
Total n-6 fatty acids	17.3 ± 8.3	16.3 ± 7.6
Arachidonic acid	0.03 ± 0.02	0.03 ± 0.02
Total n-3 fatty acids	1.8 ± 0.9	1.8 ± 0.7
Fish fatty acids	0.11± 0.09	0.14 ± 0.10

[a] Fat intake expressed as % of energy intake was 36.4 % among cases and 36.0 % among controls respectively.

Table 2 shows that total fish intake and the frequency of fish intake were inversely related with colorectal adenomas although not significantly.

Table 2: Association between fish consumption and occurrence of colorectal adenomas.

Fish consumption	Number of cases/controls	Crude OR (95% CI)	Adjusted OR (95% CI)[a]	p-value for trend
Frequency fish				
<1/month [b]	14 10	1.0	1.0	0.25
1/month-1/week	22 26	0.6 (0.2, 1.6)	0.6 (0.2, 1.8)	
>1/week	16 21	0.5 (0.2, 1.5)	0.5 (0.2, 1.6)	
Total fish intake (g/day)				
<6.4 [b]	23 18	1.0	1.0	0.12
6.4-14.0	15 20	0.6 (0.2, 1.5)	0.4 (0.2, 1.3)	
≥14.0 [c]	14 19	0.6 (0.2, 1.5)	0.4 (0.2, 1.3)	

[a] adjusted for energy intake, age, familial background and gender.
[b] reference category
[c] 14 g/day roughly corresponds to 1 serving per week

Adjusted Ors of colorectal adenomas were calculated for fatty acid intake and fatty acid composition of adipose tissue. Regarding intake of fatty acids, Table 3 shows that n-3 intake and intake of fish fatty acids (EPA and DHA) was associated with a decreasing risk of colorectal adenomas, and n-6 intake with an increasing risk, but these associations were not statistically significant. Like in other epidemiological studies we also computed the ratio of n-3 and n-6 fatty acids; this ratio was not statistically significant associated with a decreasing risk of colorectal adenomas.
Regarding fatty acid patterns in the fat biopsy Table 3 shows that n-3 and fish fatty acids (EPA and DHA) were associated with an decreasing risk of colorectal adenomas (p-value for trend 0.30 and 0.07 respectively). AA was positively, but not statistically significant associated with colorectal adenomas (p-value for trend = 0.42) and the ratio of fish fatty acids to AA was inversely associated with colorectal adenomas (p-value for trend = 0.002). These results were similar when fish fatty acids and AA status were entered in the logistic model as continuous variables.

Table 3: Association between dietary and tissue fatty acid pattern and colorectal adenomas.

	Calculated dietary intake					**Fatty acid pattern in biopsy**				
Exposure variables	Category[a]	Number of cases/ controls	crude OR (95 % CI)	adjusted OR[c] (95 % CI)	P-value for trend	Category[b]	Number of cases/ controls	crude OR (95 % CI)	adjusted OR[c] (95 % CI)	P-value for trend
Total n-3 fatty acids	<1.40	21 19	1.0	1.0	0.57	<1.50	18 19	1.0	1.0	0.30
	1.40-1.92	16 18	0.8 (0.3, 2.0)	0.5 (0.2, 1.7)[d]		1.50-1.86	18 19	1.0 (0.4, 2.5)	0.7 (0.3, 2.1)	
	≥1.92	15 20	0.7 (0.3, 1.7)	0.6 (0.2, 2.0)[d]		≥1.86	16 19	0.9 (0.4, 2.2)	0.6 (0.2, 1.7)	
Fish fatty acids	<0.08	23 19	1.0	1.0	0.28	<0.20	20 20	1.0	1.0	0.07
(EPA and DHA)	0.08-0.17	12 18	0.6 (0.2, 1.4)	0.4 (0.1, 1.2)		0.20-0.27	10 18	0.6 (0.2, 1.7)	0.4 (0.1, 1.2)	
	≥0.17	17 20	0.7 (0.3, 1.7)	0.6 (0.2, 1.6)		≥0.27	22 19	1.1 (0.4, 2.5)	0.3 (0.1, 1.1)	
Total n-6 fatty acids	<12.5	12 20	1.0	1.0	0.40	<15.4	17 19	1.0	1.0	0.32
	12.5-17.3	18 18	1.7 (0.6, 4.4)	1.5 (0.5, 4.8)		15.4-17.3	12 19	1.0 (0.4, 2.7)	1.0 (0.3, 3.3)	
	≥17.3	22 19	1.9 (0.8, 5.0)	1.7 (0.5, 6.0)		≥17.3	23 19	1.4 (0.6, 3.3)	1.7 (0.6, 5.1)	
Arachidonic acid	<0.02	19 19	1.0	1.0	0.37	<0.35	9 19	1.0	1.0	0.42
	0.02-0.04	18 19	0.9 (0.4, 2.3)	1.3 (0.4, 3.9)		0.35-0.45	19 19	1.8 (0.7, 4.9)	2.6 (0.7, 8.5)	
	≥0.04	15 19	0.8 (0.3, 2.0)	0.6 (0.2, 1.8)		≥0.45	24 19	2.1 (0.8, 5.5)	1.7 (0.5, 5.8)	
Ratio of n3/n6 fatty	<0.11	26 19	1.0	1.0	0.19	<0.09	18 20	1.0	1.0	0.33
acids	0.10-0.13	16 18	0.7 (0.3, 1.6)	0.5 (0.2, 1.6)		0.09-0.13	21 18	1.3 (0.5, 3.2)	1.1 (0.4, 3.0)	
	≥0.13	10 20	0.4 (0.1, 1.0)	0.5 (0.2, 1.6)		≥0.13	13 19	0.8 (0.3, 2.0)	0.6 (0.2, 1.7)	
Ratio of fish fatty	<3.11	21 19	1.0	1.0	0.78	<0.50	28 19	1.0	1.0	0.002
acids to AA:	3.1-6.42	15 19	0.7 (0.3, 1.8)	1.2 (0.4, 3.7)		0.50-0.70	9 18	0.3 (0.1, 0.7)	0.2 (0.1, 0.6)	
(EPA+DHA)/AA	≥6.42	16 19	0.8 (0.3, 1.9)	0.8 (0.3, 2.4)		≥0.70	15 20	0.5 (0.2, 1.2)	0.2 (0.1, 0.5)	

[a]Units g/day
[b]Units mass % methylester
[c]Adjusted for age, familial background, energy intake and gender
dAdditional adjusted for fibre intake

Discussion

When the fatty acid composition in adipose tissue was used as a biomarker of fatty acid status, adenoma risk tended to be associated with fish fatty acids (inversely) and AA (positively), while their ratio showed a significant inverse association with risk. Intake of fish and fish fatty acids were inversely associated with colorectal adenomas, although these associations were not statistically significant.

Selection and recruitment of study subjects and methods of data collection may have affected the results. The study population contained sporadic and HNPCC cases and controls with similar background. HNPCC cases and controls, both belonging to an HNPCC family, are generally more aware of their risk, but this is irrespective of their adenoma history; for this reason, sporadic and HNPCC cases were compared to controls with similar familial background. Because the size of our study is relatively small we were unable to see whether familial background might affect adenoma risk differently in both groups. Since COX-2 expression may be of less importance in HNPCC than in sporadic adenomas, this may have diluted the association (38). However this is one of the first studies to examine the relation between intake of fish and the risk of colorectal adenomas in which fish intake is measured by analysis of the fatty acid composition of adipose tissue.

Recall of dietary habits is expected to be similar in cases and controls. The cases came to the same endoscopy work up and the questionnaire asked for the food consumption during the whole year before endoscopy. So, at the time of filling out the questionnaires most patients are unaware of their diagnosis with respect to adenomas. Furthermore, the prevalence of complaints was similar in cases and controls. Results for dietary intake were less clear than for the biomarker. This may be due to the use of the food frequency questionnaire, which had a low validity and reproducibility for fish consumption. Fatty acid composition of adipose tissue is considered to be a marker of fatty acid intake of the past 2-3 years (39) and is less likely to be affected by recent dietary changes because of preclinical symptoms of adenomas. Moreover several studies found a high correlation between fish fatty acid (namely EPA and DHA) intake and these fatty acids in adipose tissue (40-42). Therefore, we give more confidence to the fatty acid composition of adipose tissue as a biomarker of fish consumption than to the food questionnaire. Moreover the correlation between intake of arachidonic acid and levels in adipose tissue is weak, probably due to conversion of linoleic acid to arachidonic acid (41). To investigate the effect of arachidonic acid on colorectal adenomas we give more confidence to the levels of arachidonic acid in adipose tissue, reflecting the availability of arachidonic acid in the human body, than to the food questionnaire.

Possibly due to the imperfect measurement of the intake of fish and n-3 fatty acids, most of the epidemiological studies did not find clear inverse associations (4-6; 8-11). Furthermore, results from epidemiological studies are not consistent, possibly due to the low range of fish consumption within study populations. The use of objective long-term biomarkers may have compensated for this, because of increased precision in exposure assessment. Despite this, some case-control studies show a protective effect of fish consumption on colorectal adenomas or carcinomas (7;13;14).

Similar to our results, Fernandez et al.(12) found an OR of 0.6 (0.5, 0.7) when comparing fish consumption higher than twice a week with lower than once a week. Although our results are not statistically significant they suggest a protection of fish intake to colorectal adenomas. To our knowledge no published papers have yet reported on fatty acid composition of adipose tissue (particular fish fatty acids) and the risk of colorectal adenomas.

The n-3 PUFAs exerts their protective effect most likely through the COX-2 pathway. However, the mechanism by which COX-2 modulation is involved in tumor development is not clear. One of the possibilities is the competition between n-3 and n-6 PUFAs. It has been shown that mice heterozygous for the $Apc^{Min/+}$ mutation, have a decreased number of intestinal tumors when fed a diet rich in the fish fatty acid EPA. These mice also have a

decrease in tissue AA and its metabolic products (21). Studies that have also examined COX-2 activity and apoptosis in animals that were fed different diets provide another link between n-3 PUFAs and COX-2. Animals fed a diet rich in n-6 PUFAs showed increased COX-2 activity and reduced apoptosis (43) Furthermore, a study in human volunteers showed that an increase in the ratio of n-3 to n-6 PUFA resulted in suppression of 2-series prostanoids production in the human rectal mucosa (17) These studies add further evidence for the hypothesis that n-3 PUFAs reduce the amount of AA and 2-series prostanoids in the tissues due to a reduced COX-2 activity, resulting in an increase in apoptosis.
In line with a role of n-3 PUFAs is a study on *cPLA2* knock-out mice; cPLA2 liberates AA from the cell membrane making it available for metabolism. $Apc^{Min/+}$ mice lacking this gene showed a decrease in tumor number compared to mice wild type for *cPLA2* (44). Together with the $Apc^{min/+}$ *COX-2* knock-out mouse mentioned before, this suggests that a reduced amount of 2 series prostanoids has a protective effect on tumor development. It is clear that the mechanism by which n-3 PUFAs protect against colon cancer is likely to be a complex picture with many other genes involved, some of which may not have been identified yet. Some other potential target mechanisms identified so far are the upregulation of *PPAR*δ, the Wnt signaling pathway (45) and the expression of the $p21^{RAS}$ oncogene, which is increased in mammary and colon tumor cells (46). Rats fed a high n-3 PUFA diet showed decreased expression of this oncogene compared to rats fed a n-6 diet. On top of this, a protein involved in post-translational modification, farnesyl protein transferase, has also been shown to be inhibited by n-3 PUFAs, thereby reducing the amount of active $p21^{RAS}$ protein (47).
In conclusion, despite limited numbers, our results suggest, in line with our hypothesis and the scientific literature that high tissue levels of fish fatty acids in adipose tissue, are associated with a lower risk of colorectal adenomas. Moreover, based on the competition between fish fatty acids (EPA and DHA) and AA for COX-2 activity, a higher ratio of fish fatty acids to AA is associated with a decreasing risk of colorectal adenomas.

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Chapter 7

Protective effect of nonsteroidal anti-inflammatory drugs on colorectal adenomas is modified by a polymorphism in peroxisome proliferators-activated receptor δ

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Abstract

OBJECTIVE: Nonsteroidal anti-inflammatory drugs (NSAIDs) are associated with a decreased risk of colorectal tumors. Single nucleotide polymorphisms (SNPs) in target genes of NSAID action, and their haplotypes, might modulate this protective effect.

A case-control study including 724 cases and 682 controls was used to evaluate the effect of NSAIDs on colorectal adenoma risk in the Netherlands, a country in which NSAID use is relatively low. METHODS: Cases and controls were classified according to presence or absence of endoscopy-proven, pathology-confirmed colorectal adenomas, ever in their lives. Thirteen SNPs in four genes (*PPARδ, PPARγ, PTGS1* and *PTGS2*) were genotyped in 787 subjects (384 cases and 403 controls).

RESULTS: Compared to non-regular users (<12 times/year), regular users of NSAIDs (≥12 times/year) had a lower risk of colorectal adenomas (odds ratio (OR): 0.75, 95% confidence interval (CI): 0.56-0.99). The results were similar for aspirin only. We found an interaction between SNP c.-789C>T in *PPARδ* and NSAID use (p=0.03). The protective effect of NSAIDs was strengthened for regular users with the PPARδ CT or TT genotypes (OR: 0.35, 95%CI: 0.11-1.13), whereas a positive association was observed for non-regular users with these genotypes (OR: 2.24, 95%CI: 1.06-4.73) as compared to non-regular users with the CC genotype. Also, a statistically significant interaction between a major haplotype containing the minor allele of this SNP and NSAID use was observed.

CONCLUSIONS: This study confirms the protective effect of NSAIDs and suggests a modulating effect of a SNP in the promoter of *PPARδ*.

Introduction

Cancer is the second leading cause of death in the Netherlands and most other developed countries. Incidence rates for colorectal cancer in the Netherlands are among the higher rates in the world and resemble the rates in other North-Western European countries and North America (1).

There is a large body of evidence, both from observational and intervention studies, suggesting that nonsteroidal anti-inflammatory drugs (NSAIDs) are associated with a reduced risk of colorectal carcinomas. Also, a 30-50% reduction has been observed for sporadic adenomatous polyps, the presumed precancerous lesion, as reviewed in Thun et al. (2).

The precise mechanism by which NSAIDs may prevent colorectal tumors is still unclear, but the enzyme prostaglandin-endoperoxide synthase (PTGS), also known as cyclooxygenase (COX), which catalyzes the first step in the conversion of arachidonic acid (AA) to prostaglandins, appears to play a major role. It has been suggested that blocking of the PTGS2 isoform is responsible for promotion of apoptosis and inhibition of tumor angiogenesis in colorectal cancer (3). Several studies have shown that treatment with selective PTGS2 inhibitors in mouse models for colorectal cancer resulted in reduced numbers of tumors (4,5). Randomized trials in patients with the inherited condition familial adenomatous polyposis have demonstrated that the selective PTGS2 inhibitor celecoxib can significantly regress existing adenomas (6). Since inhibition of the constitutively expressed PTGS1 isoform is responsible for adverse gastro-intestinal effects, selective PTGS2 inhibitors are preferred. It has been suggested however that both PTGS1 and PTGS2 are important in intestinal tumorigenesis as illustrated by the dose dependent decrease in polyp number in rats and mice after treatment with a selective PTGS1 inhibitor (7).

Besides the effect on the PTGS enzymes, NSAIDs have been shown to inhibit or stimulate other players both within the AA-pathway as well as in other pathways like the nuclear factor-κB (NK-κB) pathway (8). Two subtypes of the nuclear receptor peroxisome proliferator-activated receptors (PPARs), which are activated by the products of the AA pathway, can also act as direct targets of NSAIDs. The NSAID sulindac can bind to PPARδ

after which its activity and protein expression is down-regulated, inducing apoptosis (9). NSAIDs can also act as ligands for another receptor subtype, PPARγ (10). In contrast to PPARδ, this subtype has been shown to be activated by sulindac, which resulted in growth inhibition and apoptosis of cancer cells (11,12).
Individual differences in drug response might be due to genetic variation. The importance of the *PTGS* and *PPAR* genes in the protective mechanism of NSAIDs gives rise to the question whether single nucleotide polymorphisms (SNPs) and accompanying haploypes in these genes influence the effect of NSAIDs on colorectal tumorigenesis. To test this, we genotyped 12 SNPs in subjects participating in a case control study conducted in the Netherlands, a country in which NSAID use is relatively low. The combined effect of these SNPs, their haplotypes and regular intake of NSAIDs on colorectal adenoma risk was evaluated.

Methods

Study population

A retrospective case-control study was conducted in the Netherlands, between 1997 and 2001. The study design has been previously described (13,14). In short, this case-control study was designed to examine the association between various dietary and other lifestyle factors, genetic susceptibility and colorectal adenomas. All participants underwent endoscopy in one of ten participating hospitals because of routine screening for colorectal adenomas or gastrointestinal complaints. Cases had one or more adenomas at index endoscopy or in their past. The proportion of prevalent cases was 41% with a mean time since diagnosis of 4 years (range 0-23 years). Controls had no adenomas at index endoscopy, nor ever in their past.
The total study population included 1,477 subjects. The study was approved by the Medical Ethical Committees of all participating hospitals and all participants provided written informed consent.

Data collection

Information on use of NSAIDs, both for analgesic and other purposes, was obtained from a self-administered questionnaire including frequency of use, duration of use and brand name. Frequency of use was recorded as times per day/week/month/year/or less than once a year. Duration of use was recorded as number of years. For those who used NSAIDs for other purposes besides analgesics, duration of use was not recorded. Information on other lifestyle and demographic factors was obtained by the same questionnaire.
Participants were asked to recall their lifestyle habits in the year previous to their last endoscopy, or, in case they had changed their lifestyle habits because of complaints, the year before these complaints began. Separately, information on dietary habits was collected using a validated food frequency questionnaire (15,16).

Genetic analysis

On the basis of an inventory of the genetic variation of the selected genes in the Dutch population, as described elsewhere (Siezen et al. submitted), eleven SNPs were selected. This inventory included 100 unrelated randomly selected healthy individuals from the Dutch population. The SNP selection was based on allele frequency (with some exceptions only those SNPs with a minor allele frequency of 5% or higher were considered), position in the gene (when possible evenly distributed across the gene), possible impact on protein function (amino acid changes), and linkage between the SNPs in one gene (of two or more tightly linked SNPs only one was selected). One SNP was selected on the basis of another population study on *PTGS1* variants (17), and one was selected on the basis of a previously found interaction with NSAID use and colorectal adenomas (18).

Genotyping of twelve of the thirteen SNPs was carried out using a technique known as Pyrosequencing™. Each PCR contained 5μl 2x Hotstar master mix (Qiagen), 1μM of one primer, 0.1μM of a second primer containing a so-called universal tail of 23 nucleotides, 0.9μM of a third primer with the same sequence as the tail and labeled with biotin, and 10 ng genomic DNA, in a total volume of 10μl. PCR reactions were carried out in a Perkin-Elmer 9600 thermal cycler under the following conditions: 95ºC for 15 min, 40 cycles of 94ºC for 45 s, 57ºC for 45 s, 72ºC for 1 min, followed by 72ºC for 10 min. From the biotinylated PCR products single stranded DNA's were prepared and subsequently genotyped using the PSQ 96MA system and SNP reagent kit (Pyrosequencing AB) (19), as previously described (20). Primers for each SNP are described previously (21) Sequence primers for the pyrosequence reaction were designed using software available online (19). The *c.-765C>G* SNP in *PTGS2* was genotyped on a 7500 Realtime PCR System using the Taqman SNP genotyping assay c__11997909_10 and Taqman Fast Universal PCR Master Mix (Applied Biosystems), and 10 ng genomic DNA. Cycling was 95ºC for 20 seconds followed by 40 cycles of 95ºC for 3 seconds and 60ºC for 30 seconds. The post-read was performed at 60ºC for 30 seconds. DNA was available from 787 participants.

Statistical analysis

Seventy-one participants who only filled in a short version of the questionnaire were excluded because of incomplete data on NSAID use. Analyses on NSAID use were therefore performed including data from 1,406 participants, 724 cases and 682 controls. Analyses with genotypes were performed on 787 participants, 384 cases and 403 controls.

Aspirin and NSAIDs containing salicylic acid were grouped and analyzed separately. We considered use of 12 times per year or more as regular use. We assumed that persons who did not fill out the questions about frequency of analgesic use were not regular users. Duration of use was divided into tertiles among regular users according to distribution among controls. Those with missing data on duration of use were treated as a separate category. The analysis was repeated excluding these individuals, but this did not change the results.

Odds ratios (ORs) for the presence of colorectal adenomas and 95% confidence intervals (95% CIs) were estimated using the non-regular users of the type of drug in question as a reference group. Risk estimates stratified for genotypes, were calculated with separate genotypes where possible, with the non-regular users in combination with the homozygote major allele of the SNP of interest considered as reference group. If the numbers were insufficient heterozygote and homozygote minor genotypes were pooled.

The presence of effect modification by sex, smoking (ever/never), family history of colon cancer (yes/no) and intake of total and fatty fish (high/low) was evaluated by stratified analyses.

As confounding factors were considered: age (continuous), sex, body mass index (continuous), family history of colon cancer (yes/no), smoking (ever/never), duration of smoking (continuous), use of (other) NSAIDs (regular/non-regular), diabetes (yes/no), constipation (once a month or more, yes/no), use of hormone replacement therapy (yes/no), physical activity level (medium split, high/low), use of acetaminophen (non-regular/regular), use of antibiotics (more/less than 10 times/lifetime), education level (4 levels), indication for endoscopy (bowel complaints, screening, other/unknown), gastrointestinal symptoms (yes/no), change in dietary habits (yes/no), daily energy intake and intake of fat, fish, dietary fiber, vegetables, fruit, alcohol and coffee (all continuous). Variables were included in the multivariate model if they changed the OR by 10% or more. This applied to age, sex, duration of smoking and main indication for endoscopy.

To test whether the combination of genotypes and NSAID use deviated from multiplicativity, we calculated p-values for interaction by inclusion of a numerical term for genotype assuming either a dominant effect (homozygote major allele=1 and heterozygote or homozygote minor allele=2), a recessive effect (homozygote major allele or heterozygote =1

and homozygote minor allele=2), or a co-dominant effect (two interaction terms) multiplied by a numerical term for NSAID regular use into our multivariate models. The p-value for interaction was calculated by chi-square test comparing the -2LL values of the models with and without NSAIDs use*genotype interaction term. A p-value lower than .05 was considered statistically significant.
Haplotypes were estimated and ORs calculated using the Hplus program, available online (22). Hplus is a SNP analysis tool for performing haplotype estimations, according to the distribution of genotypes in a population. It is able to handle datasets that include case-control status as well as covariates and SNP location variables (23).
All other analyses were conducted using Statistical Analysis Software (SAS, version 8.2, SAS institute, Cary, NC).

Results

A detailed description of the total study population characteristics (14) and the subset genotyped (13,24) was published previously.
Adjusted ORs of the association between colorectal adenomas and frequency of use of total NSAIDs and aspirin are shown in table 1. Regular NSAID use confers a reduction in adenoma risk of 25%. This protective effect became more pronounced as duration of use increased, to nearly 40% in the second tertile of duration. However, there was no additional protective effect in the third tertile of duration, which includes subjects that have used NSAIDs for more than 12 years. For aspirin, a similar protective effect was observed for regular use. There was no apparent trend for duration of use of aspirin. The protective effect of aspirin even seemed diminished for individuals in the first and third tertiles, but this is likely due to chance since the number of individuals per group was small and the protective effect was retained in the second tertile.

Table 1: Regular use of NSAIDs and colorectal adenoma risk

	Cases (n=724)	Controls (n=682)	OR (95%CI)[a]
Total NSAIDs			
Non-regular[b]	531	480	1.00 (ref)
Regular[c]	193	202	0.75 (0.56-0.99)
<5 years duration[d]	40	43	0.95 (0.56-1.63)
5-12 years duration[d]	32	45	0.61 (0.34-1.10)
>12 years duration[d]	40	40	0.73 (0.42-1.27)
missing[e]	81	74	0.73 (0.48-1.10)
Aspirin			
Non-regular[b]	608	586	1.00 (ref)
Regular[c]	116	96	0.71 (0.49-1.02)
<5 years duration[d]	17	17	1.19 (0.53-2.65)
5-15 years duration[d]	19	17	0.57 (0.24-1.34)
>15 years duration[d]	21	16	1.06 (0.49-2.32)
missing[e]	57	41	0.60 (0.36-1.01)

[a] Multivariate adjustment for age, gender, indication for endoscopy and duration of smoking.
[b]Non-regular use defined as <12 times per year.
[c]Regular use defined as ≥12 times per year.
[d]Duration of use divided in tertiles according to distribution among controls.
[e]For participants who used NSAIDs for other purposes besides analgesics, data on duration of use was not collected. These were treated as a separate category in the analysis.

Genotype-NSAIDs interaction

All genotypes were in Hardy Weinberg Equilibrium. Main effects of these genotypes have been reported previously (21). Table 2 shows ORs and 95% confidence intervals for the associations between NSAIDs and colorectal adenoma risk, stratified for the genotypes of 12 SNPs.

Table 2: Regular use of NSAIDs and adenoma risk, stratified by genotype

	Non-regular use[a] (n=569)		Regular use[b] (n=218)		
Genotype	Cases/controls (280/289)	OR (95%CI)[c]	Cases/controls (104/111)	OR (95%CI)[c]	p for interaction (dominant, recessive or co-dominant effect)
PPARδ					
c.-789C>T (submitted to dbSNP)					
CC	241/254	1.00 (ref)	92/97	0.77 (0.51-1.17)	
CT+TT	23/19	2.24 (1.06-4.73)	9/14	0.35 (0.11-1.13)	0.03 (dominant effect)
N163N (rs2076167)[d]					
TT	173/180	1.00 (ref)	67/65	0.79 (0.49-1.28)	
TC	91/100	0.86 (0.56-1.33)	30/43	0.48 (0.25-0.92)	
CC	15/9	1.39 (0.48-4.01)	7/6	0.88 (0.24-3.21)	>0.05
c.2021T>C (rs3734254)[d]					
TT	179/187	1.00 (ref)	70/69	0.76 (0.48-1.22)	
TC	91/94	0.85 (0.55-1.32)	27/39	0.46 (0.23-0.91)	
CC	10/7	1.38 (0.40-4.73)	7/5	1.13 (0.29-4.39)	>0.05
c.2589G>A (rs1053046)[d]					
GG	254/263	1.00 (ref)	93/98	0.74 (0.49-1.12)	
GA+AA	26/26	1.27 (0.63-2.53)	11/16	0.54 (0.21-1.42)	>0.05
c.2806C>G (rs9794)[d]					
CC	193/209	1.00 (ref)	79/84	0.71 (0.45-1.10)	
CG+GG	79/77	0.86 (0.54-1.36)	25/29	0.63 (0.30-1.31)	>0.05
PPARγ					
P12A (rs1801282)[d]					
CC	209/217	1.00 (ref)	72/80	0.70 (0.44-1.11)	
CG+GG	68/72	1.11 (0.71-1.73)	30/34	0.73 (0.39-1.40)	>0.05
H477H (rs3856806)[d]					
CC	202/194	1.00 (ref)	74/69	0.75 (0.47-1.20)	
CT+TT	64/87	0.71 (0.45-1.11)	27/38	0.52 (0.27-1.00)	>0.05
PTGS1					
W8R (rs1236913)[d]					
CC	241/239	1.00 (ref)	93/100	0.68 (0.46-1.03)	
CT+TT	31/41	0.90 (0.49-1.65)	8/12	0.84 (0.28-2.49)	>0.05
L237M (rs5789)[d]					
CC	222/238	1.00 (ref)	78/94	0.65 (0.42-1.00)	
CA+AA	8/10	0.86 (0.28-2.67)	6/2	2.25 (0.32-15.96)	>0.05
PTGS2					
c.-1329A>G (rs689466)[d]					
AA	162/180	1.00 (ref)	56/75	0.56 (0.33-0.93)	
AG	91/92	1.20 (0.78-1.84)	40/30	1.27 (0.68-2.39)	
GG	16/12	0.95 (0.34-2.65)	6/4	2.22 (0.55-8.89)	>0.05
c.-765C>G (rs20417)[d]					
CC	172/195	1.00 (ref)	65/79	0.70 (0.43-1.12)	
CG+GG	74/68	1.12 (0.70-1.78)	26/26	0.71 (0.34-1.48)	>0.05
V102V (rs5277)[d]					
GG	214/194	1.00 (ref)	70/73	0.68 (0.43-1.08)	
GC+CC	66/95	0.65 (0.42-1.01)	34/41	0.46 (0.24-0.87)	>0.05
c.2242T>C (rs5275)[d]					
TT	116/141	1.00 (ref)	43/55	0.72 (0.41-1.27)	
TC	132/116	1.30 (0.86-1.98)	53/46	0.95 (0.53-1.68)	
CC	27/28	1.15 (0.55-2.41)	7/10	0.56 (0.16-2.00)	>0.05

[a] Non-regular use defined as <12 times per year.

[b] Regular use defined as ≥12 times per year.

[c] Multivariate adjustment for age, gender, indication for endoscopy and duration of smoking.

[d] rs number according to dbSNP (25)

A statistical significant interaction (p=0.03) was found in the dominant model between SNP c.-789C>T in *PPARδ* and regular NSAID use. Among non-regular users, CT+TT pooled genotypes increased colorectal adenoma risk (OR, 2.24; 95%CI, 1.06-4.73) compared to the CC genotype, while regular use was most strongly inversely related to risk among CT and TT genotypes although not statistically significant (OR, 0.35; 95%CI, 0.11-1.13). For the other SNPs in *PPARδ*, benefit for NSAID regular users was also more apparent among those with the minor alleles. However, no statistically significant interactions were observed.
The protective effect of NSAIDs was only apparent with the major AA genotype of SNP *c.-1329A>G* in *PTGS2*, while those with AG and GG genotypes did not appear to benefit from regular NSAID use. Interactions between regular NSAID use and SNPs in *PTGS2* however, were not statistically significant.
No statistically significant interactions were observed between any SNPs in *PPARγ* or *PTGS1*. Due to apparent selectivity of several NSAIDs for PPAR subtypes (26), interactions between ibuprofen use and SNPs in *PPARδ*, and interactions between indomethacin and diclofenac use and SNPs in *PPARγ* were analyzed separately, but no significant interactions were observed (data not shown).
An additional nine SNPs in three other genes involved in the AA pathway (15-lipoxygenase (*ALOX15*), secreted phospholipase A2 (*PLA2G2A*) and cytosolic phospholipase A2 (*PLA2G4A*)) were genotyped but no interactions with NSAID use and colorectal adenoma risk were observed (data not shown). The rs numbers (according to dbSNP (25)) and minor allele frequencies of these additional SNPs are presented in table 3.

Table 3: Additional SNPs genotyped

Gene	SNP (rs number)[a]	Minor allele frequency	
		Cases (n=384)	Controls (n=403)
ALOX15	c.-217G>C (rs2664593)	0.134	0.154
	T485T (rs743646)	0.092	0.124
PLA2G2A	c.-180C>G (rs11573156)	0.235	0.251
	T32T (rs2236771)	0.082	0.098
	c.665C>T (rs11677)	0.115	0.104
PLA2G4A	c.918+23C>T (rs2307200)	0.179	0.172
	c.1336+3G>A (rs6661772)	0.052	0.067
	R651K (rs2307198)	0.021	0.024
	c.2605G>A (rs12720707)	0.075	0.090

[a] rs number according to dbSNP (25)

Haplotype-NSAID interaction

For regular users only, the ORs of haplotypes corresponding to the 12 SNPs are shown in table 4. There was a statistically significant interaction between haplotype 11110 in *PPARδ* (the minor alleles of *c.-789C>T, N163N, c.2021T>C* and *c.2589G>A*, and the major allele of *c.2806C>G*) and regular NSAID use. Individuals with this haplotype had a statistically non significant reduced risk of adenomas of 66% as compared to the major haplotype. A very large significant reduction in risk of 73% was also seen for haplotype 01 in *PPARγ* (C allele for *P12A* and T allele for *H477H*) in regular NSAID users compared to the major haplotype 00. However, the interaction between this haplotype and NSAID use was not statistically significant.

Table 4: Regular use of NSAIDs and adenoma risk, stratified by haplotype

	Regular use of NSAIDs[a]			
Haplotype[b]	Frequency among cases	Frequency among controls	OR (95% CI)[c]	p value for interaction
PPARδ				
00000	0.780	0.781	1.00 (ref)	
01101	0.146	0.139	0.89 (0.44-1.79)	0.81
01110	0.018	0.028	0.92 (0.27-3.17)	0.37
11110	0.029	0.025	0.34 (0.08-1.37)	0.04
PPARγ				
00	0.825	0.787	1.00 (ref)	
11	0.095	0.103	1.00 (0.42-2.39)	0.74
01	0.038	0.070	0.27 (0.07-0.95)	0.49
10	0.042	0.040	0.73 (0.26-2.09)	0.44
PTGS1				
00	0.924	0.912	1.00 (ref)	
10	0.053	0.070	1.24 (0.39-3.94)	0.64
PTGS2				
0000	0.273	0.308	1.00 (ref)	
1000	0.236	0.196	2.29 (1.16-4.54)	0.12
0010	0.146	0.187	1.05 (0.50-2.17)	0.42
0001	0.185	0.175	1.43 (0.65-3.12)	0.61
0101	0.151	0.125	1.45 (0.55-3.82)	0.69

[a]Regular use defined as ≥12 times per year.
[b]0 represents major allele, 1 represents minor allele, SNP order according to table 2.
[c] Multivariate adjustment for age, gender, indication for endoscopy and duration of smoking.

Discussion

This case-control study in a population with relatively low intake of NSAIDs supports an inverse association between use of NSAIDs and colorectal adenomas. We observed that participants who used NSAIDs 12 times a year or more were about 25% less likely to develop colorectal adenomas than those using NSAIDs less than 12 times per year. This observation corresponds to findings from other studies (27).

All genes investigated in this study have been shown previously to be involved in colorectal tumorigenesis by being indirect or direct targets of NSAIDs (5,7,9,10). Some of the SNPs investigated have also been shown to modify the risk of colorectal adenomas (21). We have investigated whether these SNPs could modify the protective effect of NSAIDs on colorectal adenomas, and observed that some SNPs did have a modifying effect.

The statistically significant interaction observed between SNP *c.-789C>T* in the promoter of *PPARδ* and regular NSAID use is in line with previous findings that *PPARδ* is a direct target of NSAIDs. We have shown previously that this same SNP in the promoter of *PPARδ* interacts with fish consumption in the risk for colorectal adenomas (21). These two findings taken together strongly suggest a functional effect of the SNP when the gene interacts with a ligand. The mechanisms by which PPAR proteins are activated by ligands are becoming better understood (28). These same ligands could also be involved in regulating the expression of the *PPAR* genes, and may therefore interact with the promoter. We have investigated the possibility that this SNP interferes with a transcription factor binding site using the Genequest part of the Lasergene software package and the online tool TFSEARCH (29), but found no known consensus sites around the SNP position. Haplotype 11110 confirms the effect of SNP *c.-789C>T* in *PPARδ.* This haplotype is the major haplotype containing the minor allele of *c.-789C>T* (and the minor alleles of *N163N*, *c.2021T>C* and *c.2589G>A*, and the major allele of *c.2806C>G*). The interaction between haplotype 11110 and NSAID use was statistically significant.

PPARδ activity is suppressed in cells treated with the NSAID sulindac, by disrupting its DNA-binding ability (9). The role of PPARδ was further investigated by Kojo et al. (26), who tested 7 different NSAIDs and their selectivity for PPAR subtypes. They found that ibuprofen was the only NSAID tested that had an effect on PPARδ. We found however, that selecting for ibuprofen regular use only, did not strengthen the interactions with any SNP in *PPARδ* (data not shown). To our knowledge, no other studies have been reported in the literature on the interaction between SNPs in *PPARδ* and NSAIDs and their effect on colorectal adenomas.

The protective effect of NSAIDs was diminished in people with the G allele for SNP *c.-1329A>G* in *PTGS2*, as confirmed with the haplotype representing this allele (haplotype 1000). This effect is most likely due to the minor allele of the SNP or chance findings, and not due to an interaction, since this was not statistically significant. Moreover, a positive effect was seen for both regular and non-regular users, although the effect for regular users was somewhat stronger. No other SNPs in either *PTGS1* or *PTGS2* showed any interaction with NSAID use. This was unexpected, since the PTGS enzymes are well established targets of NSAID action. An explanation for this might be that there are other unknown SNPs in these genes, for example in intronic regions, or SNPs with a minor allele frequency below 5% and thus not tested, which interact with NSAID use. The *c.-765C>G* SNP in *PTGS2* was previously shown to interact with NSAID use in relation to colorectal adenomas (18). This result could not be replicated by us. In line with our results, in a study on *PTGS1* variants no interaction was observed between the two SNPs that correspond to the SNPs in our study. An interaction was found however, between a SNP (*P17L*) not genotyped in our study due to the low minor allele frequency reported, and NSAID use (30).

We found a decrease in risk of 80% for individuals with the 01 haplotype in *PPARγ* among regular users, as compared to the major haplotype. However, the interaction between this haplotype and NSAID use was not statistically significant, therefore the likely explanation for this is a cumulative effect of two protective effects provided by the haplotype and NSAIDs separately. A study on different NSAIDs and their selectivity for PPAR subtypes identified indomethacin and diclofenac as selective agonist for PPARγ (26). However, in our study, analysis with indomethacin and diclofenac use, albeit with limited numbers, did not strengthen the results for either SNP in *PPARγ* as compared to total NSAID use (data not shown). To our knowledge there have been no other studies reporting on the interaction between SNPs or haplotypes in *PPARγ* genes and NSAID use.

There are some limitations to the study that need to be addressed. Firstly, it cannot be ruled out that there are other genes or other SNPs in the genes studied that could interact with NSAIDs. However, we have genotyped nine other SNPs in three other genes involved in the AA pathway (*ALOX15, PLA2G2A* and *PLA2G4A*), and found no interaction between any of the SNPs and NSAID use. Recently, the more upstream target NF-κB has been implicated as one of the main regulators of NSAID action (31), therefore variants in genes encoding this transcription factor complex or within its pathway might modulate the protective effect of NSAIDs.

Second, although it is usually assumed that adenomatous polyps remain asymptomatic, in this study more cases than controls had suffered from rectal bleeding (29.0% vs 17.9%, respectively). If cases reduced their aspirin intake after observing such bleeding, because they are aware bleeding can be an aspirin side-effect, this could have falsely strengthened our results. Regular or non-regular use of aspirin, however, did not correlate with anal bleeding (25% of non-regular users experienced anal bleeding vs 23% of regular users).

Third, our case-definition included incident as well as prevalent cases. It is possible that the latter changed their eating and other lifestyle habits after diagnosis of their first polyp. We repeated the analyses with only incident cases, and this even strengthened results.

Fourth, some controls (39%) did not undergo a full colonoscopy. Therefore, it is still possible that some of the control subjects had an adenoma in the proximal colon. However, repeating

the analyses with only controls that had full colonoscopy did not change our conclusions. Last, the power of this study to detect statistically significant interactions may be limited, since stratification into genotypes according to NSAID use results in small numbers per group.
In conclusion, our results indicate a protective effect of NSAIDs against colorectal adenomas. We have shown for the first time that this effect is modulated by a polymorphism in *PPAR*δ which is apparent by analyzing the single SNP and its accompanying haplotype. However, further studies are needed to confirm our results and to explain the mechanism by which this polymorphism might interact with NSAIDs.

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Chapter 8

Main findings, concluding remarks and future prospects

Main findings, concluding remarks and future prospects

In the last two decades, family based linkage studies on colorectal cancer have been successful in identifying high penetrance genes giving rise to familial syndromes. The best known examples are the *APC* gene, mutations in which are the cause of FAP, and *MMR* genes, predisposing mutations carriers to HNPCC. However, mutations in these genes are rare as they only account for a small part of the familial clustering (3-5%) observed among colorectal cancer cases. This indicates that the majority of familial cases is likely to be the result of the interaction of many genetic variants each with a modest individual effect on tumor susceptibility, the so-called low penetrance alleles. Therefore, since a wealth of information has become available to researchers with the completion of the human genome project, much research has focused on the identification of low penetrance alleles. Genetic association studies comprise the majority of these efforts, generally evaluating the effect of single SNPs on colorectal cancer risk.
The research described in this thesis was aimed at the identification of low penetrance alleles for colorectal cancer in seven candidate genes involved in the arachidonic acid pathway. We evaluated the influence of these SNPs on colorectal adenomas and carcinomas in retrospective and prospective studies respectively. We investigated whether the observed associations were modified by fish consumption, n-3 serum levels and regular use of NSAIDs.

Main findings

Chapter 2
In an attempt to evaluate the genetic variation in our seven candidate genes, *PTGS1*, *PTGS2*, *PPARδ*, *PPARγ*, *ALOX15*, *PLA2G2A* and *PLA2G4A*, we identified fifty-eight single nucleotide polymorphisms (SNPs), including thirteen newly described SNPs. On the basis of allele frequency, position within the gene, linkage with other SNPs, and possible effect on protein function, we selected 24 SNPs to use in studies investigating associations between genotypes and risk of colorectal cancer All SNPs were used to construct haplotypes, some with higher resolution than the haplotype structures available online at that time in HapMap.

Chapter 3
First, we genotyped these SNPs in a case-control cohort, the POLIEP study, including 384 subjects with one or more adenomas and 403 controls, and considered the modifying effect of fish consumption on the observed associations. A protective effect on colorectal adenomas was found for the CT genotype of SNP *H477H* in *PPARγ* and the GC genotype of SNP *V102V* in *PTGS2* (OR, 0.63; 95%CI, 0.45-0.89 and OR, 0.65; 95%CI, 0.46-0.92 respectively) when compared to the homozygote major genotypes. An increase in adenoma risk was observed for the TC genotype of SNP *c.2242T>C* in *PTGS2* (OR, 1.47; 95%CI, 1.07-2.00) compared to the TT genotype. Analysis with estimated haplotypes confirmed these associations and revealed three additional associations with *PTGS2*, *PLA2G2A* and *ALOX15* haplotypes. Fish consumption modified the associations with *PTGS2* and *PPARδ* genotypes. For SNP *c.-789C>T* in *PPARδ*, the major genotype showed a decrease in adenoma risk for those in the highest tertile of fish consumption (T3), as compared to the lowest tertile (T1) (OR, 0.65; 95%CI, 0.41-1.02). Protective effects were also observed for SNPs *V102V* and *c.2242T>C* in *PTGS2* and high fish intake. The interaction between fish consumption and *c.2242T>C* was statistically significant, with an OR for the TT genotype and high fish consumption of 0.52 (95%CI, 0.27-1.01) as compared to low fish intake and the TT genotype.

Chapter 4
The study described in Chapter 4 was conducted to test whether the associations described in the previous chapter for colorectal adenomas could be reproduced for colorectal cancer. To this aim we used a nested case control design in a prospective cohort with 16 years of follow up, including 209 colorectal cancer cases and 418 controls frequency matched on age and sex with the cases, and only reproduced the association between SNP *V102V* in *PTGS2* and CRC risk. A protective effect of the minor allele of this SNP (CC genotype, OR, 0.37; 95%CI, 0.16-0.87) was found. However, in contrast to the adenoma study no statistically significant interactions between fish consumption and any of the SNPs analyzed were shown in this prospective study on colorectal cancer.

Chapter 5
As the interaction shown between fish consumption and SNPs for colorectal adenomas is based on the hypothesis that fish consumption acts as a proxy for n-3 PUFAs, we investigated whether the SNPs interacted in the same way with serum n-3 PUFA levels. First, we examined the association between several PUFAs, analyzed in serum, and colorectal adenomas. We found that linoleic acid as well as total n-6 PUFAs (which consists of about 85% linoleic acid) was positively associated with colorectal adenomas. The OR of the highest tertile (T3) was 1.69 (95%CI, 1.09-2.61) for total n-6 PUFAs and 1.78 (95%CI, 1.15-2.76) for LA as compared to the lowest tertile (T1), with a statistically significant trend (0.02 and 0.008 respectively). Total n-3 PUFAs were inversely, although not statistically significant associated with colorectal adenomas, (OR and 95% CI T3 versus T1: 0.71; 0.46-1.10). Results for EPA and DHA separately were similar. However, none of the SNPs tested modified these associations.

Chapter 6
In Chapter 6, we demonstrated that the ratio of n-3/n-6 fatty acids as analyzed from a fat biopsy is an important factor influencing the risk of colorectal adenomas both in sporadic as well as in genetically predisposed (HNPCC) cases. A high ratio of fish fatty acids to AA showed an OR of 0.2 in the third tertile (95%CI, 0.1-0.5), although fish intake was not significantly associated.

Chapter 7
Lastly, the modifying effect of our selected SNPs on the association between NSAIDs on colorectal adenomas was evaluated in Chapter 7. Compared to non-regular users (<12 times/year), NSAID regular users (≥12 times/year) showed a lower risk of colorectal adenomas (OR: 0.75, 95%CI: 0.56-0.99). The results were similar for aspirin only. We found an interaction between SNP *c.-789C>T* in *PPARδ* and NSAID use (p=0.03), which is the same SNP that was previously shown to interact with fish consumption. The protective effect of NSAIDs was strengthened for regular users with the *PPARδ* CT or TT genotypes (OR: 0.35, 95%CI: 0.11-1.13), whereas a positive association was observed for non-regular users with these genotypes (OR: 2.24, 95%CI: 1.06-4.73) as compared to non-regular users with the CC genotype. Also, a statistically significant interaction between a major haplotype containing the minor allele of this SNP and NSAID use was observed.

In summary, we can conclude that we have successfully identified low penetrance alleles in the *PPARγ*, *PLA2G2A* and *ALOX15* genes, conferring differential colorectal adenoma risk, and two such alleles in the *PTGS2* gene, one of which is also involved in colorectal cancer risk. These results, also in view of the interactions found between some SNPs and fish consumption and NSAID use, reinforce the importance of the role of the AA pathway in colorectal tumorigenesis, and indicate that some of these modifying alleles might contribute to a proportion of sporadic colorectal cancer cases, although the exact underlying mechanisms involved are not understood yet.

Remaining Questions

Low penetrance allele candidates

As mentioned in the introduction, there are some promising candidates in the search for low penetrance alleles associated with CRC risk. However, one of the main problems investigators keep facing is the lack of consistency between genetic association studies. What are the main factors causing these inconsistent results? These issues are extensively discussed in several recent reviews (1-3) and are shortly summarized here. From a statistical point of view inconsistent study results can be caused by false positives or false negatives. In the case of a false positive result or a type I error, the subsequent studies fail to replicate this result simply because it was due to chance. This is especially the case with studies showing borderline significance with confidence intervals including 1. It is however also possible that the first positive study is correct and subsequent studies are false negatives (type II error). This is nevertheless unlikely since subsequent studies contain generally larger cohorts.

These limitations are not insurmountable however, and can be minimized by better and more stringent statistics including corrections for multiple testing, use of haplotype tagging SNPs so as to capture all the common variation in the candidate gene, and large cohorts. Moreover, these limitations should not discourage scientists from performing genetic association studies, because studies with relatively small population sizes could still contribute to the overall evaluation of a low penetrance allele. This is illustrated by a recent meta-analysis of a variant in the *Aurora/STK15* gene in which various cancer types were considered (4). Interestingly, the majority of the 15 studies included in this meta-analysis, did not reach statistical significance on their own, but when the studies were combined to include 9549 cases and 8326 controls a statistically significant cancer promoting effect of the minor allele was suggested.

With regard to the studies described in this thesis, there are several lines of evidence indicating that the observed low penetrance alleles in *PTGS2* are plausible candidates. First, other studies have found similar associations, as discussed in Chapters 3 and 4. Very recently, another study on *PTGS2* variants reproduced the positive association with SNP *c.2242T>C* and colorectal adenomas (5). Notably, in line with our study, the increased risk was observed for the heterozygous genotype. Among males, the risk of colorectal adenomas was increased with the heterozygous genotype, although the distinction between heterozygotes and homozygotes was lost when males and females were considered as one group. However, this does point to an important and possibly functional role for the *PTGS2 c.2242T>C* SNP, or for a nucleotide change in strong LD with it. The 3'UTR of *PTGS2* contains AU rich elements (ARE) which play an important role in stabilization of the transcript when translational regulatory factors are bound to them (6). The location of this SNP, within an ARE involved in mediation of transcript degradation in the 3'UTR of the gene, suggests that it might affect the binding affinity of regulatory factors, and thus modify the stability and expression of the PTGS2 transcript (7). Since an increased risk is observed for the minor allele of this SNP, it is plausible that this allele results in a more stable transcript. The apparent effect of the heterozygous genotype in our and in the previously mentioned study, suggest there might be an interaction between the two alleles, though the exact nature of this and the reason why these allelic variants would affect colorectal adenoma risk remains to be elucidated. Further studies are needed to investigate the true functionality of this SNP.

Second, we have demonstrated a protective effect of SNP *V102V* in *PTGS2* in two independent cohorts. In the case control cohort on colorectal adenomas only the C allele appears to be sufficient for a reduction in risk of 35%, whereas for the later stages of tumor development two alleles are needed to confer a risk reduction of 63%, as analyzed in the prospective cohort. These two inverse associations make it likely that there is either some

functional effect of this SNP, for example on splicing, or that this SNP is in close LD with another functional variant, as discussed previously for SNP *c.2242T>C*. So far we have not been able to suggest any functional role of this SNP either *in silico* or experimentally. However, it cannot be ruled out that this SNP might interfere with other regulatory regions or consensus sequences within exons.

Haplotypes
An ongoing difficulty for molecular epidemiologists is the optimal selection of the number of genetic variants for their association studies. To date there is still insufficient knowledge about the functionality of SNPs, in particular non-coding or synonymous SNPs, and because of the sheer abundance of genetic variants, methods are required to aid in this selection process. Even though the number of SNPs within a gene region might be considerable, some of these SNPs will be in linkage disequilibrium, thus creating a limited number of common haplotypes per gene region. The number of SNPs that can discriminate these haplotypes is considerably less than the total number of SNPs in that region, and can therefore serve as so-called haplotype tagging (ht) SNPs. This is defined as the indirect approach to select candidate SNPs. The great advantage to this approach is that it optimizes the amount of genetic variation included in the association study.
In this thesis, several results also support the importance of analyzing haplotypes. First, there was an apparent lack of associations between single SNPs in *PLA2G2A* and colorectal tumors. However, when all SNPs were combined and haplotypes estimated, the haplotypes containing the minor allele of SNP *c.665C>T* located in the 3'UTR of the gene, was positively associated with colorectal adenomas.
Secondly, analysis of the haplotype containing the major allele of *P12A* and the minor allele of *H477H* strengthened the association found between the *H477H* SNP in *PPAR*γ and the risk of colorectal adenomas. The importance of the haplotypes encompassing both SNPs was also illustrated by two other studies on the association between risk alleles and body weight and type 2 diabetes. However, the statistically significant association in the second study concerned the haplotype containing the minor allele of *P12A* and the major allele of *H477H* (also known as *C1431T*) (8,9). This does indicate however, a likely functional effect resulting from the combination of these SNPs, either on protein function or on the relative amount of protein expressed.
No previous studies have been reported evaluating associations between SNPs or haplotypes at the anticarcinogenic *ALOX15* gene and colorectal tumors. However, associations with a specific haplotype in another subtype of the lipoxygenase enzyme, the procarcinogenic *ALOX5* gene, have been reported recently (10), indicating again the importance of this family of enzymes in colorectal tumorigenesis.

Fish consumption and biomarkers of fish intake
We investigated retrospectively as well as prospectively whether increased fish consumption has a protective effect on colorectal tumor formation and have not demonstrated a clearly inverse association. In a relatively small case-control study on colorectal adenomas, we observed an inverse, though not statistically significant association (OR 0.4; 95%CI, 0.2-1.3, T3 vs T1) between fish consumption and colorectal adenomas (Chapter 6), which could not be confirmed when this study was extended and contained a larger population (OR 0.98; 95%CI, 0.72-1.32, T3 vs T1) (Chapter 3). A non-significant inverse association was observed between fish consumption and colorectal cancer in a prospective cohort study (RR 0.83; 95%CI, 0.57-1.20, high vs low) (Chapter 4). Fatty acid levels as measured from fat biopsies and serum samples were analyzed for their association with colorectal adenomas. Whereas increased n-3 PUFAs content in serum suggested a slight protection against adenomas (OR 0.71; 95%CI, 0.46-1.10, T3 vs T1) (Chapter 5), the ratio of EPA+DHA/AA seemed most important as protective factor when fat biopsies were analyzed (OR 0.2; 95%CI, 0.1-0.5, T3 vs T1) (Chapter 6). These apparent inconsistencies might be the result

of small population sizes, or the limited range of intake and relatively low fish consumption in the study populations investigated. However, inconsistencies similar to our findings have been reported in the literature. Some prospective epidemiological studies investigated the association between fish consumption and cancer and found inverse associations (11-15), whereas other studies have not (16-22). The same inconsistencies apply to studies using nutrient calculations from food frequency questionnaires from which n-3 PUFA intake is deduced. One case-control study showed an inverse association between estimated n-3 PUFA intake and colorectal cancer though exclusively in women (23), whereas two additional studies showed no association (24,25). Only one recent prospective study has employed serum measurements of fatty acids and found an inverse association between total n-3 PUFA, EPA and DHA content and colorectal cancer, also only in women (26). Recently, a meta analysis of 38 studies evaluating consumption of n-3 PUFAs and its effect on tumor incidence provided no evidence for a protective effect on any tumor type studied, including colorectal tumors (27).
Several animal and *in vitro* studies indicate more consistently that n-3 PUFAs suppress the development of cancer, as reviewed by Roynette et.al (28). It has been shown that diet supplementation with fish oil decreases tumor number in animals with chemically induced colorectal tumors (29) as well as in genetically engineered mouse models of intestinal cancer (Apc^{Min} and $Apc^{\Delta 716}$) (30,31). In search of a mechanism of action by which n-3 PUFAs might exert its effect on tumorigenesis, it has been shown that treatment of cancer cell lines with n-3 PUFAs induces apoptosis and suppresses cell growth (32,33). In humans, supplementation with fish oils containing n-3 PUFAs in patients with sporadic adenomatous polyps resulted in reduced intestinal hyperproliferation (34,35). In summary, these experimental data suggest a protective role of n-3 PUFAs against intestinal tumorigenesis, which has not been consistently confirmed in human observational studies (27).

Comparing fish consumption and n-3/n-6 PUFAs
A second remaining question concerning fish consumption and biomarkers of fish intake, arises from the discrepancy between the results obtained from the analysis with SNPs and fish consumption (Chapter 3), and the analysis with SNPs and n-3 PUFAs (Chapter 5). We found that fish consumption interacted with SNPs in *PPAR*δ and *PTGS2*, while n-3 PUFA levels did not. One can debate whether serum is the most appropriate medium for measuring fatty acids as a biomarker for dietary intake. It has been shown that adipose tissue is the most suitable medium for the reflection of long-term dietary intake (36). However, adipose tissue samples are difficult to obtain and might lead to reduced power due to small sample size because of non-responders. The most practical medium for fatty acid measurements is therefore considered to be serum, of which the cholesterol and phospholipids fractions reflect the dietary intake over the last few days (36). This time window might be representative for long-term intake however, as also supported by our observation of a clear correlation between fish consumption as assessed from food frequency questionnaires and n-3 PUFA serum levels. Therefore, the different results obtained from fish consumption and fatty acid analysis in sera could indicate that there may be another bioactive agent in fish that interacts with the genes in the AA pathway. Although it is far from clear which agent that might be, we hypothesize that vitamin D could be one of the candidate bioactive agents present in fish, as explained hereafter.
Vitamin D has been shown to be associated with colorectal cancer risk in several epidemiological studies which is supported by *in vitro* and animal studies. The epidemiological studies conducted so far that have solely considered diet as a source of vitamin D have been inconsistent. Studies also considering vitamin D supplements or those examining circulating levels of the intermediate metabolite $25(OH)D_3$ in serum are more consistent and most show significant decreases in colorectal cancer or adenoma risk among individuals with increased vitamin D intake or serum $25(OH)D_3$ levels, as reviewed by Grant

and Garland (37). Numerous animal studies have also implicated a role of vitamin D as a bioactive compound with a protective potential in intestinal tumorigenesis. Where supplementation with vitamin D in the diets of rodent models has been shown to decrease tumor numbers in the intestine, low vitamin D status resulted in increased tumor numbers (38).
Vitamin D is either ingested with the diet where it is present in fatty fish, meat and eggs, or as a precursor (pre-vitamin D_3) it can be produced endogenously in the skin after exposure to UVB. The actions of vitamin D are mediated by binding of its metabolite ($1,25(OH)_2D_3$) to the vitamin D receptor (VDR) which is part of the nuclear hormone-receptor superfamily and is present in normal colon as well as in colorectal tumor cells (39). Activated VDR forms a complex with the retinoic X receptor (RXR) which in turn binds to a vitamin-D response element (VDRE) present in the promoter of target genes, inducing their expression. Recently it has been shown that PPARδ is a direct target gene of activated VDR thereby causing increased expression of this gene in breast and prostate cells lines (40). Another study however has demonstrated that vitamin D inhibits β-catenin signaling which in turn leads to decreased expression of Wnt target genes, including *PPARδ* (41). Recently *PPARδ* has been identified as a focal point of crosstalk between prostaglandin and Wnt signaling pathways (42). How and if known effects of vitamin D on cell proliferation, differentiation and apoptosis are the results of interactions with these or other pathways has to be addressed in future research. Although the above mentioned studies are contradictory regarding the effect of vitamin D on *PPARδ* expression, they do indicate that there may be an interaction between vitamin intake and the *PPARδ* gene, which may account in part for the interaction found between a SNP in the *PPARδ* promoter and fish consumption. Whereas there is an interaction between vitamin D and *PTGS2* remains to be elucidated in future research. It should be noted that the role of vitamin D as active agent in fish is a hypothesis, and that other agents present at high concentrations in fish, e.g. selenium, might also be important.

N-6 polyunsaturated fatty acids
It has been widely accepted that n-3 PUFAs not only have a protective effect on colorectal cancer but also on cardiovascular disease. The health effects of n-6 PUFAs however are less unequivocal. It has been hypothesized that intake of PUFAs, including n-6 PUFAs, instead of saturated fatty acids, lowers LDL cholesterol and therefore reduces the risk of cardiovascular diseases (43). Although several studies have supported this hypothesis by investigating the association between total PUFAs and cardiovascular disease (43), the role of n-6 PUFAs in tumorigenesis is cause for concern. We have shown that an increased amount of LA results in an increased risk of colorectal adenomas as compared to low LA content (chapter 6). This is line with several studies indicating a tumor promoting role of n-6 PUFAs (44,45). LA is an essential fatty acid and therefore needs to be present in the diet. The conflicting effects of n-6 PUFAs on human health however, suggest that it should be present in limited amounts, and at a ratio with n-3 PUFAs closer to 1:1 instead of the current average of 15:1 (46).

NSAIDs
We have shown an inverse association between regular NSAID use and colorectal adenomas (OR, 0.75), which is further strengthened by the minor allele of SNP *c.-789C>T* in *PPARδ* (OR, 0.35) (Chapter 7). As with n-6 PUFA intake, the effect of certain NSAIDs on health status seems to be tissue specific. Whereas n-6 PUFAs seem to have a positive effect on the cardiovascular system, high amounts are also likely to be tumor promoting. In contrast, PTGS2 selective inhibitors, the so-called Coxibs, have been proven to be protective against colorectal tumors. However recent data about serious cardiovascular side-effects have resulted in the withdrawal of rofecoxib (Vioxx) off the market by its manufacturer Merck (47). Clearly, a more tailored advice aimed at individual circumstances

is necessary to obtain maximum efficiency and safety. This is also illustrated by several studies, including the study described in Chapter 7 of this thesis, describing the role of differences in genetic background between individuals in modulating the extent of the preventive effect of NSAIDs.

Gene-environment interactions and CRC

The above mentioned inconsistencies observed in genetic association studies might also be due to population heterogeneity between studies. In this respect, we must consider not only population admixture due to different ethnic backgrounds, but also differences in environmental or lifestyle factors (for example diet) that could influence the effect of the allele. Examples of interactions in relation to colorectal cancer found by two or more studies, although in most cases not consistently in all studies, have been summarized in table 2.

Table 2: Gene-environment interactions in relation to colorectal cancer

Gene symbol	Variant	Environmental factor	Nature of interaction	Ref
MTHFR	C677T	Folic acid	Homozygotes Val allele are at different risk with low folate intake	(48)
TS	High vs low expression	Folic acid	Decrease in risk due to increased folate intake only for allele conferring increased expression of *TS*	(49)
ADH3	γ2	Alcohol/Folic acid	Increased risk for γ2 allele in combination with high alcohol and low folate intake	(50)
NAT2	Rapid vs slow acetylator	Heterocyclic amines in cooked meat	Stronger association with red meat intake among rapid acetylators	(51)
mEH	High vs low activity	Meat intake	High activity in combination with well done meat increases risk	(52)
XRCC1	Arg194Trp and Arg399Gln	MUFAs	Arg/Arg and Glu/Glu only protective with low levels of MUFAs	(53)
VDR	Bsm1 and long vs short poly-A site	Calcium	Reduced risk with BB and SS genotypes only with low calcium intake	(54)
UGT1A6	Slow vs fast metabolizing	Aspirin	Increased benefit of aspirin use for slow metabolizers	(55)

The results from this thesis add to the associations and interactions found so far for candidate low penetrance alleles and environmental factors. We found an interaction between SNPs *c.-789C>T* and *N163N* in *PPARδ* and fish consumption that modified the risk of colorectal adenomas. Our original hypothesis was that n-3 PUFAs present in fish are converted into eicosanoids, including prostaglandins. Since prostaglandins act as ligands for PPARδ (56), fish consumption may interact with PPARδ by modifying the spectrum of PPAR ligands. This can be influenced by SNPs, explaining the interactions founds between SNPs in *PPARδ* and fish consumption. A third interaction was shown between SNP *c.2242T>C* in *PTGS2* which also modified adenoma risk. A high concentration of n-3 PUFAs, which could result from high fish consumption, has been shown to directly inhibit PTGS2 causing a decrease in the overall production of prostaglandins (57), as measured in mammary gland tissue. SNPs in *PTGS2* might interfere with these processes.

However, we were unable to replicate the interaction found for adenomas between the *PPARδ* and *PTGS2* alleles and fish consumption, for colorectal cancer. Neither were we able to show this interaction with n-3 PUFA levels measured in serum instead of fish

consumption in the same population. This does not necessarily mean that the observed interactions are false positive results, since, as previously discussed, n-3 PUFAs might not be the active agent in fish that interacts with these genes. Moreover, what is important in the early stages of tumor initiation might not be as important in tumor progression. It is clear that it is difficult to demonstrate gene-environment interactions in a consistent manner, and to establish interactions beyond reasonable doubt. Clearly, at present only a small proportion of cases can be explained by specific low penetrance alleles and their interaction with environmental factors, and more research is still needed.

Concluding remarks

In the studies described in this thesis we have been able to make a number of comparisons. First, we have been able to assess candidate low penetrance alleles within the AA pathway and their influence on colorectal adenoma risk, after which these candidates were evaluated for their role at later stages of tumor development. We found that the minor allele of *V102V* in *PTGS2* indeed influenced the risk of both adenomas and adenocarcinomas, thus suggesting that other low penetrance alleles in *PTGS2* and *PPAR*γ are only involved in modulating adenoma risk. Second, as previously discussed here (Chapter 5), we have also been able to compare fish consumption as a marker of n-3 PUFA intake with actual serum fatty acid analysis from the same subjects as a biomarker. We found that fish consumption correlates with n-3 PUFA content in serum, but their quantitative effects on adenoma risk are different. These comparisons add extra value as compared to other molecular epidemiology studies that only evaluate one endpoint or environmental (dietary) factor.

Although we have attempted the evaluation of specific gene-environment interactions, we have not considered gene-gene interactions simply because the number of individuals carrying a combination of alleles is too small to properly analyze the data. Therefore, we cannot rule out that some of the SNPs for which we could not demonstrate an association with colorectal adenomas or cancer, could still have an important influence on adenoma or carcinoma risk when combined with SNPs in other genes.

In conclusion, despite its limitations, this thesis represents a first attempt to identify low penetrance alleles in genes in the AA pathway, including their interactions with fish fatty acids and NSAIDs.

Future prospects

Currently, most genetic association studies employ the pathway-driven candidate gene approach, in which one or more candidate genes are chosen for their biological relevance in pathways important to the disease, and polymorphisms in these genes are tested for allele or genotype frequency differences between cases and controls. As technology improves and genotyping becomes affordable, the ultimate goal of whole genome association studies might become a realistic possibility. As the current total of deposited SNPs in public databases approaches 10 million, it is clear that for a whole genome scan a selection needs to be made to exclude redundant SNPs. At present there are no set rules for this but certain SNP characteristics can be taken into consideration. One could for example opt for selecting SNPs exclusively within coding regions, which would reduce the total number to around 20,000 SNPs, and increase the possibility of including SNPs with functional effects. It has been shown however, that evolutionary conservation within non-coding regions is as strong as within exonic sequences, thus indicating the putative functional relevance of SNPs within these regions (58,59). A more robust approach would be to capture the major variation within the genome by the creation of a haplotype map, which is currently underway (60). It has recently been shown that the human genome is organized in discrete haplotype blocks spanning tens to hundreds of kilobases bounded by recombination hotspots (61). Within the blocks little or no recombination occurs resulting in a limited number of common haplotypes (2 to 4), despite the large number of SNPs within each block. The haplotypes can be identified by genotyping only a small subset of the SNPs within a block, termed haplotype

tagging (ht) SNPs. It is estimated that approximately 300,000 htSNPs are sufficient to cover all of the common variation within the genome (62) but estimates of 100,000 to 200,000 htSNP have also been mentioned (60).
Although the whole-genome haplotype approach has the advantage that no prior hypothesis is needed about candidate genes or SNPs within them, and if strong risk or protective alleles exist it is likely that they, or a SNP in LD with the causal variant, are genotyped, some limitations are also evident. The so called common disease common variant (CDCV) hypothesis states that common variants are more likely to contribute substantially to common disease than rare variants (63), but opponents of this hypothesis warn that rare variants might have a detrimental effect on for example protein function and when these SNPs are not taken into consideration, substantial information is missed (64). Another point to consider is the effect of gene-gene interactions, which will be missed when the single alleles do not show a main effect on their own. It has even been suggested that genetic predisposition to sporadic cancer is the result of a network of interacting tumor susceptibility alleles (65), which will be virtually impossible to determine through genotyping alone, even if all the genetic variation in the network is included. New strategies to overcome this problem still need to be developed.
Another potential problem of a whole-genome scan is the reduction of power due to multiple testing. This problem becomes even greater when gene-gene and gene-environment interactions are also considered. Including prior biological knowledge about the gene or the effect of the SNP (Bayesian statistics) might help to make the associations more plausible, and in the same way, biologically credible interactions make statistical interactions more plausible. Nevertheless, small effects of single SNPs remain difficult to detect, and large studies are needed to generate sufficient power to reach statistical significance.

In the case of colorectal cancer, before whole genome studies become feasible, future genetic association studies should focus on candidate genes from important colorectal cancer pathways, such as Wnt-, TGF-β-, RTK driven ERK MAPK- and PI3K/AKT signaling pathways. The Wnt signaling pathway, which is deregulated in virtually every colorectal tumor, could be a prime target for such association studies. Moreover, one should not just consider the main players in this pathway, the *APC* and *β-catenin* genes, but also include genes encoding other proteins capable of modulating the levels of the β-catenin/TCF transcription complex, e.g. *GSK3-β* and *TCF-1*. Another major pathway involved in colorectal tumorigenesis is the transforming growth factor (TGF) β signaling pathway. The *SMAD* genes involved in TGF-β signaling are also promising target genes for genetic association studies, as well as the *TGF-β* receptor and specific TGF-β signaling target genes. One of the pathways that plays an important part in colorectal cancer progression is the receptor tyrosine kinase induced extracellular-signal-regulated mitogen-activated protein kinases (ERK MAPK) pathway, which could form the basis of a candidate gene association study. A pathway found to be activated in many cancers, including colorectal cancer, is the phosphatidylinositol 3-kinase (PI3K)/AKT pathway. This pathway provides a link between metabolic signals and other pathways (for example PGE_2 and the activation of PPARδ (66)), and is therefore another prime target for association studies. These pathways are only examples towards the identification of possible future candidates, and merely represent a selection of important colorectal cancer pathways which should also include pathways involving RAS, p53 and cytochrome p450, among others. At present, much research is aimed at identifying and refining pathways involved in colorectal carcinogenesis which may not only be useful in the identification of novel drug targets but may also act as candidate low penetrance genes for genetic association studies.
In the field of nutrigenomics in relation to colorectal cancer risk, where gene-diet interactions are evaluated, future efforts will be considerably improved once more knowledge on the effects on gene expression of specific food components is obtained. This might again be

achieved by micro-array experiments using genetically engineered mouse models of colorectal cancer, as well as human intervention studies .

Exciting recent research in the field of lipid metabolism has further linked two of the environmental factors investigated in this thesis, n-3 PUFAs and NSAIDs, with regard to inflammation. It is now hypothesized that increased n-3 PUFA intake not only results in decreased production of pro-inflammatory eicosanoids due to competition with AA , but also produces mediators with potent anti-inflammatory effects of their own (67). Moreover, new evidence suggests that acetylation of PTGS2 by aspirin does not only result in complete inhibition of the COX activity of the enzyme, but induces a conformational change resulting in a shift to a lipoxygenase function, producing potent lipoxins (68). This prompted Serhan and colleagues to study the effect of n-3 PUFA metabolites derived from acetylated PTGS2 on inflammation. They found that these mediators could resolve inflammation, and subsequently called them resolvins (69). An orphan receptor ChemR23 was identified as a specific receptor of the EPA derived resolvin RvE1, which inhibits activation of NF-κB by TNF-α, and is among other tissues also expressed in the gastrointestinal tract (70). It is likely that these mediators play an important role in reducing inflammation in the GI tract, and that n-3 PUFAs and aspirin exert their protective effect on colorectal cancer partly through this pathway. In relation to this thesis, it would be useful to analyze the combination of regular aspirin use and high levels of plasma n-3 PUFAs to test whether this has a greater protective effect then expected from simply adding the two effects. The modulating effects on PTGS2 of other NSAIDs should be evaluated, after which more n-3 PUFA derived mediators can be identified and assessed on their function on inflammation and colorectal cancer.

In conclusion, although together with other fields like animal modeling and *in vitro* analysis, the whole genome approach holds great promise in the identification of low penetrance alleles, this does not necessarily represent the ultimate goal every scientist should aim for. The immense costs and infrastructure needed to implement such a project makes it not affordable for most laboratories. With increased knowledge about interactions between specific pathways and the environment, a more educated selection of candidate genes can be made. Prior knowledge about gene function and regulation in normal and tumor cells, from for example expression profiling studies, will also aid in gene selection. We will also need larger sample sizes, implying collaboration among different laboratories, and improved statistical methods allowing correct interpretation the data. The time when we will unravel the role of all genetic and environmental factors and their interactions in the etiology of complex diseases might still be some distance away, but it is safe to assume that these studies will significantly contribute to the achievement of this goal.

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Nederlandse samenvatting

Nederlandse Samenvatting

Dikke darmkanker is één van de meest voorkomende soorten van kanker en is de oorzaak van meer dan 4000 sterfgevallen per jaar in Nederland. Net als bij andere soorten kanker het geval is, ontstaat een darmtumor in een stapsgewijs proces waarbij in tumorcellen steeds meer genen ontregeld raken door zowel genetische (DNA mutaties) als epigenetische (inactivering van genen via onder andere promoter hypermethylering) oorzaken. In het geval van darmkanker ontstaat er in een eerste fase een zogenaamd goedaardig adenoom, ook wel poliep genoemd. Deze poliep kan dan uitgroeien tot een kwaadaardig adenocarcinoom, die uiteindelijk in de laatste fase van het kankerproces kan uitzaaien (metastaseren). Het samenspel tussen (epi)genetische factoren en omgevingsfactoren, waaronder voeding, lijkt van invloed op zowel de eerste als de latere fasen in het ontstaan van darmkanker.
Bij erfelijke gevallen van darmkanker worden specifieke mutaties in bekende kankergenen overgedragen aan het nageslacht wat de kans op het verkrijgen van darmkanker sterk verhoogd. Deze mutaties kunnen echter maar een klein gedeelte van het aantal patiënten met een familiehistorie van darmkanker verklaren, en soms is het zelfs niet duidelijk of er meer darmkanker in de familie voorkomt dan gemiddeld. Het optreden van darmkanker in deze patiënten, maar ook bij de meerderheid van patiënten met 'sporadische' darmtumoren, kan wellicht verklaard worden door een combinatie van vele kleine variaties in ons DNA. Kortweg door de genetische verschillen tussen individuen. De meest voorkomende vorm van kleine verschillen in ons DNA zijn verschillen van een enkele nucleotide, ofwel single nucleotide polymorphisms (SNPs). Hierbij kunnen op een specifieke positie in het DNA twee verschillende nucleotiden voorkomen, resulterend in twee verschillende allelen. Deze SNPs vormen tegenwoordig vaak de basis voor genetische associatie studies. In deze studies wordt onderzocht of er een verschil bestaat in de frequentie van de allelen van een SNP tussen mensen met een ziekte (patiënten), en mensen zonder ziekte (controles).

Resultaten van zowel epidemiologisch als experimenteel onderzoek geven aan dat het metabolisme van vetzuren, in het bijzonder het arachidonzuur metabolisme, een belangrijke rol speelt bij het ontstaan van (dikke) darmkanker. Hieruit is de hypothese geformuleerd dat polymorphismen in genen op sleutelposities in dit metabolisme de gevoeligheid voor het ontstaan van darmkanker zouden kunnen beïnvloeden. In dit proefschrift is onderzocht wat de invloed is van SNPs in arachidonzuur metabolisme genen op de kans op het verkrijgen van darmkanker. De genen die geselecteerd zijn voor dit onderzoek betreffen een zevental kandidaatgenen die coderen voor eiwitten die onder andere betrokken zijn bij het omzetten van vetzuren uit het dieet naar prostaglandinen en leukotrienen (het arachidonzuur metabolisme), moleculen betrokken bij onder andere ontstekingsreacties en tumorvormende processen. Vetzuren uit ons dieet worden in eerste instantie omgezet naar fosfolipiden, die worden ingebouwd in celmembranen. Deze lipiden kunnen weer worden vrijgemaakt door de fosfolipase-A2 familie van enzymen. De genen *PLA2G2A* en *PLA2G4A* coderen twee vormen van deze enzymen. De vrijgekomen vetzuren worden daarna omgezet door cyclo-oxygenase enzymen (gecodeerd door *PTGS1* en *PTGS2*) in prostaglandinen en door lipoxygenase enzymen (onder andere ALOX15) in leukotrienen. Deze producten kunnen, naast de vetzuren zelf, fungeren als liganden voor twee nucleaire receptoren (PPARδ en PPARγ), die de expressie van andere genen kunnen regelen.

Behalve de invloed van genetische factoren op het risico op het ontstaan van darmkanker, zijn er een aantal risicofactoren die de kans vergroten, waaronder een Westerse voeding en leefwijze (darmkanker komt meer voor in Westerse landen). Dit laatste wordt geïllustreerd met roken, weinig lichaamsbeweging en overgewicht. Ook zijn er (voedings)factoren die beschermend kunnen werken, waaronder het eten van vis en regelmatig gebruik van

nonsteroide ontstekingsremmers (NSAIDs), waar aspirine en ibuprofen bekende voorbeelden van zijn. Deze twee factoren en de wijze waarop ze het risico op darmkanker kunnen beïnvloeden, al dan niet in combinatie met genetische variaties in genen betrokken bij het arachidonzuur metabolisme, zijn eveneens onderzocht in dit proefschrift.

Ten eerste hebben we de genetische variatie van de zeven kandidaatgenen in de vorm van SNPs in kaart gebracht voor de Nederlandse bevolking, door de basevolgorde van alle coderende en regulatoire gebieden te bepalen in het DNA van 100 willekeurig geselecteerde Nederlandse personen. Van de gevonden 58 variaties hebben we er 21 geselecteerd voor verder onderzoek. Vervolgens hebben we, in een cohort van ongeveer 400 patiënten met darmpoliepen en 400 controles (POLIEP cohort), de genotypen van de geselecteerde SNPs bepaald. Er zijn verschillen in de verdeling van genotype tussen patiënten en controles waargenomen voor een aantal SNPs. Een SNP in exon 3 van *PTGS2* kwam minder vaak voor in patiënten, en mensen die het minst voorkomende allel minstens één keer bevatten hebben een verlaagde kans op darmkanker van 35%. Een andere SNP in *PTGS2*, in een regulatoir gebied, resulteerde in een verhoging van het risico met ongeveer 50%. Deze SNPs hebben dus mogelijk invloed op de functie van het gen en zorgen ervoor dat het proces van omzetting minder goed of juist beter verloopt. Ook een SNP in *PPAR*γ gaf een verlaging in risico van ongeveer 35%. Eveneens werden verschillen waargenomen in distributie van combinaties van allelen, genaamd haplotypes, in *PTGS2*, *PLA2G2A* en *ALOX15*. Ook werd onderzocht of visconsumptie, als proxy (benadering) voor n-3 meervoudig onverzadigde vetzuren die omgezet worden door bovengenoemde enzymen, het effect van deze allelen en genotypes kon beinvloeden. Bij mensen met het meest voorkomende genotype van een SNP in *PPAR*δ was het risico verlaagd voor het ontstaan van darmkanker in combinatie met een relatief hoge consumptie van vis. Ook mensen met het meest voorkomende genotype van de eerder genoemde SNP in het regulatoire gebied van *PTGS2* hadden een sterk verlaagd risico als ze veel vis aten. Om te onderzoeken of de bovengenoemde associaties voor darmpoliepen (adenomen) ook in patiënten met dramkanker (adenocarcinomen) aanwezig zijn, werden de genotypen van dezelfde SNPs, en twee bijgevoegde SNPs, in twee cohorten (DOM en PPHV cohorten) met een totaal van ongeveer 600 patiënten en 800 controles bepaald. Ook in deze cohorten werd een associatie tussen de SNP in exon 3 van *PTGS2* en darmkanker gevonden, wat aangeeft dat deze SNP wellicht ook een rol speelt in de latere fase van progressie van darmpoliepen naar kwaadaardige tumoren. Er werden echter, waarschijnlijk door het kleine aantal patiënten (161) waar visconsumptie data van beschikbaar waren, geen interacties aangetoond tussen deze SNPs en visconsumptie.
Onze hypothese was dat de n-3 meervoudig onverzadigde vetzuren de darmkanker beschermende bestanddelen zijn in vis, en dat deze vetzuren een interactie aangaan met de eerder genoemde genen. Om dit te onderbouwen hebben we de n-3 en n-6 vetzuurgehaltes gemeten in het serum van de 400 patiënten met darmpoliepen en 400 controles. Hoewel de op deze wijze bepaalde vetzuurgehaltes representatief zijn voor de vetzuurinname van de laatste paar dagen, werd aangetoond dat serum n-3 vetzuren representatief waren voor visconsumptie zoals deze was aangegeven door de deelnemers zelf in een voedselvragenlijst. Bovendien leek een verhoogd n-3 vetzuurgehalte beschermend te werken tegen darmpoliepen, terwijl een verhoogd n-6 vetzuurgehalte, met name in de vorm van linolzuur, een verhoogd risico op darmpoliepen teweeg bracht. De n-3 vetzuren hadden echter niet dezelfde interactie met de SNP in *PPAR*δ als visconsumptie. Een van de mogelijke verklaringen hiervoor kan zijn dat de actief beschermende bestanddelen in vis, die een interactie aangaan met *PPAR*δ en *PTGS2*, niet de n-3 meervoudig onverzadigde vetzuren zijn. Desondanks is uit andere metingen gebleken dat de verhouding van n-3/n-6 vetzuren een informatieve parameter kan zijn voor het risico op darmkanker. Gemeten waardes van deze vetzuren in vetweefsel afkomstig van een kleiner aantal patiënten met

darmpoliepen en controles liet zien dat een hoge n-3/n-6 verhouding resulteerde in een verlaging van het risico op darmkanker met 80%.
De 400 patiënten met darmpoliepen en controles uit de POLIEP studie hadden aangegeven op hun vragenlijst hoeveel NSAIDs ze slikten, en het bleek dat mensen die meer dan 12 keer per jaar een tablet slikten, 25% minder kans op het krijgen van darmkanker hadden. Dit resultaat bevestigd wat al eerder gevonden is in andere studies en populaties. De SNP in *PPARδ*, die ook een interactie liet zien met visconsumptie, moduleert ook het effect van NSAIDs op darmpoliepen. Mensen met de minst voorkomende genotypen waren extra beschermd als ze regelmatig NSAIDs slikten, terwijl deze mensen juist een verhoogd risico hadden als ze weinig slikten. Deze resultaten suggereren dat deze SNP zich op een voor *PPARδ* belangrijke positie in de promoter bevindt.
Samenvattend kan worden geconcludeerd dat sommige genetische polymorphismen in een aantal genen betrokken bij het arachidonzuur metabolisme van invloed lijken op het ontstaan van darmkanker, zowel in vroege als latere fasen. Met name allelen in *PPARγ*, *PLA2G2A, ALOX15* en *PTGS2* lijken van invloed op het risico voor het ontstaan van darmpoliepen. Hetzelfde *PTGS2* allel lijkt ook van invloed op het risico voor darmkanker. In combinatie met de interacties die werden aangetoond tussen een SNP in *PPARδ* en visconsumptie en gebruik van NSAIDs, ondersteunt dit zowel nader onderzoek naar de functionaliteit van deze SNPs als onderzoek naar bevestiging van de in deze studie aangetoonde associaties in veel grotere cohorten.

Curriculum Vitae

Curriculum Vitae

Christine Louise Ernestine werd op 8 maart 1974 geboren te Schagen. In juni 1992 behaalde zij het VWO diploma aan de Rijks Scholengemeenschap in Schagen. Na een oriëntatie en reis periode van drie jaar startte zij in 1995 met de studie Biomedical Sciences aan de University of Wolverhampton in Engeland. Tijdens deze opleiding heeft zij een stage gelopen bij het Regional Genetics laboratorium van het Women's Hospital in Birmingham, Engeland. Tijdens deze stage heeft zij onderzoek gedaan naar het verband tussen de ziekten hemochromatose en type II diabetes, door te onderzoeken of er een relatie is tussen hemochromatose gen (HFE) mutaties en type II diabetes. In mei 2000 behaalde zij een first class honours Bachelor of Science degree, en in september van datzelfde jaar startte ze als Assistent in Opleiding bij het Laboratorium voor Toxicologie, Pathologie en Genetica van het RIVM te Bilthoven, in samenwerking met de afdeling Humane Genetica van de Universiteit Leiden en later met de afdeling Experimentele Pathologie van de Erasmus Universiteit Rotterdam. Tijdens dit promotie onderzoek heeft zij de genetische aanleg voor dikke darmkanker en de invloed van bepaalde omgevings-(voedings)factoren hierop bestudeerd. Dit onderzoek vormt de basis voor het huidige proefschrift. Op dit moment is Christine Siezen werkzaam als wetenschappelijk onderzoeker op het RIVM, op de afdeling Toxicologie, Pathologie en Genetica.

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