Fish consumption, does it beneficially affect markers of colorectal carcinogenesis?

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Thesis

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To my parents

Abstract

Background: Fish consumption is possibly associated with a decreased risk of colorectal cancer, as has been shown in several observational studies. However, most of these studies did not discriminate between the effects of oil-rich and lean fish. To date, no randomized controlled trials (RCTs) have examined the possible beneficial effects of fish intake on colorectal cancer risk.

Aim: The aim of this thesis was to investigate whether fish consumption beneficially affects markers of colorectal carcinogenesis.

Methods and Results: In a case-control study (363 cases, 498 controls), we studied the association of serum n-3 polyunsaturated fatty acid (PUFA) levels as a marker of oil-rich fish intake with colorectal adenomas, a precursor lesion of colorectal cancer. We found that individuals with high serum long chain n-3 PUFA levels had a decreased risk of colorectal adenomas (odds ratio (OR) 0.67, 95% confidence interval (CI) 0.46; 0.96), whereas individuals with high serum n-6 PUFA levels had an increased risk of colorectal adenomas (OR 1.68, 95% CI 1.17; 2.42).

In an RCT, we studied the effects of 3.5g/d fish oil (~1.5g/d n-3 PUFA) for 12 weeks on 19 serum inflammation markers in 77 healthy subjects and found that serum levels of these cytokines and chemokines were not changed.

Finally, we studied the effects of increasing fish consumption compared with no additional fish, on markers of colorectal carcinogenesis in an RCT. Subjects (n=242), at an increased risk of colorectal cancer and those with no macroscopic signs of disease, were randomly allocated to receive dietary advice (DA) plus either two additional weekly portions of oil-rich fish (salmon, ~1.4g/d n-3 PUFA) or lean fish (cod, ~0.09 g/d n-3 PUFA), or only DA for six months. We observed no change in apoptotic and mitotic cell numbers after the 6-months intervention with either salmon or cod compared with DA. Furthermore, colorectal genotoxicity, levels of cytokines and chemokines in colonic biopsies and feces, and fecal calprotectin were also not markedly changed after fish consumption. Only serum C-reactive protein (CRP) levels were statistically significantly decreased after consumption of salmon (-0.5 mg/l, 95% CI -0.9; -0.2) and cod (-0.4 mg/l, 95% CI -0.7; 0.0) compared with DA.

Conclusion: The results of this thesis do not provide strong evidence for beneficial effects of fish consumption on markers of colorectal carcinogenesis.

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1 Introduction

Background

Currently, cancer is the leading cause of death in the Netherlands and colorectal cancer (CRC) is one of the most prevalent types of cancer in the Netherlands and other Westernized countries [1]. The incidence of CRC is particularly high in countries with a typical Western lifestyle, such as Europe and North America, which implies that lifestyle factors and more specifically diet, are important factors in the development of CRC. Of all environmental factors which appear to play a role in the etiology of CRC, diet and physical activity seem to be most important [2]. One of the dietary factors that is associated with a reduced CRC risk is the consumption of fish.

In the next paragraphs, the development and progression of CRC is briefly described, as well as the possible mechanisms of how n-3 polyunsaturated fatty acids (PUFA) and other fish constituents could affect markers of colorectal carcinogenesis. This is followed by the current evidence from observational studies and human intervention trials on fish consumption and colorectal cancer risk, and closes with a short introduction to the chapters of this thesis.

Colorectal carcinogenesis

To understand the process of colorectal carcinogenesis, it is important to be aware of the normal processes in the colon. The colonic mucosa is completely renewed every 5-7 days [3]. Renewal occurs by cell growth, or cell proliferation, in the lower parts of the colonic crypt while cells are shed at the top of the crypt by cell death, or apoptosis [4] (**Figure 1.1**). Gastrointestinal epithelial cell homeostasis is maintained by the balance between cell growth and cell death.

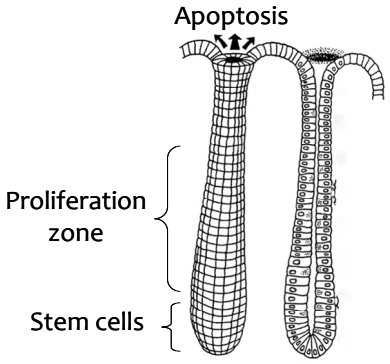

Figure 1.1 Schematic overview of a colonic crypt, derived from [5].

Disruption of this balance contributes to colorectal carcinogenesis. Regulation of this balance is controlled by several types of genes, including oncogenes and tumor suppressor genes, and factors in the cellular environment that influence their expression. In this process, maintenance of the DNA sequence and structure is important, though gene expression can also be altered without changing the DNA sequence, which is called epigenetic modulation. Colorectal carcinogenesis may gradually progress over several decades (10- 40 years) and is considered to be a multi-stage process, with multiple underlying molecular and (epi)genetic alterations [6]. These alterations could lead to a growth advantage and clonal expansion of the altered cells. This growth advantage is stimulated by increased cell proliferation, and by decreased apoptosis. More specifically, an increase in cell proliferation or mitosis indicates a shorter cell cycle time, thereby decreasing time for cells to repair any replication errors. In contrast, lower levels of apoptosis indicate slower removal of damaged cells.

Subsequently, this growth advantage can lead to the development of benign adenomatous polyps [7]. Over time, adenomas can grow and become more disorganized and eventually some (about 15%) may develop into carcinomas [8]. This process is called the adenoma-carcinoma sequence (**Figure 1.2**) [9,10].

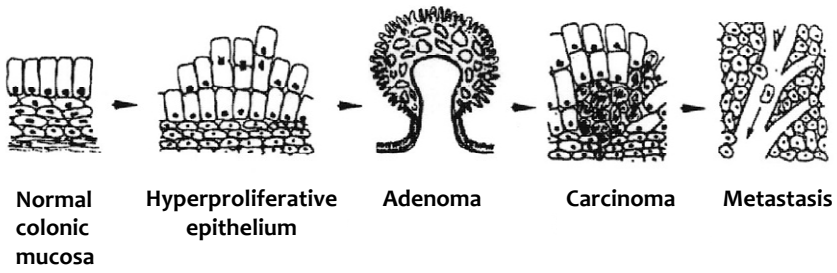

Figure 1.2 Adenoma-Carcinoma sequence derived from [10].

In addition, the presence of prolonged or chronic, low-grade inflammation in the gut could indicate an increased risk of CRC [11-15]. Chronic inflammation can create a local tissue microenvironment where reactive oxygen and nitrogen species released from inflammatory cells could cause malignant DNA alterations, or promote tumor growth [13,16-18]. Patients with chronic inflammatory bowel disease (ulcerative colitis or Crohn's disease) have an increased risk of developing CRC [19-21].

Colorectal cancer may be caused by many factors, both exogenous as well as endogenous. As early as 1981, Doll and Peto estimated that between 10-70% of CRC could be attributed to diet [22], and this has subsequently been adjusted to 65-75% [23]. The World Cancer Research Fund (WCRF) and the American Institute for Cancer Research (AICR) have recently systematically reviewed all literature on diet and cancer and found

convincing evidence that several dietary factors increase CRC risk, namely red and processed meat intake, and consumption of alcoholic drinks, especially in men [2]. In addition, increased body fat and abdominal fat is associated with an increased CRC risk and increased levels of physical activity are related to a decreased CRC risk [2,24,25]. Another dietary factor that could beneficially affect colorectal carcinogenesis is the consumption of fish [2,26].

In summary, the process of colorectal carcinogenesis is a multi-stage and multi-factorial process. Most important factors that affect colorectal cancer risk include diet and physical activity, and one of the dietary factors that could decrease colorectal cancer risk is consumption of fish.

Potential mechanisms how fish consumption reduces colorectal cancer risk

Fish contains a number of constituents, including n-3 polyunsaturated fatty acids (PUFA), also called omega-3 fatty acids, vitamin D, selenium, and proteins, which could potentially reduce CRC risk through a variety of mechanisms.

N-3 PUFA

Fish is the major source of eicosapentaenoic acid (EPA; C20:5n-3) and docosahexaenoic acid (DHA; C22:6n-3), which are considered the most important n-3 PUFA in terms of nutritional benefits. In Europe, the average intake of EPA + DHA varies between 50-950 mg per day [27]. In the Netherlands, the general current recommendation is to consume at least 450 mg n-3 PUFA from fish per day [28].

The human body cannot synthesize n-3 PUFA *de novo,* but it can form EPA and DHA from the 18-carbon n-3 PUFA alpha-linolenic acid (ALA; C18:3n-3) through processes of desaturation and elongation. Hence the term *semi*-essential fatty acid is applicable for EPA and DHA. In healthy individuals, only 5-10% of ALA is converted into EPA, and 1-5% of ALA is converted into DHA; women and children seem to be more efficient in this conversion than men [29-33]. The conversion of ALA into EPA and DHA depends on the amount of other fatty acids consumed [34] and occurs in competition with n-6 PUFA, as the same enzymes are used for elongation and desaturation (**Figure 1.3**).

N-3 PUFA can affect colorectal carcinogenesis in a number of ways. Firstly, the effects of n-3 PUFA on CRC risk may be mediated by the eicosanoid pathway, which involves the cyclooxygenase (COX) enzymes. COX-2 is known to play a major role in the development and progression of CRC, by promoting cell survival, cell growth, migration, invasion and angiogenesis [35-37]. Also, n-3 PUFA lead to less potent pro-inflammatory eicosanoid metabolites, such as prostaglandin E3 (PGE3) and leukotriene B5 (LTB5) compared with n-6 PUFA derived eicosanoids, such as prostaglandin E2 (PGE2) and leukotriene B4 (LTB4) (Figure 1.3), which could indirectly decrease CRC risk.

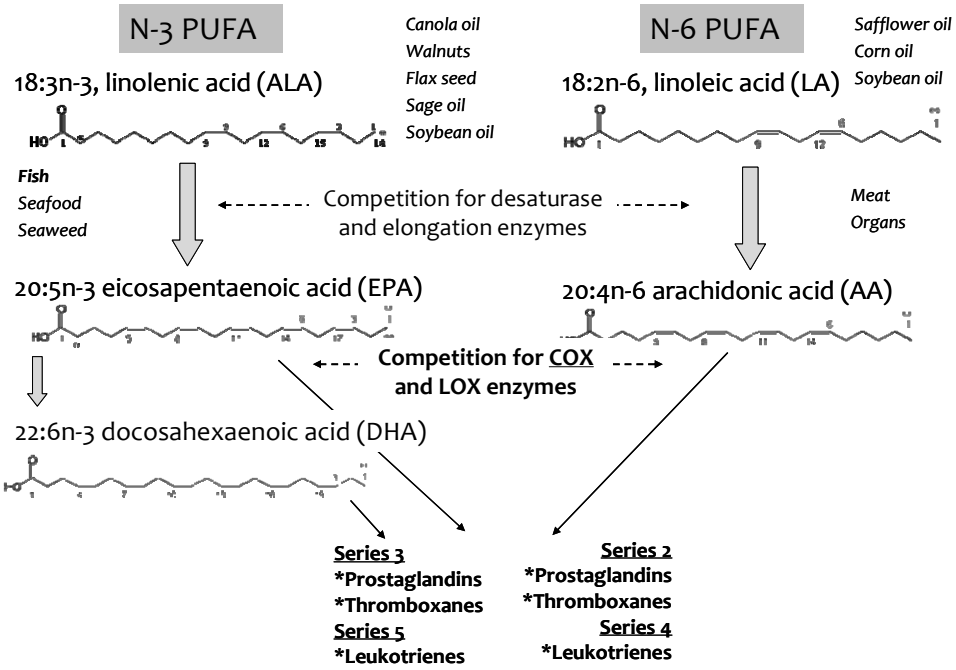

Figure 1.3 Conversion of n-3 and n-6 PUFA adapted from [38,39].

Secondly, n-3 PUFA could affect intracellular pathways which can lead to the activation of one or more transcription factors such as nuclear factor kappa beta (NF-κβ), which plays a key role in regulating the immune response, or peroxisome proliferator-activated receptors (PPARs) [40], which play a role in lipid metabolism and cellular differentiation. Thirdly, n-3 PUFA could affect oxidative stress, which in its turn can induce cell proliferation or apoptosis, depending on the level of oxidative stress; with mild oxidative stress probably leading to cell proliferation whereas substantial levels of oxidative stress could lead to apoptosis [41,42]. Lastly, it has also been argued that n-3 PUFA could affect genotoxicity, since n-3 PUFA are more readily oxidized and could enhance lipid peroxidation [17,43], which ultimately could cause endogenous DNA damage under oxidative stress [44].

Other nutrients present in fish

Besides n-3 PUFA, fish contains other nutrients that could have beneficial effects on CRC risk, such as vitamin D, selenium, or protein.

The D of vitamin D originally was derived from the German word '*Dörschleberöl*', which means cod liver oil, indicating the historical link between vitamin D and seafood. The intake of fish contributes 9-12% to the daily vitamin D intake in the Netherlands and is mainly derived from fatty fish [45]. In the Netherlands, the current recommendation is to

consume 2.5-5 mcg/d vitamin D [28]. Several epidemiological studies have shown that vitamin D could reduce CRC risk [2,46,47]. Vitamin D can have anti-proliferative and pro-differentiation effects in some cells, mediated by the vitamin D receptor, which could reduce CRC risk [2,48,49]. Potential anti-cancer properties are mainly attributed to the hormonal form of vitamin D, calcitriol. Vitamin D can induce differentiation and apoptosis in intestinal cells and thereby influence growth [2].

Fish also contains the trace element selenium (Se), varying from 20-45 mcg/100g fish in both lean and oil-rich fish [50]. However, fish only contributes a minimal amount to the total daily intake with plant foods accounting for more than 90% of daily intake [45]. The current recommendation in the Netherlands is to consume at least 50-150 mcg/d Se [28]. Inverse associations of selenium with colorectal cancer or adenomas have been found in a few observational studies [51,52] and one randomized controlled trial (RCT) with colorectal mortality as outcome [53]; however, the evidence thus far is sparse [2]. Selenium could affect colorectal carcinogenesis by altering phase I and II enzymes leading to reduced DNA adduct formation, and by inhibiting cell proliferation and inducing apoptosis [54-56]. Moreover, selenium has been found to reduce COX-2 protein levels and PGE2 levels *in vitro* [57].

Fish is also a source of high quality proteins. Fish protein contains lysine, the sulfur-containing amino acids cysteine and methionine, and glutamic acid [58]. Cysteine and methionine can improve the anti-oxidant status of the body [59], modulate nitric oxide (NO) synthase and NO production [60], and can affect the inflammatory response [61-63], which consequently may reduce CRC risk. Moreover, cysteine analogues can induce apoptosis [59]. Fish proteins are readily digested which can contribute to formation of less nitrogen in the colon. A reduction in N-fermentation in the colon is associated with a reduction in phenols and indols, which could also reduce inflammation by inhibiting COX [64,65].

In summary, the consumption of fish could beneficially affect colorectal carcinogenesis in various ways which are schematically summarized in ***Figure 1.4****.*

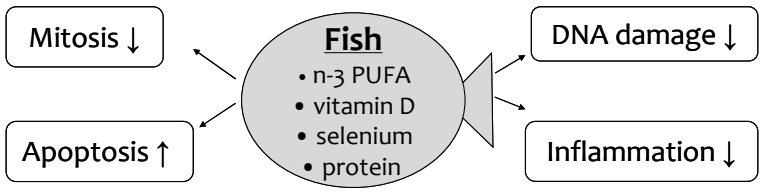

Figure 1.4 How fish can beneficially affect processes related to colorectal carcinogenesis: a schematic overview adapted from [2].

Fish consumption and colorectal cancer: evidence so far

The first suggestions that fish consumption could be related to a decreased CRC risk were derived from ecological studies, demonstrating that mortality and incidence of CRC were lower in populations consuming large amounts of fish, for example Inuit or Japanese people [66-71]. Moreover, migrant studies showed that the incidence of CRC dramatically increased in Japanese people who relocated to the United States, compared with those who stayed in Japan [72]. This difference may be related to a change in diet, including a possible reduction in fish consumption, or an increase in red meat consumption, both of which could increase CRC risk [2]. In recent years, Japan has been adopting more Western-style dietary habits, which has been reflected by an increase in the incidence of CRC in this population [73]. Conversely, not all ecological studies have found an association between fish consumption and colorectal cancer mortality [74-76] and colorectal cancer incidence [77,78].

Results from 17 case-control studies investigating the association between fish consumption and colorectal cancer also showed conflicting results: nine studies confirmed this beneficial effect of fish consumption on CRC risk [68,79-86] while eight other case-control studies did not observe this [87-94]. Additionally, the results of prospective cohort studies were also not equivocal. Recently, two meta-analyses were performed [25,26], both showing an inverse association of fish consumption and CRC risk. Geelen et al found a relative risk (RR) of 0.88 (95% confidence interval (CI) 0.78; 1.00) comparing the highest quartile of fish consumption with the lowest (~70g/d difference between the highest and lowest quartile) [26].

A general limitation of case-control and prospective cohort studies is that many of these studies were not specifically designed to study the association between fish intake and CRC risk, which could have affected the study outcomes in different ways. In many studies, the intake of fish is assessed by a food frequency questionnaire and often no distinction is made in the type of fish consumed, nor are other factors related to fish consumption taken into account, such as preparation of the fish. Observational studies are also limited by the fact that they cannot establish a causal relationship. This requires randomized controlled trials (RCTs) that are considered the 'gold standard' to provide causal evidence [95].

To date, no RCTs have been performed on fish consumption and markers of colorectal carcinogenesis, however three RCTs have studied the effects of fish, either salmon or cod, on inflammation markers [63,96,97]. These studies found that CRP levels were decreased after cod consumption [63] but not after salmon consumption [96]. Levels of cytokines were decreased after salmon consumption [96,97], but not after cod consumption [63].

Further evidence from RCTs is restricted to RCTs studying the effects of specific fish components, mainly n-3 PUFA. In **Tables 1.1** & **1.2** the effects of fish components on intermediate markers of colorectal carcinogenesis are summarized.
Eleven RCTs studied the effects of n-3 PUFA on apoptosis and mitosis, showing conflicting results: six studies demonstrated a reduction in cell proliferation [98-101] and an increase in apoptosis [99,102] in response to fish oil supplementation, whilst five other studies failed to show any effect [103-107] (**Table 1.1**). The studies varied in study duration (1-6 months), doses of n-3 PUFA (ranging from 2-8g/d EPA +DHA), type of subjects included (healthy, colorectal adenoma or carcinoma patients). However, not one feature could explain the observed differences in results.
Many RCTs have been conducted on n-3 PUFA and inflammation; an overview of 22 RCTs is given in **Table 1.2**. Four RCTs showed an effect of n-3 PUFA on all inflammatory markers studied [108-112], three studies showed an effect on a limited number of inflammation markers studied [113-115], whilst fifteen studies demonstrated no effect whatsoever [116-129]. The different doses (varying from 0.3-8 g/d n-3 PUFA) and study durations (varying from 4-52 weeks), the different populations (men/women, healthy vs. diseased), and the numerous different markers of inflammation studied (serum markers, ex vivo stimulated markers), could have contributed to these inconsistent results.
To the best of our knowledge, no RCTs on vitamin D, Se, or protein and apoptosis, mitosis or inflammation have been performed, other than two studies supplementing vitamin D in combination with calcium that showed no effect on cell proliferation [130,131] (Table 1.1).

In summary, the evidence on fish consumption and colorectal cancer risk is restricted to observational studies, showing limitedly suggestive evidence that fish consumption is associated with reduced colorectal cancer risk. Randomized controlled trials using fish components showed conflicting results. Thus, an RCT studying the effects of fish consumption on markers of colorectal carcinogenesis is needed.

Rationale and outline of this thesis

The overall aim of this thesis was to study whether fish consumption can beneficially affect markers of colorectal carcinogenesis.

The evidence from observational studies on fish consumption and CRC thus far suggests that fish consumption is associated with a reduced CRC risk, but the evidence is limitedly suggestive and not equivocal. Therefore, we used serum n-3 PUFA levels as biomarker of fish intake, in combination with serum n-6 PUFA levels, to study the association with colorectal adenomas as precursor lesion of CRC, in a case-control study (**Chapter 2**).

One of the potential mechanisms by which n-3 PUFA could reduce CRC risk is by favorably affecting inflammation. Several RCTs on n-3 PUFA and inflammation markers have been conducted, although the evidence thus far is inconclusive and no RCT included a large panel of inflammation markers, or their interrelationships. Therefore, we have studied the effects of fish oil supplementation on a large panel of serum inflammation markers in an RCT (**Chapter 3**).

To answer the question whether increasing fish consumption beneficially affects markers of colorectal carcinogenesis, we performed an RCT on fish consumption and several intermediate markers of colorectal carcinogenesis. We included oil-rich and lean fish to also study whether different types of fish lead to differential effects on markers of colorectal carcinogenesis. **Chapter 4** describes the results of the effects of fish consumption on the CRC risk markers apoptosis and mitosis in colonic crypts. The effects of the fish intervention on markers of genotoxicity in the colon are presented in **Chapter 5**. **Chapter 6** describes the effects of the fish intervention on inflammatory markers in serum, feces, and colonic tissue. Finally, in **Chapter 7** the main outcomes and implications are discussed.

Table 1.1 Overview of randomized controlled trials (RCTs) on n-3 PUFA & vitamin D and effects on apoptosis and mitosis. No RCTs on selenium or fish protein and apoptosis and mitosis have been performed.

Reference	Population	Exposure	Duration	Endpoint (method)	Main results
n-3 PUFA					
Courtney 2007 [99]	Colorectal adenomas patients (n=30), UK	2g/d EPA , purified	3m	Cell proliferation (Ki67) Apoptosis (M30)	Significantly reduced Significantly increased
Cheng 2003 [102]	Patients polypectomized for adenomas/tumors (n=41), Japan	Fish advice + 100mg EPA + 400mg DHA/d	2y	Cell proliferation (Ki67) Apoptosis (TUNEL)	No effect Apoptosis affected after 2y, not 1y
Gee 2001 [103]	Patients diagnosed with left-sided colon carcinoma (n=7), UK	2.4g/d n-3	7-21d	Apoptosis (morphological)	No effect
Gee 1999 [104]	Patients undergoing surgery for colonic carcinoma (n=49), UK	1.4g EPA+ 1.0g DHA/d	12.3 ± 0.5d	Mitosis (morphological)	No effect
Huang 1996 [107]	Patients stage 1 or 2 colon carcinoma or adenomatous polyps (n=27), US	9 g/d n-3 PUFA	6m	Cell proliferation (BrdU)	No significant effect Only in those with abnormal baseline
Bartram 1995 [105]	Healthy people (n=12), Germany	4.4 g/d n-3 PUFA	4wk	Cell proliferation (BrdU)	No significant effect
Anti 1994 [98]	Patients with polyps (n=15), Italy	1.4g EPA+ 1.1g DHA/d	6m	Mitosis ([3H] thymidine labelling in paraffin sections, labelling index)	Significant decrease (NB only within groups)
Anti 1994 [98]	Patients with sporadic adenomas (n=60), Italy	1.4g EPA+ 1.1g DHA/d 2.7g EPA + 2.4g DHA/d 4.1g EPA + 3.6g DHA/d	30d	Mitosis ([3H] thymidine labelling in paraffin sections, labelling index)	Only significantly changed in those with abnormal baseline levels
Bartram 1993 [101]	Healthy people (n=12), Germany	4.4g/d n-3	4wk	Cell proliferation (BrdU)	Significant decrease
Bartoli 1993 [100]	High risk population CRC (n=40), Italy	2.5g EPA+DHA/d 5.1g EPA+DHA/d 7.7g EPA+DHA/d	30d	Cell proliferation ([3H] thymidine labelling in paraffin sections)	Significant effect of n-3 PUFA most pronounced in highest dose
Anti 1992 [106]	Patients sporadic adenomas (n=20), Italy	4.1g EPA + 3.6g DHA/d	12wk	Mitosis ([3H] thymidine labelling in paraffin sections, labelling index)	No significant differences between groups
Vitamin D					
Holt 2006 [130]	Polyp patients (n=19), US	1500 mg x3/d calcium plus 400IU vitamin D3	6 m	Proliferation (MIB-1 staining), apoptosis (TUNEL) in normal tissue and polyp tissue	Normal tissue no effect Polyp tissue no treatment effect
Holt 2002 [131]	Polyp patients (n=39), US	1250mg calcium, or Ca with 400IU 1.25-OH vitamin D3,	6m	Cell proliferation (Ki67)	No effect

Abbreviations: wk (week), y (year), d (day), PUFA (polyunsaturated fatty acid), EPA (eicosapentaeonic acid), DHA (docosahexaenoic acid), UK (United Kingdom), US (United States), BrdU (Bromodeoxyuridine), TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labelling), IU (international unit)

Table 1.2 Overview of randomized controlled trials (RCTs) on n-3 PUFA or fish protein and inflammation. No RCTs using vitamin D or selenium and inflammation have been performed.

Reference	Population	Exposure	Duration	Endpoint (method)	Main results
n-3 PUFA					
Kelley 2009 [108]	Hypertriglyceridic men (n=34), aged 39-66, US	7.5g/d DHA oil	90d	CRP (high sensitivity (hs)) Cytokines circulating serum	CRP reduced IL-6 reduced
Yusof 2008 [109]	Healthy middle aged men (n=21), UK	1.8g EPA + 0.3g DHA/d	8wk	Plasma IL-6, E-selectin, sICAM-1, sVCAM-1 (ELISA), and CRP (hs)	sICAM-1 reduced rest not affected
Browning 2007 [110]	Women (n=30), UK	1.3g EPA + 2.9g DHA/d	12wk	Serum cytokine production: IL-6, TNF-a (PBMC supernatant, ELISA), CRP (hs)	IL-6 reduced TNF-a non-significant decrease CRP < within group
Fujioka 2006 [116]	Middle aged men and women, normal to high triglyceride (n=214), Japan	0.6g EPA + 0.26g DHA n-3 enriched foods/d	12wk	Plasma CRP (hs) Plasma soluble TNF-receptor 1&2 (ELISA)	No changes in CRP or TNF-receptor 1&2
Mehra 2006 [113]	Patients with heart failure (n=14), US	8 g/d n-3 PUFA	18wk	Cytokine production: TNF-a, IL-1 (Ex vivo PBMC LPS stimulated, ELISA)	TNF-a reduced IL-1 not affected
Murphy 2006 [117]	Apparently healthy men and women (n=30), Australia	173 mg /d n-3 PUFA 199 mg/d n-3 PUFA	6wk	Cytokine production: IL-1 beta, TNF-a (ex vivo monocyte, ELISA)	No changes in any marker
Rees 2006 [118]	Young and older men (n=169), UK	1.35, 2.7 or 4.05 g/d EPA	12wk	Cytokine production: TNF-a, IL1b, IL6 (ex vivo PBMC, LPS stimulated)	Ex vivo No effect with any dose on TNF-a, IL1b, IL6
Geelen 2004 [129]	Men and post-menopausal women (n=84), NL	1.5g/d n-3 PUFA	12wk	CRP (hs)	No effect
Kew 2004 [119]	Healthy adults (n=42), UK	4.7g EPA or 4.9g/d DHA oil	4wk	Cytokine production: TNF-a, IL-1b, IL-4, IL-5, IL-6, IL-8, IL-10 , IFN-g (ex vivo PBMC, LPS or ConA stimulated)	No effect
Miles 2004 [120]	Men (n=74), UK	2.0g/d EPA	12wk	Cytokine production : TNF-a, IL-1b IL-2, IFN-g, IL-4, IL-10	No effect
Kew 2003 [121]	Men and women (n=180), UK	1.7g/d EPA+DHA	26wk	Cytokines IL2, IL4, IFN-g or LPS-stimulated TNF-a, IL1b, IL6,IL10 (ex vivo, PBMC, LPS stimulated)	No effect on IL2, IL4, IFN-g or LPS stimulated TNF-a, IL1b, IL6,IL10

Table 1.2 (Cont) Overview of randomized controlled trials (RCTs) on n-3 PUFA or fish protein and inflammation. No RCTs using vitamin D or selenium and inflammation have been performed.

Reference	Population	Exposure	Duration	Endpoint	Main results
Trebble 2003 [122]	Men (n=16), UK	0.3, 1.0 and 2.0 g/d EPA +DHA/d	5-12wk	Cytokine production TNF-a, IL-6, IFN-g (ex vivo)	No significant effects
Wallace 2003 [123]	Adults (n=40), UK	0.5g EPA + 1.4g DHA/d	12wk	Cytokine production : TNF-a, IL-1b, IL-2, IL-4, IL-10, IFN-g (ex vivo, whole blood, LPS or ConA stimulated)	No effect
Grimble 2002 [124]	Men (n=111), UK	1.3 g EPA + 0.5g DHA/d	12 wk	TNF-a (ex vivo, PBMC, LPS stimulated)	No effect
Hawkes 2002 [125]	Pregnant women (n=120), Australia	0.07g EPA+ 0.3g DHA/d 0.14g EPA + 0.6g DHA/d	12wk	Cytokine production: TNF-a, IL-1b, IL-6 (in vivo, HMC, PBMC LPS stimulated)	No effect
Thies 2001 [126]	Healthy people (n=48), UK	0.7g EPA+ 0.3g DHA/d	12wk	Cytokine production: TNF-a, IL-1b, IL-6 (ex vivo PBMC, LPS stimulated, ELISA)	No effect
Yaqoob 2000 [127]	Healthy men and women (n=40), UK	2.1g EPA + 1.1g DHA/d	12wk	Cytokine production: IL-1a, IL-1b, IL-2, IL-10, TNF-a, IFN-g (PBMC and whole blood)	All no significant effect
Kelley 1999 [114]	Men aged 20-40 (n=11), US	2.8 energy% n-3 PUFA	13wk	Cytokine production: IL-1b, TNF-a (ex vivo, PBMC, LPS stimulated)	Production IL1b reduced Production TNF-a reduced (~45%)
Blok 1997 [128]	Healthy men (n=58), Belgium	0, 1.1, 2.1, or 3.2g/d n-3 PUFA	52wk	Cytokines in serum (IL-6) and production (IL-1b, TNF-a, IL-1Ra) (in vivo ELISA, ex vivo whole blood, LPS stimulated)	No effect
Wigmore 1997 [111]	Cancer cachexia patients (n=61), Germany	EPA		CRP (hs) IL-6 (PBMC, LPS stimulated)	CRP reduced IL6 by PBMC reduced
Caughey 1996 [112]	Healthy people (n=30), Australia	1.6g EPA+ 1.1g DHA/d	4wk	TNF-a, IL-1beta (ex vivo, ELISA)	TNF-a reduced IL-1beta reduced
Cooper 1993 [115]	Men and women (n=31), UK	1.5g EPA + 0.9g DHA/d	6-8wk	Cytokine production: IL-6, IL-1b, TNF-a (ex vivo whole blood, LPS stimulated)	Only effect on IL-1b and IL-6 when LPS > 1000pg/l No effect on TNF-a synthesis

Abbreviations: wk (week), y (year), CRP (C-reactive protein), hs (high sensitivity), IL (interleukin), TNF (tumour necrosis factor), ELISA (enzyme linked immunoassay), IFN(interferron), LPS (lipopolysaccharide), PBMC (peripheral blood mononuclear cell), PUFA (polyunsaturated fatty acid), EPA (eicosapentaeonic acid), DHA (docosahexaenoic acid), UK (United Kingdom), US (United States)

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2

Opposing associations of serum n-3 and n-6 polyunsaturated fatty acids with colorectal adenoma risk: An endoscopy-based case-control study

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Abstract

Several human and animal studies have shown that n-3 polyunsaturated fatty acids (PUFA) might be associated with a decreased risk, whereas other studies showed that n-6 PUFA may be associated with an increased risk of colorectal cancer. However, results from these studies are not consistent.

We evaluated the associations between serum n-3 and n-6 PUFA levels and colorectal adenoma risk in an endoscopy-based case-control study, conducted in the Netherlands between 1997 and 2002. We included 363 cases of colorectal adenomas and 498 adenoma-free controls. Serum fatty acids were measured in cholesteryl esters. Logistic regression models were used to calculate odds ratios (OR), which were adjusted for age, gender and alcohol intake.

Total serum n-3 PUFA levels were inversely associated with colorectal adenoma risk, the OR comparing the third tertile with the first tertile was 0.67 [95% confidence interval (CI) 0.46; 0.96, p for trend=0.03]. Serum eicosapentaenoic acid (EPA; C20:5n-3) and docosahexaenoic acid (DHA; C22:6n-3) and the n-3/n-6 ratio were inversely associated with colorectal adenoma risk, but these were not statistically significant. In contrast, the risk of colorectal adenomas was increased by total n-6 PUFA with an OR of 1.68 (95% CI, 1.17; 2.42, p for trend=0.006) and by linoleic acid (LA; C18:2n-6) with an OR of 1.65 (95% CI, 1.15; 2.38, p for trend=0.007). This is the first observational study that simultaneously finds an inverse association of serum n-3 PUFA and a positive association of n-6 PUFA with colorectal adenoma risk.

Introduction

Consumption of fish might be associated with a reduced risk of colorectal cancer, as was shown in a recent meta-analysis [1]. It could be hypothesised that a possible protective effect could be due to the n-3 polyunsaturated fatty acids (PUFA) eicosapentaenoic acid (EPA; C20:5n-3) and docosahexaenoic acid (DHA; C22:6n-3). N-3 PUFA can work through several actions to protect against the initiation and early stages of colorectal cancer, including reducing cell proliferation, enhancing tumor cell apoptosis, promoting cell differentiation and reducing inflammation [2,3]. However, results from human studies evaluating colorectal cancer risk and n-3 PUFA measured in serum, have not been consistent, 1 study did observe a negative association [4], whereas another study did not find an association [5], or only in men [6]. Studies on colorectal adenomas, which can be considered as a precursor stage of colorectal cancer and n-3 PUFA are even more scarce. Oh et al found no association between n-3 PUFA intake obtained by a food frequency questionnaire and colorectal adenoma risk [7].

In contrast to n-3 PUFA, n-6 PUFA could be related to an increased risk of colorectal cancer. Animal studies have shown that n-6 PUFA may enhance the risk of colorectal carcinogenesis [8,9]. However, the evidence of a relationship between n-6 PUFA and colorectal cancer from human studies is not consistent: positive associations have been found [10,11], some studies have shown no association [5,6,12-14] whereas other studies showed

inverse associations [4,15]. To the best of our knowledge, no human studies on colorectal adenomas and n-6 PUFA measured in serum have been performed.

Based on the opposing effects of n-3 and n-6 PUFA on tumor initiation, cell proliferation, and inflammation, it could be hypothesized that n-3 and n-6 PUFA could have opposing effects on colorectal cancer risk in human studies. However, studies which assessed n-3 and n-6 PUFA intake by food frequency questionnaire [10,12-14] or by serum levels [4-6], did not show opposed associations.

As far as we know, no studies have been performed evaluating serum n-3 and n-6 PUFA and colorectal adenoma risk, as a precursor lesion of colorectal cancer, simultaneously. Therefore, we studied the association between colorectal adenomas and serum levels of n-3 and n-6 PUFA in an endoscopy-based case-control study.

Material and methods

This Dutch case-control study has been described in detail elsewhere [16]. Briefly, participants were recruited among those undergoing an endoscopy in 10 outpatient clinics between June 1997 and October 2002. Cases and controls were Dutch speaking, Caucasian, aged 18- 75 at the time of the endoscopy, who did not suffer from hereditary colorectal cancer syndromes or chronic inflammatory bowel diseases, and did not have a history of colorectal cancer or (partial) bowel resection. The response rates in different hospitals varied from 35-87%. The study protocol was approved by the Medical Ethical Committees of all participating hospitals and all participants provided written informed consent.

The total study population included 768 colorectal adenoma cases and 709 polyp-free controls. Cases were defined as those with at least 1 histologically confirmed colorectal adenoma ever in their life. For the current analyses, we redefined cases as those who were diagnosed with a first adenoma at the index endoscopy conducted at the start of the study when blood samples were collected and questionnaires were filled in. We only included recently diagnosed cases, because it could be hypothesised that serum fatty acid levels may be influenced by the presence of colorectal adenomas. In controls, diagnosis of any type of adenoma was negative at the index endoscopy, and they had no history of any type of adenomas, based on medical records. For the current analyses, we excluded controls who did not undergo a full colonoscopy to exclude the chance of misclassification due to possible proximal adenomas. From the total study population, we excluded 169 subjects because analysis of serum PUFA could not be performed, due to limited samples or technical reasons. Finally, this resulted in a study population of 861 participants: 363 colorectal adenoma cases and 498 polyp-free controls.

Nonfasting venous blood samples were obtained for the assessment of fatty acids in cholesteryl esters. Serum cholesteryl fatty acids were analyzed by gas chromatography as

previously described [17]. The total coefficients of variation of n-3 and n-6 PUFA of the assay ranged between 3.7 and 5.9%. Serum PUFA are expressed as mass percentage of total fatty acid methyl esters in gram per 100-g fatty acids methyl esters.

Participants filled in dietary and lifestyle questionnaires regarding their habits in the year before the index endoscopy. The lifestyle questionnaire included questions on family history of colorectal cancer (only first degree family members), physical activity, smoking and use of medications, as non steroidal anti-inflammatory drugs (NSAIDs). To assess dietary habits a standardized semi-quantitative food frequency questionnaire, was used (EPIC FFQ) [18]. Intakes of total energy and various nutrients were calculated using the Dutch food composition table [19].

Pearson's correlation coefficients were used to calculate correlations between serum total n-3 PUFA and reported fish consumption and between serum n-3 and n-6 PUFA.

To evaluate the association between serum PUFA and colorectal adenoma risk, we used logistic regression models to calculate odds ratios (ORs) and 95% confidence intervals (CI) per tertile of serum PUFA based on the distribution among controls. To test for linear trend, representing potential dose-response relationships over the ordered categories of exposure, the median value among controls was assigned to each tertile.

Effect modification by gender, family history of colorectal cancer, age and regular NSAIDs use was evaluated by comparing stratified ORs. The following variables were evaluated for confounding: family history of colorectal cancer, body mass index, indication for endoscopy, physical activity, ever smoker, regular use of NSAIDs, use of hormone replacement therapy, diet change due to gastrointestinal complaints, and daily intake of energy, alcohol, fat, fiber, red meat, vegetables and legumes and cholesterol. Variables were included as confounding variables in the multivariate model if they changed the OR by 10% or more. The variables age and gender differed markedly between cases and controls in this study population [16] and therefore remained in the models at all times. As a separate and additional analysis, n-3 and n-6 PUFA levels were included 1 model simultaneously to test for independence of effects. All statistical analyses were carried out using the SAS statistical software program (version 9.1, SAS Institute, Cary, NC).

Results

Cases were more likely to be male, older, and smoked more than controls (**Table 2.1**). Furthermore, the percentage of participants who had a colonoscopy for screening purposes was somewhat higher among cases compared to controls. Cases also had a higher alcohol intake (Table 2.1). Subjects in the highest tertile of serum n-3 PUFA levels were more likely to be male, older and had a higher intake of fish, alcohol and total energy than those in the lowest tertile of n-3 PUFA (data not shown). Hormone replacement therapy was more common among women in the highest tertile of serum

n-3 PUFA. Subjects in the highest tertile of n-6 PUFA levels smoked less than those in the lowest tertile of n-6 PUFA, but there were no differences in gender distribution or age. Additionally, their total fish intake and consequently their n-3 PUFA serum levels were lower, as was their intake of alcohol (data not shown).

Table 2.1 Characteristics of 363 cases and 498 controls (total n=861)

	Cases (n=363)	**Controls (n=498)**
Demographic variables		
Gender (% men)	53.7	39.2
Age (years, mean ± SD)	58.1 ±10.3	52.0 ±13.5
BMI (kg/m^2, mean ± SD)	26.2 ± 4.1	25.6 ±4.1
Family history of CRC (% yes)	22.0	21.7
Education (%low)	39.2	32.1
Lifestyle variables		
Ever smoker (% yes)	66.2	58.2
Physical activity (% low)	58.4	51.8
Regular use of NSAID* (%)	34.7	37.6
HRT among women (% yes)	15.5	17.1
Indication for endoscopy (%)		
Complaints/ Screening/ Other	70.3/ 18.5/ 11.0	75.9/ 13.1 / 11.0
Serum PUFA [1†]		
n-3 PUFA	2.0 (1.7 – 2.5)	2.0 (1.6 – 2.4)
ALA	0.5 (0.5 – 0.7)	0.6 (0.5 – 0.7)
EPA	0.8 (0.6 – 1.2)	0.8 (0.6 – 1.1)
DHA	0.6 (0.4 – 0.7)	0.6 (0.4 – 0.7)
n-6 PUFA	61 (59 - 64)	61 (58 – 64)
LA	53 (49 - 55)	52 (49 – 55)
AA	6.9 (5.9 – 8.0)	6.9 (5.9 – 7.9)
n-3/n-6 ratio	0.03 (0.03- 0.04)	0.03 (0.03- 0.04)
Dietary intake (mean ± SD)		
Energy (MJ/d)	8.8 ± 2.5	8.4 ± 2.5
Fat (g/d)	83.6 ± 29.2	78.5 ± 29.2
Total fish intake (g/d) [†]	8.4 (3.3 – 16.3)	8.8 (3.3 – 16.8)
Red meat (g/d)	59.9 ± 34.1	54.9 ± 32.4
Total fruit (g/d)	190 ± 131	185 ± 129
Total vegetables and legumes (g/d)	129 ± 51	120 ± 47
Fibre (g/d)	23.9 ± 6.2	22.9 ± 6.5
Alcohol (g/d) [†]	8.8 (1.0 – 23.3)	4.4 (0.3 – 15.1)
Cholesterol (mg/d)	233 ± 96	214 ± 81
Diet change due to gastrointestinal complaints (% yes)	19.9	36.1

[*] > 12 times per year; [1] expressed in mass % of fatty acids in cholesteryl esters; [†] Values are medians (25th percentile-75th percentile); Abbreviations: SD (standard deviation), BMI (body mass index), CRC (colorectal cancer), NSAID (non steroidal anti-inflammatory drug), HRT (hormone replacement therapy), PUFA (polyunsaturated fatty acid), ALA (alpha-linolenic acid; C18:3n-3),, EPA (eicosapentaenoic acid; C20:5n-3) , DHA (docosahexaenoic acid; C22:6n-3) , LA (linoleic acid; C18:2n-6), AA (arachidonic acid; C20:4n-6), MJ/d (Mega Joule per day), g/d (gram per day)

No modifying effect was observed for gender distribution, age, family history of colorectal cancer or regular NSAIDs use. Identified confounding variables were age, gender, and alcohol intake.

The correlation coefficient between serum total n-3 PUFA and total fish consumption was 0.37, and for serum total n-3 PUFA and fatty fish consumption it was 0.44. The correlation coefficient between total n-3 PUFA and total n-6 PUFA was -0.41.

For total n-3 PUFA, the OR of colorectal adenomas comparing the third tertile with the first was 0.67 (95% CI 0.46; 0.96, *p* for trend 0.03) (**Table 2.2**). Serum EPA and DHA were also inversely associated with colorectal adenoma risk, but not statistically significantly. In contrast, the risk of colorectal adenomas was increased by total n-6 PUFA with an OR of 1.68 (95% CI, 1.17; 2.42, *p* for trend 0.006) and by linoleic acid (LA; C18:2n-6) with an OR of 1.65 (95% CI, 1.15; 2.38, *p* for trend 0.007).

Table 2.2 Association between serum PUFA and colorectal adenomas in an endoscopy-based case-control study including 363 cases and 498 polyp-free controls

Serum PUFA*	Tertiles[1]	Number of cases/ controls	Model OR (95% CI) [2]	p for trend
Total n-3 PUFA	≤ 1.8	115/166	1.00 (ref)	0.03
	1.8-2.3	124/166	0.80 (0.56-1.15)	
	≥ 2.3	124/166	0.67 (0.46-0.96)	
ALA	≤ 0.5	127/166	1.00 (ref)	0.66
	0.5-0.6	109/166	0.86 (0.61- 1.22)	
	≥ 0.6	127/166	0.93 (0.66-1.31)	
EPA	≤ 0.7	106/166	1.00 (ref)	0.24
	0.7-1.0	122/166	0.86 (0.60- 1.24)	
	≥ 1.0	135/166	0.79 (0.55- 1.15)	
DHA	≤ 0.5	112/166	1.00 (ref)	0.05
	0.5-0.6	140/166	1.02 (0.73- 1.45)	
	≥ 0.6	111/166	0.71 (0.49- 1.02)	
Total n-6 PUFA	≤ 58.9	103/166	1.00 (ref)	0.006
	58.9-62.8	127/166	1.55 (1.08- 2.23)	
	≥ 62.8	133/166	1.68 (1.17- 2.42)	
LA	≤ 50.0	111/166	1.00 (ref)	0.007
	50.0-54.1	117/166	1.27 (0.89- 1.83)	
	≥ 54.1	135/166	1.65 (1.15- 2.38)	
AA	≤ 6.2	123/166	1.00 (ref)	0.83
	6.2-7.5	112/166	0.91 (0.64- 1.30)	
	≥ 7.5	128/166	0.96 (0.68- 1.35)	
n-3/n-6 ratio	≤ 0.028	116/166	1.00 (ref)	0.07
	0.028- 0.038	127/167	0.81 (0.57- 1.16)	
	≥ 0.038	127/165	0.71 (0.49- 1.02)	

***** mass% of fatty acid methyl esters; [1] Tertiles based on distribution among controls; [2] Model : Adjusted for gender, age and alcohol intake; Abbreviations: PUFA (polyunsaturated fatty acid), ALA (alpha-linolenic acid; C18:3n-3), EPA (eicosapentaenoic acid; C20:5n-3), DHA (docosahexaenoic acid; C22:6n-3), LA (linoleic acid; C18:2n-6), AA (arachidonic acid; C20:4n-6)

No marked association with colorectal adenomas was found for serum α-linolenic acid (ALA; C18:3n-3) and for serum arachidonic acid (AA; C20:4n-6) (Table 2.2). The n-3/n-6 ratio was inversely associated with colorectal adenoma risk, OR 0.71 (95% CI 0.49; 1.02, *p* for trend 0.07). When we also adjusted for n-6 PUFA, we found that the association between n-3 PUFA and colorectal adenomas was slightly attenuated, OR 0.79 (95% CI 0.53; 1.18). When the associations of n-6 PUFA or LA were adjusted for n-3 PUFA, these were also slightly attenuated, OR 1.51 (95% CI 1.02; 2.24) and 1.48 (95% CI 1.01; 2.17), respectively.

Discussion

In this endoscopy-based case-control study, we observed an inverse association of serum n-3 PUFA and a positive association of serum n-6 PUFA and LA with colorectal adenoma risk. Although there were indications for opposing associations of n-3 and n-6 PUFA on colorectal cancer risk in animal studies [2,3,8,9], other human studies on colorectal cancer have not found this simultaneous opposing effects of n-3 and n-6 PUFA [4-6,10,12-14]. It is hypothesised that n-3 and n-6 PUFA can have opposing effects on colorectal tumor formation via modulation of the AA pathway; by changing the substrates and products, like eicosanoids, or by a direct effect on the genes involved in this pathway, although the precise mechanisms are far from clear [3].

One could question whether the observed association of n-3 PUFA and colorectal adenomas could partly be explained by n-6 PUFA, and vice versa. However, when we adjusted the appropriate models for n-3 PUFA with n-6 PUFA or vice versa, results would only slightly attenuate.

One of the strengths of this study is that we used serum PUFA levels to determine the association of PUFA with colorectal adenoma risk. Serum PUFA levels take into account the bioavailability of PUFA from foods and the metabolism of PUFA in the human body and are not dependent on memory, awareness of fat intake or willingness to report details of diet and are therefore considered to be a better marker of PUFA intake than dietary questionnaires [20,21]. A second strength is that our control population did not have any colorectal adenomas as confirmed by a full colonoscopy. This reduces the chance of misclassification due to possible proximal adenomas. Moreover, the cases were all recently diagnosed with colorectal adenomas. This reduces the possibility that the presence of colorectal adenomas may have influenced serum fatty acid levels [22].

A limitation of this study is that endoscopies are not routinely conducted for screening purposes in the Netherlands, but only on clinical indication. Therefore, the reason for most subjects' initial endoscopy was either bowel complaints, faecal blood loss or a family history of colorectal cancer. Bowel complaints may lead to changes in dietary habits. However, when we excluded participants who had indicated that they had

changed their diet due to these complaints (72 cases, 178 controls), it did not change our results. Also, we do not have sufficient data on serum PUFA levels of patients not participating in the study to further evaluate the possible selection bias due to nonresponse. However, from a short questionnaire distributed upon invitation inquiring after age, gender, body weight, height, education level, smoking and consumption of alcohol and meat, we know that there were no large differences in these characteristics between responders and nonresponders [23]. We tried to limit information bias by including only recently diagnosed cases in these analyses, by asking the participants to fill out the questionnaires regarding their habits in the year before their complaints or last endoscopy, and by using serum markers of PUFA.

Furthermore, we studied colorectal adenomas instead of colorectal cancer. One could question whether all colorectal adenomas would eventually develop into carcinomas. If we would include only 'high-risk' adenomas in our analyses, defined as villous adenomas, more than 3 adenomas or adenomas with severe dysplasia, results would only slightly attenuate for n-6 PUFA. Also, colorectal adenomas might be a better marker for the earlier processes of colorectal carcinogenesis, especially villous adenomas. It could be hypothesized that dietary effects may especially play a role in these early processes.

Genetic factors could possibly influence the association between n-3 or n-6 fatty acids and the risk of colorectal adenomas [24,25], which could have contributed to the observed discrepancies in human studies. For instance, single nucleotide polymorphisms in cyclooxygenase (COX)-2 and peroxisome proliferator-activated receptor (PPAR)-delta could modify the association between colorectal adenomas and fish consumption [24].

In conclusion, this is the first observational study that simultaneously finds an inverse association of serum n-3 PUFA and a positive association of serum n-6 PUFA with colorectal adenoma risk. Prospective studies are needed to further investigate the role of PUFA, particularly the opposing effects of n-3 and n-6 PUFA, in colorectal carcinogenesis taking genetic polymorphisms into account.

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3

No effect of fish oil supplementation on serum inflammatory markers and their interrelationships: a randomized controlled trial in healthy, middle-aged individuals

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Abstract

Background: High intake of n-3 polyunsaturated fatty acids (PUFA), mainly present in fish, may be associated with decreased inflammation. Previous intervention studies on fish PUFA and inflammatory markers in healthy subjects did not analyze a broad spectrum of inflammatory cytokines, chemokines, and cell adhesion molecules, or their interrelationships. Therefore, we determined the effects of fish oil supplementation on 19 serum inflammatory markers and their interrelationships in healthy, middle-aged individuals.

Methods: Individuals (n=77) aged 50-70 years completed a randomized, double-blind placebo controlled intervention study. Participants received 3.5g/d fish oil (1.5g/d total n-3 PUFA) (n=39) or placebo (high oleic sunflower oil) (n=38) during 12 weeks. Serum concentrations of 19 inflammatory markers were determined using a multiplex immunoassay pre- and post-intervention. Changes in concentrations were analyzed using analysis of covariance (ANCOVA) and differences in patterns in inflammatory markers between the fish oil and placebo group were analyzed by principal components analysis (PCA).

Results: Fish oil supplementation did not significantly affect serum concentrations of cytokines, chemokines, or cell adhesion molecules as compared with placebo. However, there was a trend that all inflammatory markers were increased after fish oil supplementation. PCA did not result in markedly distinctive patterns of inflammatory markers for the fish oil and placebo group.

Conclusion: This 12-week randomized, double-blind placebo-controlled intervention trial did not show that 1.5g/d n-3 PUFA significantly affected the serum inflammatory response in healthy individuals, nor patterns of inflammatory markers. Thus, a healthy middle-aged population may not benefit from fish oil as anti-inflammatory agent.

Introduction

Chronic inflammation underlies a variety of human diseases, including cardiovascular disease and inflammatory bowel disease. Consumption of the n-3 polyunsaturated fatty acids (PUFA) eicosapentaenoic acid (EPA, C20:5 n-3) and docosahexaenoic acid (DHA, C22:6 n-3), as mainly present in fish, may be associated with anti-inflammatory effects [1,2]. A generally accepted mechanism behind this is that higher dietary intake of EPA increases its inflammatory cell membrane concentration at the expense of arachidonic acid (AA, C20:4 n-6). This can lead to a shift in production of cytokines via an altered eicosanoid production, resulting in a less pro-inflammatory state [3]. Numerous studies have investigated the effects of n-3 PUFA on inflammatory markers in different types of populations. If we only focus on studies on inflammatory markers in healthy subjects, the evidence of effects of n-3 PUFA on inflammatory markers is not very solid, as is shown in two recent reviews [4,5]. Fritsche concluded that n-3 PUFA supplementation studies with healthy human subjects did not provide consistent or compelling evidence to support the hypothesis that dietary PUFAs affect inflammatory responses in a manner that is likely to have clinical consequences [4]. And also Sijben and Calder concluded in their review that most studies in healthy subjects did not show effects on immune markers such as

cytokine production by lymphocytes and monocytes [5]. In most of the studies evaluating the effect of n-3 PUFA on inflammatory cytokines, isolated cells stimulated with stimuli like endotoxin were studied, while studying circulating soluble markers in serum might be more closely linked with the *in vivo* situation. In addition, in most of these studies only a few inflammatory markers were studied, like in a recent study of Yusof and Calder [6] who studied six systemic inflammatory markers or Fujioka et al [7] who studied only two inflammatory markers. Moreover, since cytokines are interrelated in regulatory networks, a wide spectrum of cytokines rather than a few selected cytokines should be analyzed including their interrelationships. Besides cytokines, other forms of soluble inflammatory markers could be added to this spectrum of inflammatory markers, such as chemokines, and cell adhesion molecules. Chemokines are chemotactic cytokines which regulate the process of inflammation by controlling the homeostasis of circulation of leukocytes [8]. Soluble forms of cell adhesion molecule-1, and vascular cell adhesion molecule-1 are found in serum and are elevated during inflammatory conditions [9]. So far, few studies have addressed the effects of fish oil supplementation on cytokines in combination with cell adhesion molecules or chemokines in a healthy, middle-aged population, showing conflicting results: some studies showed a decrease in cell adhesion molecules [6,10] while other studies showed no effect [11,12]. Therefore, our main aim was to study the effects of fish oil supplementation on a large range of serum inflammatory markers including cytokines, chemokines, and cell adhesion molecules using a multiplex immunoassay in middle-aged individuals. In addition, we evaluated the interrelationships of these inflammatory markers in the two treatment groups.

Subjects and methods

Subjects and Study design

This study was primarily designed to investigate the effects of fish oil supplementation on heart rate variability and baroreflex sensitivity in healthy subjects and power calculations were based on the primary outcome of changes in heart rate variability and baroreflex sensitivity [13].

We included 81 subjects aged 50-70 years in a parallel, double-blind, placebo-controlled intervention trial that was performed between January and May 2001. After a run-in period of 4 weeks, in which subjects were provided with placebo capsules and were instructed not to consume fish, seafood, or fish oil capsules, subjects were randomized to receive either a daily dose of 3.5g of fish oil or placebo oil (high oleic sunflower oil) during a 12-week intervention period. Subjects were stratified by habitual fish consumption, diastolic blood pressure, and gender and then randomized to receive either fish oil or placebo by a person independent of the study. Both researchers and participants were

blinded to the treatment and fish oil and placebo capsules were indistinguishable from each other.

The daily dose of fish oil provided approximately 700 mg of EPA, 560 mg of DHA, and 260 mg of other n-3 fatty acids (in total 1.5 g/d n-3 PUFA). Exclusion criteria were mainly related to problems of the cardiovascular system including past or present cardiovascular disease, diabetes mellitus, and hypertension (> 170 mm Hg systolic or > 100 mm Hg diastolic). Only post-menopausal women not receiving hormone replacement therapy were included. Compliance was checked and confirmed by analyzing n-3 fatty acids in serum cholesteryl esters and by counting the number of leftover capsules. The study protocol was approved by the Medical Ethical Committee of Wageningen University and all subjects gave written informed consent.

Characteristics of the study population including height, weight, and physical activity [14,15] were measured at baseline and published previously [13]. Habitual fish intake was assessed by interviewing the subjects using a questionnaire on the frequency of fish intake. Participants also kept a diary on their general health status. High sensitivity serum C-reactive protein (CRP) concentrations were measured at baseline and after intervention using an enzyme immunoassay and results were published before [16].

Measurements of inflammatory markers

Non-fasting blood samples were collected at the start and end of the intervention, for each participant at the same time of day pre- and post-intervention. Also, subjects were instructed to eat low fat meals on the day of the blood draw. Blood samples were stored at -80°C until further processing. Multiplex immunoassays were performed as previously described [17,18] to measure serum concentrations of soluble interleukin (IL)-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8 (or CXCL8), IL-10, IL-12p70, IL-13, tumor necrosis factor alpha (TNF-α), interferon-gamma (IFN-γ), macrophage migration inhibitory factor (MIF), monocyte chemo-attractant protein-1 (MCP-1 or CCL2), intracellular adhesion molecule-1 (sICAM-1), vascular adhesion molecule-1 (sVCAM-1), macrophage inflammatory protein-1-alpha (MIP-1α or CCL3), Regulated upon Activation, Normal T-cell Expressed and Secreted (RANTES or CCL5), and Eotaxin (CCL11). Samples were analyzed using the Bio-plex system in combination with the Bio-plex Manager software version 3.0 (Bio-Rad Laboratories, Hercules CA, USA). Concentrations of analytes were quantified using a standard curve that was generated using five parametric curve fittings to the series of known concentrations of analytes. Due to technical reasons, results of one participant were excluded from analyses (**Figure 3.1**). Intra-assay variability expressed as coefficient of variation (CV) of the multiplex immunoassay has been published previously and varied between 6.5- 22% [17,18].

Statistical methods

We excluded three subjects from the analyses based on a serum concentration of CRP > 10 mg/l, either at baseline or at the end of the intervention resulting in a study population of 77 subjects (Figure 3.1). These three excluded subjects reported flu-like symptoms in their diary preceding blood collection [16]. We assigned a value equal to half of the detection limit of a given cytokine or chemokine if any values were below detection limit. A sensitivity analysis was performed to determine whether the results were sensitive to this arbitrary choice by comparing analyses where one quarter of the detection limit was assigned to such data points.

Since data were not normally distributed, medians and interquartile ranges (25^{th} percentile, 75^{th} percentile) are presented for baseline and end values of all inflammatory markers. The end values of the outcomes were used in a model of analysis of covariance (ANCOVA) comparing the fish oil supplemented group with the placebo group adjusted for baseline values.

As additional and explorative analysis, we applied principal components analysis (PCA) to analyze patterns of cytokines, chemokines, and cell adhesion molecules by using the Factor procedure in SAS [19]. We also included CRP measures which were measured and published previously in these patterns [16]. Responses of inflammatory markers (after – before measurement) were entered into the model and based on evaluation of eigenvalues and the Scree plot, patterns were derived and rotated using the VARIMAX option in SAS. Individual factor loadings for the main three factors were plotted to explore whether two clusters would be visible representing the two treatment groups.

For the inflammatory markers, we considered a p-value of < 0.01 as statistically significant since we performed multiple comparisons. All statistical analyses were carried out using the SAS statistical software program (version 9.1, SAS Institute Inc., Cary, North Carolina, USA).

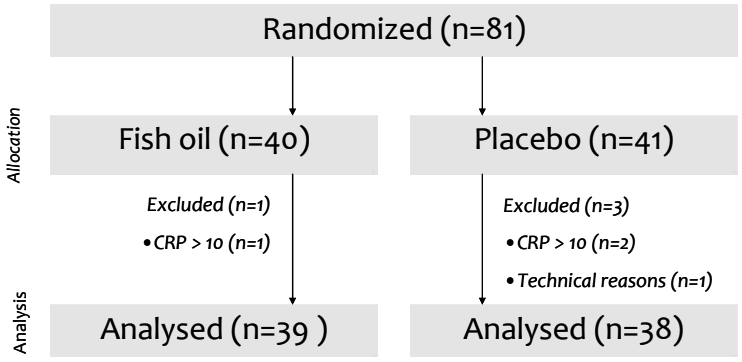

Figure 3.1 Flow chart of participants

Results

Baseline characteristics of the participants are presented in **Table 3.1.** Subjects receiving fish oil and placebo were similar in terms of gender, age, BMI, habitual fish intake, smoking status, and degree of physical activity. One serious adverse event was reported during the study but this was not related to the study. This person was not included in the analysis.

Table 3.1 Baseline characteristics of all subjects (n=77) presented as mean ± SD, or in n (%)

	Fish oil (n=39)	**Placebo (n=38)**
Gender (n, % male)	19 (49%)	20 (53%)
Age (y)	58.0 ± 4.3	59.5 ± 5.3
BMI (kg/m^2)	26.5 ± 3.2	26.6 ± 3.6
Habitual fish intake (freq/month)	3.4 ± 2.4	3.4 ± 3.0
Current smokers (n, %)	7 (18%)	8 (21%)
Physical activity (PASE)[1]	134.1 ± 54.3	136.9 ± 58.4

[1] PASE=Physical Activity Scale for the Elderly, a higher score indicates more physical activity

Serum cytokine concentrations at baseline and after the 12-week intervention are presented in **Table 3.2** and serum chemokines, and cell adhesion molecules are presented in **Table 3.3**. The percentage of subjects with a measurement below the detection limit varied from 0% for some cytokines and adhesion molecules to 87% for IL-10 (Table 3.2 and 3.3).

Serum concentrations of cytokines after treatment did not differ statistically significantly between the fish oil treated and placebo treated subjects (Table 3.2). In addition, serum concentrations of chemokines and cell adhesion molecules were not significantly different between the two intervention groups after the 12-week fish oil intervention (Table 3.3). Overall, it appeared that all serum inflammatory markers were increased rather than decreased after fish oil supplementation as compared with placebo, however, these increases were not statistically significant. Results from the sensitivity analysis, using a different value for replacing values below the detection limit, were comparable with the original analysis (data not shown).

Table 3.2 Serum cytokines (pg/ml) at baseline and after 12-week fish oil (n=39) or placebo oil supplementation (n=38), presented as median and interquartile range (25th percentile, 75th percentile), and the percentage of subjects with a measurement below detection limit

Cytokine	Treatment	Baseline		% Below Detection limit	Week 12		% Below Detection limit	p-value*
		Median	IQR		Median	IQR		
IL-1α	Placebo	3.05	[1.11; 23.1]	13	1.77	[1.11; 5.28]	13	
	Fish oil	3.05	[1.11; 39.2]	28	5.61	[1.19; 60.8]	21	0.72
IL-1β	Placebo	2.46	[1.17; 2.46]	68	2.46	[1.17; 2.46]	79	
	Fish oil	1.26	[1.17; 2.46]	85	2.46	[1.17; 2.46]	82	0.26
IL-2	Placebo	2.53	[1.16; 10.6]	24	2.32	[1.16; 4.57]	34	
	Fish oil	2.60	[1.16; 10.4]	23	3.09	[1.16; 15.6]	23	0.13
IL-4	Placebo	2.52	[1.31; 2.52]	63	2.52	[1.31; 2.52]	82	
	Fish oil	1.31	[1.31; 2.52]	69	2.01	[1.31; 2.52]	74	0.92
IL-5	Placebo	1.29	[0.97; 1.94]	29	1.16	[0.97; 1.46]	32	
	Fish oil	1.63	[0.97; 2.48]	54	1.63	[0.97; 2.48]	44	0.36
IL-6	Placebo	3.42	[2.64; 50.0]	47	3.42	[2.06; 37.1]	50	
	Fish oil	8.00	[1.66; 31.3]	33	6.84	[1.66; 35.6]	31	0.05
IL-10	Placebo	2.51	[1.09; 2.51]	79	2.51	[1.09; 2.51]	87	
	Fish oil	2.51	[1.09; 2.51]	67	2.51	[1.09; 2.51]	72	0.34
IL-12p70	Placebo	26.6	[8.68; 596]	5	19.2	[5.99; 188]	11	
	Fish oil	27.7	[9.14; 168]	13	52.0	[16.0; 210]	10	0.14
IL-13	Placebo	10.4	[4.87; 27.6]	5	7.07	[4.87; 25.4]	8	
	Fish oil	9.68	[4.87; 20.6]	13	9.66	[3.88; 23.0]	13	0.25
TNF-α	Placebo	2.74	[2.74; 30.4]	61	2.74	[0.99; 21.4]	61	
	Fish oil	2.74	[1.20; 20.1]	51	2.74	[1.20; 19.2]	54	0.49
IFN-γ	Placebo	4.84	[4.84; 42.5]	50	4.84	[4.84; 28.9]	50	
	Fish oil	4.84	[2.55; 35.6]	33	6.00	[2.55; 36.8]	41	0.14

* p-value of ANCOVA comparing fish oil vs. placebo after intervention adjusted for baseline values

Table 3.3 Serum chemokines (pg/ml) and cell adhesion molecules (ng/ml) at baseline and after 12-week fish oil (n=39) or placebo oil supplementation (n=38), presented as interquartile range (Q1, Q3), and the percentage of subjects with a measurement below detection limit

Inflammatory marker	**Treatment**	**Baseline**		**% Below Detection limit**	**Week 12**		**% Below Detection limit**	**p-value***
		Median	IQR		Median	IQR		
Chemokines								
MIF	Placebo	10.1	[1.30; 11.8]	84	5.0	[1.30; 11.8]	82	
	Fish oil	11.8	[1.30; 11.8]	79	4.6	[1.30; 11.8]	79	0.36
MCP-1/CCL2	Placebo	26.2	[12.0; 40.8]	8	22.7	[13.9; 38.5]	5	
	Fish oil	24.6	[13.8; 38.8]	5	31.0	[48.2; 179]	3	0.50
MIP-1α/CCL3	Placebo	88.1	[48.2; 219]	8	87.1	[80.7; 183]	0	
	Fish oil	129.3	[80.7; 180]	3	156.0	[80.7; 183]	0	0.29
RANTES/CCL5	Placebo	209.0	[155; 503]	0	250.0	[136; 421]	0	
	Fish oil	266.0	[193; 398]	0	273.0	[166; 399]	0	0.57
Eotaxin/CCL11	Placebo	45.9	[24.7; 111]	0	63.8	[22.0; 99.6]	0	
	Fish oil	62.4	[33.6; 177]	0	63.6	[27.7; 137]	0	0.54
IL-8/CXCL8	Placebo	5.1	[4.92; 13.7]	29	6.8	[4.9; 12.8]	34	
	Fish oil	10.2	[6.62; 14.5]	10	9.5	[6.80; 13.9]	8	0.13
Cell adhesion molecules								
sVCAM-1/sCD106	Placebo	116.9	[59.1; 157.5]	0	104.8	[19.6; 154.3]	0	
	Fish oil	117.9	[83.6; 184.9]	0	118.7	[96.4; 173.6]	0	0.10
sICAM-1/sCD54	Placebo	58.6	[11.3; 75.7]	0	58.2	[9.73; 78.0]	0	
	Fish oil	63.2	[30.2; 85.5]	0	67.4	[40.4; 91.7]	0	0.09

* p-value of ANCOVA comparing fish oil vs. placebo after intervention adjusted for baseline values

Principal components analysis

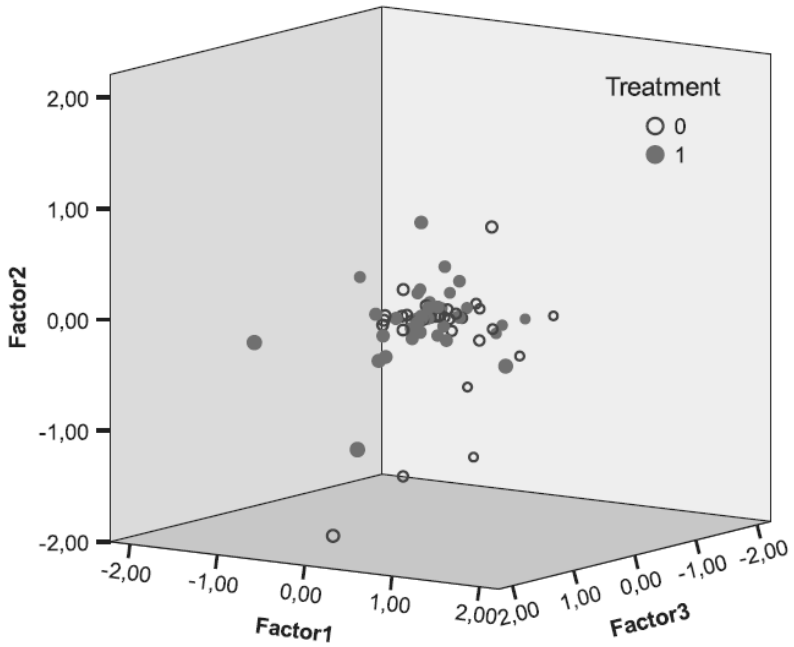

Figure 3.2 Three-dimensional plot of factor loadings with each color representing different treatment group (treatment 0=placebo, 1=fish oil)

In **Figure 3.2** the individual results of the PCA are plotted for the first three factor loadings, each dot represents one person. Factor 1 consisted of IL-1β, IL-2, IL-5, IL-6, IL-10, IL-13, TNF-α, IFN-γ, MIP1a, IL-8, and sVCAM and explained 43.4% of the total variance. Factor 2 consisted of IL-1α, IL-2, IL-4, and MIF and explained 17.6% of the total variance. Factor 3 consisted of MCP-1 and RANTES and explained 10.7% of the total variance. The first three factors together explained 71.7% of the total variance. We observed no explicit clusters for subjects treated with either fish oil or placebo (Figure 3.2).

Discussion

This randomized, double-blind placebo-controlled intervention trial showed no effect of 1.5g n-3 PUFA daily for 12 weeks on serum inflammatory markers in healthy middle-aged individuals. If anything, there was a trend that all serum inflammatory markers tended to be increased after fish oil supplementation but this was not statistically significant. No marked differences in patterns of serum inflammatory markers between treatment groups were observed.

Our results contribute to the body of evidence that in studies with healthy individuals generally no effects of n-3 PUFA supplementation on inflammatory markers were observed [4,5]. Healthy individuals, like the participants of this study, generally have low levels of serum inflammatory markers. Thus, the chance that low levels of inflammation are reduced by an intervention with fish oil is very small. In addition, low levels of serum inflammatory markers are not easy to detect, which is reflected in the number of values below detection limit in this study. Chemokines and cell adhesion molecules were in the detectable range, but no effect of fish oil supplementation was observed for these inflammatory markers. Therefore, it may well be possible that in a middle-aged population like this no beneficial effect of fish oil supplementation on serum inflammatory markers is to be expected, although it may be possible that other markers of immune function, like the expression of cell adhesion markers on monocytes and respiratory burst response in neutrophils could be affected, as was shown in previous studies [20-22].

A limitation of our study is that blood samples were collected throughout the day which could have introduced some variation due to possible natural fluctuations in cytokine concentrations [23]. To counteract this potential problem, blood samples per participant were collected at the same time of day before and after intervention. In addition, fluctuations in cytokine concentrations could be expected after meals, especially high fat meals [24-26]. However, subjects were asked to consume low fat meals before the measurement. Nevertheless, the additional variation caused by fluctuations over the day may have weakened our results. Moreover, we used a multiplex immunoassay rather than enzyme-linked immunosorbent assay (ELISA), which is generally considered the gold standard. Nevertheless, the multiplex immunoassay used in our study was validated against ELISA [17] and sensitivity, accuracy, and reproducibility of the two methods were similar. Using different methods of measuring inflammatory markers could lead to different results [27,28]. However, if we compare studies using different methods for measuring inflammatory markers in healthy individuals, the conclusions are the same for these studies, i.e. no beneficial effects of fish oil were observed in healthy individuals.

A clear strength of our study is the compliance of our subjects to the intervention, which was very high [13]. Another advantage is that we measured a large panel of serum inflammatory markers, including chemokines, cell adhesion molecules, and previously

CRP. This might better reflect the overall inflammatory response compared with other studies, investigating only a few markers. When studying a large range of markers it should be kept in mind that correction for multiple testing might be necessary. In this study we choose a p-value of <0.01 as statistically significant. We chose not to use the Bonferroni correction as this might be too conservative since the inflammatory markers are also interrelated [29]. Additionally, we studied the coherence and interrelationships of the inflammatory markers using PCA. While studying patterns of inflammatory markers, one could make the distinction between pro- and anti-inflammatory markers. However, we could not identify any specific pro- or anti-inflammatory pattern, or specific patterns for chemokines or cell adhesion molecules. It could be argued that the classification of pro- and anti-inflammatory is too simplistic since the function of a specific cytokine depends on the amount, the target cell, the producing cell, and the sequence of actions of cytokines. Furthermore, we used PCA to analyze the possible patterns in our data set since PCA is a commonly used method to analyze possible patterns [19]. We realize that other types of factor analysis can be applied as well, although these other methods have other underlying assumptions. Due to the number of participants, the PCA was rather explorative.

For future large-scale studies, the use of the multiplex immunoassay has two big advantages: first only 50µl of biological fluid is necessary for the measurement of the multiplex immunoassays and second, its relatively low costs. Future observational and experimental studies could use this approach to measure a large range of inflammatory markers and using factor or cluster analysis. It would be most interesting to include subjects with an inflamed status, like patients with cardiovascular disease or inflammatory bowel disease, especially since inflammatory markers are elevated and possible patterns of interrelationships are easier to detect. In addition, besides serum inflammatory markers also more local markers of inflammation, such as markers in colonic tissue, could be considered to study the effects of diet on inflammation [28].

In conclusion, the results of this randomized, double-blind, placebo-controlled intervention are in agreement with previous studies that suggest that fish oil supplementation has no effect on serum inflammatory markers in a healthy middle-aged population. Moreover, no effects on the interrelationships of these inflammatory markers were found.

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4

Fish consumption and markers of colorectal cancer risk: a multicentre randomized controlled trial

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Abstract

Background: Diet is a major factor in the etiology of colorectal cancer, with high fish consumption possibly decreasing colorectal cancer risk, as is shown in several observational studies. To date, no intervention trials have examined the possible beneficial effects of fish intake on colorectal cancer risk.

Objective: The objective was to investigate the effects of a 6-mo intervention with oil-rich or lean fish on apoptosis and mitosis within the colonic crypt.

Design: In a multi-centre, randomized, controlled intervention trial, patients with colorectal polyps, inactive ulcerative colitis, or no macroscopic signs of disease were recruited (n=242) and randomly allocated to receive dietary advice plus either 300g oil-rich fish (salmon) per week (n=82), 300g lean fish (cod) per week (n=78), or only dietary advice (DA) (n=82). Apoptosis and mitosis were measured in colonic biopsy samples collected before and after intervention (n=213).

Results: The total number of apoptotic cells per crypt did not increase in the salmon or cod group: -0.10 (95% CI -0.36; 0.16) and -0.06 (95% CI -0.32; 0.20) respectively compared with DA. The total number of mitotic cells per crypt decreased non-significantly in the salmon group (-0.87, 95% CI -2.41; 0.68) and in the cod group (-1.04, 95% CI -2.62; 0.53) compared with DA. Furthermore, the distribution of mitosis within the crypt did not significantly change in either group.

Conclusion: An increase in the consumption of either oil-rich or lean fish to 2 portions weekly over 6 mo does not markedly change apoptotic and mitotic rates in the colonic mucosa. The trial has been registered at www.clinicaltrials.gov under identifier NCT00145015.

Introduction

Colorectal cancer is one of the cancers most strongly related to dietary habits [1,2], with 65-75% of the incidence of colorectal cancer attributed to dietary factors [3]. One of the dietary habits that may reduce colorectal cancer risk is consumption of fish [1]. Several observational studies have shown that fish consumption could be related to a decreased risk of colorectal cancer [1]. Recently, a meta-analysis of 19 prospective cohort studies showed a 12% decrease in relative risk (RR) of colorectal cancer (RR 0.88, 95% CI 0.78; 1.00) in a comparison of high fish consumption with low fish consumption [4]. The largest contributing study to this meta-analysis was the European Prospective Investigation into Cancer (EPIC) study, with a hazard ratio of 0.69 (95% CI 0.54; 0.88) and a contrast of 70g fish/d between the lowest vs. highest intake category [5]. The beneficial effects of fish consumption are generally attributed to their very long chain n-3 or omega-3 polyunsaturated fatty acid (VLC-PUFA) content, as established in animal and *in vitro* studies [6]. However, fish contains other nutrients that have been associated with a reduced colorectal cancer risk, such as vitamin D and selenium [1,7]. Observational studies are currently unable to assess whether the possible protective effect of fish on colorectal cancer risk is only associated with the consumption of oil-rich fish or with fish in general, as no discrimination in type of fish is normally made in these studies. Hence, there is a need for intervention studies involving different types of fish. Because the assessment of

colorectal cancer incidence as outcome is not feasible, intermediate endpoints of colorectal cancer risk have to be used, despite their recognized limitations.

An intermediate endpoint often used for colorectal cancer risk in intervention studies is cell proliferation [8]. A decrease in cell proliferation or mitosis indicates a longer cell cycle time, thereby increasing time for cells to repair any replication errors [9]. For many years, this has been considered one of the few available early biomarkers of colorectal cancer risk. Patients with ulcerative colitis (UC) and those with colorectal polyps have increased mitotic rates [9,10] and are at an increased risk of developing colorectal cancer. Gastrointestinal epithelial cell homeostasis is maintained by the balance between cell growth and apoptosis; therefore, more recently apoptosis has been considered to be a useful marker of colorectal cancer risk [11]. Higher levels of apoptosis indicate faster removal of damaged cells which can possibly prevent clonal expansion, and higher apoptotic levels have been linked to reduced colorectal cancer risk in both animal models [12] and humans [13]. Patients with UC [14,15] and those with polyps [16] have decreased apoptotic rates.

Mitosis and apoptosis have been examined in several studies that investigated dietary supplementation with fish oil and have shown inconsistent results. Some studies showed a reduction in cell proliferation [17-20] and an increase in apoptosis [18,21] in response to fish oil supplementation, whereas other studies failed to show an effect [22-26]. Also, a number of studies only confirmed an effect of fish oil in adenomatous tissue and not in normal tissue [17,19,25] or only showed an effect of fish oil within and not between treatment groups [17,25].

To our knowledge, no intervention trials have examined the possible beneficial effects of fish consumption on colorectal cancer risk. Therefore, the aim of the current study was to investigate the effects of an intervention using either oil-rich or lean fish, on apoptosis and mitosis in colonic crypts as markers of colorectal cancer risk in a high-risk population of patients, with either colorectal polyps or inactive UC, and in healthy control patients. We hypothesized that increased fish consumption would lead to increased apoptotic levels and decreased mitotic levels and that oil-rich fish would result in more pronounced effects than would lean fish.

Subjects and methods

Subjects and sample size

The trial (FISHGASTRO study) was carried out by two research centers, Wageningen University (WU), Wageningen, the Netherlands (NL), and the Institute of Food Research (IFR), Norwich, United Kingdom (UK). Potential participants were recruited from outpatient colonoscopy clinic lists in eight clinical centers (6 in the Netherlands, 2 in the United Kingdom). The primary outcome of this trial was the change in apoptosis. Based on findings in a previous study, a sample size of 90 subjects per intervention group was calculated to provide power of at least 80% to detect a change of 0.2 apoptotic cells per

crypt with a standard deviation (SD) of 0.46, using a two-sided statistical significance level of $p < 0.05$ [23]. To account for a dropout rate of 10%, we needed 100 subjects per intervention group (n=300).

Between November 2004 and July 2007, we recruited male and female volunteers aged 18- 80 years for the study from outpatient clinic attendees visiting the hospital for a colonoscopy, which was part of their regular medical care. Three groups of subjects were recruited: (i) those with (previous) colorectal polyps which were histologically confirmed, (ii) those diagnosed with non-active UC, and (iii) those without any macroscopic signs of disease in the colon, whose reasons for attending included irritable bowel syndrome, hemorrhoids, unexplained anemia, bowel complaints, or changes in defecation pattern. Approximately 10% of the invited patients were willing to participate in the trial. The main reasons for not participating were an unwillingness to increase their fish consumption or to undergo an extra sigmoidoscopy at the end of the trial, which was additional to their regular medical care. Subjects were excluded if they were: allergic to fish, taking fish oil supplements, taking non-steroidal anti-inflammatory drugs (NSAIDs) or acetylsalicylic acid, organ transplant recipients receiving immuno-suppression therapy, type I diabetics, or patients with an elevated infection risk.

The Dutch study protocol was approved by the Medical Ethical Committee of Nijmegen University Medical Centre St. Radboud (reference 2004/111), and the English study protocol was approved by King's Lynn Local Research Ethics Committee (reference 04/Q0105/8). All subjects gave written informed consent after the study was explained to them both in writing and verbally.

Design and Treatment

The FISHGASTRO study is a multi-centre parallel randomized controlled intervention trial (RCT). After an initial colonoscopy procedure, eligible subjects were randomly allocated by an independent person to 1 of 3 dietary intervention groups: (i) oil-rich fish group receiving two 150g portions of farmed salmon per week for 6 mo, (ii) lean fish group receiving two 150g portions of Icelandic cod per week for 6 mo, or (iii) dietary advice (DA) group. All 3 intervention groups received general dietary advice to achieve a healthy diet [27,28]. Treatment codes were generated by country and patient group in blocks of six using a computer-generated randomization schedule. The fish was provided to the participants at their home and they were asked to consume it in addition to any regular fish consumption. Salmon and cod provided approximately 1.4g/d and 0.09g/d of n-3 VLC-PUFA respectively [29]. We chose a study duration of 6 mo since this would be long enough to incorporate n-3 PUFA in the colonic epithelium [23].

Volunteer compliance was checked using food diaries, regular phone calls every 2-4 wk, and for the salmon group by pre- and post-intervention measurements of serum n-3 VLC-PUFA concentrations.

Data collection

Colonic biopsy samples were collected before intervention during a routine colonoscopy procedure and after intervention during a sigmoidoscopy procedure. The preparation of the colonoscopy procedure consisted of macrogol (Kleanprep, Norgine BV, Amsterdam, NL) in NL, or Picolax (Ferring Pharmaceuticals Limited, Berkshire, UK) in UK; the preparation of sigmoidoscopy procedure consisted of an enema in both NL and UK. Distal colon biopsies were obtained from mucosa of normal-appearance at ~20- 30cm from the anal verge during the colonoscopy or sigmoidoscopy.

Fasted blood samples were taken on the day of the colonoscopy or sigmoidoscopy procedure and serum was stored at -80°C prior to analysis. Serum cholesteryl fatty acids were measured as previously described [29]. Furthermore, we determined serum 25-hydroxy vitamin D (enzyme-immunoassay, Immunodiagnostics Systems Ltd, UK) and serum selenium concentrations using an Agilent 7500ce ICP/MS (Agilent UK Ltd, Stockport, UK) following UV assisted wet digestion in a Metrohm 705 UV digester (Metrohm, Buckingham, UK). Dietary habits were assessed before and at the end of the intervention period by a self-administered food frequency questionnaire (FFQ) [30,31], and additionally by a 7-day food diary in the UK. Information on lifestyle, including physical activity [32] and smoking, weight, and height measures was obtained at baseline by questionnaire. Participants were asked to report any changes in well being, and medication and supplement use during the intervention period.

Analysis of crypt cell apoptosis and proliferation in colonic biopsies

After being collected, colonic biopsies were immediately fixed in ethanol: acetic acid (3:1) and stored at 4°C before analysis. Apoptosis and mitosis were determined in intact micro-dissected crypts using morphological criteria [33-35]. Biopsies were dissected under low power microscopy to yield thin strips of crypts which were gently squashed beneath a cover-slip. Ten to twenty randomly selected intact crypts were viewed under a light microscope (x400). The length of each crypt was determined by comparison with a calibrated linear eyepiece graticule (Nikon UK, Kingston, UK) and the positions of mitotic cells were recorded along the length of the crypt. Data were expressed as the total number of apoptotic or mitotic cells per crypt. The microscopist was blinded to both treatment and patient group and all analyses were performed in the same research center (IFR).

Statistical analyses

Data analysis was carried out according to a predefined analysis plan. Subject compliance of the salmon group, based on serum n-3 VLC-PUFA changes, was tested using a paired Students t-test within the salmon group. After the intervention, changes in outcome variables were evaluated by using an analysis of covariance (ANCOVA), with adjustment

for baseline values. We compared the changes in outcome measures in the salmon and cod group with the changes in the DA group; therefore changes are presented as mean change compared with the DA group (95% CI). The distribution of mitosis within the crypt was also analyzed. Crypt lengths were normalized in tenths and ANCOVA was used to compare mitotic rates in the bottom 40% and top 40% of the crypts, as previously described by Anti et al [17]. For all analyses, we explored whether results were different per patient group and per country. We performed analyses using the SAS statistical software program (SAS version 9.1) and considered a p-value of <0.05 as statistically significant. The researchers performing the statistical analyses were blinded to the treatment and patient group during the analyses.

Results

Subjects and compliance

Of the 242 randomized participants, 216 completed the 6-mo intervention, as is shown in **Figure 4.1**. Reasons for discontinuation are depicted in Figure 4.1 and consisted mainly of not wanting an extra sigmoidoscopy (n=5), not willing to eat fish (n=3), pregnancy (n=2), or occurrence of prostate cancer (n=2). Four serious adverse events were reported during the study period but none were related to the study.

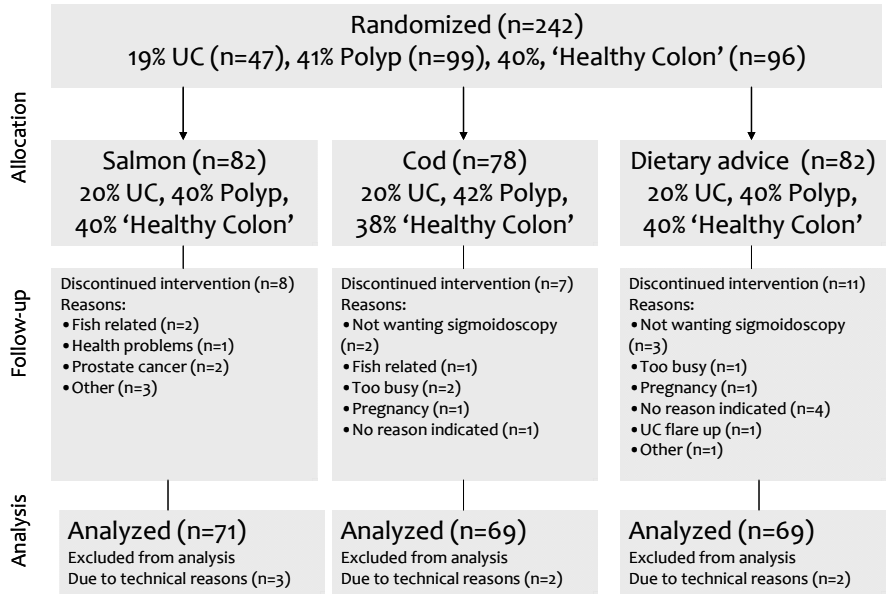

Figure 4.1 Flow chart of the Fishgastro intervention study.

Baseline characteristics of all subjects that completed the intervention are shown in **Table 4.1**. Overall, no differences in baseline characteristics were observed between the 3

intervention groups, although subjects receiving cod included fewer women and fewer smokers. Baseline characteristics of the dropouts were similar to those participants who finished the intervention (data not shown).

Table 4.1 Baseline characteristics of the FISHGASTRO study population (n=213) (mean ± SD) [1]

Intervention group: Characteristic	Salmon (n=74)	Cod (n=70)	Dietary advice (DA) (n=69)
Age (year)	55.1 ± 11.5	57.4 ± 10.3	55.3 ± 9.5
Sex (% female)	51	41	54
BMI (kg/m^2)	26.5 ± 4.4	26.8 ± 4.3	26.7 ± 3.5
Smoking (% current)	26.0	11.4	15.9
Family history of CRC (% yes)	1.3	4.3	4.4
Indication for baseline colonoscopy (% screening)	41	44	46
Research Centre (% NL)	76	74	78
Patient groups (% polyp/ UC/ healthy controls)	38/ 19/ 43	46/ 20/ 34	42/ 19/ 39

Abbreviations: SD (standard deviation), BMI (body mass index), CRC (colorectal cancer), NL (Netherlands), UC (ulcerative colitis)

[1]There were no statistical significant differences among the three intervention groups

Before the start of the intervention, fish was consumed on average 1.5 times per week, as is shown in **Table 4.2**. Dietary assessment data indicated that fish consumption increased by 1.4 portions per week in the salmon group and by 1.3 times per week in the cod group during the intervention (Table 4.2); no increase in fish consumption was observed in the DA group. Furthermore, nutrient intake indicated that dietary habits other than fish consumption did not significantly change in any of the intervention groups, and specifically that meat consumption did not decrease in the salmon or cod group (data not shown).

Table 4.2 Fish intake at baseline and the end of the intervention presented as frequency per week (mean ± SD)

Intervention group: Fish intake (freq./wk)	Salmon	Cod	Dietary advice (DA)
Baseline	1.6 ± 1.3	1.6 ± 1.1	1.5 ± 1.1
End	2.8 ± 1.3 [1]	2.9 ± 1.3 [1]	1.6 ± 1.3
Change	1.4 ± 1.1 [2]	1.3 ± 1.4 [2]	0.1 ± 1.0
Difference in change compared with DA (mean (95% CI))	1.3 (0.9; 1.7)	1.2 (0.8; 1.7)	

[1] Significantly different compared with baseline (p<0.0001, paired t-test)
[2] Significantly different change compared with DA (p<0.05, ANCOVA)
Abbreviations: freq. (frequency), wk (week), DA (dietary advice), CI (confidence interval)
Measured at baseline in n=71 salmon, n=65 cod, n=69 DA, after intervention n=59 salmon, n=61 cod, n=60 DA

Participants in all 3 intervention groups had similar levels of serum n-3 VLC-PUFA at baseline, as is shown in **Table 4.3**. Serum n-3 VLC-PUFA levels significantly increased in the salmon group (p<0.0001; Table 4.3) and not in the cod or DA group compared with baseline. This indicated that subjects in the salmon group generally complied with the salmon intervention.

Table 4.3 Serum measures of very long chain n-3 PUFA (EPA + DHA) per intervention group of participants that completed the intervention (mean ± SD mass% of total fatty acids in cholesteryl esters)

Intervention group: **Serum very long chain n-3 PUFA**	**Salmon** **n=71** [1]	**Cod** **n=69** [1]	**Dietary advice** **(DA) n=70**
Baseline	2.85 ± 1.41	2.71 ± 1.07	2.64 ± 1.20
End	3.59 ± 1.41 [2]	2.68 ± 1.04	2.49 ± 1.06
Change	0.74 ± 1.35	-0.04 ± 0.94	-0.14 ± 0.80
Difference in change compared with DA (mean (95%CI))	0.88 (0.52; 1.24)[3]	0.11 (-0.27; 0.47)	

[1] missing values due to technical reasons, in salmon group n=3, in cod group n=1, and in DA n=1
[2] Significantly different compared with baseline (p<0.0001, paired t-test)
[3] Significantly different change compared with DA (p<0.05, ANCOVA)

Serum selenium concentrations were significantly increased after intervention compared with baseline within the cod group (+ 4.6 ± 14.5 ng/ml, p 0.02). However, compared with the DA group, changes in the cod and salmon group were not statistically significant, for cod 4.7 ng/ml (95% CI -1.6; 11.1) and for salmon 1.3 ng/ml (95% CI -4.9; 7.4). Serum vitamin D concentrations were non-significantly increased in the fish intervention groups, by 1.5 nmol/l (95% CI -8.4; 11.3) in the salmon group and by 1.5 nmol/l (95% CI -8.9; 11.7) in the cod group as compared with DA.

Markers of colorectal cancer risk

Fish consumption had no effect on the number of apoptotic cells per crypt after 6-mo intervention compared with DA, as is indicated in **Table 4.4**. Similarly, the changes in the number of mitotic cells per crypt were not significantly different after intervention with fish compared with dietary advice, although a non-statistically significant decrease was observed of -0.9 mitotic cells per crypt (95% CI -2.4; 0.7) in the salmon group and -1.0 mitotic cells per crypt (95% CI -2.6; 0.5) in the cod group compared with the DA group (Table 4.4).

Table 4.4 Markers of colorectal cancer: number of apoptotic cells per crypt, and number of mitotic cells per crypt in the FISHGASTRO study (mean ± SD)

Intervention group: **Number of apoptotic cells per crypt (nr/crypt)**	**Salmon (n=74)**	**Cod (n=70)**	**Dietary advice (DA) (n=69)**
Baseline	0.8 ± 1.0	0.7 ± 0.9	0.6 ± 0.7
End	0.6 ± 0.7	0.5 ± 0.7	0.5 ± 0.6
Change	-0.2 ± 0.9	-0.2 ± 0.8	-0.1 ± 0.6
Difference in change compared with DA (mean (95% CI))	-0.1 (-0.4; 0.2)	-0.1 (-0.3; 0.2)	
Number of mitotic cells per crypt (nr/crypt)			
Baseline	7.0 ± 3.9	7.1 ± 4.5	6.2 ± 4.2
End	5.1 ± 3.3	5.0 ± 3.1	5.1 ± 3.3
Change	-1.9 ± 5.0	-2.1 ± 4.6	-1.1 ± 4.5
Difference in change compared with DA (mean (95% CI))	-0.9 (-2.4; 0.7)	-1.0 (-2.6; 0.5)	

After intervention, the percentage of mitotic cells in the bottom of the crypt non-significantly increased in the salmon group with 2.7 (95% CI -2.9; 8.3), and in the cod group with 5.5 (95% CI -0.2; 11.2) compared with DA, and no changes were observed for the percentage of mitotic cells at the top of the crypt, as is shown in **Table 4.5**.

Comparison of the results per country at baseline showed statistically significant differences between countries in the number of apoptotic cells per crypt (NL 0.5 ± 0.8; UK 1.1 ± 0.9, $p < 0.0001$) and in the number of mitotic cells per crypt (NL 6.1 ± 3.6; UK 9.1 ± 5.0, $p < 0.0001$). However, compared with the DA group, the changes in the number of apoptotic cells per crypt in the intervention groups did not differ between countries: -0.2 (95% CI -0.5; 0.2) for salmon and -0.2 (95% CI -0.5; 0.1) for cod in the Netherlands and 0.1 (95% CI -0.4; 0.6) for salmon and 0.3 (95% CI -0.2; 0.8) for cod in the United Kingdom. The non-significant decrease in mitotic cells in the salmon and cod group was more pronounced in NL participants compared with UK, although the decrease in mitotic cells in NL was not statistically significant, changes in number of mitotic cells per crypt were in -0.1 (95% CI -1.3; 1.2) for salmon and -0.7 (95% CI -2.0; 0.6) for cod in the Netherlands and 0.0 (95% CI -3.3; 3.2) for salmon and 1.5 (95% CI -1.7; 4.7) for cod in the United Kingdom.

Table 4.5 Results of the percentage of mitotic cells in the 40% upper part of the crypt and in the 40% bottom part of the crypt (nr/crypt) (mean ± SD)

Intervention group: **Percentage of mitotic cell numbers per crypt** **40% upper part crypt**	**Salmon (n=74)**	**Cod (n=70)**	**Dietary advice (DA) (n=69)**
Before	3.5 ±5.9	2.8 ± 4.3	2.5 ± 4.4
After	2.7 ± 5.1	2.8 ± 5.3	2.2 ± 4.3
Change	-0.8 ±5.9	-0.1 ± 5.6	-0.3 ± 3.8
Difference in change compared with DA (mean (95% CI))	-0.5 (-2.2; 1.2)	0.2 (-1.6; 1.9)	
Percentage of mitotic cell numbers per crypt **40% bottom part crypt**			
Before	81.7 ± 15.0	80.5 ± 13.4	86.0 ± 16.5
After	82.9 ± 12.2	84.4 ± 13.3	84.5 ± 10.8
Change	1.1 ± 15.5	4.0 ± 17.3	-1.5 ± 18.1
Difference in change compared with DA (mean (95% CI))	2.7 (-2.9; 8.3)	5.5 (-0.2; 11.2)	

Comparison of the different patient groups at baseline showed that the number of apoptotic cells per crypt was significantly higher in UC patients (0.9 ± 1.2, p=0.04), and non-significantly higher in polyp patients (0.7 ± 0.9, p=0.11) as compared with control subjects (0.6 ± 0.6). The number of mitotic cells per crypt was significantly higher in UC patients (8.1 ± 4.2, p=0.02) but not in patients with polyps (6.6 ± 4.3, p=0.33), as compared with healthy controls (6.3 ± 4.0). The conclusions from our study did not change when we analyzed changes in apoptosis and mitosis stratified for patient group. We observed no change in the number of apoptotic cells after intervention, and this result was not affected by patient groups (data not shown).

Compared with the DA group, the number of mitotic cells per crypt changed non-significantly in polyp patients for salmon (-1.6, 95% CI -4.0; 0.8) and for cod (-0.4, 95% CI -2.7; 1.9), in UC patients for salmon (-1.7, 95% CI -5.4; 2.1) and for cod (-2.9, 95% CI -6.6; 0.8), and in healthy controls for salmon (0.1, 95% CI -2.5; 2.7) and for cod (-0.9, 95% CI -3.6; 1.9).

Discussion

The results of this trial do not support the hypothesis that additional fish consumption over a 6-mo period changes the number of colonic apoptotic and mitotic cells or the distribution of mitotic cells within the crypt. Furthermore, no marked differences in these markers were observed between oil-rich and lean fish. To the best of our knowledge, this is the first RCT to have studied the effects of fish consumption on markers of colorectal cancer risk. It is generally considered preferable by many health professionals to encourage dietary change rather than the use of supplements. Therefore, the use of fish in this study better reflects the effects of the current advice of 2 portions of fish per week, as compared with previous fish oil studies intervening with n-3 PUFA doses corresponding to 1-4 portions of salmon per day [17,19]. Another advantage of our study was that we compared oil-rich fish to lean fish. This allowed us to explore whether or not the possible effects of fish were mainly associated with n-3 PUFA.

There may be several reasons for not finding significant effects of fish on apoptotic and mitotic cell numbers per crypt. First, the baseline fish consumption of 1.5 portions/wk was already high in these subjects, which was also reflected in their higher serum n-3 VLC-PUFA levels, compared with those of subjects in other fish oil supplementation studies [36,37]. Perhaps a more pronounced effect of increasing fish consumption would be expected in a population of non-fish consumers. Second, it appeared that subjects did not consume the salmon or cod in addition to their habitual fish consumption, as requested, but partly substituted the fish they would normally consume with the study fish. Therefore, the intended increase of 2 additional portions of fish per week actually only resulted in an increase of 1.4 and 1.3 extra portions per week of salmon and cod respectively. This resulted in an additional intake of 0.99 and 0.05g/d n-3 VLC-PUFA for salmon and cod, respectively. Thus, the contrasts in our study between the intervention groups may not have been large enough to observe a beneficial effect of additional fish consumption. On the other hand, an increase of 1.4 portions/wk may be the maximally achievable dietary modification in a population of fish eaters. The study duration of 6 mo was chosen as being of sufficient length to allow incorporation of n-3 VLC-PUFA in the colonic epithelium [23]. Such changes were expected to increase eicosapentaenoic acid and docosahexaenoic acid in mitochondrial phospholipids of colonocytes at the expense of n-6 PUFA, also known as omega-6 PUFAs, thereby enhancing the deletion of colonic cells through apoptosis and reducing the level of DNA adducts through cell proliferation [38]. Of all 7 studies that evaluated the effects of fish oil supplementation on cell proliferation, 4 studies lasting 1-6 mo observed an effect [17-20]. As far as we know, 3 studies assessed the effects of fish oil supplementation on apoptosis: 2 studies observed an effect after 3 [18] and 24 months [21]. Thus, for both markers 6 mo intervention appears to be long enough, however, for apoptosis only one short-term study was not able to do so [22].

To measure apoptosis and mitosis, a morphological method was used that has 2 advantages over other methods. Ki67 [8] and TUNEL [11] require well orientated histological sections to allow identification of full length longitudinal crypt sections [39], whereas apoptosis, mitosis, and the distribution of mitosis within the crypt can be measured simultaneously using the morphological method. In a whole crypt mount it is easier to detect the relatively rare apoptotic cells. However, the whole crypt mount approach does not identify all apoptotic cells on the luminal surface whereas TUNEL and M30 stains may preferentially detect this normal programmed cell death [11]. It is a moot point as to whether crypt associated apoptosis or luminal apoptosis is of most clinical significance [40]. Different methods have been used to measure apoptosis and mitosis in various studies which makes it difficult to compare our results to those of others. Although, compared with a previous study using the same method to measure mitosis, our subjects have comparable levels of cell proliferation [41], yet the variation in mitotic levels was much higher despite the larger number of subjects in this study; and this could have affected the power of our study. One possible explanation as to why the variation was higher than in a previous study, might be that the patient group used in this study was less homogenous than in this previous study, in which all patients were undergoing resection for colorectal cancer [23]. Ideally, we would have studied incidence of colorectal cancer as outcome measure; however because this is not feasible in an RCT, we studied apoptosis and mitosis as intermediate markers of colorectal cancer risk. In addition, other potential early markers of colorectal cancer risk may also be relevant, eg, DNA damage leading to key mutations, DNA methylation, mitochondrial dysfunction, or presence of aberrant crypt foci. However, in this study we chose to focus on apoptosis and mitosis as the most well-established markers for relatively short-term RCTs.

Because UC and polyp patients are at an increased risk of developing cancer, it could be hypothesized that the most pronounced effects would occur in these subjects. However, the number of subjects we were able to recruit in the UC group was lower (n=41) than was originally planned (n=90). Recruitment of patients with inactive UC was difficult because the number of eligible patients from those visiting the hospital for a colonoscopy was relatively low; thus not reaching 90 subjects as planned would have decreased the power of our study. The changes in apoptotic numbers did not differ between patient groups for either salmon or cod. However, the most pronounced decrease in the number of mitotic cells was found in the UC patients, although it was not statistically significant. Thus, this data suggests that fish consumption might decrease the number of mitotic cells in UC patients but this should be investigated in a larger UC population. It should also be noted that of the patients referred to as having a 'healthy colon', ~60% had bowel complaints as indication for their colonoscopy, whereas the reasons for the initial endoscopy included familiar occurrence of CRC, hemorrhoids or anemia in the other 40%. Although their colonoscopy did not show any colonic

abnormalities, these 'healthy colon' patients do not all represent truly healthy individuals. Indeed, it has previously been shown that a similar group of symptomatic patients with apparently normal mucosa had more mitotic cells per crypt than those assessed as part of a routine screening program [42].

Another factor that could have affected our results, was the use of different bowel preparations before intervention in the 2 countries, and also after intervention compared with pre-intervention. First, pre-intervention Kleanprep was used in the Netherlands and Picolax in the UK as preparation for colonoscopy; Picolax has recently been reported to give higher levels of cell proliferation than Kleanprep [43]. Indeed, we found significantly higher levels in the UK as compared to NL for both apoptosis and mitosis at baseline, suggesting that this could well have influenced our results. However, changes in apoptosis and mitosis were not significantly different between countries. Second, post-intervention colonic biopsy samples were collected by means of sigmoidoscopy, which was additional to patient's regular medical care because it was considered unduly invasive to require subjects to undergo an additional colonoscopy. The switch from colonoscopy at baseline to sigmoidoscopy after the intervention might explain the reduction in mitosis seen in all three intervention groups, although this might only explain the decrease in the UK since for Picolax a decrease in cell proliferation has been found switching from Picolax to no bowel preparation while this has not been observed for Kleanprep [43]. In designing future studies using colonoscopy or sigmoidoscopy procedures, especially in multi-centre studies, attention should be paid to standardizing bowel preparations if feasible.

To further elucidate the association of fish consumption with colorectal cancer, future intervention studies should also try to include nonfish eaters or individuals with a relatively low fish consumption, although this would practically be a major challenge. Alternatively, observational studies could be performed, using more detailed questionnaires on fish intake and specifically inquiring as to the different types of fish and the preparation of fish, since this could affect the possible beneficial effects of fish.

In conclusion, this randomized, controlled intervention study on fish and markers of colorectal cancer showed that additional fish consumption of ~1.4 portions of either oil-rich or lean fish per week over 6 mo does not markedly change apoptotic and mitotic rates of the colonic mucosa in a population of fish eaters.

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5

Increasing fish consumption does not affect genotoxicity markers in the colon in an intervention study

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Abstract

Observational studies suggest that fish consumption is associated with a decreased colorectal cancer (CRC) risk. A possible mechanism by which fish could reduce CRC risk is by decreasing colonic genotoxicity. However, concerns have also been raised over the levels of toxic compounds found in mainly oil-rich fish, which could increase genotoxicity. Therefore, the objective was to investigate the effects of fish on genotoxicity markers in the colon in a randomized controlled parallel intervention study. For a period of six months, subjects were randomly allocated to receive two extra weekly portions of (i) oil-rich fish (salmon), (ii) lean fish (cod), or (iii) just dietary advice. The Comet Assay was used to measure the DNA damage-inducing potential of fecal water (n=89) and DNA damage in colonocytes (n=70) collected pre- and post-intervention as markers of genotoxicity.

Genotoxicity of fecal water was not markedly changed after fish consumption: 1.0% increase in tail intensity (TI) (95% confidence interval (CI) -5.1; 7.0) in the salmon group and 0.4% increase in TI (95% CI -5.3; 6.1) in the cod group compared with the dietary advice group. DNA damage in colonocytes was also not significantly changed after fish consumption, in either the salmon group, (-0.5%TI, 95% CI -6.9; 6.0), or cod group (-3.3%TI, 95% CI -10.8; 4.3) compared with the dietary advice group. Measurements of genotoxicity of fecal water and DNA damage in colonocytes did not correlate (r=0.06, n=34). In conclusion, increasing consumption of either oil-rich or lean fish did not affect genotoxicity markers in the colon.

Introduction

Colorectal cancer (CRC) is one of the most commonly occurring cancers worldwide and has been associated with dietary habits [1]. CRC develops over many years as a result of accumulation of DNA damage and mutations, resulting in a loss of control of cell proliferation and failure of damaged cells to undergo apoptosis [1,2]. DNA damage is thought to be caused by genotoxic insults and factors in the diet may modulate genotoxicity in the colon. One of the dietary habits that possibly influences the risk of CRC is consumption of fish. Several observational studies have shown that high intakes of fish could be related to a decreased risk of CRC [1,3-7]. This potential benefit on CRC could be mediated by apoptosis and mitosis, which has been shown in several intervention studies [8-12]. Genotoxicity could be decreased by the intake of fish by modulation of enzymes involved in detoxification of phase I or II enzymes like glutathione S-transferase [13], by a decrease in inflammatory processes via oxidative stress pathways [14,15], or by decreasing the bacterial conversion of bile acids into more genotoxic secondary bile acids [16,17].

Whilst the focus has been on the beneficial effects of fish consumption, concerns have been raised as to whether it could also have unfavorable effects, due to the possible presence of toxins. Toxic compounds such as dioxins or polychlorinated biphenyls (PCBs), which can accumulate in the food chain and which are mostly found in oil-rich fish [18,19], could increase colonic genotoxicity. Although mostly associated with beneficial effects, n-3 polyunsaturated fatty acids (PUFA), highly abundant in oil-rich fish, could

potentially increase genotoxicity as they are readily oxidized and could enhance lipid peroxidation [20] and oxidative stress [21] leading to an increase in endogenous DNA damage. Thus, oil-rich fish could have differential effects on CRC risk compared with lean fish. To the best of our knowledge, no intervention study has been performed examining the genotoxic effects of consumption of either oil-rich or lean fish in the colon.

Colorectal genotoxic effects can be measured indirectly by determining the DNA-damage inducing potential of fecal water in human colon adenocarcinoma cells (e.g. HT29 cells) *in vitro*, or directly by measuring DNA damage in colonocytes extracted from colorectal biopsies *in vivo*. Fecal water represents the aqueous fraction of the feces and diet has been shown to affect fecal water genotoxicity [22-25]. Moreover, it has been demonstrated that fecal water can influence processes related to colorectal carcinogenesis, such as apoptosis [26] and proliferation [27]; patients with colorectal polyps differ in fecal water biochemistry compared with healthy controls [27]. However, it is not clear how genotoxicity of fecal water translates to DNA damage in the colonic epithelium, and ultimately we are interested in the processes in the colon. Therefore, we also included measurements of DNA damage in colonocytes. To sum up, the aim of the current study was to study the effects of fish consumption on markers of genotoxicity in the colon.

Subjects and Methods

Subjects and study design

The design of the study was a multi-centre parallel randomized controlled intervention study and has been described in detail elsewhere [28]. Three groups of subjects were recruited: those with (previous) colorectal polyps, those diagnosed with non-active ulcerative colitis (UC), and those without any macroscopic signs of disease in the colon. After an initial colonoscopy procedure, 242 eligible subjects were randomly allocated by an independent person to one of three dietary intervention groups: (i) oil-rich fish group receiving two 150g portions of salmon per week for six months, (ii) lean fish group receiving two 150g portions of cod per week for six months, and (iii) dietary advice (DA) group. All three intervention groups received dietary advice on achieving a healthy diet [29,30]. The fish was delivered to the participants in their home and they were instructed to consume it in addition to their regular fish intake. Salmon provided approximately 3.3g of long chain n-3 PUFA (eicosapentaenoic acid [EPA] + docosahexaenoic acid [DHA]) per 100g fish and for cod this was 0.2g/100g fish [31]. Furthermore, salmon contained 0.45 TCDD equivalents (TEQ)/g fish and cod contained 0.04pg TEQ/g fish [32]. We chose a study duration of six months since this would be sufficient to incorporate n-3 PUFAs into the colonic epithelium [33]. Compliance was checked by food diaries and regular phone calls every two to four weeks, and in the salmon group by serum levels of the long chain n-3 PUFA.

Feces was collected by a subgroup (n=128) of the subjects 1-3 weeks prior to the collection of colonic biopsy samples; 89 pairs of pre- and post-intervention fecal samples were randomly selected among those who collected feces and processed for the Comet Assay. Colorectal biopsy samples were collected at baseline during a colonoscopy procedure and post-intervention during a sigmoidoscopy procedure. A subset of complete sets of pre- and post-intervention samples collected in the Netherlands (NL) could be used for the Comet Assay (n=70) based on practical reasons associated with the need to process colorectal biopsies within 24h. A total of 34 subjects had DNA damage measured both in fecal water and in colonocytes at baseline. Life style factors including smoking, weight and height measures were obtained by questionnaire at the start; the frequency of fish consumption was assessed pre- and post-intervention.
Ethical approval was obtained from the Medical Ethical Committee of Nijmegen University Medical Centre St. Radboud in NL (reference 2004/111) and King's Lynn Local Research Ethics Committee in the United Kingdom (UK) (reference 04/Q0105/8). The trial has been registered at www.clinicaltrials.gov under identifier NCT00145015. All subjects gave their written informed consent and a subset of subjects consented separately for collection of fecal samples.

Preparation of fecal water and treatment of HT29 cells

The DNA-damage inducing potential of fecal water in the colonic HT29 cell line was determined as previously described [34]. A total fecal sample from one bowel movement was collected and stored in a cooled container for transport to the laboratory within 4h. At the laboratory, samples were homogenized, aliquoted and stored at -80°C until further processing. Samples were defrosted, homogenised by stirring, mixed with the same amount (w/v) of ice chilled PBS and centrifuged at 25,000xg for 2h at 4°C as described previously [35]. The pre- and post-intervention samples from individual volunteers were analyzed in parallel. After incubation of the cells with fecal water (50% concentration) or control treatments (PBS or 75µM H_2O_2) an aliquot was taken and cell viability was assessed by staining the cells with Trypan blue and dead and viable cells were counted using a haemacytometer.

Preparation of colonic biopsies

Primary colonocytes were isolated from colonic biopsies within 24h of the endoscopy procedure, as described previously [36]. Briefly, biopsy samples were incubated with 1mg/ml collagenase P and 2mg/ml proteinase K in Hank's balanced salt solution (HBSS), for approximately 90min at 37°C. The suspension was centrifuged for 5min at 400xg. The pellet was re-suspended in fresh HBSS for further processing. Cell numbers and viability were determined using the Trypan blue exclusion test [35].

Comet Assay

DNA damage was determined by the Comet Assay as previously described [37] and expressed as % tail intensity (TI), with a higher score indicating more DNA damage. For the Comet Assay in colonocytes, a single batch of HT29 cells which had been treated with or without a known genotoxin (75µM H_2O_2) served as positive and negative control. Colonocytes ($2x10^6$) were mixed with 50µl 0.7% low-melting agarose and distributed onto microscope slides (Trevigen, Gaithersburg, US). Alternatively, fecal water treated HT29 cells ($2x10^6$) were distributed in 90µl 0.7% low-melting agarose on agarose pre-covered microscope slides and after 10min covered with another layer of agarose. Only slides with colonocytes or controls were treated with 99% ethanol for 10min allowing them to dry prior to storage and shipment. All slides were stained with SYBR Green (2 µg/ml, Trevigen Inc.) and microscopical images were quantified using the image analysis system of Perspective Instruments (Halstead, UK); 50 images were evaluated per slide and the percentage of fluorescence in the tail, % TI was scored. For fecal water genotoxicity mean values of three parallel slides were determined and for colonocytes mean values of four replicate slides were determined. All slides were scored by a single scorer in a blinded manner.

Statistical analyses

Changes in outcome variables were evaluated using an analysis of covariance (ANCOVA) adjusting for baseline values. We compared the changes in outcome measures in the salmon and cod group with the changes in the DA group and therefore changes are presented as mean change compared to DA (with a 95% confidence interval). We explored if genotoxicity measured in HT29 cells after incubation with fecal water correlated with measurements of DNA damage in colonocytes using the Spearman correlation coefficient (n=34) and by cross-classification based on the median value. We also explored whether results were different between smokers and non-smokers as it has been shown that smoking could affect genotoxicity [24,38]. We performed analyses using the SAS statistical software program (SAS version 9.1) and considered a p-value <0.05 as significant. The researchers performing the statistical analyses were blinded to the treatment and patient group.

Results

Baseline characteristics of subjects whose fecal water was used for the Comet Assay are shown in **Table 5.1**. The DA group included more women compared with the salmon and cod group. The cod group had a lower percentage of current smokers and was less physically active. Further, subjects in all three intervention groups did not differ in terms of age or BMI. Subjects in whom DNA damage was determined in colonocytes (n=70, data not shown) were comparable in terms of age and BMI. The cod group included

somewhat fewer females, no current smokers, and were more physically active. The DA group included fewer polyp patients compared with the salmon or cod group.

Table 5.1 Baseline characteristics of the FISHGASTRO population for whom fecal water genotoxicity was determined by the Comet Assay (n=89)

Intervention group: **Characteristic**	**Salmon (n=26)**	**Cod (n=34)**	**Dietary advice (DA) (n=29)**
Age (year, mean ± SD)	57.8 ± 12.6	57.9 ± 8.5	55.6 ± 10.5
Sex (% female)	46	47	58
Smoking (% current)	31	9	15
BMI (kg/m^2, mean ± SD)	25.2 ± 3.7	26.0 ± 4.3	25.9 ± 3.2
Physical activity (% high)	46	24	31
Type of patient (% polyp/ UC / Healthy)	50/ 19/ 31	50/ 21/ 29	42/ 15/ 42
Country (% NL)	58	56	69

Abbreviations: SD (standard deviation), BMI (Body Mass Index), UC (ulcerative colitis), NL (Netherlands)

Baseline values of genotoxicity in fecal water was 9.9 ± 7.5% TI in the salmon group, 7.5 ± 6.2% TI in the cod group, and 13.9 ± 10.4% TI in the DA group. DNA damage in colonocytes was 17.8 ± 11.1% TI in the salmon group, 17.6 ± 7.0% TI in the cod group, and 15.0 ± 7.5% TI in the DA group at baseline.

The Spearman correlation coefficient between genotoxicity measured in fecal water and DNA damage in colonocytes was 0.06 (n=34). The overlap of the cross-classification based on the median values of genotoxicity of fecal water and DNA damage of colonocytes was 19 out of 34 subjects (56%).

At baseline genotoxicity was higher in smokers than in non-smokers: for fecal water genotoxicity values were 13.6 ± 8.3% TI for smokers (n=15) and 9.3± 8.2% TI for non-smokers (n=74, p=0.07); for colonocytes these values were 18.0 ± 13.9% TI for smokers (n=14) and 16.6 ± 8.2% TI for non-smokers (n=55, p=0.63).

Before the start of the intervention, subjects (n=89) consumed on average 1.1 ± 0.8 portions fish per week. Fish consumption increased by 0.7 ± 0.6 weekly portions in the salmon group, 0.9 ± 0.9 in the cod while the DA group changed their fish consumption with 0.2 ± 0.9 weekly portions of fish.

Subjects in whom DNA damage was measured in colonocytes (n=70) consumed on average 0.8 ± 0.6 portions fish per week at baseline. Fish consumption increased by 0.8 ± 0.7 weekly portions in the salmon group and 0.4 ± 0.7 weekly portions in the cod group while the DA group changed their fish consumption by 0.0 ± 0.4.

Fecal water

At baseline, viabilities of the HT29 cells after 30min incubation with 50% fecal water ranged from 62-100%, post-intervention this ranged from 61-100%.

Changes in genotoxicity of fecal water are presented in **Figure 5.1A**. We observed changes of 5.8 ± 10.6% TI (mean ± SD) in the salmon group, 5.3 ± 10.4% TI in the cod group, and 4.8 ± 11.9% TI in the DA group. The changes in the salmon and cod group compared with DA were 1.0%TI (95% CI -5.1; 7.0) and 0.4%TI (95% CI -5.3; 6.1), respectively.

Colonocytes

At baseline, viabilities of the fresh colonocytes ranged from 70-100%, post-intervention this ranged from 84-100% indicating that cells were viable for DNA damage measurement.

Changes in DNA damage measured in colonocytes are presented in **Figure 5.1B**. We observed changes of -0.1 ± 14.3% TI in the salmon group, -2.9 ± 9.8% TI in the cod group, and 0.3 ± 8.4% TI in the DA group. After intervention, levels of DNA damage in colonocytes were not significantly changed compared with DA in the salmon group, -0.5% TI (95% CI -6.9; 6.0), or in the cod group, -3.3% TI (95% CI -10.8; 4.3).

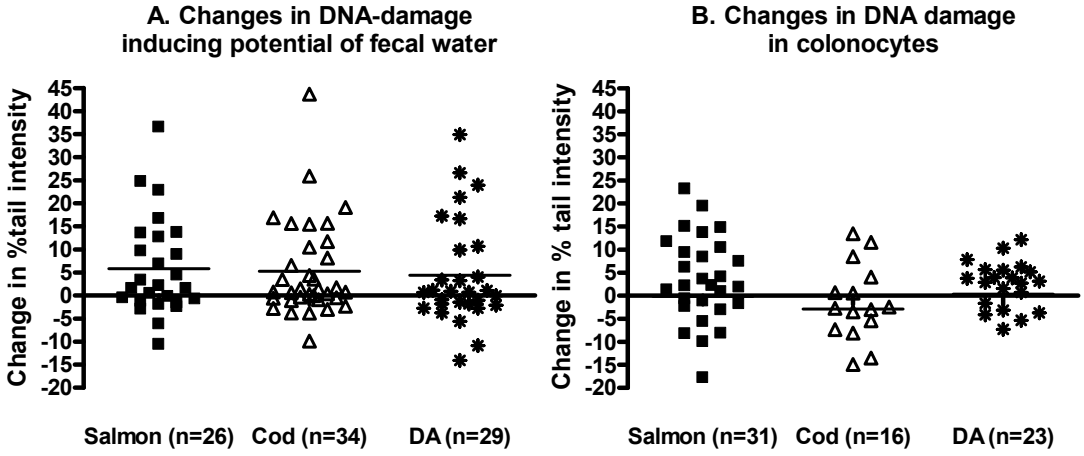

Figure 5.1 Results of changes in DNA-damage inducing potential of fecal water (Figure 5.1A, n=89) and DNA damage of colonocytes (Figure 5.1B, n=70). The horizontal line indicates the mean value. Abbreviation: DA (dietary advice)

Discussion

This intervention study showed that increasing fish consumption for six months neither positively nor negatively altered the genotoxicity of fecal water or DNA damage in colonocytes and that results of both measurements did not correlate. To the best of our knowledge, this is the first intervention study that has examined the effects of increasing fish consumption on genotoxic effects in the colon, in both fecal water and colonocytes. We included two types of fish in this trial to study the possible differential effects of oil-rich and lean fish but observed no marked differences between the two types.

The Comet Assay, which we used in this trial, is considered to be a rapid, simple, economical, and sensitive method to measure DNA damage [39-41]. However, the Comet Assay also has a limitation [42], since it does not specifically identify the type or site of DNA damage. In general, there are several aspects that contribute to the internal validity of the Comet Assay [43]. By including positive and negative controls throughout the study by including a control group (DA group) to compare our results with, and by scoring the slides and analyzing the data in a blinded manner, we have optimized our internal validity. In most dietary intervention studies, blood lymphocytes have been used to study the effects of diet on DNA damage. However, DNA damage in lymphocytes does not necessarily represent DNA damage in all cells, tissues, or organs [42-44]. Since we are specifically interested in colorectal carcinogenesis, we measured the genotoxicity of fecal water and DNA damage in colonocytes. In previous studies, genotoxicity of fecal water has been considered a suitable marker for the assessment of the rapid changes in genotoxicity in the gut in response to diet [27,37,45]. Previous studies demonstrated that several specific dietary modifications could influence fecal water genotoxicity [23,24,37,46,47], while other studies showed no effect [24,25,37,46,48]. An advantage of using fecal water genotoxicity in intervention studies is that this does not require an invasive procedure to obtain biological material, which decreases the burden on participants. However, the use of fecal water as a marker of genotoxicity also is restricted. For example, for patients with active UC, constipation or other bowel complaints it might not be feasible to collect feces. In our study, we were only able to collect feces from about half of the subjects; a possible reason for this could that subjects who did not collect feces had more bowel complaints compared with those who did collect feces. It may be possible that more pronounced effects would have been observed in their feces.

More importantly, we would like to know the effect of diet specifically in the colon itself. Only one previous dietary intervention study has assessed DNA damage in colonocytes, which demonstrated that levels of colorectal DNA damage were reduced after intervention with a synbiotic preparation containing both pre- and probiotics for 12 weeks [49]. Thus, measuring DNA damage in colonocytes is applicable in intervention studies, though due to the invasive method of obtaining tissue this method is not always preferred or possible. Therefore, we also explored the correlation between

measurements of genotoxicity in fecal water and DNA damage in colonocytes, but found no agreement between these methods. One reason for this could be that genotoxicity of fecal water and DNA damage in colonocytes do not measure exactly the same endpoint; the fecal water induced DNA-damage represents the genotoxic burden of excreted feces, which is mainly modulated by dietary exposure and processes of the gut flora [50], whereas the DNA damage measured in colonocytes also reflects the effects in the cells which additionally depends on the expression of biotransformation enzymes or the extent of DNA repair mechanisms [51]. However, since the number of subjects in whom both outcomes were measured was low (n=34), this analysis was explorative, and needs to be confirmed in larger studies.

A limitation of this study was that subjects were all fish consumers at baseline. Subjects in whom fecal water genotoxicity was determined consumed 1.1 ± 0.8 portions of fish week before the start of the intervention. It seems inevitable that in recruiting subjects to an intervention study with fish, mainly fish consumers will volunteer. Another limitation was that whilst the subjects were asked to increase their consumption of salmon or cod by two portions per week, the actual average increase was ranged from 0.7-0.9 weekly portions of fish, probably due to the relatively high habitual fish consumption at baseline. This resulted in smaller differences between the fish intervention groups and the DA group than anticipated which could have led to smaller effects of the intervention. An additional limitation was the coincidental imbalance in numbers of smokers between the intervention groups. We observed higher levels of DNA damage in current smokers compared with non-smokers in both fecal water and colonocytes; however, the study lacked power to further investigate the possible effect modification of smoking in our data. It is known that smokers may differ in their enzyme expression of detoxifying enzymes [38] and it could be that smokers respond differently to a possible beneficial diet compared with non-smokers [24]. A strength of this study was that we included two types of fish, salmon and cod, though we did not observe differential effects on genotoxicity.

It has been hypothesized that the possible beneficial effects of fish could be outweighed by potential unfavorable effects by toxins, peroxidation, or oxidative stress [52]. We only measured the levels of dioxin equivalents and found that the levels of dioxin equivalents in intervention fish were well below the current maximum tolerable intake of 8 pg TEQ/100g fish [53], and thus the unfavorable effects in this fish intervention due to toxins were considered to be small. However, more studies are needed to further investigate the effect of fish consumption on genotoxicity.

In conclusion, increasing consumption of oil-rich and lean fish over six months did not result in genotoxic effects in the colon.

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6

Fatty and lean fish consumption reduce C-reactive protein levels but do not affect inflammation markers in feces and colonic biopsies

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Submitted

Abstract

Background: Fish consumption is associated with a reduced colorectal cancer risk. A possible mechanism by which fish consumption could decrease colorectal cancer risk is by reducing inflammation. However, thus far intervention studies investigating both systemic and local gut inflammation markers are lacking.

Objective: The objective of the current study was to investigate the effects of fatty and lean fish consumption on inflammation markers in serum, feces, and gut.

Design: In an intervention study, subjects were randomly allocated to receive dietary advice (DA) plus either 300g of fatty fish (salmon) or 300g of lean fish (cod) per week for six months, or only DA. Serum C-reactive protein (CRP) levels were measured pre- and post-intervention (n=161). In a subgroup (n=52), we explored the effects of the fish intervention on fecal calprotectin and a wide range of inflammation markers in fecal water and in colonic biopsies: cytokines (interleukin (IL)-1α, IL-β, IL-4, IL-6, IL-10, IL-13, TNF-α) and chemokines (MCP-1, MIP-1α, Rantes, Eotaxin, IL-8).

Results: Serum CRP concentrations were decreased after consuming salmon (-0.5mg/l, 95% confidence interval (CI) -0.9; -0.2) and cod (-0.4mg/l, 95% CI -0.7; 0.0), compared with DA. None of the inflammation markers in fecal water and colonic biopsies were changed after fish consumption compared with DA.

Conclusions: Increasing salmon or cod consumption over six months decreased the systemic inflammation marker CRP. Exploratory analysis of local markers of inflammation in the colon or feces did not reveal an effect of fish consumption.

Introduction

Fish consumption has been associated with a decreased risk of colorectal cancer, as was shown in several observational studies [1,2]. One of the mechanisms by which fish consumption could lead to a decreased colorectal cancer risk is by favorably altering inflammatory processes. Chronic inflammation is part of the pathophysiology of a variety of human diseases, including colorectal cancer [3-7]. In the gut, prolonged or chronic, low-grade inflammation can create a local tissue microenvironment where reactive oxygen and nitrogen species released from inflammatory cells could cause malignant DNA alterations or promote tumor growth [4,8-10], and thus increase colorectal cancer risk [11]. It is hypothesized that the effects of fish intake on inflammation are mostly mediated by n-3 polyunsaturated fatty acids (PUFA), also called omega-3 PUFA, as has been extensively reviewed [12-14]. The intake of very long chain n-3 PUFA could decrease the production of inflammatory eicosanoids, such as prostaglandin E3 (PGE3) or leukotriene B5 (LTB5), and cytokines, such as interleukin (IL)-6 or tumor necrosis factor alpha (TNF)-α, which are inducers of C-reactive protein (CRP) [12,13]. The eicosanoids formed from n-3 PUFA are less potent pro-inflammatory metabolites compared with the eicosanoids derived from n-6 PUFA, such as PGE2 and LTB4 [15]. Conversely, also an increased cod consumption has been shown to decrease inflammation [16]. Cod contains low amounts of n-3 PUFA compared with salmon [17] but high amounts of protein and selenium. It is hypothesized that cod

could affect inflammation by its specific amino acid content, which could down-regulate the production of pro-inflammatory mediators such as TNF-α and IL-6 [18], or by its selenium content, which could affect cyclooxygenase (COX)-2 pathways [19] and by that route, also inflammation.

Currently, the evidence for a relationship between fish consumption and inflammatory processes is restricted to a few intervention studies, all studying effects of consumption of a single type of fish on inflammatory markers in serum [16,20,21]. Intervention studies addressing the effects of fish consumption on local inflammatory markers in the colon are lacking. Therefore, we aimed to study the effects of consumption of two weekly portions of fatty and lean fish over six months on serum levels of CRP, as well as on fecal calprotectin and on cytokines and chemokines in fecal water and in colonic biopsies. Furthermore, we explored the correlation between cytokines and chemokines measured in fecal water with those measured in colonic biopsies.

Subjects and Methods

Subjects and study design

The design of this study was a multi-centre parallel randomized controlled intervention study which has been described in detail elsewhere [22]. Briefly, the trial was carried out by two research centers, Wageningen University (WU), Wageningen, the Netherlands (NL), and the Institute of Food Research (IFR), Norwich, United Kingdom (UK). Potential participants were recruited from outpatient colonoscopy clinics in eight clinical centers (six in NL, two in UK).

Three groups of subjects were recruited: those with previous colorectal adenomas, those diagnosed with non-active ulcerative colitis (UC), and those without any macroscopic signs of disease in the colon. After an initial colonoscopy procedure, 242 eligible subjects were randomly allocated to one of three dietary intervention groups: (i) fatty fish group receiving two 150g portions of farmed salmon per week during six months, (ii) lean fish group receiving two 150g portions of Icelandic cod per week during six months, and (iii) dietary advice only (DA) group. All three intervention groups received general dietary advice to achieve a healthy diet [23,24]. Two weekly portions of fish are in concordance with the current dietary recommendation [23,24]. The fish was provided to the participants at their home and had to be consumed in addition to the fish they normally consumed. The fish was analyzed for its fatty acid content [25]: salmon provided on average 3.3g/100g and cod 0.2g/100g of very long chain n-3 PUFA (eicosapentaenoic acid [EPA] + docosahexaenoic acid [DHA]). The study duration of six months was based on the time required for incorporation of n-3 PUFA in the colonic epithelium [26]. Compliance was checked using food diaries, regular phone calls every two to four weeks, and for the salmon group by pre- and post-intervention measurements of serum n-3 PUFA levels [25].

Ethical approval was obtained from the Medical Ethical Committee of Nijmegen University Medical Centre St. Radboud (reference 2004/111) and King's Lynn Local Research Ethics Committee (reference 04/Q0105/8). All subjects gave written informed consent after the study was explained to them both in writing and verbally.

Blood samples from fasted subjects were taken on the day of the endoscopy procedure pre- and post-intervention and serum was stored at -80°C prior to analysis. A sub-sample of the population (n=128) agreed to collect feces. Total feces from one bowel movement was collected one to three weeks before the endoscopy procedure pre- and post-intervention, and stored in a cooled container for transport to the laboratory within 4h. Samples were homogenized, aliquoted and stored at -80°C until further processing. Colonic biopsy samples were collected from normal-appearing mucosa at 20-30cm from the anal verge during a colonoscopy procedure pre-intervention and during a sigmoidoscopy procedure post-intervention. Information on smoking, weight, and height was obtained by questionnaire at baseline; the frequency of fish consumption was assessed pre- and post-intervention.

The main outcome of this study was the change in serum CRP levels which was measured in 197 subjects. Due to technical reasons, CRP could not be determined in 19 of the original 216 subjects whom completed the intervention (see **Figure 6.1** for a flow chart of subjects). Additionally, inflammation markers were measured in feces and in colonic biopsies in a random sample (n=52) of those who collected feces.

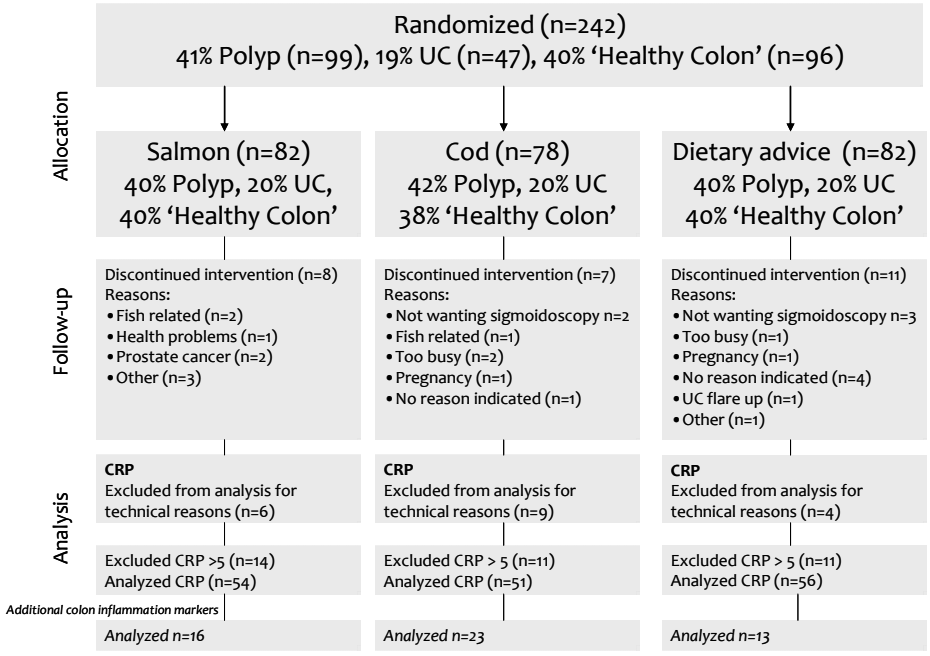

Figure 6.1 Flow chart of subjects . Abbreviations: UC (ulcerative colitis), CRP (C-reactive protein)

Analysis of serum C-reactive protein

High-sensitivity CRP concentrations were measured in serum with an enzyme immunoassay using a high sensitivity CRP protein kit (Immulite, Siemens Medical Solutions Diagnostics) and are presented in mg/l. The inter-assay variation was 4.8- 10.0% and the intra-assay variability was 4.2- 6.0%.

Analysis of fecal calprotectin

Calprotectin concentrations were measured in fecal samples using the Calpro-kit (Calpro AS, Orange Medical, the Netherlands) as previously described [27]. Results of fecal calprotectin are presented as mg per kg feces dry weight. The inter-assay variation was 5.1% and the intra-assay variability was 2.1%.

Analysis of cytokines and chemokines in fecal water and in colonic biopsies

To obtain fecal water, fecal samples were centrifuged (Optima LE-80K Ultracentrifuge, Beckmann) at 25,000xg for 2 hours at 4°C. Fecal water samples were filtered before further use. Frozen colonic biopsy samples were ground with a pestal and mortar in liquid nitrogen and re-suspended in 100µl ice-cold PBS, containing 10 mg/ml cocktail of protease inhibitors (Sigma, Zwijndrecht, NL); 50µl saponin containing permeabilization buffer (Becton Dickinson) was added and the homogenates were subsequently centrifuged (1min, 14000g, 4°C) to obtain the supernatants which were used for cytokine determinations.

In fecal water and colonic biopsy supernatants, cytokines and chemokines were determined using a multiplex immunoassay, as previously described [28,29]. Concentrations of the cytokines interleukin (IL)-1α, IL-1β, IL-4, IL-6, IL-10, IL-13, tumor necrosis factor alpha (TNF-α), and the chemokines IL-8 (or CXCL8), macrophage migration inhibitory factor (MIF), monocyte chemo-attractant protein-1 (MCP-1 CCL2), macrophage inflammatory protein-1-alpha (MIP-1α or CCL3), 'Regulated upon Activation, Normal T-cell Expressed and Secreted' (RANTES CCL5), and Eotaxin (CCL11) were determined. Samples were analyzed using the Bio-plex 100 system in combination with the Bio-plex Manager software version 3.0 (Bio-Rad Laboratories, Hercules CA, USA). Cytokine and chemokine concentrations were calculated by means of a standard curve that was generated using five parametric curve fittings to the series of known concentrations of analytes. Intra-assay variability expressed as coefficient of variation (CV) of the multiplex immunoassay varied between 6.5 and 22% [28,29].

Results are presented as ng cytokine or chemokine per g feces or per g protein present in biopsies. Protein content of the colonic biopsies was determined using the Bicinchoninic Acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, US).

Statistical analyses

Outcome measures were evaluated using an analysis of covariance (ANCOVA) adjusting for baseline values. We tested whether the outcome measures in the salmon and cod group differed from those in the DA group. Results are presented as mean and standard error (SE) of the outcome measure post- intervention adjusted for baseline value in each of the three intervention groups. Also, the differences of the mean outcome variables with 95% CI in the salmon and cod group vs. DA are presented. For all analyses, we explored whether results differed between patient groups. We assigned a value equal to half of the detection limit of a given cytokine or chemokine if any values were below detection limit. For the cytokines and chemokines, we did not report the results if more than 50% of the samples had a value below the detection limit. As a sensitivity analysis, we explored whether using multiple imputation for cytokine and chemokine values below the detection limit would change our results [30]. Factors that were used for multiple imputation included gender, BMI, country, hospital, clinical diagnosis, treatment, and compliance. We evaluated Spearman correlations between the baseline levels of cytokines and chemokines measured in fecal water and in colonic biopsies. We performed analyses using the SAS statistical software program (SAS version 9.1) considering a p-value of <0.05 as significant. Statistical analyses were performed blinded for treatment and patient group.

Results

CRP is an acute phase protein of inflammation and subjects with CRP levels above 5 mg/l (either pre- or post-intervention) are expected to suffer from acute inflammation and were therefore excluded from our analyses (n=36). Baseline characteristics of the resulting 161 subjects are presented in **Table 6.1**. The distribution of patient groups over the three intervention groups was not completely balanced. The salmon group included somewhat more healthy colon patients compared with the cod and DA group (48% vs. 28% and 39%) and the cod group included somewhat more UC patients compared with the salmon and DA group (26% vs. 17% and 20%). Subjects receiving cod included fewer women (43% vs. 46% and 52%) and fewer smokers (10% vs. 23% and 16%) compared with the salmon and DA group. At baseline, serum CRP concentrations were comparable in the three intervention groups (on average 1.6 ± 1.0 mg/l) and levels did not differ (p=0.41) between UC (1.5 ± 1.0 mg/l, n=33) or polyp patients (1.6 ± 1.1mg/l, n=66) and healthy controls (1.6 ± 0.9 mg/l, n=62).

Before the start of the intervention, subjects consumed on average 1.7 times fish per week in all three intervention groups (Table 6.1). Fish consumption increased by on average 1.5 ± 1.1 times per week in the salmon group, 1.4 ± 1.5 in the cod group and 0.1 ± 1.2 in the DA group compared with baseline.

Table 6.1 Baseline characteristics of the FISHGASTRO for whom C-reactive protein (CRP) was determined (n=161)

Intervention group: Characteristic	Salmon (n=54)	Cod (n=51)	Dietary advice (n=56)
Age (year, mean ± SD)	54.1 ± 12.1	57.4 ± 9.0	56.1 ± 9.3
Sex (% female)	46	43	52
BMI (kg/m^2, mean ± SD)	25.7 ± 3.7	25.8 ± 3.3	26.9 ± 3.5
Smoking (% current)	23	10	16
Research Centre (% NL)	76	75	79
Patient groups (% polyp/ UC/ Healthy controls)	35/ 17/ 48	47/ 26/ 28	41/ 20 / 39
Fish consumption (freq./week, mean ± SD)	1.7 ± 1.4	1.7 ± 1.1	1.6 ± 1.2
CRP (mg/l, mean ± SD)	1.5 ± 1.0	1.6 ± 1.1	1.7 ± 1.0

Abbreviations: SD (standard deviation), BMI (body mass index), NL (Netherlands), UC (ulcerative colitis), freq (frequency), CRP (C reactive protein)

In the subgroup (n=52) in which additional inflammation markers were determined, the percentage of UC patients was higher in the DA group compared with the salmon or cod group (31% vs. 12% and 13%). The three intervention groups were comparable in terms of age and habitual fish intake. The cod group had a somewhat higher BMI compared with the salmon and DA group (27.0 kg/m^2 vs. 24.4 kg/m^2 and 25.8 kg/m^2) and the salmon group included more smokers compared with the cod and DA group (31% vs. 13% and 15%).
We observed that levels of calprotectin were higher (p=0.008) in UC patients (274.6 ± 451.4 mg/kg (n=9) compared with polyp patients (71.9± 72.8 mg/kg, n=30) or healthy colon patients (50.7 ± 30.1 mg/kg, n=28). No differences between patient groups were seen for any of the cytokines and chemokines measured in feces. IL-10 measured in biopsies was significantly lower in healthy subjects (p=0.02) compared with polyp and UC patients and Eotaxin measured in biopsies was significantly higher in UC patients (p=0.0002) compared with polyp and healthy subjects (results not shown).
In this subgroup (n=52), subjects consumed on average 1.9 ± 1.5 times fish per week before the start of the intervention. The salmon and cod group increased their fish consumption by on average 1.4 ± 1.1 and 1.5 ± 1.2 times fish per week respectively, while the DA group increased their fish consumption with 0.1 ± 1.4 times per week.

C-reactive protein (n=161)

Compared with the DA group, serum CRP concentrations decreased significantly by -0.5 mg/l (95% CI -0.9; -0.2) in the salmon group and by -0.4 mg/l (95% CI -0.7; 0.0) in the cod group (**Table 6.2**).
As the percentage of UC patients was not equal in the three intervention groups, we explored whether results were different when UC patients (n=33) were excluded from

the analysis; however this did not change our conclusions. In this case, serum CRP concentrations decreased significantly by -0.4 mg/l (95% CI -0.8; -0.1) in the salmon group and by -0.4 mg/l (95% CI -0.8; 0.0) in the cod group when compared with the DA group.

Table 6.2 Results of serum CRP levels (mg/l) in those with CRP below 5 mg/l (n=161)

Intervention group:	Salmon (n=54)	Cod (n=51)	Dietary advice (DA) (n=56)
CRP (mg/l) post-intervention [1] mean (SE)	1.3 (0.1)	1.5 (0.1)	1.8 (0.1)
Differences compared with DA mean (95% CI)	-0.5 (-0.9; -0.2) [2]	-0.4 (-0.7; -0.0) [2]	

[1] adjusted for baseline values
[2] statistically significant difference (p ANCOVA < 0.05)
Abbreviations: SE (standard error), CI (confidence interval), CRP (C-reactive protein), DA (dietary advice)

Fecal calprotectin (n=52)

Fecal calprotectin levels post-intervention adjusted for baseline values were 108.1 ± 21.0mg/kg for salmon, 92.0 ± 17.6 mg/kg for cod, and 87.2 ± 23.8 mg/kg for the DA group. Compared with the DA group, no significant changes in fecal calprotectin concentrations were observed after intervention with salmon (20.9 mg/kg, 95% CI -41.3; 83.1) or cod (4.7 mg/kg, 95% CI -53.2; 62.6).

When the analysis was repeated excluding UC patients (n=9), this did not change our conclusions: changes in the salmon and cod group compared with DA were -12.3 mg/kg (95% CI -58.4; 33.7) and -6.9 mg/kg (95% CI -50.1; 36.3) respectively.

Cytokines and chemokines in fecal water and colonic biopsies (n=52)

In fecal water, more than 50% of the samples had IL-1β, IL-4, IL-6, IL-13, MIF, and Eotaxin concentrations below the detection limit and therefore these inflammation markers were not included in our further analyses. The concentrations of cytokines (IL-1α, IL-10, and TNF-α) and chemokines (MCP-1, MIP-1α, RANTES, and IL-8) in fecal water were not significantly changed after intervention with either salmon or cod compared with DA (**Table 6.3**).

In colonic biopsies, more than 50% of the samples had MIP-1α concentrations below the detection limit and therefore this inflammation marker was not included in our analyses. The levels of the detectable cytokines (IL-1α, IL-4, IL-6, IL-10, IL-13, and TNF- α) and chemokines (MCP-1, RANTES, Eotaxin, and IL-8) did not change significantly in colonic biopsies after intervention with either salmon or cod compared with DA (**Table 6.4**).

Table 6.3 Results of cytokines and chemokines measured in fecal water (n=52) [1a]

	Outcome variables post- intervention, mean (SE) [2]			Differences compared with DA, mean (95% CI) [3]	
Intervention group:	**Salmon (n=16)**	**Cod (n=23)**	**Dietary Advice (DA) (n=13)**	**Difference Salmon vs. DA**	**Difference Cod vs. DA**
Cytokines					
IL-1α (ng/ml)	451 (120)	432 (100)	644 (133)	-192 (-548; 169)	-213 (-548; 123)
IL-10 (pg/ml)	14.1 (15.9)	25.6 (13.2)	29.6 (17.9)	-15.4 (-64.0; 33.2)	-3.9 (-49.0; 41.7)
TNF-α (pg/ml)	18.6 (18.5)	24.7 (15.5)	55.5 (20.8)	-36.9 (-93.2; 19.5)	-30.8 (-83.2; 21.6)
Chemokines					
MCP-1 (pg/ml)	92.6 (63.3)	115 (52.7)	126 (71.3)	-33.2 (-226; 160)	-10.7 (-190; 169)
MIP-1α (pg/ml)	255 (74.1)	186 (61.7)	267 (82.4)	-11.9 (-235; 211)	-80.3 (-287; 128)
RANTES (pg/ml)	30.2 (7.8)	28.1 (6.5)	38.3 (8.7)	-8.1 (-31.5; 15.3)	-10.1 (-31.8; 11.6)
IL-8 (pg/ml)	25.5 (18.8)	37.9 (15.6)	47.9 (21.0)	-22.4 (-79.5; 34.7)	-10.0 (-63.0; 43.0)

Table 6.4 Results of cytokines and chemokines in biopsies (n=52) [1b]

	Outcome variables post- intervention, mean (SE) [2]			Differences compared with DA, mean (95% CI) [3]	
Intervention group:	**Salmon (n=16)**	**Cod (n=23)**	**Dietary Advice (DA) (n=13)**	**Salmon vs. DA**	**Cod vs. DA**
Cytokines [4]					
IL-1 α (ng/mg)	441 (79.0)	532 (66.0)	582 (87.6)	-141 (-378; 95.6)	-49.6 (-271; 171)
IL-1β (pg/mg)	1.05 (0.59)	2.22 (0.49)	2.79 (0.66)	-1.74 (-3.53; 0.04)	-0.57 (-2.24; 1.09)
IL-4 (ng/mg)	65.9 (19.4)	67.7 (16.2)	69.1 (21.9)	-3.16 (-62.6; 56.3)	-1.40 (-56.6; 53.8)
IL-6 (ng/mg)	132 (25.7)	105 (21.4)	122 (28.3)	9.8 (-67.0; 86.5)	-16.7 (-88.0; 54.7)
IL-10 (pg/mg)	0.40 (0.04)	0.40 (0.04)	0.41 (0.05)	-0.01 (-0.13; 0.12)	-0.01 (-0.12; 0.11)
IL-13 (pg/mg)	0.25 (0.02)	0.24 (0.02)	0.21 (0.03)	0.03 (-0.04; 0.11)	0.02 (-0.04; 0.09)
TNF-α (pg/mg)	1.90 (0.19)	2.13 (0.15)	2.03 (0.21)	-0.14 (-0.73; 0.45)	0.10 (-0.41; 0.62)
Chemokines [4]					
MCP-1 (pg/mg)	4.45 (1.05)	5.17 (0.87)	6.29 (1.15)	-1.83 (-4.95; 1.29)	-1.12 (-4.01; 1.78)
RANTES (pg/mg)	107 (14.6)	88.6 (12.2)	86.5 (16.2)	20.5 (-23.5; 64.4)	2.03 (-38.8; 42.8)
Eotaxin (pg/mg)	3.94 (0.97)	3.92 (0.81)	3.56 (1.06)	0.39 (-2.51; 3.29)	0.37 (-2.31; 3.05)
IL-8 (pg/mg)	1.88 (0.34)	1.84 (0.28)	1.61 (0.37)	0.27 (-0.74; 1.28)	0.23 (-0.72; 1.17)

[1a] Results of IL-1β, IL-4, IL-6, IL-13, MIF and Eotaxin are not presented since > 50% of the values were < detection limit
[1b] Results of MIP-1α are not presented since > 50% of the values were < detection limit
[2] Adjusted for baseline values
[3] None of these markers were statistically significantly different from DA (ANCOVA)
[4] per mg protein
Abbreviations: SE (standard error), CI (confidence interval), IL (interleukin), TNF-α (tumor necrosis factor alpha), MCP-1 (monocyte chemo-attractant protein-1), MIP1α (macrophage inflammatory protein-1-alpha), RANTES (Regulated upon Activation, Normal T-cell Expressed and Secreted), DA (dietary advice)

We explored whether changes in cytokine and chemokine concentrations were different excluding UC patients (n=9) from the analyses. For fecal water, this did not change our conclusions (data not shown). For colonic biopsies, excluding UC patients did not change our conclusions except for Eotaxin, for which we now observed a significant decrease of -0.9 pg/ml (95% CI -1.7; -0.1) in the cod group compared with DA.
Results from the sensitivity analysis, using multiple imputations for those samples with a value below the detection limit, were comparable with results from the original analysis (data not shown).

Correlations of cytokines and chemokines in fecal water and colonic biopsies
Spearman correlation coefficients of measurements of cytokines and chemokines in fecal water and in colonic biopsies at baseline were below 0.1 for all inflammation markers except for IL-1α, for which we found a Spearman correlation coefficient of 0.2 (p=0.15).

Discussion

This intervention study showed that increased consumption of both salmon and cod for six months reduced the systemic inflammation marker CRP by 25-30%. This suggests that not only oil-rich fish beneficially affects inflammation, but also lean fish. Exploration of a broad range of local gut inflammation markers, i.e. cytokines and chemokines measured in feces and colonic biopsies and fecal calprotectin did not reveal an effect of fish consumption. In addition, the correlation between cytokines and chemokines measured in fecal water and colonic biopsies was poor.
We hypothesize that the main reason why we did not observe effects of fish consumption on levels of cytokines and chemokines in the colon and on fecal calprotectin is the large variation in these markers. This variation may be explained by technical aspects of the assays, as well as variations throughout the day [31]. Also, a shorter half life of cytokines and chemokines [32,33] may explain the greater variation as compared with CRP [34]. A specific reason for the variation in fecal inflammation markers could be that we used a stool sample from a single bowel movement, which does not reflect complete daily fecal output. Altogether, we cannot rule out the possibility that fish consumption affects inflammation in the colon, which could be detected in a much larger study where averages of replicate marker measurements are used to decrease variation.
A strength of this study was that we explored the effects of fish on a broad range of local inflammation markers in feces and colonic tissue, besides looking at a systemic marker of inflammation (CRP). Also, this was the first study that measured cytokines and chemokines in colorectal biopsies, i.e. specifically in the tissue of interest. The poor correlation between cytokines and chemokines measured in fecal water and colonic

biopsies indicated that measuring inflammation in fecal water could not be assumed to be a useful and less invasive substitute of the measurements in colonic biopsies.

For the measurement of cytokines and chemokines, we used a multiplex immunoassay [28,29]. Previous studies have shown that results of a multiplex immunoassay are comparable to those of an enzyme-linked immunosorbent assay (ELISA), which is considered the 'gold standard' for cytokine measurement [35-37]. However, a disadvantage of the multiplex immunoassay might be that it is less sensitive to detect levels in the lower ranges and thus more samples are below the detection limit compared with ELISA. In this study, concentrations of IL-4, IL-6, IL-13, MIF, and Eotaxin were below the detection limit in fecal water in more than 50% of the subjects. Conversely, most cytokines and chemokines, except MIP-1α, were detectable in colonic biopsies in over 50% of the subjects. It has been argued whether to assign the value of half of the detection limit to values below the detection limit, or to use multiple imputations [30,38]. Therefore, we performed a sensitivity analysis for cytokines and chemokines with values extrapolated below the detection limit and found that in this RCT these two methods did not lead to different conclusions.

UC is a chronic inflammatory disease and we indeed found that UC patients had significantly higher baseline calprotectin levels compared with healthy controls and polyp patients. Since the number of UC patients was imbalanced among the three intervention groups, we explored whether the results of the inflammation markers were different excluding these patients. For CRP and calprotectin, this did not change our conclusions. The single change we found for cytokines and chemokines was that we now observed a small but significant decrease in Eotaxin in colonic tissue in the cod group compared with DA. However, considering the total number of analyses this could well have been a chance finding. Also, gender, smoking status and BMI were not equally balanced over the three intervention groups; however, we do not expect that these factors would substantially affect responses in inflammation markers.

Another limitation of this study was that the subjects at baseline consumed on average 1.7 ± 1.2 times fish per week, which is relatively high compared with the average consumption in the Netherlands [39,40] but normal for the Norfolk (UK) population [40]. This was inevitable since only people who like to consume fish were willing to increase their fish consumption and thus to participate. Thus, the results of this intervention reflect the effects of increasing fish consumption from moderate to high baseline levels to even higher levels of fish consumption. Therefore, it remains possible that increasing fish consumption in subjects who hardly consume fish would reduce local inflammation markers in the colon.

To the best of our knowledge, this is the first RCT that simultaneously studied the effects of both fatty and lean fish on serum CRP and local gut inflammation markers in feces and in colonic biopsies. Previously, three intervention trials studied the effects of a single type

of fish on serum inflammation markers [16,20,21]. Of these three studies, only two studied the effects on serum CRP levels [16,20]. Our results are in concordance with those of the study by Ouellet et al, who found that serum CRP levels decreased by 25% after 4 weeks of consuming 58-68% of the daily protein intake as cod [16]. They also found no effects of cod on additional inflammation markers: plasma IL-6, TNF-α, and adiponectin concentrations. Conversely, Seierstad et al did not find an effect of 700g salmon per week on serum CRP levels in 60 patients with coronary heart disease [20]. Possible reasons why this study did not find an effect of salmon on CRP could be related to the relatively short study duration of 6 weeks or the type of subjects included. Additionally, we excluded those subjects with CRP concentrations above 5 mg/l to avoid interference of acute inflammation, which might also have contributed to differences in results. Furthermore, Seierstad et al found a decrease in serum vascular cell adhesion molecule-1 (VCAM-1) and IL-6, but not in TNF-α, IL-10, E-selectin, intercellular adhesion molecule-1 (ICAM-1) [20], whereas Meydani et al found that the production of IL-1β, TNF-α and IL-6 by mononuclear cells was significantly reduced after consumption of salmon [21]. Thus, the two salmon intervention studies did find effects on some cytokines levels in blood, something we cannot directly compare to our results as we measured cytokines in fecal water and colonic biopsies.

We observed that CRP levels dropped by about 25-30% after the salmon and cod intervention. The question that remains is whether the decrease in CRP levels is related to a decrease in colorectal cancer risk. The association between serum CRP levels and colorectal cancer risk has been studied in a meta-analysis of 8 prospective studies including 1159 colorectal cancer cases and 37986 controls, showing a relative risk of 1.12 (95% CI 1.01; 1.25) per unit change in natural log-transformed CRP levels [41]. More recently, a prospective cohort study found an hazard ratio of 1.9 (95% CI 0.8; 4.6) for colorectal cancer, comparing individuals with high CRP levels (> 3mg/l) to those with low levels (< 1mg/l) [42]. So based on the results of observational studies a decrease in CRP levels is related to a decrease in colorectal cancer risk. However, it remains to be further investigated how changes in CRP concentrations are associated with inflammation in the colon and colorectal cancer risk.

In conclusion, this trial showed that increasing salmon or cod consumption decreased the systemic inflammation marker CRP but did not reveal an effect on a broad range of local inflammation markers in colonic biopsies and feces. To further elucidate the effects of fish consumption on colorectal inflammation, future intervention studies should include a large population of subjects with a low habitual fish intake and measure markers of colorectal inflammation in colonic biopsies.

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General Discussion

7

"I have a dream that one day we will have performed the ideal study to show that fish consumption decreases the risk of colorectal cancer. In this ideal study, people consume fish once to twice per week and as an endpoint, the incidence of colorectal cancer is studied.
I have a dream that after the intervention with fish the incidence of colorectal cancer has declined. I have a dream that this ideal study will provide evidence based dietary guidelines for fish, not only based on cardiovascular disease risk, but also based on colorectal cancer risk …"

Then I woke up and realized that the ideal study on the effects of fish consumption on colorectal cancer does not exist.

The aim of this thesis was to investigate whether fish consumption beneficially affects markers of colorectal carcinogenesis. In this chapter, the main findings of this thesis are summarized and discussed, and recommendations for future research are given.

Main findings

Table 7.1 Overview of the results of this thesis

Study	Exposure	Endpoint	Results	Chapter
Case-control study (POLIEP-study)	Serum levels n-3 and n-6 PUFA	Colorectal adenoma risk 363 cases, 498 controls	High n-3 PUFA in serum: 33% lower risk High n-6 PUFA in serum: 68% higher risk	2
Intervention trial (CardioN)	Fish oil 3.5g/d (1.5g n-3 PUFA), 12 weeks	Serum cytokines and chemokines (n=77)	No effect of fish oil	3
Intervention trial (FISHGASTRO)	Fish consumption, salmon or cod (300g/wk) vs. only dietary advice, 6 months	Apoptosis and mitosis in colonic crypt (n=216)	No effect of fish	4
Intervention trial (FISHGASTRO)	Fish consumption, salmon or cod vs. only dietary advice, 6 months	Genotoxicity of fecal water (n=89) or DNA damage in colonocytes (n=70)	No effect of fish	5
Intervention trial (FISHGASTRO)	Fish consumption, salmon or cod vs. only dietary advice, 6 months	Inflammatory markers: Serum CRP (n=195); Fecal calprotectin Cytokines and chemokines in feces and biopsies (n=52)	CRP reduced after salmon and cod No effect on other inflammatory markers	6

The main findings of this thesis are summarized in **Table 7.1**. Initially, in a case-control study we observed that individuals with high serum n-3 polyunsaturated fatty acid (PUFA) levels had a decreased risk of colorectal adenomas (odds ratio (OR) 0.67, 95% confidence interval (CI) 0.46; 0.96), whereas individuals with high serum n-6 PUFA levels had an increased risk of colorectal adenomas compared with controls (OR 1.68, 95% CI 1.17; 2.42) (**Chapter 2**). Next, we studied the effects of fish oil supplementation on serum inflammatory markers in healthy subjects in a randomized controlled trial (RCT) and found that supplementation of 3.5g/d fish oil (~1.5g/d n-3 PUFA) for 12 weeks did not affect a panel of 19 serum cytokine and chemokine concentrations compared with placebo (**Chapter 3**).

Finally, we performed an RCT on the effects of fish consumption on markers of colorectal carcinogenesis, in a population at increased risk of CRC and those with no macroscopic

signs of disease in the colon. In this RCT, increasing fish consumption with either salmon (300g/week, providing ~1.4 g/d n-3 PUFA) or cod (300g/week, providing ~0.09 g/d n-3 PUFA) over six months did not affect apoptosis and mitosis in colonic crypts (**Chapter 4**), genotoxicity of fecal water or DNA damage in colonocytes (**Chapter 5**), or inflammatory markers measured in colonic biopsies and in feces as compared with dietary advice (**Chapter 6**). However, a reduction in serum C-reactive protein (CRP) levels was observed after intervention with salmon (-0.5 mg/l, 95% CI -0.9; -0.2) and cod (-0.4 mg/l, 95% CI -0.7; 0.0) compared with the dietary advice group (**Chapter 6**). Thus, we only observed an effect of salmon and cod on the systemic inflammation marker CRP, while other markers of colorectal carcinogenesis were not affected.

In the next paragraphs, methodological considerations of the RCT on fish consumption are discussed. We chose to focus on the fish RCT in this chapter since issues regarding methodological considerations of the case-control study and the CardioN study have been extensively discussed by for example van den Donk [1], Tiemersma [2] and Geelen [3], and have been summarized in Chapters 2 and 3, respectively.

Why our fish intervention trial was not a 'dream' study: methodological considerations

As dreams are usually not true [4], also the RCT described in this thesis has its limitations which need to be taken into account when interpreting the results. In the following paragraphs, issues related to design, exposure, and endpoints are discussed.

Design

The intervention study had a parallel, randomized, controlled design, as shown in **Figure 7.1**, including three groups of subjects and three intervention arms. An advantage of a parallel study design is that the study duration could be six months, which would not have been sufficient in a cross-over design, with two or three successive intervention periods and wash-out periods in between. Another advantage was that less endoscopy procedures per subject were needed as compared with a cross-over design, which reduced the burden to the subjects. However, a limitation of a parallel study design is that large inter-individual variation affects the power of the study and thus a larger sample size is needed, compared with a cross-over design.

The sample size of the fish RCT was based on an expected change in number of apoptotic cells of 0.2 with a standard deviation of 0.46 [5], and it was estimated that with 90 subjects per intervention group the power would be 80%.

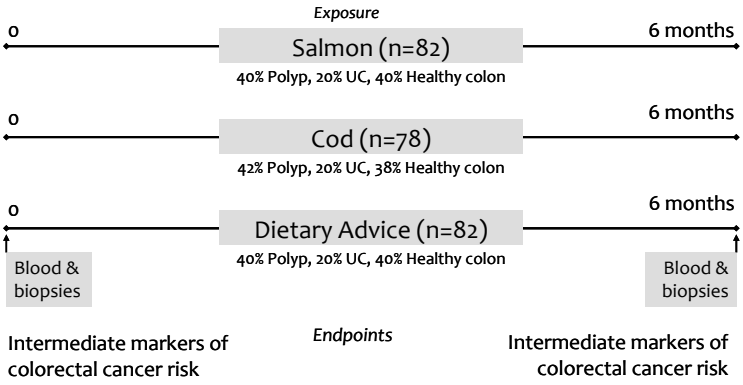

Figure 7.1 Overview of the design of the FISHGASTRO study

After three years of intense recruitment, we had achieved the inclusion of 242 subjects. Thus, we did not reach 270 subjects as anticipated. This has decreased the power to detect statistical significant changes and which has increased the chance of type II error ('false negatives'). Moreover, the variation in response of the outcome variables was very large, which also contributed to a decreased power to detect statistically significant changes.

Subjects were recruited from outpatient clinic attendees visiting the hospital for a colonoscopy, which had several implications for both the recruitment and the subjects. The recruitment of subjects for this fish RCT proved to be very difficult since many people were not willing to increase their fish consumption (~20% of those invited to participate). Especially individuals who consume little or no fish were not willing to participate in this particular RCT. Consequently, the baseline fish consumption of those who participated was relatively high compared with the general Dutch population [6,7]. Another important reason for reclining the invitation for participation in this trial was the fact that participants needed to undergo an additional sigmoidoscopy (~40% of those invited), and another ~30% reclined for other reasons. Eventually, only about ten percent of the people we invited actually participated in the trial, which was considerably lower than anticipated.

Subjects with polyps and ulcerative colitis (UC) are at an increased risk of colorectal cancer [8-10] and a greater effect of fish on colorectal carcinogenesis markers might be expected to occur in these patients. For the main study outcome mitosis, subgroup analysis indicated that if any effects of fish were to be observed, these would occur in polyp and UC patients; however, we lacked power to draw conclusions within subgroups.

Furthermore, the question could be raised as to how 'healthy' the healthy colon patients were. Our 'healthy' subjects were coming to the hospital for a colonoscopy. Approximately 60% of these subjects had bowel complaints as indication for endoscopy, whilst in the other 40% reasons for the initial endoscopy included familiar occurrence of

CRC, hemorrhoids or anemia. Their colonoscopy did not show any colonic abnormalities, although it has previously been shown that a similar group of symptomatic patients with apparently normal mucosa had more mitotic cells per crypt than those assessed as part of a routine screening program [11]. Thus, these 'healthy colon' patients did not all represent truly healthy individuals.

Another point to take into consideration is the 'control' group, which was the dietary advice (DA) group. It was not feasible to perform a blinded study in such a way that participants did not know which type of fish they were consuming. Therefore, it is conceivable that subjects in the DA group also might have increased their fish intake; however, results from dietary questionnaires indicated that their fish intake did not increase. It could also be argued that specifically subjects in the DA group changed their dietary patterns following the dietary advice they received [12,13]. However, all three intervention groups received dietary advice and nutrient intake did not reflect changes in dietary intake in any of the three groups, other than changes in fish consumption in the salmon and cod group. Thus, we consider our control group appropriate.

The data generated in this RCT were analyzed using analysis of covariance (ANCOVA). We tested the end value and adjusted for baseline value. This increased power as opposed to testing the response (end minus baseline) values or non-parametric testing [14,15]. Another advantage of ANCOVA is that possible imbalances at baseline are accounted for. It is debatable whether one should adjust analyses for randomization factors [16], i.e. here hospital or patient group. In many RCTs, no adjustments were made for the randomization factors. In this study, we also chose not to adjust our analyses for hospital, since this would decrease interpretability of the results and power to detect statistically significant changes [17]. Conversely, we explored whether results were different per patient group and per country and found differences in baseline values of some outcomes, e.g. mitosis, CRP, or fecal calprotectin. However, the conclusions of our studies did not change if we explored the results per patient group or per country, but power was obviously limited.

Another factor to be taken into account is the per-protocol analysis we performed. An alternative approach would be to analyze the data according to the intention-to-treat principle. However, this would answer a different research question, related to the clinical effectiveness of a fish intervention [18].

In summary in terms of design, the recruitment of subjects in this parallel randomized controlled trial proved to be very difficult and resulted in a smaller study with reduced power, including subjects with relatively high habitual fish consumption.

Exposure

The exposure in this RCT was the additional consumption of two weekly portions of salmon or cod for six months. We estimated that the intervention period was long enough to incorporate n-3 PUFA in the colonic epithelium [5]. Six months is relatively short-term considering the long latency period of CRC, though six months is probably long enough to affect intermediate markers of CRC risk. For example, the four RCTs on n-3 PUFA and mitosis that observed an effect lasted 1-6 months [19-22]. In addition, intervention studies with study durations of longer than six months pose a major burden on the subjects and are likely to result in lower compliance, especially when considering foods rather than supplements. Thus, we consider the six months intervention an acceptable study duration, since we used intermediate markers of CRC risk.

During intervention, subjects were instructed to consume the fish provided on top of their habitual fish consumption. Adherence to the intervention or compliance, was assessed using a combination of approaches: regular phone calls, dietary assessment, and in the salmon group by serum levels of the very long chain n-3 PUFA eicosapentaenoic acid (EPA; C20:5n-3) and docosahexaenoic acid (DHA; C22:6n-3). Serum n-3 PUFA levels were significantly increased in the salmon group and not in the other two intervention groups, which indicated that overall the subjects consumed the salmon provided within the study, and that the other two intervention groups did not significantly increase their n-3 PUFA intake. However, dietary questionnaires indicated that subjects in the fish intervention groups replaced some of their habitual fish consumption by the intervention fish. Therefore, one could question as to whether adherence to the intervention protocol was adequate. In this light, 'compliance' might not have been satisfactory, but it might also be the maximum achievable in a fish intervention study. This reduced compliance resulted in a smaller contrast between the fish groups and the DA group.

A possible confounding factor that could have affected our results is the consumption of meat, which could have declined during intervention, especially in the fish intervention groups. A decrease in meat consumption is also associated with a reduced CRC risk [23]. However, our subjects in the salmon and cod group did not report lower meat consumption, or an increased total energy intake or body mass index.

Intervening with fish, instead of fish oil, introduces another factor that could have influenced our results, namely the preparation of the fish. From a subgroup of Dutch participants (n=85), we know that most fish was fried (~73% of salmon, and ~84% of cod), mostly in margarine or vegetable oil. Serum n-6 PUFA levels were increased after the cod intervention compared with the dietary advice group, but not after salmon intervention. This could have weakened the possible beneficial effect of cod, since increased n-6 PUFA could possibly increase CRC risk [24-26].

The possible beneficial effects of fish consumption could have been outweighed by the presence of toxins in the fish. As an example of an often found toxin, we measured dioxin equivalents in the salmon and cod provided to the participants and these were well below the daily tolerable intake [27]. Moreover, we did not observe an increase in genotoxicity in the colon, as measured by the Comet assay in colonocytes and fecal water. Thus, the chance that the potential beneficial effects of fish were outweighed by the presence of toxins is regarded as small.
An advantage of this RCT was that we included two types of fish, oil-rich and lean fish. This made it possible to study whether the potential beneficial effects of fish could be attributed to oil-rich fish, possibly mediated by n-3 PUFA, or to fish in general. In this RCT, we did not observe any effects of salmon and cod for all but one outcome measure. We observed that both salmon and cod reduced serum CRP levels, indicating that it might not only be the very long chain n-3 PUFA that are beneficially affecting inflammation, but also other constituents in fish, such as vitamin D, selenium or protein.

In summary in terms of exposure, the contrasts between the fish intervention groups and the dietary advice group may not have been large enough to observe an effect, while the six month intervention period seemed adequate. The chance that results were biased by other variables such as meat consumption, fish preparation, or toxic compounds in fish, is regarded as small.

Endpoints
Since colorectal cancer incidence as such could not be studied as the main outcome measure, we studied the effects of fish consumption on intermediate or surrogate outcomes. In general, Schatzkin and Gail proposed that three conditions are required to establish a surrogate marker for cancer risk: (i) a surrogate endpoint marker should be associated with cancer, (ii) the exposure (=treatment) should be associated with the surrogate endpoint marker, and (iii) the surrogate endpoint marker should 'mediate' the association between exposure and cancer [28], as illustrated in **Figure 7.2**. Another important consideration, besides reproducibility and variability, is whether the magnitude of the association between exposure and surrogate marker predicts the magnitude of the association between exposure and true outcome [29].

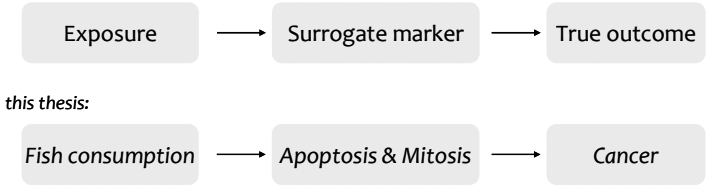

Figure 7.2 Relationship between a surrogate marker and true outcome, adjusted for this thesis [28]

In this RCT, we studied the effects of fish consumption on several intermediate endpoints of CRC risk, including apoptosis and mitosis as primary endpoints. However, it is currently debated whether available intermediate endpoints of CRC risk are actually associated with colorectal cancer. For example, apoptosis and mitosis have been considered by many to be reliable intermediate markers of CRC risk [30,31], but they have also been criticized for not representing an actual increased CRC risk [28,32].

For apoptosis, having decreased levels does not necessarily lead to an increased CRC risk as is shown in literature [33]: some studies found that apoptotic rates were inversely related to colorectal adenomas [34,35] or CRC risk [36-40], whereas other studies observed no association whatsoever [41-43], or only in distal tumors [44]. Notably, the colorectal adenomas studies included mostly familial adenomatous polyposis (FAP) patients, which may not be comparable to other forms of CRC [34,35]. For mitosis, high proliferative activity in colonic mucosa has been found to be associated with higher CRC risk [44-48], although another study did not find this association [37]. The latter study included colorectal carcinoma cases, whilst the former studies also included cases with colorectal adenomas [45,47,48]. Thus, how well the surrogate endpoints apoptosis and mitosis are associated with CRC risk remains uncertain.

Another point to take into consideration regarding apoptosis and mitosis is that methods to assess apoptosis and mitosis may differ between studies. Of the available methods to measure apoptosis and mitosis, we used the morphological method, which is generally accepted [49-51], though other methods like TUNEL, Ki67 or M30 expression are also commonly used [32,52]. These methods make use of histology in longitudinal crypt sections, whereas the morphological method has the advantage that it measures apoptosis, mitosis, and distribution of mitosis, simultaneously. A disadvantage of the whole-crypt-mount approach is that it does not identify all apoptotic cells on the luminal surface, whereas TUNEL and M30 may preferentially detect this normal programmed cell death [53]. Moreover, the use of these different methods to assess apoptosis and mitosis complicates comparisons between studies.

Taken together, the use of apoptosis and mitosis as intermediate endpoints of colorectal cancer risk in randomized controlled trials is generally accepted despite their recognized limitations, though it should be considered that presently a better intermediate marker of colorectal cancer risk is lacking.

In addition to apoptosis and mitosis, we also studied other intermediate markers of colorectal carcinogenesis. To measure DNA damage, we used the Comet assay, in both colonic tissue and in fecal water. The Comet assay is generally considered a rapid, simple, economical, and sensitive method to measure DNA damage [54-57]. Conversely, the Comet assay also has limitations. Factors that could contribute to variation in results of the Comet assay in human studies include differences in laboratory conditions and the use of

different Comet parameters, e.g. tail moment or % tail intensity. We performed the Comet assay in such a way that it only detects DNA damage in the form of strand breaks, while it could not detect other forms of DNA damage, such as base oxidation and DNA adduct formation [57]. Single strand breaks are quickly repaired and are not regarded as mutagenic [55], and therefore possibly not related to CRC risk. Thus, this makes the use of DNA damage measured by the Comet assay as surrogate marker of CRC questionable.
Only two observational studies have studied the association between colorectal DNA damage and CRC risk, showing that DNA damage in the colon is related to CRC [58,59]. Both studies measured DNA damage using the Comet assay, although the former study compared DNA damage in tumor tissue to normal tissue [58], while the latter study compared the DNA in adenocarcinoma cases with healthy controls [59]. Whether a decrease in levels of DNA damage actually represents a decreased CRC risk should be further investigated.

As markers of inflammation, we included high-sensitivity serum C-reactive protein (CRP), which is a sensitive and reliable marker of inflammation, yet it only represents systemic inflammation [60]. Therefore, we also measured fecal calprotectin and cytokines & chemokines in fecal water and colonic biopsies, as local gut inflammation markers. The main reason why we did not observe effects of fish consumption on levels of cytokines and chemokines in the colon and on fecal calprotectin may be related to the large variation in these markers. This variation might be explained by technical aspects of the assays, as well as variations throughout the day [61]. Also, a shorter half life of cytokines and chemokines [62,63] may explain the greater variation as compared with CRP [60]. A specific reason for the variation in fecal inflammation markers could be that we used a stool sample from a single bowel movement, which does not reflect complete daily fecal output.
For CRP, a meta-analysis of eight prospective studies showed a 12% increased colorectal cancer risk (95% CI 1%; 25%) per unit increase of natural log-transformed CRP levels [64]. More recently, a prospective cohort study found a hazard ratio of 1.9 (95% CI 0.8; 4.6) for colorectal cancer, comparing individuals with high CRP levels (> 3mg/l) to those with low levels (< 1mg/l) [65]. Similar to the previous surrogate endpoints of CRC risk, it is still not fully known how changes in inflammatory markers are related to CRC risk [66,67].

To summarize in terms of endpoints, due to the near impossibility of actually studying colorectal cancer incidence as an outcome in randomized controlled trials, we studied several intermediate markers of colorectal carcinogenesis, each with its limitations. We observed that increasing fish consumption did not affect these intermediate markers of colorectal carcinogenesis, other than a decrease in serum CRP levels after intervention with salmon and cod.

Future research, a 'fishing expedition'?

What was already known?
▪ Fish consumption is associated with a reduced risk of colorectal cancer, though the evidence from observational studies was limitedly suggestive
▪ No randomized controlled trials (RCTs) had been performed on fish consumption and the markers of colorectal carcinogenesis apoptosis, mitosis or genotoxicity
▪ RCTs on fish and fish oil supplementation have shown that fish and fish oil can reduce inflammation though results were not consistent
▪ Fish contains a number of nutrients that could be beneficial, including n-3 PUFA, vitamin D, selenium, and protein

What does this thesis add?
▪ People with higher serum n-3 PUFA levels have a 33% reduced risk of colorectal adenomas and people with higher serum n-6 PUFA levels have an 68% increased risk of colorectal adenomas
▪ Supplementation of 1.5g/d n-3 PUFA for 12 weeks did not affect a large panel of serum inflammation markers in healthy subjects
▪ Increasing fish consumption by two weekly portions for six months did not affect colorectal apoptosis, mitosis, genotoxicity, and inflammation markers in a population of fish consumers, except for a significant decrease in serum CRP levels
▪ Salmon and cod did not differentially affect markers of CRC risk

How should we proceed with studies on fish consumption and CRC risk? Our results together with evidence from literature do not provide strong evidence that fish consumption can beneficially affect markers of colorectal carcinogenesis. However, we cannot disregard the limitedly suggestive evidence that has been observed for fish consumption and CRC.

In order to continue the research on the effects of fish consumption, or any food product, on colorectal cancer, the first step should be to focus on the likely mechanisms of action and to develop valid intermediate risk markers of CRC, since the current markers of CRC risk lack validity, as previously mentioned.
Promising candidates for intermediate markers of colorectal carcinogenesis, besides apoptosis and mitosis, include chromosomal instability [68,69], aberrant crypt foci [49,70-72], or more specifically epigenetic markers, like DNA methylation [29,73] or histone acetylation [74]. Nevertheless, it remains to be investigated whether these intermediate markers comply with the conditions to be an established surrogate marker of cancer risk, as proposed by Schatzkin and Gail [28].
To study intermediate markers of CRC risk, we could follow a systems biology or systems epidemiology approach, which is especially attractive now with the wide availability of affordable human microarrays [75,76]. Systems biology is 'a discipline that seeks to

determine how complex biological systems function by integrating experimentally derived information through mathematical and computing solutions' [77]. Using a systems biology approach, information from human microarrays could be combined with functional outcome markers, such as protein levels of these markers, to verify mechanistic information and possibly to discover new markers.

If we were to perform RCTs with valid intermediate markers of CRC risk, there are a few aspects to take into consideration. Regarding the exposure, different doses and different types of fish should be used. The different doses of fish consumption could reveal the dose-response relationship between fish consumption and CRC risk. The inclusion of different types of fish per individual has been shown to increase compliance of participants [78].
Regarding the participants, individuals with a low habitual fish consumption should be included, although this poses a major challenge since it is difficult to find participants willing to change their dietary habits and their fish intake in particular. An option to improve recruitment of subjects consuming little or no fish could be to focus on finding those people who do not consume fish due to costs, e.g. students or people with a low socio-economic status. The consumption of fish could be made more attractive by including more types of (popular) fish like sushi and provide the participants with ready-to-consume fish to increase compliance.

In summary, using valid intermediate markers of colorectal carcinogenesis in new fish randomized controlled trials, also including subjects with a low habitual fish consumption, future studies on fish consumption and colorectal cancer would not be a mere 'fishing expedition'.

Concluding remarks

The main message from this thesis is that we found no strong evidence that increasing fish consumption could beneficially affect markers of colorectal carcinogenesis, and that oil-rich and lean fish used in this RCT did not have differential effects on CRC risk markers. However, fish consumption has also been associated with beneficial effects related to other metabolic diseases, such as cardiovascular disease [79,80], and therefore the current dietary recommendation to consume two portions of fish weekly does not need to be altered.
Finally, consumption of fish is not the only possible beneficial strategy for colorectal cancer prevention. Low intake of red and processed meat, low intake of alcohol, reduced body & abdominal fatness, and increased physical activity have been shown to reduce colorectal cancer risk [23].

"I have a dream that one day the ideal study has shown that by increasing fish consumption, the incidence of colorectal cancer has declined.

I have a dream that the fish stocks have been restored and that there is enough fish to feed the world. I have a dream that for those who do dislike fish or who do not want to consume fish equivalent alternatives are available.

I have a dream that fish as part of a healthy diet helps to reduce the incidence of colorectal cancer."

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Summary in Dutch
(Samenvatting)

Samenvatting

Kanker is momenteel de belangrijkste doodsoorzaak in Nederland. Dikke darmkanker is daarbij één van de meest voorkomende vormen van kanker. Voeding speelt een belangrijke rol bij het ontstaan van dikke darmkanker; overgewicht, weinig lichaamsbeweging, het eten van rood vlees en het consumeren van alcohol verhogen het risico om dikke darmkanker te krijgen. In dit proefschrift is onderzocht of de consumptie van vis kan bijdragen aan een verlaagd risico op dikke darmkanker. Daarnaast is onderzocht of het uitmaakt welke soort vis geconsumeerd wordt. Vis bevat namelijk een aantal stoffen die mogelijk kunnen beschermen tegen dikke darmkanker. Zo is vis rijk aan visvetzuren, ook wel omega-3 of n-3 vetzuren genoemd, die vooral in vette vis (bijvoorbeeld zalm of haring) worden aangetroffen. Naast visvetzuren bevat vis vitamine D, selenium en eiwitten, die mogelijk ook een bescherming kunnen bieden tegen dikke darmkanker.

Om de invloed van (vette) vis op dikke darmkanker te onderzoeken, waren gegevens van twee studies beschikbaar. De eerste studie was een observationeel onderzoek waarbij mensen met poliepen (n=363) zijn vergeleken met mensen zonder poliepen (n=498) (**Hoofdstuk 2**). Poliepen, ook wel adenomen genoemd, zijn een soort voorstadium van dikke darmkanker. Mogelijk zouden mensen die meer vis eten een lager risico hebben op het krijgen van dikke darmkanker. De hoeveelheid vis die mensen gegeten hebben kan worden gemeten aan de hoeveelheid n-3 waardes in het bloed. Uit deze studie bleek dat mensen die hoge n-3 waardes in het bloed hadden 33% minder kans hadden op het krijgen van poliepen ten opzichte van mensen met lage visvetzuur waardes in het bloed.

De tweede studie was een interventie studie naar de effecten van visolie in 77 gezonde vrijwilligers (**Hoofdstuk 3**). Beschikbare bloedmonsters zijn gebruikt om het effect van visolie op ontstekingsmarkers in het bloed te onderzoeken. Ontstekingen kunnen mogelijk het risico op dikke darmkanker verhogen. Visolie bleek geen effect te hebben op de ontstekingsmarkers in het bloed van deze gezonde mensen. Een mogelijke verklaring is dat de proefpersonen dermate gezond waren dat er geen verdere gezondheidsbevorderende effecten zijn waargenomen.

Om de effecten van visconsumptie op het risico van dikke darmkanker te onderzoeken, zijn vis-interventie studies nodig. Tot nu toe zijn dergelijke vis-interventie studies niet uitgevoerd en in dit proefschrift zijn de resultaten van het eerste vis-interventie onderzoek beschreven: de FISHGASTRO studie. Om het risico op dikke darmkanker te onderzoeken wordt idealiter bestudeerd of gezonde mensen wel of geen dikke darmkanker ontwikkelen nadat ze wel of geen vis hebben gegeten. Het ontwikkelen van dikke darmkanker kan echter vele jaren duren, waardoor een dergelijke studie erg lang zou duren. Daarom is gekeken naar voorspellers van darmkankerrisico, zoals verhoogde celdeling, verminderde celdood, de mate van DNA schade in de darm, en verhoging van ontstekingsmarkers.

Aan de FISHGASTRO studie deden in totaal 242 mensen mee met dikke darmpoliepen, een chronische darmontsteking, of mensen die gezond waren. Zij kregen zes maanden lang ofwel twee porties zalm per week, ofwel twee porties kabeljauw, ofwel geen extra vis (de controle groep). Alle proefpersonen ontvingen informatie over gezonde voeding. Voor en na de zes maanden interventie is een darmbiopt en bloed afgenomen; daarnaast zijn ook een aantal vragenlijsten ingevuld door de proefpersonen en hebben sommige proefpersonen ontlasting verzameld. In darmbiopten is gekeken naar de mate van celdeling en celdood. Deze waren niet veranderd na het eten van zalm of kabeljauw ten opzichte van de controle groep (**Hoofdstuk 4**). De DNA schade in het darmbiopt en in de ontlasting was niet veranderd na het eten van zowel zalm als kabeljauw (**Hoofdstuk 5**). De ontstekingsmarkers in het darmbiopt en in de ontlasting waren ook niet veranderd, behalve één ontstekingsmarker, C-reactive protein (CRP) in het bloed. Deze was gedaald na het eten van zowel zalm als kabeljauw (**Hoofdstuk 6**). Een lager niveau van CRP in het bloed kan mogelijk duiden op een lager risico op dikke darmkanker, maar de precieze functie van CRP moet nog verder worden onderzocht. De conclusie op basis van deze eerste vis-interventie studie is dat er geen duidelijk bewijs is gevonden dat het verhogen van de visconsumptie intermediaire markers van dikke darmkanker gunstig beïnvloedt.

In **Hoofdstuk 7** zijn de resultaten van dit onderzoek in perspectief geplaatst. Het belangrijkste deel van dit proefschrift omvat de vis-interventie studie. Bij de interpretatie van dit onderzoek is belangrijk te realiseren dat de proefpersonen voordat ze meededen aan de studie al een vrij hoge visinname hadden, dat de proefpersonen in de visgroepen wel iets meer zijn gaan consumeren maar niet de twee porties per week die wij voor ogen hadden, en dat de steekproefgrootte mogelijk niet groot genoeg was om verschillen te kunnen aantonen. Daarnaast is het onduidelijk in hoeverre de gebruikte voorspellers van dikke darmkanker risico daadwerkelijk het risico op dikke darmkanker kunnen voorspellen. Vervolgonderzoek zou zich daarom in eerste instantie moeten richten op het ontwikkelen van gevalideerde risicomarkers van dikke darmkanker. Tevens zou in vervolgstudies gebruik gemaakt moeten worden van meerdere dosis vis en meer variatie in soorten vis. De belangrijkste boodschap van dit proefschrift is dat er geen sterk bewijs is gevonden dat het verhogen van visconsumptie voorspellers van dikke darmkanker gunstig kan beïnvloeden. Daarnaast is consumptie van vis niet de enig mogelijke factor ter vermindering van het risico op dikke darmkanker. Zo kan een dieet met matige consumptie van rood vlees en alcohol, een verlaagde hoeveelheid abdominaal lichaamsvet, en voldoende lichamelijke activiteit bijdragen aan een verlaagd risico op dikke darmkanker. Aangezien visconsumptie wel bijdraagt aan het verminderen van andere ziekten, zoals hart- en vaatziekten, houdt de huidige aanbeveling om twee maal per week vis te eten stand.

Dankwoord

Bedankt! Thank you! Gracias!

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gerda

Regie	
Promotor-1	Ellen Kampman
Promotor-2	Gertjan Schaafsma
Co-promotor	Anouk Geelen
Scenario	
Paranimf-1	Akke Botma
Paranimf-2	Ondine Nieuwerth- van de Rest
Kamergenoten	Renate Van de Borne- Winkels, Martinette Streppel
FISHGASTRO onderzoek	
Deelnemers	Alle FISHGASTRO deelnemers
Assistenten	Yvonne ter Telgte, Janneke van Wijngaarden, Celine Brattinga
Studenten	Margriet Smits, Annemieke Kok, Liza van Steenbergen, Monique Jöris, Susann Bellmann, Marlieke Visser, Else-Mariëtte van Heijningen, Cathelijne Mieloo, Jantina Stol, Maaike Walters, Anke Enneman, Anne Maria Hilbers
Wageningen Lab	Lucy Okma, Pieter Versloot, Paul Hulshof, Betty van der Struijs
Wageningen NMG groep	Guido Hooiveld, Jan Harryvan, Hanneke Vermeulen, Shohreh Keshtkar
Dietetiek	Els Siebelink, Karin Borgonjen, Saskia Meyboom
Secretariaat	Marie Jansen, Karen Zweers, Gea Brussen, Gabriëlle van den Broek, Cornelia van Bree-Evers
FISHGASTRO op locatie	
Location IFR, Norwich, UK	Liz Lund, Gosia Newman, Linda Harvey, Joanne Doleman, Jack Dainty, Kasia Przybylska-Phillips, Andrew Hart, Matthew Williams
Location FSU, Jena, Germany	Beatrice Pool-Zobel †, Michael Glei, Nina Habermann
Locatie Radboud Nijmegen	Fokko Nagengast, Maria van Vugt & Afdeling Maag-Darm-Lever, endoscopie, en lab
Locatie Gelderse Vallei, Ede	Ben Witteman & Afdeling Maag-Darm-Lever, endoscopie, en lab
Locatie Slingeland Doetinchem	Paul van de Meeberg & Afdeling Maag-Darm-Lever, endoscopie, en lab
Locatie Antonius Nieuwegein	Robin Timmer & Afdeling Maag-Darm-Lever, endoscopie, en lab
Locatie CWZ Nijmegen	Adriaan Tan & Afdeling Maag-Darm-Lever, endoscopie, en lab
Locatie Rijnstate Arnhem	Peter Wahab & Afdeling Maag-Darm-Lever, endoscopie, en lab
Locatie WKZ, Utrecht	Ger Rijkers
Overige ondersteuning	
Overige coauteurs	Marge Ocke, Henk van Kranen, Chris Siezen, Ingeborg Brouwer
Collega's/ Colleagues	Renate, Martinette, Sandra, Gertrude, Marja, Mariëlle, Simone, Carla, Cora, Mariken, Alina, Maureen, Petra, Pascalle, Geert, Heleen, Meike, Andrea, Du, Janette, Linda, Elise, Nicolien, Antonie, Esmée, Pleunie, Pauline, Sophie ,Angélique, Marieke, Mark, Edith, and Pieter
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	Oma
	Papa & mama

About the author

Curriculum Vitae

Gerda Karolien Pot was born on November 10, 1978 in Maastricht, the Netherlands. In 1995, after four years of secondary school at 'Stedelijke Scholengemeenschap' in Maastricht, she went to secondary school for one year in Mérida, Venezuela. In 1998, she graduated from secondary school (gymnasium B) at Trichter College in Maastricht. In the same year she started her studies on Nutrition and Health at the former Wageningen Agricultural University. She focused on Toxicology by her MSc thesis and an internship at Unilever, Colworth, United Kingdom. Afterwards, she decided to focus more on human studies by an internship at former TNO Nutrition in Zeist. In 2003 she received her MSc degree with honours and started working at the Division of Human Nutrition. She was involved in writing two grant proposals, and data entry for the FACIT study. In April 2004, she started her PhD project described in this thesis, which was carried out at the Division of Human Nutrition, Wageningen University. She was a member of the organizing committee of the PhD study tour to England, Ireland, and Scotland in 2005, and joined the PhD study tour to the United States in 2007. She was a member of the daily board of the committee of temporary scientific staff within the Division of Human Nutrition from 2006 to 2008. In 2009, she was selected to join the European Nutrition Leadership Programme. During this period, she also attended several courses and conferences.

Publications

Publications in peer-reviewed journals

1. Pot GK, Majsak-Newman G, Geelen A, Harvey LJ, Nagengast FM, Witteman BJM, van de Meeberg PC, Timmer R, Tan A, Wahab PJ, Hart A, Williams MP, Przybylska K, Dainty JR, Schaafsma G, Kampman E, Lund EK. Fish consumption and markers of colorectal cancer risk: a multi-centre randomized controlled trial. *AJCN 2009;90(2):354-361*
2. Pot GK, Brouwer IA, Enneman A, Rijkers GT, Kampman E, Geelen A. No effect of high dose fish oil supplementation on systemic inflammatory markers and their interrelationships: a randomized controlled trial in healthy, middle-aged subjects. *EJCN (in press)*
3. Pot GK, Geelen A, van Heijnigen EMB, Siezen CL, van Kranen HJ, Kampman E. Serum n-3 and n-6 polyunsaturated fatty acids and the risk of colorectal adenomas: an endoscopy-based case-control study. *Int J Cancer. 2008;123(8):1974-7*
4. Visser M, Geelen A, Pot GK, van Bergeijk JD, Brehler HD, Kampman E, Witteman BJM. Stress, anxiety, and depression in patients with Inflammatory Bowel Disease (IBD) and Irritable Bowel Syndrome (IBS). *Psychologie en Gezondheid (Psychology and Health in Dutch), 2008;36(2):56-62.*
5. de Haan LH, Pot GK, Aarts JM, Rietjens IM, Alink GM. In vivo relevance of two critical levels for NAD(P)H:quinone oxidoreductase (NQO1)-mediated cellular protection against electrophile toxicity found in vitro. *Toxicol In Vitro. 2006 ;20(5):594-600.*
6. Pot GK, Kampman E, Antoine JM, Milner JA, Maman C, Jones PJ, Cummings JH, Kok FJ. 'Let food be thy medicine': similar actions and similar health benefits for foods and drugs? *Submitted*
7. Pot GK/ Habermann N, Majsak-Newman G, Harvey LJ, Geelen A, Przybylska-Philips K, Nagengast FM, Witteman BJM, van de Meeberg PC, Hart AR, Schaafsma G, Hooiveld G, Glei M, Lund EK, Pool-Zobel B, Kampman E. Increasing fish consumption does not affect genotoxicity in the colon in an intervention study. *Submitted*
8. Pot GK, Geelen A, Majsak-Newman G, Harvey LJ, Nagengast FM, Witteman BJM, van de Meeberg PC, Hart A, Schaafsma G, Lund EK, Rijkers GT, Kampman E. Fatty and lean fish consumption reduce C-reactive protein levels but do not affect inflammation markers in feces and in colonic biopsies. *Submitted*

Selection of Abstracts in scientific journals or proceedings

1. Majsak-Newman G, Pot GK, Maxwell E, Geelen A, Harvey LJ, Nagengast FM, Witteman BJ, van de Meeberg PC, Timmer R, Tan A, Wahab PJ, Hart A, Williams MP, Kampman E, Lund EK. Vitamin D levels in 70% of Patients Attending For Colonoscopy in The UK and Netherlands are Sub-Optimal but Consumption of Salmon Does Not Provide Any Significant Improvement . *Gut (in press)*
2. Pot GK, Majsak-Newman, G, Geelen A, Harvey LJ, Przybylska K, Hart A, Williams MP, Dainty J, van t' Veer P, Schaafsma G, Nagenast FM, Witteman BJM, van de Meeberg PC, Timmer R, Kampman E, Lund EK. Effect of a fish intervention on markers of colorectal carcinogenesis: the FISHGASTRO study. *Gut (in press)*
3. Pot GK, Majsak-Newman G, Geelen A, Harvey L, FISHGASTRO Study team gastroenterologists, van 't Veer P, Schaafsma G, Kampman E, Lund E. Effect of a fish intervention on markers of colorectal carcinogenesis: the FISHGASTRO study. Dutch Association of Gastroenterology, Veldhoven, the Netherlands Oct 2008
4. Pot GK, van Heijningen EMB, Geelen A, Siezen CL, van Kranen HJ, Kampman E. Opposing associations of serum n-3 and n-6 fatty acids with colorectal adenoma risk: an endoscopy-based case-control study. WCRF launch conference, Washington DC, US 2007
5. Pot GK, Geelen A, Nagengast FM, Witteman BJM, Van 't Veer P, Schaafsma G, Lund EK, Kampman E. Fish consumption and inflammation in colorectal carcinogenesis: the FISHGASTRO study. Keystone Symposium Mechanisms Linking Inflammation and Cancer, Santa Fe, New Mexico, US 2007

Overview of completed training activities

Discipline specific activities

Courses

- Masterclass Nutrition and Cancer, VLAG Graduate School, Wageningen, NL, 2007
- Masterclass Dietary Influences on Blood pressure, VLAG/NZO, Wageningen, NL, 2006
- Masterclass Molecular biology of intestinal microbiota, probiotics and Food chain bacteria, ABS Graduate School, Helsinki, Finland, 2006
- Eco-physiology of the gastrointestinal tract, VLAG Graduate School, Wageningen, NL, 2005
- Masterclass Nutrition and Communication, VLAG Graduate School, Wageningen, NL, 2005

Meetings

- Wageningen Nutritional Sciences Forum, 2009
- NWO Nutrition days, Papendal/ Deurne, NL, 2004- 2008
- Mechanisms linking Inflammation and Cancer, Keystone Symposium, Sante Fe, New Mexico, US, 2007
- Food, Nutrition, Physical Activity, and the Prevention of Cancer: a Global perspective. Launch Conference, Washington DC, US, 2007
- Rank Prize Fund Symposium Omega-3, Windermere, UK, 2006
- SEAFOODplus conferences, Copenhagen Denmark 2004, Tromso Norway 2006, Copenhagen Denmark 2008
- Bowel-days (Darmendag), NL, 2005-2008
- Dutch Society of Gastroenterology (NVGE) annual meetings 2005 & 2008

Training period at other institutes

- Department of Toxicology, Friedrich Schiller University, Germany, 2004
- Institute of Food Research, Norwich, UK, 2007 and 2008

General courses

- European Nutrition Leadership Programme (ENLP), Luxembourg, 2009
- Career perspectives, Wageningen University, NL, 2008
- Scientific Writing, Wageningen University, NL, 2007
- Organizing and supervising MSc students, Wageningen University, NL, 2006
- Project and Time Management, NL, 2005
- VLAG PhD week, 2004

Optional courses and activities

- Literature Study Programs (Journal Club, N-3 club, Oldsmobiles)
- Epi-research meetings
- Staff seminars at Division of Human Nutrition, Wageningen University
- PhD study tour to England, Ireland, and Scotland 2005 & United States 2007
- Preparation research proposal

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