BIOMARKERS FOR COLON CANCER:
Applications in human and rat studies

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ABSTRACT
Colon cancer is one of the major causes of cancer in industrialized countries, and it is caused by a combination of hereditary, environmental and dietary factors and lack of physical activity. Of these factors, diet is one of the most important. To increase our knowledge about the diet-colon cancer link, it is important to conduct human intervention studies using good cancer markers. Presently, the most reliable marker is occurrence of polyps in the colon. As this method is invasive, time demanding and very costly, it is important to have access to early/intermediate biomarkers for testing hypotheses relating diet and cancer. Approximately 25 biomarkers for colon cancer risk are presently available, for colon mucosa, faeces and blood. Faecal water is the aqueous phase of faeces, and this phase is believed to mediate many of the effects of diet on colon cancer. The composition of faecal water is not fully understood, but it probably contains both tumour promoters and anti-cancer agents. The overall aim with this thesis was to further characterize a selection of early/intermediate biomarkers and to assess the colon cancer preventative effects of; polyethylene glycol (PEG), probiotic bacteria, prebiotic carbohydrates, and natural cyclooxygenase-2 (COX-2) inhibitors. In the first study, we investigated whether three selected biomarkers differed between a high risk (adenoma patients) and a low risk group for colon cancer. Colonic cell proliferation induced by faecal water was significantly higher in the high risk group compared to the low risk group, and may therefore be a useful biomarker for future intervention studies. Two additional biomarkers; preneoplastic lesions (ACF) and the inflammatory protein GMP were used in a rat study to study the cancer preventative effects of the demulcent PEG. PEG significantly reduced ACF formation and faecal inflammation measured by GMP. In order to evaluate the colon cancer preventive properties of synbiotics (probiotics and prebiotics), a double-blind intervention study was carried out in humans. Patients with polyps or a history of cancer were recruited, and a large number of biomarkers were assessed. The synbiotic supplements significantly altered the colonic microflora in a favorable way. The intervention also significantly reduced colorectal proliferation and DNA damage, and the capacity of colonic contents (faecal water) to induce necrosis and DNA damage in cells. Earlier studies have indicated that faecal water can influence COX-2, which is a marker for colon cancer. A small pilot study with vegetarians was carried out to assess if natural COX-2 inhibitors were present in faecal water. Natural COX-2 inhibitors were present in human faecal water, and further characterisation indicated that the COX-2 inhibitors are polar and water soluble, and that they act in synergy. Some data also indicated a possible role for phenolic compounds in these effects. In conclusion, our work will aid in the selection of appropriate biomarkers in future human intervention trials to assess the effect of dietary components on colon cancer risk. PEG and synbiotics are two agents that may be useful in reducing risk for tumours in the colon. Lastly, natural COX-2 inhibitors are present in human faeces, and may also be a target for dietary recommendations in the future.

Key words: Colon cancer biomarkers, faecal water, polyethylene glycol, synbiotics, COX-2 inhibitors

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ABBREVIATIONS

AA = arachidonic acid
ACF = aberrant crypt foci
AOM = azoxymethane
AP-1 = activator protein 1
APC = adenomatous polyposis coli
COX = cyclooxygenase
DCA = deoxycholic acid
DMEM = Dulbecco’s modified eagle medium
DMSO = dimethyl sulphoxide
EGCG = epigallocatechingallate
ELISA = enzyme-linked immunospecific antibody assay
FACs = fluorescence activated cell sorter
FAP = familial adenomatous polyposis
FBS = foetal bovine serum
GC-MS = gas chromatography-mass spectrometry
GI = gastrointestinal tract
GMP = granulocyte marker protein
IBD = inflammatory bowel disease
LA = lithocholic acid
NFκB = nuclear factor kappa B
NMR = nuclear magnetic resonance
NS-398 = [N-2-(cyclohexyloxy)-4-nitrophenyl]methane-sulfonamide
NSAIDs = non-steroidal anti-inflammatory drugs
PEG = polyethylene glycol
PGs = prostaglandins
PI = propidium iodide
PS = phosphatidylserine
SCFA = short chain fatty acid
TJs = tight junctions
TNF-α = tumour necrosis factor alpha
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BACKGROUND

COLON CANCER EPIDEMIOLOGY

The incidences of colorectal cancer vary widely around the world. In Europe and other developed countries, colon cancer is one of the major causes of cancer death (Silverberg 1985; Boyle and Langman 2000; Gill and Rowland 2002). Among the factors that contribute to the development of colon cancer, diet is thought to be one of most importance, although the genetic predisposition also plays a role. Interestingly, factors of dietary origin in the colon can alter the expression of genes that are involved in the carcinogenesis process.

Epidemiological studies have shown that people on a typical Western-style high-fat diet and a sedentary lifestyle are at high risk for colon cancer (Armstrong and Doll 1975; Willett 1998). Furthermore, populations migrating from countries with a low risk of cancer to westernized countries, show an increased risk for colon cancer (Haenzel and Kurihara 1968; Potter, Slattery et al. 1993). A diet low in saturated fat, and high in fruit and vegetables and fiber is believed to decrease the risk of colon cancer (Howe, Benito et al. 1992; WCRF 1997). In line with this, vegetarians show a lower risk of developing colon cancer compared to omnivores (Frentzel-Beyme and Chang-Claude 1994; Almendingen, Trygg et al. 1995). However, several clinical trials have failed to prove that such a diet prevents the occurrence of colonic polyps and cancer (MacLennan, Macrae et al. 1995; Alberts, Martinez et al. 2000; Schatzkin, Lanza et al. 2000).

Taken together, this strongly indicates that diet plays an important role in the aetiology of colon cancer, although it is still not clear which dietary factors are most important. To increase our understanding of this diet-colon cancer link it is; first, important to develop new methods (biomarkers) for testing hypotheses relating diet and cancer; second, to clarify underlying mechanisms.

Figure 1. The morphology of a crypt in the colon (Potten 1998)
COLON CANCER MECHANISMS

Morphology of the colon

In a healthy colon there is a fine balance between cell growth and cell death, and the epithelial cells are normally renewed every 4-8 days (Lipkin 1973). However, in a situation of stress, cell division and proliferation can take over and disrupt the balance. This imbalance can contribute to a higher frequency of mutations, which is a risk factor for cancer development.

There are several types of cells in the colon; stem cells and daughter cells in the bottom of the crypt, and more specialized cells in the upper part of the crypt (Potten 1998; Ponz de Leon and Percesepe 2000). The stem cells are meant to differentiate into more specialized cells like goblet cells, absorptive cells and endocrine cells (Fig. 1). Normally, proliferative cells are only found in the bottom of the crypt, whereas differentiation takes place in the middle of the crypts (Butler, Hewett et al. 1999). Cells on top of the crypt have a very short life span, and mutations in these cells are probably without impact for development of cancer (Potter 1999).

Colon carcinogenesis

Colorectal tumours appear as a consequence of loss of growth control. An imbalance in proliferation, differentiation and apoptosis results in an accumulation of hyperproliferative cells in the luminal surface (Stadler, Yeung et al. 1988). Such a hyperproliferative zone with undifferentiated cells in the upper crypt, constitutes a risk for progression into benign tumours (Fig. 2).

In 1988, Vogelstein et al. introduced a multistep genetic model of colorectal carcinogenesis (Vogelstein, Fearon et al. 1988). According to this model, most carcinomas in the colon origin from pre-existing adenomas, which initially are induced by activation of protooncogenes and/or inactivation of tumour suppressor genes, (whereof the last tend to predominate). At least four to five mutations in different genes are normally required for the formation of a malignant tumour. Tumour suppressor genes can be inactivated by two different mechanisms; first by mutations and second, by loss of specific chromosomal regions. Familial adenomatous polyposis (FAP) is a hereditary syndrome caused by an inherited mutation in the adenomatous polyposis coli (APC) gene on chromosome 5q (Jen, Powell et al. 1994). This mutation is believed to cause the hyperproliferative zones that proceed into polyps and adenomas in the colon, acting via modulation of cadherin activity and closure of TJs (tight junctions) (Troxell, Chen et al. 1999). Alterations in the same region may also be important in the early carcinogenesis in patients without polyposis. A second form of genetically predisposed colon cancer is hereditary non-polyposis colorectal cancer (HNPCC) (Lynch and Lynch 1994). Mutations in DNA mis-match genes are involved in this condition (de Leeuw, Dierssen et al. 2000; Muller and Fishel 2002). Another frequently observed alteration in the early stage is loss of methyl groups in DNA (DNA hypomethylation).
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Normal Hyperproliferation/ dysplasia Small adenoma Large adenoma Carcinoma/ metastasis

Toxic, genotoxic compounds

18q deletion p53 inactivation APC mutation/ inactivation K-ras activation IRq deletion p53 inactivation

Figure 2. Formation of carcinoma in the colon. Adapted from Gill and Rowland (Gill and Rowland 2002).

This change may lead to aneuploidy with loss of gene alleles (Goelz, Vogelstein et al. 1985). Another common feature in early carcinogenesis is mutations in the protooncogene ras. These adenomas constitute a risk, as they are likely to develop into larger and more dysplastic tumours through clonal expansion (Bos, Fearon et al. 1987). Although the genetic changes often occur in the order as described above, the total accumulation of mutations and deletions seems to be more important for carcinomas to metastasize rather than their order with respect to each other (Fearon and Vogelstein 1990). Polyps and cancer are furthermore, more common in the left distal part of the colon, and affect men and women equally (Wiese, Thompson et al. 2003; Nasir, Kaiser et al. 2004).

Inflammation and colon cancer

The incidence of colon cancer and inflammatory bowel disease (IBD) vary among the world in a similar pattern. This variation is probably partly due to different diets, since developing countries such as Asia, Africa and South America have a lower incidence of cancer and IBD compared to Western countries (NCI 2000; Sandler and Eisen 2000). A recent hypothesis suggests that a high risk diet (high in saturated fat and phosphate and low in calcium), such as is common in western countries, increases the permeability of tight junctions (TJs) between colonocytes and thereby exposes them to toxic agents in the lumen (Bruce, Giacca et al. 2000). Various components of dietary origin can increase or decrease the leakage of TJs. Increased permeability makes it possible for growth factors and toxic agents to act on the exposed epithelium and induce inflammation. Growth factors and products of inflammation are also known to increase proliferation, the latter also induces mutations and apoptosis. All these factors are believed to be important in carcinogenesis, and are summarized in Fig. 3. Mutations of the APC gene can affect the function of cadherin and the closure of TJs (Troxell, Chen et al. 1999), and an abundance of growth factors can stimulate proliferation. The accessibility of calcium in the lumen markedly affects the function of the TJs. A decreased calcium level keeps the TJs open and allows components/microbes to migrate from the lumen into the epithelial mucosa and vice versa (Ma, Hollander et al. 1992).
There are several lines of evidence that support an association between colonic inflammation and colon cancer. First, patients with IBD are at markedly increased risk of developing colon cancer (Gillen, Walmsley et al. 1994; Biasco, Brandi et al. 1995). Second, non-steroidal anti-inflammatory agents (NSAIDs) decrease the risk of colon cancer and reduce the formation of polyps in cancer patients (Giardiello, Hamilton et al. 1993; Phillips, Wallace et al. 2002). Third, infiltration of *Escherichia coli* has been reported in tumours and surrounding tissues (Swidsinski, Khilkın et al. 1998). Fourth, colon cancer patients have increased levels of inflammatory proteins in faeces (Kristinsson, Roseth et al. 1999).

**Faecal water**

In recent years, there has been considerable interest in the role of the aqueous phase of human faeces (faecal water) in studies examining the mechanisms underlying the dietary aetiology of colon cancer. This aqueous phase of faeces is called faecal water and this concept was introduced by Rafter et al. in 1987 (Rafter, Child et al. 1987). Components in faecal water are more frequently in contact with the colonic epithelium than components of the solid phase. They are therefore more likely to give rise to cell damage, which constitute a risk for initiation and progression of colon cancer (Rafter, Geltner et al. 1987). The composition of faecal water is not fully understood, but there are indications that both tumour promoters and anti-cancer preventative agents are present. The main cytotoxic compounds are believed to be the secondary bile acids; deoxycholic acid (DCA) and lithocholic acid (LA). These are formed by anaerobic bacteria from the primary bile acids cholic and chenodeoxycholic acid. Secondary bile acids are cytotoxic and induce cell proliferation in the colon, and are believed to play an important role in colon carcinogenesis (Stadler, Yeung et al. 1988; Lapre and Van der Meer 1992).

Several intervention studies have demonstrated that it is possible to alter the concentration of soluble bile acids in faecal water by a change in diet (Rafter, Child et al. 1987; Allinger, Johansson et al. 1989), while leaving the total amount of bile acids uninfluenced (Rafter, Child et al. 1987). This effect can probably be explained by an increased amount of fiber and calcium in the diet, which is thought to protect against cell damage in the colon. Fiber can bind bile acids and decrease their solubility. Calcium can precipitate acidic lipids (as bile acids) as calcium soaps and thereby protect the epithelium in the colon from possible toxic effects (Rafter, Eng et al. 1986; Lapre, De Vries et al. 1993; van Faassen, van den Bogaard et al. 1996). A diet high in fat increases the excretion of bile acids into the lumen and consequently also the production of secondary bile acids. The concentration of bile acids (primary and secondary) and pH in faecal water differ between vegetarians and omnivores (Allinger, Johansson et al. 1989; van Faassen, Hazen et al. 1993). Other components in faecal water are; neutral steroids, fatty acids, short chain fatty acids (SCFA), minerals and vitamins, (van Faassen, Hazen et al. 1993; Dolara, Caderni et al. 2002). SCFA are produced by lactic acid bacteria in the colon and the main SCFA is butyrate (Scheppach 1994). More recently, small phenolic
compounds and derivatives thereof were identified in faecal water (Jenner, Rafter et al., 2005).

Figure 3. Two pathways involved in focal epithelial defect mechanism inferred from inhibition of carcinogenesis by demulcents, NSAIDs and antioxidants (Bruce, Giacca et al., 2000).
Diets high in fruit and vegetables

Epidemiological studies indicate that there is a strong link between diet and cancer (Armstrong and Doll 1975; Potter, Slattery et al. 1993; Willett 1998). The cancer risk can either be reduced by minimizing the exposure to carcinogens or by adding chemopreventive agents to the diet.

A high intake of saturated fat and meat, and low intake of fiber (a typical Westernized diet) is believed to contribute to cancer in the large bowel (WCRF 1997). Amino compounds and nitrosamines and to some extent secondary bile acids, formed in the gastrointestinal tract after ingestion of meat and saturated fat may contribute to this effect (Mirvish 1995; Silvester, Bingham et al. 1997). These compounds and metabolic products thereof, may be cytotoxic or genotoxic to the colonocytes and thereby induce mutations and/or destroy the fine balance of cell growth/death in the colon (Venturi, Hambly et al. 1997; Rieger, Parlesak et al. 1999). Bile acids and inflammation in the colon can furthermore stimulate breakdown of phospholipids in the membrane of the colonocytes and release of arachidonic acid (Craven, Pfanstiel et al. 1986; Reddy 2000). High availability of arachidonic acid activates cyclooxygenase-2 (COX-2) with a subsequently higher production of free radicals, which both are risk factors for colon cancer. This Westernized diet is also rich in iron, which lead to access of the pro-oxidant in the colon and production of hydroxyl radicals (Craven, Pfanstiel et al. 1986; Erhardt, Lim et al. 1997). In addition, there animal studies indicating that red meat promotes colorectal carcinogenesis (Pierre, Tache et al. 2003; Pierre, Freeman et al. 2004). The balance of fatty acids in the diet also seems to play an important role for the development of cancer. Too much of w-6 fatty acids increase tumour formation in rats, whereas w-3 fatty acids in fish oil prevents inflammation and possibly the formation of tumours in humans (Caygill, Charlett et al. 1996; Calder 2002).

Appropriate lifestyle changes and a change in diet towards a higher intake of fruit and vegetables can prevent cancer development (Steinmetz and Klaunig 1996). This is further confirmed by the fact that vegetarians have a lower risk of developing colon cancer than omnivores (Frentzel-Beyme and Chang-Claude 1994; Almendingen, Trygg et al. 1995). A vegetarian diet is rich in fiber, antioxidants and phytochemicals, and low in saturated fat.

The National cancer institute (NCI) has identified more than 1000 different phytochemicals that possess cancer-preventive activity, and many of these can be found in common foods; soybeans, ginger, grapes, green tea, citrus fruits, tomatoes and cruciferous vegetables are some examples (Surh 2003). The dominating group of phytochemicals is polyphenols, whereof phenolic acids and flavonoids are the most important subgroups. Resveratrol, quercetin, catechin, genistein, hesperetin, anthocyanidin are some examples of dietary polyphenols (Scalbert and Williamson 2000). Recent studies have shown that faeces and faecal water have a remarkable antioxidant capacity (Garsetti, Pellegrini et al. 2000; Jenner, Rafter et al. 2005). Some of
the antioxidant capacity in faeces was correlated with the intake of beverages rich in polyphenols (especially coffee and wine) (Garsetti, Pellegrini et al. 2000).

Only a minor part of the ingested polyphenols are found in plasma, and the concentration rarely exceeds 1 μM, even if the intake is 1000 times larger (Hollman, de Vries et al. 1995; Scalbert and Williamson 2000). Most of the polyphenols are poorly absorbed due to glycosylation of the phenolic ring and high molecular weight and the elimination rate in plasma is very fast. The vast majority of the polyphenols are conjugated in the liver and excreted with the bile into the intestinal lumen (Scalbert and Williamson 2000). The highest concentrations of polyphenols are consequently found in the colon, where the gut microflora can convert them into more simple compounds such as phenolic acids. These phenols are very likely to exert a local direct effect in the gastrointestinal tract (Garsetti, Pellegrini et al. 2000; Halliwell, Rafter et al. 2005). Polyphenols are reducing agents and can together with vitamin C and vitamin E protect the gastrointestinal cells from oxidative damage (Scalbert and Williamson 2000; Halliwell, Rafter et al. 2005). A high availability of phenols in the lumen may also upregulate toxin metabolizing and antioxidant enzymes (van Lieshout, Posner et al. 1998). They can also together with phytate from fiber, chelate catalytic iron and thereby decrease the production of harmful hydroxyl radicals (Garsetti, Pellegrini et al. 2000).

Other nutrients in faeces that may act locally as antioxidants are β-carotene, and selenium. Vitamin E exists in many different isoforms, but α-tocopherol is the only biologically active form. The other forms of tocopherols and tocotrienols are excreted into the lumen via the enterohepatic shunt, which will give rise to a high local concentration. These may like the phenols, act locally in the lumen as oxygen scavengers (Stone and Papas 1997; Halliwell, Rafter et al. 2005).

The microflora

The human species live in symbiosis with microbes, and the host-microbe interaction is extremely important as the microbial flora constitutes approximately 90% of our cells in the body (Savage 1977). The microbial density in the proximal small intestine is relatively sparse, but the density increases drastically in the distal part to approximately 10^8 bacteria/g luminal contents. The majority of the bacteria is, however, found in the colon, where the concentration has been estimated to 10^{11}-10^{12}/g luminal contents (Savage 1977; Hopkins, Sharp et al. 2001). At birth the intestinal tract is sterile, and thereafter the microbiota is gradually built up during the postnatal development (Bryant 1997; Mackie 1997). An adult intestine harbours around 500 bacterial species (Savage 1977; Suau, Bonnet et al. 1999; Blaut, Collins et al. 2002). Many of these bacteria can digest complex polysaccharides (cellulose, xylan, and undigested starch, mucins) into monosaccharides, which our body cannot readily absorb (Hooper, Midtvedt et al. 2002). These can, however, be further fermented by the flora resulting in SCFA, which can be used by the host (preferably butyrate used by the epithelial cells see below) (Roediger 1980). Butyrate is the main energy source for the colonocytes and it is metabolized to glucose or glutamine to produce energy (Scheppach 1994).
Probiotic bacteria

The microflora in the intestine can possibly be manipulated through the diet. Lactic acid producing bacteria like Lactobacilli is one big family of bacteria, which has been used for a very long time to preserve food. These bacteria are nowadays believed to improve the intestinal microflora balance and thereby prevent intestinal disorders such as cancer and inflammatory bowel disease. It has been shown that the endogenous microflora is important in the onset of colon cancer; a favourable change in the flora is therefore likely to prevent tumour development. Thus, considerable attention has focused on dietary components that can influence the gut microflora as a strategy for colon cancer prevention. The definition of probiotic bacteria is “live microbial feed supplements which beneficially affect the host animal by improving its intestinal microbial balance”. There is accumulated evidence from animal studies that support a protective role for probiotics, prebiotics (non-digestible carbohydrates that stimulate lactic acid bacteria numbers in the gut, see below) and synbiotics (combinations of pro- and prebiotics).

*Lactobacillus acidophilus, Lactobacillus rhamnosus* (GG), *Lactobacillus paracasei, Lactobacillus salvaricus* and *Lactobacillus plantarum* are all bacteria species found naturally in the human intestinal system (Molin 2001). Some of these are also used in fermented food products. *L. paracasei* and *L. rhamnosus* are usually found in dairy products, whereas *L. plantarum* is found in fermented plant products. In western countries, fermented food products are often associated with dairy products. Fermented plant products are, however, very common in other cultures as in Indonesia, Tanzania, Japan, China and African countries.

Several studies have indicated that addition of probiotic cultures (e.g. *Lactobacillus* GG, *L. salivarius* or *Bifidobacterium longum*) to the diet of rats treated with colon carcinogens reduces colon tumour incidence and multiplicity (Hirayama and Rafter 2000). The precise mechanisms by which lactic acid bacteria inhibit colon cancer are presently unknown. However, the mechanisms most likely vary with species and involve a complex interaction between the bacteria and the colonic environment. Gram-negative anaerobes, which normally are involved in inflammation and in production of toxins and carcinogens in the colon, are suppressed by lactic acid bacteria (Fuller 1991). There are also speculations that lactic acid bacteria produce antitumourigenic compounds. Some lactic acid bacteria suppress specific bacterial enzyme activities, for example β-glucuronidase, β-glucosidase, nitroreductase, and azoreductase (Cole, Fuller et al. 1989; Ling, Korpela et al. 1994). These enzymes may be important in activation of procarcinogens in the colon. Lactic acid bacteria also increase the intestinal immunity, via increased number of immunoglobulin-secreting cells in the intestinal mucosa (Mao, Nobaek et al. 1996). Growth of lactic acid bacteria in the colon increases the production of SCFA, and production of lactic acid, which lead to a lower colonic pH. A low pH in the colon is believed to control the proliferation of pathogens and protect against colonic tumourigenesis. A specific binding and degrading of carcinogens has also been suggested as a likely protective mechanism of lactic acid bacteria (Hirayama and Rafter 1999). The hypothesized protective mechanisms are summarized in Fig. 4.
Presently, there is no direct clinical evidence for colon cancer prevention by probiotic bacteria, but there is indirect evidence from in vitro and animal studies supporting this theory (Koo and Rao 1991; Renner and Munzner 1991; Reddy and Rivenson 1993). However, probiotics have been shown to decrease inflammatory bowel disease symptoms (Gionchetti, Rizzello et al. 2000; Nobaek, Johansson et al. 2000). As previously mentioned, inflammatory bowel disease is a known risk factor for development of colon cancer. There are some studies examining the effect of probiotics on biomarkers for colon cancer risk in healthy volunteers and patients. Consumption of lactic acid bacteria by volunteers has been shown to reduce the mutagenicity of urine and faeces associated with the ingestion of carcinogens in cooked meat (Lidbeck, Overvik et al. 1992). Mucosal cell proliferative activity in upper colonic crypts of patients with colon adenomas (believed to be a risk factor for tumour development) significantly decreased after the administration of *L. acidophilus* and *B. bifidus* cultures (Biasco, Paganelli et al. 1991). There is also some indirect evidence that *L. acidophilus* decreases the genotoxicity of faecal water (Oberreuther-Moschner, Jähres et al. 2004).

**Figure 4.** Proposed protective mechanisms mediated by probiotic bacteria. Adapted from Hirayama et al (Hirayama and Rafter 1999).
Prebiotics & synbiotics

Recently, there has been a growing interest in prebiotics, indigestible carbohydrates that can be fermented by the intestinal bacterial flora. Fermentation of prebiotic carbohydrates results in a lowering of the pH and a higher production of the short chain fatty acids; acetate, propionate, and butyrate (Kleessen, Hartmann et al. 2001). The latter provide an energy source for the intestinal cells and may be important for a normal apoptotic function in the colon (Collard, Guy et al. 2003; Heavey and Rowland 2004).

There are several types of potential prebiotics available, but only the inulin-type fructans, galactooligosaccharides and lactulose strictly fulfil the criteria for prebiotics (Van Loo 2004). Inulin can be found in many food plants; garlic, onion, tomatoes and bananas, but one of the richest sources is chickory root, and it appears that a combination of different lengths of the fructans is most effective in changing the microbial flora in a favourable way (Van Loo 2004). Prebiotics are believed to be a good substrate for lactic acid producing bacteria (probiotic bacteria), which results in a high production of butyrate and lactic acid. A decline in pH will prevent growth of many pathogenic bacteria (Gibson and Wang 1994; Kleessen, Hartmann et al. 2001). Dietary prebiotics also improve the bowel function and efficacy of mineral absorption from food (mainly Ca and Mg) (Coudray, Tressol et al. 2003).

A wide range of studies have been conducted in rodent models, including transgenic mice, on the cancer-modulating effect of dietary prebiotics. These have consistently demonstrated a reduction in the endpoints measured; colonocyte DNA damage, aberrant crypt foci (ACF) in colon (believed to be preneoplastic lesions), number of tumours per animal, size and growth rate of the tumours, mean survival time, increase in life span (Reddy 1998; Rowland, Rumney et al. 1998). In all animal experiments where mixtures of probiotics (Bifidobacterium spp.; L. casei; L. rhamnosus) and prebiotics (lactulose, long chain inulin, short chain fructo-oligosaccharides) were tested, per definition synbiotics, the effect of the mixture on the measured biomarkers was greater than the sum of the two separately, suggesting a synergistic advantage of synbiotics (Rowland, Rumney et al. 1998; Gallaher and Khil 1999).

There are also some indications that inulin and oligofructose have anti-inflammatory properties and that they potentiate the effects of cytostatica utilized in human cancer treatment (Taper and Roberfroid 2002; Cavin, Delannoy et al. 2005). Taken together, prebiotics alone or in combination with probiotic bacteria are dietary ingredients that can change the microflora in a favourable way and potentially prevent colon cancer in humans.
**Polyethylene glycol**

Polyethylene glycols (PEGs) are demulcients or soothing agents that are used as laxatives to treat chronic constipation and as food additives to improve the consistency of foods (Attar, Lemann et al. 1999; DiPalma, DeRidder et al. 2000). Recent studies suggest that they might also be useful as chemopreventive agents for colorectal cancer. The suggestion is based on the observation that PEGs with molecular weights of around 8000 (PEG 8000) dramatically reduce the incidence of carcinogen-induced colonic tumours, as well as the number of preneoplastic lesions, aberrant crypt foci (ACF) in rats (Corpet and Parnaud 1999; Parnaud, Tache et al. 1999; Corpet, Parnaud et al. 2000; Naigamwalla, Chia et al. 2000). The precise mechanism by which PEG prevents carcinogenesis in rats is not known, but several mechanisms have been suggested to explain this protective effect of PEGs.

One of the earliest hypotheses was that PEG increases the volume and water in faeces, and thus dilutes the concentration of potentially toxic compounds in the faecal stream. This theory alone is not very likely as other polymers also can increase the volume of water in the faeces without reducing colon carcinogenesis (Parnaud, Tache et al. 1999). Another suggestion is that PEG protects epithelial colonic cells from irritation and mechanical abrasion in the same way it protects mammalian cells grown in suspension cultures in vitro (Corpet and Parnaud 1999; Parnaud, Tache et al. 1999). In the development of colon cancer there is a deficiency in goblet cells that make mucin. It is possible that PEG coats the epithelial surface and replaces the function of lost mucin (Parnaud, Tache et al. 1999). This explanation could explain the reduced proliferation and, perhaps, the increased apoptosis of colon cells exposed to PEG in vitro and in vivo (Roy, DiBaise et al. 2001; Wali, Stoiber et al. 2002; Wali, Koetsier et al. 2003; Roy, Gulizia et al. 2004). A further suggestion is that PEGs increase cell-to-cell communication and restore differentiation of cells in the colon (Laboisse, Maoret et al. 1988), reducing the impact of the carcinogen treatment. This explanation is consistent with the focal loss of tight junctions and increased permeability of the epithelial membranes in colon carcinogenesis (Soler, Miller et al. 1999). PEG could potentially reduce this process or reduce its significance in some way. A still further hypothesis, involving some of the hypotheses above, is inflammation caused by a disruption of the epithelial membrane is reduced by PEGs. This explanation would predict a close association between colonic inflammation and colonic carcinogenesis. This scenario is very likely as patients with inflammatory bowel disease have an increased likelihood of colon cancer; animals with chemically-induced colonic inflammation often develop colon cancer (Gillen, Walmsley et al. 1994; Biasco, Brandi et al. 1995; Kristinsson, Armbruster et al. 2001) and animals treated with anti-inflammatory agents often show a reduced colon carcinogenesis (Kawamori, Rao et al. 1998).
BIOMARKERS FOR COLON CANCER

To increase our knowledge about the diet-colon cancer link, it is important to conduct human intervention studies using good cancer markers. Presently, the most reliable marker is occurrence of polyps in the colon. As this method is invasive, time demanding and very costly, it is important to have access to early/intermediate biomarkers for testing hypotheses relating diet and cancer. Approximately 25 biomarkers for colon cancer risk are presently available, for colon mucosa, faeces and blood, and these are validated to varying degrees (Table 1).

Thus, in designing a dietary intervention study/clinical trial to study anticancer effects (colon cancer), it is recommended to use as many of the below "state of the art" biomarkers as is feasible.

Calprotectin

Calprotectin is a calcium-binding protein found mainly in neutrophils (granulocytes), where it constitutes approximately 60% of the soluble cytosolic proteins (Dale, Brandtzaeg et al. 1985). Smaller amounts of calprotectin are also found in monocytes, macrophages and keratinocytes. This protein consists of two light and one heavy chain and has a molecular weight of 36 kDa (Dale, Fagerhol et al. 1983). Each chain can bind two calcium ions (Johne, Fagerhol et al. 1997). Calprotectin is also a very stable protein in the presence of calcium (Dale, Fagerhol et al. 1983; Fagerhol 1990), and in stool the protein is stable for at least 3 days at room temperature (Steinbakk, Naess-Andresen et al. 1990; Ton, Brandsnes et al. 2000). Stool samples stored at -20°C have a stable calprotectin level for at least 1 year (Steinbakk, Naess-Andresen et al. 1990). The function of calprotectin is not clearly understood, although several authors have reported that it has antimicrobial activity (Steinbakk, Naess-Andresen et al. 1990; Clohessy and Golden 1995) and anti-tumour properties in vitro (Yui, Mikami et al. 1995; Mikami, Yamazaki et al. 1998).

Table 1. An overview of endpoints and biomarkers used in the SYNCAN project

<table>
<thead>
<tr>
<th>Faeces</th>
<th>Faecal water</th>
<th>Blood</th>
<th>Biopsies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total anaerobes¹</td>
<td>Tight junction integrity</td>
<td>CEA</td>
<td>Proliferation</td>
</tr>
<tr>
<td>Total bifidobacteria¹</td>
<td>Mannitol flux</td>
<td>CA19-9</td>
<td>MMP9 gene</td>
</tr>
<tr>
<td>Total lactobacillus</td>
<td>Cell invasion</td>
<td>Total cholesterol</td>
<td>MMP2 gene</td>
</tr>
<tr>
<td>Clostridium perfringens¹</td>
<td>Cell cytotoxicity</td>
<td>LDL</td>
<td>TIMP1 gene</td>
</tr>
<tr>
<td>Coliforms¹</td>
<td>Early apoptosis</td>
<td>HDL</td>
<td>TIMP2 gene</td>
</tr>
<tr>
<td>Bacteroides¹</td>
<td>Necrosis</td>
<td>Triglycerides</td>
<td>TIMP3 gene</td>
</tr>
<tr>
<td>Enterococci¹</td>
<td>Comet assay</td>
<td>GST activity</td>
<td>Comet assay</td>
</tr>
<tr>
<td>Calprotectin</td>
<td>TGF-beta</td>
<td>NK activity</td>
<td>GST expression</td>
</tr>
<tr>
<td></td>
<td>PGE₂</td>
<td>Phagocytosis</td>
<td>Phagocytosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phagocytosis MF</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Burst fMLP</td>
<td></td>
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<td></td>
<td></td>
<td>Burst PMA</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Burst fMLP MF</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>IFN-gamma</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>IL-10</td>
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<tr>
<td></td>
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<td>IL-12</td>
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<td>IL-2</td>
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<tr>
<td></td>
<td></td>
<td>TNF-alpha</td>
<td></td>
</tr>
</tbody>
</table>

¹ Endpoint measurement rather than a biomarker for colon cancer.
The antimicrobial activity seems to be dependent on the availability of calcium and zinc (Steinbakk, Naess-Andresen et al. 1990; Clohessy and Golden 1996). Both minerals can bind calprotectin, and one possible defence mechanism might be due to depriving microorganisms of zinc (Brandtzaeg, Dale et al. 1992). Since calprotectin is a calcium-binding protein it may also interfere with the intracellular calcium-dependent signalling (Nisapakultorn, Ross et al. 2001). Presently, it is not known by which mechanism calprotectin is transferred from the cytosol of neutrophils to the extra cellular matrix, where it combats microorganisms. Calprotectin is only secreted from the cell when its membrane is damaged or when the cell is dying (Bjerke, Halstensen et al. 1993; Voganatsi, Panyutich et al. 2001). However, it cannot be excluded that calprotectin is transferred to the cell membrane surface, wherefrom it protects the cell from various invasive microorganisms. Furthermore, calprotectin may be involved in a defence mechanism against tumour development. It has been shown that calprotectin; independent of the presence of calcium induces apoptotic cell death in various tumour cells (Mikami, Yamazaki et al. 1998).

The amount of calprotectin in faeces is probably dependent on the leakage of colonic TJs. A high level of faecal calprotectin is a response to an acute focal colonic inflammation and can be detected in faeces with an enzyme-linked immunospecific antibody (ELISA) assay (Roseth, Fagerhol et al. 1992). Neutrophils containing calprotectin can migrate from the blood stream into the gut lumen through TJs, as a consequence of a defective closure mechanism. Pathologic conditions such as colon cancer and IBD increase the epithelial permeability and allow migration of neutrophils into the lumen (Berstad, Arslan et al. 2000). The involvement of a defective TJs function is further supported by the fact that high calprotectin levels are correlated with increased epithelial permeability in patients with ulcerative colitis (Roseth, Aadland et al. 1997).

Faecal calprotectin is elevated in both patients with colorectal cancer and IBD, which supports an association of inflammation and colon cancer (Roseth, Kristinsson et al. 1993; Roseth, Aadland et al. 1997; Kronborg, Ugstad et al. 2000; Tibble, Sigthorsson et al. 2000). Calprotectin has been suggested as a new non-invasive marker in screening for colon cancer. At present faecal occult blood testing (FOBT) is the most frequently used method in such studies. One disadvantage with FOBT is, however, a low detection rate, may be as low as 26% (Ahlquist 1997). This is probably due to the fact that all adenomas and cancers do not bleed (Macrae and St John 1982). In comparison, calprotectin is a simpler and more sensitive marker for colon cancer. The sensitivity for faecal calprotectin in patients with colorectal cancers and adenomatous polyps is 79%, the sensitivity for haemoglobin in FOBT is only 42% (Kronborg 1999). The specificity is somewhat lower for detection of calprotectin, as an increased level of faecal calprotectin may also be a response to a transient or permanent colonic inflammation.
Granulocyte marker protein

The rat equivalent to human calprotectin is granulocyte marker protein (GMP). An animal model has been established to study GMP in rat stool during colon carcinogenesis (Kristinsson, Roseth et al. 1999; Kristinsson, Nygaard et al. 2002). GMP, as well as calprotectin, originates from granulocytes (neutrophils) and is increased in stool as a consequence of increased permeability and inflammation in colon. This rat protein binds iron and has an amino acid sequence identical to rat transferrin (Kristinsson, Nygaard et al. 2002), and can be detected in stool with a similar ELISA technique as described above.

Genotoxicity

There has been a great interest in genotoxicity of faecal compounds since the early 1980s. DNA alterations increase the risk for neoplastic cells, which can develop into cancer. The first studies carried out in this area investigated how diet influenced the excretion of faecal mutagens in faeces (Reddy, Sharma et al. 1984). At this time the best available technique for screening for possible carcinogens, was Ames test. It is, however, uncertain if mutagenicity of bacteria is relevant for genotoxicity of human cells.

Nowadays, there are more relevant methods available. The comet assay, a method, which allows visualization of DNA damage in individual cells, is one popular method for assessing genotoxicity of faecal water in human cells (Fig. 5). This method can be used both in vitro in cell cultures or on biopsy cells in a more in vivo-like situation (Olive, Banath et al. 1990). The genotoxic activity of faecal water has been shown to be highly influenced by the diet (Glinghammar, Venturi et al. 1997). A diet high in meat and fat increases the genotoxic potential of faecal water (Rieger, Parlesak et al. 1999), whereas a high consumption of vegetables reduces genetic damage (Pool-Zobel and Leucht 1997). Further mechanistic studies have shown that oxidative damage is involved in genotoxicity induced by faecal water (Venturi, Hambly et al. 1997). The intra and interindividual variability for genotoxicity of faecal water seem to be very high.

Figure 5. The figure shows DNA strand breaks in a single cell measured by the Comet assay. The majority of the DNA is situated in the cell nucleus. DNA fragments outside the nucleus form a tail (comet), which intensity is an estimate of the total DNA strand breaks in the cells. (SYNCAN 2004)
This high interindividual variability cannot alone be explained by different diets, as the interindividual variability was not lowered when subjects consumed identical diets (Osswald, Becker et al. 2000). One possible explanation is that the gut microflora differs between individuals.

**Cytotoxicity & proliferation**

The colonic epithelium is a homeostatic tissue with strict control of proliferation, growth arrest and apoptosis. Disruption of the balance between cell growth and cell death can result in clonal expansion and development of carcinomas (Bedi, Pasricha et al. 1995). A gradual inhibition of apoptosis has been reported in the conversion of normal colonic epithelium into carcinomas (Bedi, Pasricha et al. 1995). Ageing is one factor that enhances proliferation and reduces apoptosis in the colonic mucosa (Xiao, Moragoda et al. 2001). These alterations may be responsible for the high incidence of colon cancer in old people. Cell cytotoxicity and proliferation are accepted biomarkers for colon cancer risk. Cytotoxicity of faecal water has previously been reported to be influenced by the diet. A decrease in calcium intake increases the cytotoxicity (Glinghammar, Venturi et al. 1997). Secondary bile acids, as DCA in faecal water may be responsible for this cytotoxic effect (Glinghammar and Rafter 2001). These bile acids can together with fatty acids cause damage on the colonic epithelial cells and epitheliolysis. Compensatory cell proliferation will be an unwanted effect of this cell death (Stadler, Yeung et al. 1988). The cytotoxic and proliferative effects of bile acids have also been demonstrated in animal models and colonic cell lines (Lapre, De Vries et al. 1993; van Munster, Tangerman et al. 1993; Glinghammar, Holmberg et al. 1999). Cell proliferation has been shown to occur when cells are exposed to faecal water for a prolonged incubation time (longer than 24 h) (Glinghammar, Holmberg et al. 1999). Increased proliferation is also correlated to activation of activator protein-1 (AP-1). AP-1 is a protein complex composed of 2 proteins, members of the fos and jun family. This complex acts as a transcription factor for various genes involved in growth control.

**Necrosis and apoptosis**

A balance between cell division and cell death is important to maintain a normal cellular homeostasis in the colon. Resistance to cell death (e.g. apoptosis) is a key event in expansion of tumour cells, while accelerated cell death is evident in acute and chronic degenerative diseases (Danial and Korsmeyer 2004). Cell division is normally strictly regulated by critical control points (G1, G2, and M) in the cell cycle. If a cell is damaged, it will either be arrested in G0 where reversible DNA damage will be repaired before it enters S phase or the cell can undergo unspecific cell death (necrosis) or programmed cell death (apoptosis).

Necrosis can be evoked by non-physiological disturbances as high doses of toxins, ischemia or viruses (Butler, Hewett et al. 1999). Necrosis is characterized by a loss of membrane integrity, swelling of the cytoplasm and mitochondria, and eventually...
ruptures of the membrane, which leads to cell lysis. No energy is required for this process and it can easily be detected by the extensive DNA fragmentation that occurs after cell lysis. The release of cellular components will also lead to a significant inflammatory response in the surrounding tissue (Fadeel, Orrenius et al. 1999).

Apoptosis on the other hand, is induced by physiological stimuli such as limitation of growth factors, oxygen or nutrients, DNA damage or activation of oncogenes (al-Rubeai and Singh 1998). Apoptosis is furthermore an active process which requires energy, and it is characterized by membrane blebbing, shrinking of the cytoplasm, condensation of the nucleus and finally formation of small apoptotic bodies (Fadeel, Orrenius et al. 1999). An early event in apoptosis is asymmetry of the plasma membrane resulting in the exposure of phosphatidyl serine (PS) residues at the outer plasma membrane. A common technique to detect early apoptosis is the annexin V assay for flowcytometry. The annexin V protein labelled with FITC recognizes the PS on the outside of the membrane (van Engeland, Nieland et al. 1998). To be able to distinguish between dead cells and apoptotic cells, annexin V is added simultaneously with propidium iodide (PI). There are two distinct pathways that can be activated by apoptotic signals. Firstly, death receptors on the cell surface can be activated by binding of tumour necrosis factor (TNF) or Fas ligand (FASL). Secondly, intrinsic stress responses induced by for example limitation of growth factors or DNA damage, which leads to release of cytochrome c from the mitochondria. Both pathways involve activation of the key proteolytic enzymes caspases.

It is well known that the rate of apoptosis in the colon epithelium cells is influenced by factors in the lumen, originating from the diet and drugs. Since apoptosis is thought to be a protective mechanism against cancer, it is important to point out that many factors that enhance apoptosis have been characterized in plant food sources (Avivi-Green, Polak-Charcon et al. 2000; Bonnesen, Eggleston et al. 2001; Moragoda, Jaszewski et al. 2001). One of these substances is butyrate, a SCFA produced by fermentation of dietary fibers by the intestinal flora as discussed above. The composition of faecal water is a reflection of the ingested diet and its components are consequently very interesting to investigate for their ability to induce apoptosis in the colon. DCA is found in the lipid phase of faecal water and is known to be tumour promotor in the large bowel (Hori, Matsumoto et al. 1998). Surprisingly, several in vitro studies have reported induced apoptosis in colonic carcinoma cells after treatment with DCA (Hague, Elder et al. 1995; Glinghammar, Inoue et al. 2002). The tumour promotor effect may be a result of a selection and expansion of cells resistant to DCA induced apoptosis (Hague, Elder et al. 1995). Intact faecal waters increase the rate of apoptosis to a higher degree than the lipid extracts from the same faecal waters (Haza, Glinghammar et al. 2000). For that reason there must be other components in the faecal water besides bile acids responsible for the induction of apoptosis. If apoptosis, induced by faecal waters, is preventive or promotive for colon carcinogenesis is still to be ascertained.
CYCLOOXYGENASES

The cyclooxygenase family

Cyclooxygenase (COX) exists in two different isoforms: COX-1 and COX-2. Both enzymes are involved in the production of prostaglandins, but they are expressed in different tissues and have separate functions. COX-1 is constitutively expressed in nearly all tissues and its major function is to produce PGs for homeostatic regulation in the body (Schafer 1995; Vane, Bakhle et al. 1998). Recently, a splice variant of COX-1 was reported (COX-3). Drugs which decrease pain and fever as acetaminophen and phenacetin, may act via inhibition of this new splice variant called COX-3 (Chandrasekharan, Dai et al. 2002; Schwab, Schluesener et al. 2003).

The other isoform, COX-2 is inducible and is expressed in gastrointestinal cells, macrophages and other inflammatory cells in response to cytokines, phorbol esters, mitogenic agents, growth factors and bacterial lipopolysaccharides (Kujubu, Fletcher et al. 1991; Smith and Dewitt 1996). Anti-inflammatory cytokines and corticosteroids decrease induction of COX-2 (Onoe, Miyaura et al. 1996; Vane, Bakhle et al. 1998). COX-2 is not normally expressed in the human colon, but an overexpression has been reported in colon tumours (Eberhart, Coffey et al. 1994; Dimberg, Samuelsson et al. 1999). COX-2 is, on the other hand, constitutively expressed in tissues where the need for prostaglandin production is high, e.g. in the brain, kidneys, spinal cord and in the placenta (Vane, Bakhle et al. 1998; Hinz and Brune 2002). The induction of prostaglandin production in response to activation of the COX-2 gene is very rapid. A single stimulus can increase or decrease the protein levels in a matter of hours (Herschman 1996).

Prostaglandin biosynthesis

Cyclooxygenase is the key enzyme in the conversion of arachidonic acid to prostaglandins and tromboxanes (Fig. 6). Both isoforms of cyclooxygenase (COX-1/COX-2) are often co-expressed in the same cell, and they catalyse the same reaction. In the catalytic domain of the enzyme, there are two distinct sites, the first has cyclooxygenase activity which incorporates two oxygen molecules into polyunsaturated fatty acids (for example arachidonic acid), and the second has peroxidase activity which reduces peroxides to corresponding alcohols (Kulmacz, van der Donk et al. 2003).

The cyclooxygenase and peroxidase sites are functionally and physically separate, but an interplay between them is important for their optimal function. In the presence of oxygen a PGH2 precursor is formed, which rapidly will be reduced by the peroxidase site into PGH2. PGH2 is then readily converted into a wide range of prostacyclins, prostaglandins, and tromboxane A2 by synthases and isomerases (other than COX-1/COX-2).

The main substrate for cyclooxygenases is arachidonic acid, but as COX-1 and COX-2 are found in different tissues and in different locations within the same cell, they
use separate substrate pools and react locally to different stimuli (Reddy and Herschman 1996; Smith, Garavito et al. 1996). COX-2 has, furthermore, a wider substrate specificity than COX-1, e.g. it accepts even shorter fatty acids which may give rise to other signalling molecules than prostanoids (Kozak, Crews et al. 2002).

**COX-2 and cancer**

Inflammation is an important part of the body’s defence system (Maslinska and Gajewski 1998), but prostaglandins which mediate inflammatory responses are also thought to play a role in tumour development in the lung, colon, and breast (Soslow, Dannenberg et al. 2000; Koki and Masferrer 2002; Ermert, Dierkes et al. 2003). The link between COX-2 and cancer is more evident in the colon than in other organs. The induction of COX-2 is a key event in the increased production of prostaglandins observed in colon cancers in human and animals (Rigas, Goldman et al. 1993; Eberhart, Coffey et al. 1994; DuBois, Radhika et al. 1996). Up regulation of COX-2 protein levels and enhanced PGE2 levels can influence important parameters for tumour development, among those are; inhibition of apoptosis, induction of cell proliferation, promotion of angiogenesis and increased invasiveness of malignant cells, (Leahy, Koki et al. 2000; Koki and Masferrer 2002; Leahy, Ornberg et al. 2002).

Results from studies assessing the effect of NSAIDs on colon carcinogenesis further confirm this hypothesis. NSAIDs are cyclooxygenase inhibitors, and regular intake of NSAIDs is associated both with a reduced mortality and decreased incidence of colon cancer (Thun, Namboodiri et al. 1991; Giovannucci, Rimm et al. 1994). In 1999 the US Food and Drug Administration approved celecoxib (a selective COX-2 inhibitor) for reduction of polyps in patients with FAP.

**COX inhibitors**

It is evident that a regular intake of NSAIDs markedly decreases the risk for colon cancer (Thun, Namboodiri et al. 1991; Giovannucci, Rimm et al. 1994). This effect is at least partly due to inhibition of the COX-1 and COX-2 enzymes. NSAIDs are effective agents, but their use is limited due to undesirable side effects as gastrointestinal ulcerations and bleeding, and renal toxicity (Silverstein, Faich et al. 2000). These side effects are mainly due to inhibition of COX-1 (Wolfe, Lichtenstein et al. 1999). Specific COX-2 inhibitors (coxibs) are now widely spread and used against inflammatory disorders.

One such specific COX-2 inhibitor is celecoxib (celebrex), it has, as mentioned above, been used to prevent polyps in FAP patients, and other coxibs act as antitumourigenic agents in rats (Steinbach, Lynch et al. 2000; Davies, Gudde et al. 2001). However, a similar COX-2 inhibitor, rofecoxib (also called VIOXX), was recently withdrawn from the market, due to crucial side effects (Davies and Jamali 2004). It all started in 2000, when Merck and Co. began a long-term prospective clinical trial to test rofecoxib in adenomatous polyposis patients (APPROVe). The result was striking;
patients who received VIOXX had a doubled risk of myocardial infarction and stroke (Fitzgerald 2004). Celecoxib (Celebrex) is not as strong COX-2 inhibitor as VIOXX, but preliminary studies show that Celecoxib has similar adverse myocardial effects (Couzin 2004; Topol and Falk 2004). An extensive evaluation of all coxibs and their potential side effects is now being carried out. There are some speculations that co-administration with low doses of aspirin will protect against adverse myocardial effects, as inhibition of COX-1 prevents these effects (Levesque, Brophy et al. 2005).

An inhibition of the COX-2 enzyme (with NSAIDs) results in an accumulation of unesterified arachidonic acid (AA). Unesterified AA has been shown to be a signal for induction of apoptosis and to be protective against colon cancer development (Cao, Pearman et al. 2000)

![Figure 6. Major pathways for production of prostaglandins (Huss 2003)](image-url)
**Natural COX-2 inhibitors**

COX-2 inhibitors are not only available as drugs, many food stuffs and medicinal plants contain compounds with COX-2 inhibitory effects. In Sweden, over 30% of the common food plants have anti-inflammatory effects, and there is a wealth of evidence in the literature that spices and medicinal plants from Asia and Africa contain similar anti-inflammatory compounds (Jager, Hutchings et al. 1996; Kim, Yamada et al. 1999; Flodmark, Bruhn et al. 2001; Lee, Cuendet et al. 2001; Murakami, Takahashi et al. 2002).

Non-polar fatty acids like eicosapentaenoic acid (EPA), docosapentaenoic acid (DHA), and ursolic acid markedly inhibit COX-2 in mice and in in vitro experiments (Ringbom, Segura et al. 1998; Ringbom, Huss et al. 2001; Dommels, Haring et al. 2003; Calviello, Di Nicuolo et al. 2004). In line with this, olive oil and fish oil, which are complex mixtures of fatty acids have been reported to inhibit COX-2 protein expression (Llor, Pons et al. 2003). This effect is probably mediated via inhibition of apoptosis. Other active COX-2 inhibitors are ajoene in garlic and resveratrol in grapes. These components inhibit both COX-1 and COX-2 activity but have no effect on the protein level (Subbaramaiah, Chung et al. 1998; Dirsch and Vollmar 2001). On the other hand, there are natural compounds which specifically inhibit COX-2. Curcurmin is one such example. It is a pigment which gives turmeric its yellow bright colour, and it inhibits both COX-2 activity and COX-2 protein levels in cells (Zhang, Altorki et al. 1999; Goel, Boland et al. 2001; Surh, Chun et al. 2001). This effect is mediated via phosphorylation (inactivation) of phospholipase A₂, which catalyzes the release of arachidonic acid from the cell membrane (Kase, Saitoh et al. 1998; Hong, Bose et al. 2004). There are also some indications that β-carotene inhibits both COX-2 expression and prostaglandin production in cells (Palozza, Serini et al. 2005).

Another large group of inhibitors is flavonoids. These occur naturally in plants, and when consumed as medicinal drugs have mild if any side effects. Polyphenols from red wine, the phenol epiogallocatechingallate (EGCG) in green tea, and prenylated flavonoids found in Chinese medicine are some examples (Chi, Jong et al. 2001; Hong, Smith et al. 2001; Luceri, Caderni et al. 2002). Apigenin, genistein and kaempferol are also well known flavonoids derived from the diet, which have anti-inflammatory activities (Liang, Huang et al. 1999). Most polyphenols inhibit COX-2 activity by competing for the arachidonic acid binding site on the enzyme (Alanko, Riutta et al. 1999). Some phenols stimulate PGE₂ formation at low concentrations and inhibit the formation at higher concentrations (Alanko, Riutta et al. 1999).

Most studies concerning natural COX-2 inhibitors have been carried out with plant extracts or pure compounds, but the actual effect in the colon might be questioned as many compounds are metabolized by the intestinal flora. The water phase of faeces (faecal water) may better reflect the real composition of compounds in the colon.
AIM OF THE PRESENT STUDY

Overall aims:

- Evaluate biomarkers for colon cancer
- Assess if substances in a healthy diet can prevent colon cancer
METHODS

Methods of relevance to this thesis are discussed below.

Faecal water preparation

In the first paper, we used a standard procedure to prepare faecal water (Venturi, Hambly et al. 1997). Briefly, faeces was directly centrifuged and the water phase was decanted and sterile filtered through a 0.45 μm filter. This procedure was not suitable in the third paper as the volume of the faecal waters was a strong limiting factor. To increase the volume of faecal water, faeces was homogenized with PBS (weight ratio 1:1) prior to centrifugation, and thereafter sterile filtered (Klinder, Karlsson et al. 2005). This procedure was developed to make it feasible to analyse as many as possible of the intermediate biomarkers used in the SYNCAN intervention study. In the fourth and fifth paper, the methodology from the first paper was further refined. Firstly, the faeces was ultra centrifuged a second time to make the water phase more clear. Secondly, the faecal water was diluted 1:4 in PBS prior to sterile filtration. Thirdly, all faecal waters were sterile filtered through 0.8/0.2 μm filters. The new filters were chosen as we previously noticed that some faecal waters filtered with 0.45 μm filters had a tendency to be toxic to the cells (e.g. increased the cell detachment from the culture plate surface). We believe that this toxicity could be explained by unsterile samples.

Cell lines

In order to evaluate and use intermediate biomarkers in the field of colon cancer, it is important to use a well established system, where it is easy to control the test variables. Cultured cell lines are a common model system used in this sense. A large number of different colonic cell lines exist. The majority of the cell lines originate from adenomas or carcinomas from patients. The greatest advantage with these transformed cells is that these are possible to cultivate over a long period of time. Presently, no normal adult cell line is available for long term culture. However, there is one cell line classified as normal; FHT (foetal colon). These cells have previously been used in our lab to confirm results obtained from studies carried out in carcinoma cell lines (Haza, Glinghammar et al. 2000). The majority of the work in this thesis was carried out using the colorectal carcinoma cell lines; HT29, HCT116, and Caco-2.

The HT29 cell line originates from a female Caucasian with colorectal adenoma. It harbours a mutation in the p53 gene and mutation in the APC gene (Kutchera, Jones et al. 1996; Chendil, Oakes et al. 2000). The HCT116 cell line originates from a male Caucasian, and harbours a mutation in the ras-proto oncogene, whereas the p53 and APC genes are normal (Kutchera, Jones et al. 1996; Chendil, Oakes et al. 2000). The Caco-2 cells originate from a male Caucasian with colorectal adenoma. These cells have
a mutation in the β-catenin and APC genes (Ilyas, Tomlinson et al. 1997). Upon reaching confluency, these cells express characteristics of enterocytic differentiation.

**Apoptosis & necrosis in cells**

A balance between cell division and cell death is important to maintain a normal cellular homeostasis in the colon. Resistance to cell death (e.g. apoptosis) is a key event in expansion of tumour cells, while accelerated cell death is evident in acute and chronic degenerative diseases (Danial and Korsmeyer 2004).

HCT116 cells were seeded out into six-well plates and incubated for 48 hours. Faecal water samples were diluted 1:10 in media and applied to the cells for 2 hours. The cells were harvested by trypsinization, and the cells were resuspended in binding buffer, and stained with annexin V and propidium iodide. The samples were subjected to FACS analysis within an hour. Early apoptosis was quantified as the percentage of the cell population, which was positively stained by Annexin V-FITC but unstained by propidium iodide (Q4 in Fig. 7) (van Engeland, Nieland et al. 1998). Necrotic cells were identified as percentage of cells, which was positively stained by propidium iodide (Q1 in Fig. 7). Ten thousand cells were analyzed for each sample.

![Flowcytometry of HCT116 cells with PI and annexin V staining. Cells were treated with DCA or faecal water for 2 hours. The top panel shows control cells (untreated), and the bottom panel shows fw-treated cells, and DCA treated cells.](image)

**Figure 7.** Flowcytometry of HCT116 cells with PI and annexin V staining. Cells were treated with DCA or faecal water for 2 hours. The top panel shows control cells (untreated), and the bottom panel shows fw-treated cells, and DCA treated cells.
Faecal calprotectin/granulocyte marker protein

Mucosal inflammation and increased levels of faecal calprotectin is evident in patients with inflammatory bowel disease and cancer in the large bowel (Roseth, Kristinsson et al. 1993; Roseth, Aadland et al. 1997). Calprotectin/GMP was extracted from faeces according to a generally accepted procedure (Roseth, Fagerhol et al. 1992; Kristinsson, Roseth et al. 1999). Briefly, 100 mg of human faeces was vortexed and homogenized in extraction buffer in a weight/volume ratio of 1:50, followed by centrifugation. The rat faeces samples were homogenized in a weight/volume ratio of 1:3, followed by centrifugation, and dilution 1:50 in assay buffer. The calprotectin/GMP concentrations of faecal supernatants were assessed by a calprotectin/GMP enzyme linked immunosorbent assay (Oslo, Norge). Standards and positive controls were included in the kit and performed according to manufacturer’s instructions and were included in each run of the assay. The optical density was read at 405 nm in a spectrophotometer. The calprotectin/GMP concentrations in the faecal samples were calculated from the standard curve obtained with the standards. The concentrations of extracted calprotectin/GMP were expressed as μg/g stool.

Inhibition of COX-2 activity

Effects on COX-2 enzymatic activity were assayed using a radiochemical COX-2 in vitro assay (Noreen, Ringbom et al. 1998), with minor modification (i.e. the test compounds/samples and enzyme were incubated for 3 min at 37°C, and heptane replaced n-hexane in the eluent). Purified COX-2 enzyme was incubated with the faecal water sample (non-diluted) or test compound for 10 min before addition of [1-14C]-arachidonic acid. The inhibition of COX-2 catalyzed prostaglandin biosynthesis was calculated as the relative decrease in radioactivity (disintegrations per minute) of the samples containing the test substance as compared to the solvent vehicle. In this assay, a 20% or greater inhibition was regarded as the cut off point for an actual effect. Inhibition in % was calculated as:

\[
\frac{(MaxPG^1 - \text{background}^2) - (PG^3_{\text{test}} - \text{background})}{(MaxPG - \text{background})} \times 100
\]

^1Maximum production of prostaglandins with only solvent present, measured in disintegrations per minute (DPM); ^2Background measured in DPM; ^3Amount of prostaglandins produced with test faecal water present, measured in DPM.
Inhibition of COX-2 and PGE$_2$ production in cells

In order to test the faecal waters in a more “natural like” assay, we evaluated them for COX-2 inhibition in the human cell model HT29. Colonic HT29 cells exhibit a low basal level of COX-2 protein, which can be upregulated by TNF-α stimulation. Cells were simultaneously treated with TNF-α (50 ng/mL) and with faecal water (diluted 1:20) or test agent.

Proteins were essentially isolated as previously described (Glinghammar and Rafter 2001), and separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (8%) as described by Laemmli (Laemmli 1970). The membranes were incubated for 1 hour with mouse monoclonal antibody against COX-2 and actin followed by a 1 h-incubation with anti-mouse IgG conjugated with horseradish peroxidase. The ECL+ Western blot detection system was used to detect the bands and the light emitted was quantified using a FUJI LAS 100. Protein bands were quantified densitometrically and expressed as percentage inhibition of the TNF-α treated cell band intensity (COX-2/actin). The values are means ± SEM (n=3). Values are normalized for enabling comparison between different experiments.

PGE$_2$ is a major product produced by COX-2 from arachidonic acid and is often used to estimate COX-2 activity in cells (Calviello, Di Nicuolo et al. 2004). HT29 cells were plated in 12 well plates, and treated with 100 μmol/L aspirin for 10 hours in order to inactivate COX-1. At day 3, the cells were incubated with TNF-α (50 ng/ml) in the absence or presence of faecal water (1:20) or test agents for 5 h. After treatment, the test solutions were removed and replaced with 100 μmol/L arachidonic acid diluted in 0.1% DMEM and left for 1hour. The PGE$_2$ concentration in the cell supernatants was determined by radioactivity immunoassay using [$^3$H]PGE$_2$, and polyclonal antiserum to PGE$_2$. The samples were assayed undiluted and a standard curve with PGE$_2$ was included in each run. Each faecal water was tested at least twice in the cell system and later analyzed in duplicate in the radioimmunoassay and expressed as percentage inhibition of the TNF-α treated cells. Values are normalized for enabling comparison between different experiments.
RESULTS & DISCUSSION

FAECAL WATER INDUCED PROLIFERATION MAY BE A POTENTIAL BIOMARKER FOR COLON CANCER (paper I)

Study design

Subjects were recruited from two hospitals in the Netherlands, the Academic Hospital Maastricht and the Maasland Hospital Sittard. Patients were randomly selected from the endoscopy control schedule and were asked to provide a 48-hour stool sample. The low risk group consisted of individuals showing no neoplastic bowel conditions after endoscopic examination indicated by complaints of blood per anus. Subjects with previous bowel surgery, cholecystectomy, familial adenomatous polyposis and recent use of antibiotics or suppositories were excluded from this study.

Subjects with colorectal polyps with a diameter less than 1 cm, regardless of their number or pathology, were assigned to the low risk adenoma I group. Those who suffered from polyps larger or equal to 1 cm were assigned to the high risk group adenoma II. In the low risk group, 21% showed moderate dysplasia, whereas 55% of the high risk group showed polyps with moderate or severe dysplasia. The aim with the study was to assess if three different intermediate biomarkers (effects of faecal water on cell proliferation, activator protein -1 or genotoxicity) differed between a high and a low risk group for colon cancer.

Results & discussion

Several recent studies suggest that the aqueous phase of human faeces (faecal water) mediates many of the effects of diet on colonic tumour development. It has been demonstrated that the biochemical composition of this faecal fraction is diet-dependent, being altered by dietary factors known to influence colon cancer risk, e.g. fat, fiber, calcium, probiotic bacteria (Rafter, Geltner et al. 1987; Allinger, Johansson et al. 1989; Glinghammar, Venturi et al. 1997), and that components of this faecal fraction can influence signalling pathways and colonic cell kinetics (Ha, Sirisoma et al. 1998; Haza, Glinghammar et al. 2000). If the components of faecal water are influenced by diet and possibly other unknown factors, it is a plausible hypothesis that the biochemistry of the faecal water may differ between healthy individuals and patients having colonic adenomas. This can be assessed by examining relevant responses of colonic cells in culture to faecal waters ex vivo from healthy controls and adenoma patients.

We, thus, have measured the genotoxicity of the faecal water samples in HT29 cells, using the comet assay, a method to measure the extent of DNA strand breaks in isolated cells (McKelvey-Martin, Green et al. 1993). An increased proliferative activity in the colonic epithelium is believed to be an early event in colonic tumourigenesis and this parameter has also been used as a ‘risk marker’ for the disease (Holt, Atillasoy et al.
1998). It has also been suggested that prolonged deregulated expression of AP-1 activity (a transcription factor whose activation has been associated with the promotion of neoplastic transformation) in colon cells by faecal water components may contribute to tumour promotion in the colon (Glinghammar, Holmberg et al. 1999). It has previously been shown that lipid extracts of human faecal water can induce AP-1 activity in colonocytes (Glinghammar, Holmberg et al. 1999) and the bile acids, deoxycholic acid (DCA) and chenodeoxycholic acid (CDCA), have also been shown to have this capacity (Qiao, Chen et al. 2000).

We report that induction of cell proliferation by human faecal waters in colonic cells was significantly higher (180\%±65, p<0.01) in adenoma group I towards the controls (125\%±51) and for adenoma group II (173\%±85, p<0.05) and could to a large extent be explained by the concentrations of deoxycholic and chenodeoxycholic acids in the faecal water using regression models. No difference between the groups was observed for induction of Activator protein-1 or DNA strand breaks induced by faecal water as measured by the COMET assay. However, interestingly breakage of isolated DNA in vitro was higher for faecal water originating from patients than controls (p<0.05) which could be explained to some degree, in a regression model, by faecal concentrations of lithocholic acid and fecapentaene-12. This study gives support to the hypothesis that the biochemical composition of the fluid (faecal water) in contact with the epithelial cells in the colon is different for control and adenoma patients and that induction of colonic cell proliferation by faecal water may be a useful biomarker in dietary intervention studies.
POLYETHYLENE GLYCOL REDuces ACF & INFLAMMATION IN RATS (paper II)

Study design

A total of 28 male Fisher 344 rats (5 weeks old) were housed individually in wire-bottom cages. After two weeks of acclimatization the rats were randomized by weight to an experimental group with 18 animals and a control group with 10 animals, and received a modified AIN-93 diet with 20% (w/w) beef tallow. Animals in the experimental group were initiated weekly with an intraperitoneal injection of azoxymethane (AOM, 15 mg/kg body weight) for two weeks. Seven days after the last AOM injection they were again randomized by weight to the control modified AIN-93 diet or to the control diet with 5% (w/w) PEG 8000 (Fig. 8). The rats were killed 9 weeks after the dietary intervention started, and their colons were examined for ACF. 24 hour-stool samples were collected every second week throughout the study. The aim with the study was to investigate whether supplementation with PEG reduces aberrant crypt foci (ACF) in carcinogen-initiated rats via effects on colonic inflammation.

Results & discussion

Recent studies suggest that the demulcent and soothing agent polyethylene glycol (PEG) might be useful as a chemopreventive agent for colon cancer. PEG 8000 at 5% w/w in the diet markedly reduced the levels of GMP in the faeces of both untreated and carcinogen-treated rats. The reduction was evident within 2 weeks and reached nearly 10-fold, from 77 to 8 mg/day, at 9 weeks (Fig. 9). Under the same conditions PEG 8000 decreased the number of ACF in the carcinogen-treated animals, nearly 2-fold for large ACF. As faecal GMP is evidence of granulocyte migration into the faecal stream, the reduction by PEG indicates a marked reduction of colonic inflammation. These same conditions reduced colon carcinogenesis as assessed by a reduction of ACF, and of colonic tumours in previous studies (Parnaud, Tache et al. 1999). These two observed effects of PEG - on inflammation and on colon carcinogenesis are thus consistent with the notion that PEG reduces colon carcinogenesis through a mechanism involving reduced colonic inflammation.
PEGs could reduce inflammation by protecting the integrity of the colonic epithelium. To determine if the reduction of faecal GMP was associated with a decrease in the permeability of colonic tight junctions (TJ), rat faecal waters were prepared and assessed in an in vitro cell system. It was found that faecal waters with a high content of PEG did not reduce colonic permeability. The mechanism responsible for the protective effects of PEG thus remains unknown. It is reasonable to assume that the reduction of inflammation is responsible for the reduction of carcinogenesis, but we cannot exclude the possibility that the two phenomena are a consequence of a third unknown factor. Our initial suggestion, that the faecal stream in PEG-treated animals contains factors that reduce colonic permeability, was not supported, though our test of that hypothesis could be made more rigorous. Other possibilities still remain. Dilution of irritants and/or carcinogens in the lumen may still play an important protective role. The increased osmolality in faeces, selective cytostatic effects or effects on apoptosis in cancer cells could also be important (Corpet and Parnaud 1999; Parnaud, Tache et al. 1999; Parnaud, Corpet et al. 2001; Roy, DiBaise et al. 2001; Wali, Stoiber et al. 2002; Wali, Koetsier et al. 2003). Preliminary results in from our laboratory indicate that PEG may inhibit COX-2 at higher concentrations (Fig. 10). This is in agreement with the theory of reduced inflammation.

In summary, our results show that a 5% PEG diet reduces colonic inflammation as well as inhibits colon carcinogenesis in the rat. Although the mechanism responsible for this phenomenon is not known, the observation may still be very important. If PEG similarly reduces faecal calprotectin and colonic inflammation in man, it may well also reduce colon carcinogenesis. As PEG is a non-toxic agent, the next natural step would then be to evaluate PEG as a chemopreventive agent in a human intervention trial.

**Figure 9.** The left figure shows the mean of the number of ACF per rat in three different groups; control group (no AOM), AOM-treated animals, and AOM-treated animals which received a 5% PEG diet. The right figure shows the concentration of faecal GMP in same three groups at two different time point (2 and 9 weeks).
Figure 10. 10% PEG inhibits prostaglandin E$_2$ production in colonic cells (HT29 cells). HT-29 cells stimulated with TNF-$\alpha$ (50 ng/ml) for 5 hours (TNF); HT-29 cells simultaneously treated with TNF-$\alpha$ (50 ng/ml) and (25 $\mu$mol/L) (NS-398) or PEG (0-10%). Results are presented as relative inhibition of PGE$_2$ production in cells (values are normalized for an easy comparison between different experiments).
SYNBIOTICS MAY REDUCE THE RISK FOR COLON CANCER (paper III)

Study design

SYNCAN was an EU funded project relying on collaboration between 8 research groups around Europe. The major aim with the study was to evaluate the colon cancer preventing properties of synbiotics (pre- and probiotics) in human volunteers. (Fig. 11).

In vitro fermentations were carried out to identify the synbiotic combination that had the best competitive advantages in the colonic system (Klinder, Gietl et al. 2004). A mixture of $10^{10}$ *Lactobacillus rhamnosus* (LGG) and $10^{10}$ *Bifidobacterium lactis* (Bb12) in combination with synergy 1 (enriched inulin mixture) was chosen. Synergy 1 was produced by ORAFTI in Belgium and the probiotic bacteria strains by Valio in Finland. The diet intervention study was carried out by the team of Dr. Collins, Cork Ireland. Patients with history of colon cancer (DUKES B) and patients with high risk for colon cancer (polypectomised) were recruited and randomized to placebo or synbiotic supplementation (LGG, Bb12 and Synergy 1) (Fig. 12). 80 subjects (37 cancer, 43 polyp) were recruited to the intervention, and 34 cancer subjects and 40 polyp subjects completed the trial.

![SYNCAN project strategy](image)

*Figure 11. The SYNCAN project strategy (SYNCAN 2004)*
The daily supplement was distributed as one capsule containing the probiotic bacteria and a 12 g sachet containing the prebiotic mixture. The supplementation period lasted 12 weeks. Blood, faeces and colon biopsy samples were collected before the intervention, after 6 weeks and after 12 weeks (T1, T2, and T3), and distributed to the partners in the biomarker network (Table 1 and Fig. 12). 6 of the 8 partners were part of the biomarker network. Our laboratory was responsible for analyzing faecal water biomarkers (e.g. calprotectin in total faeces, and effects on cytotoxicity, early apoptosis and necrosis in cells). A small pilot study (healthy human transit study) with healthy volunteers was carried out to check that the practical procedure worked. Three subjects received rifampicin-resistant (rif\(^\text{R}\)) mutants of Bb12 and LGG together with a 12 g sachet of Synergy1 for a 7-day period.

### Faecal water preparation

A pilot study was carried out to further refine faecal water use as a biomarker by selecting the technically most feasible and sensitive preparation method (Klinder, Karlsson et al. 2005). Total faeces of one passage from 7 volunteers (5 female, 2 male) were collected and faecal waters were prepared by three different methods, A: direct centrifugation, B: extraction of faeces in PBS before centrifugation (either 1 g faeces + 1 ml PBS (B.1) or 1 g faeces + 2 ml PBS (B.2)) and C: centrifugation of lyophilised and reconstituted faeces. The preparation of faecal water by method B was at least as sensitive as method A for the in vitro assays of faecal water genotoxicity, but was superior to method A regarding volume of faecal water obtained. Method B.1 was chosen for the SYNCAN project.
Figure 13. A decrease in necrosis was detected in cells after exposure to faecal water from polyp patients with synbiotic supplementation compared to placebo. The two groups were compared with the GLM method (general linear model). P=0.89 for cancer patients and P=0.04* for polyp patients respectively (Q1).

Results & discussion

The rat study carried out in parallel with the human trial showed that a synbiotic supplementation (same as used in human trial) significantly reduced the numbers of colonic tumours and increased the concentration of short chain fatty acids (SCFA) in the colon (Femia, Luceri et al. 2002). These data indicate that synbiotic supplementation might be a useful preventative agent in man also. The healthy human transit study carried out prior to the intervention study, showed that rif^{R} lactobacilli and rif^{R} bifidobacteria were recovered in faeces after 7 days of feeding synbiotics (average counts of $4.17 \times 10^6$ and $2.65 \times 10^7$ cfu/g of faeces), indicating good survival properties in the intestine.

The SYNCAN human study showed that the synbiotic intervention clearly had an impact on the patient’s faecal flora. *Bifidobacterium* increased in numbers in both polyp and cancer patients, whereas *lactobacillus* only increased in polyp patients. Other groups of bacteria decreased significantly in numbers (Coliforms in both groups and *Cl. Perfringens* in polyp patients). In the polyp patients, but not the cancer patients, the synbiotic intervention significantly decreased the capacity of the faecal waters to induce necrosis in colonic cells (Fig. 13). Increased cytotoxicity of luminal contents and mucosal inflammation have been suggested to be associated with increased risk for colon cancer (Lapre and Van der Meer 1992; Glinghammar and Rafter 2001; Poullis, Foster et al. 2004). Cytotoxicity of faecal waters has been reported to be diet-dependent and correlated with colonic cell proliferation (see below). All faecal waters were in addition to cytotoxic and apoptotic effects investigated for their ability to induce DNA damage, affect epithelial barrier function and tumour cell invasion. The first parameter, DNA damaging capacity of faecal water was significantly decreased in polyp patients after synbiotic intervention, measured by the Comet assay. No effect of the intervention on the ability of faecal water to affect epithelial barrier function or tumour cell invasion was observed. A decrease in DNA damage was also seen in the colonic epithelium (biopsies) of polyp
patients after intervention, together with a significant decrease in proliferative activity. Lastly, the effect of synbiotic intervention on several immunological markers was examined. No effect of the synbiotic intervention on the inflammatory marker calprotectin in faeces was observed in the colorectal cancer or polyp patients. No strong systemic immunomodulatory effects were observed by the synbiotic intervention. However, supplementation prevented an increased secretion of IL-2 by peripheral blood mononuclear cells (PBMC) in the polyp group, and increased the production of IFN-\(\gamma\) in the cancer group.

Presently, the strongest surrogate marker for colon cancer is polyp recurrence, but as the method is invasive and very costly, there is a great need for rapid and reliable non-invasive “intermediate” markers for testing hypotheses relating diet and cancer risk. In the SYNCAN human intervention study as many as possible of the available “intermediate” biomarkers were used to assess the effect of a synbiotic supplementation (Table 1). The synbiotic intervention resulted in significant alterations in the composition of the colonic bacterial ecosystem, which must have consequences for the metabolic activity of this ‘organ’. This results in a decreased exposure of the epithelium to cytotoxic and genotoxic agents and one consequence of this appears to be a decreased colonic cell proliferation. Our observation that overall the polyp patients appeared more susceptible to biomarker effects may be explained by the fact that the synbiotic intervention had a greater impact on the faecal flora in these patients than the cancer patients. Future research should focus on the effects of prebiotics and probiotics separately.
NATURAL COX-2 INHIBITORS ARE PRESENT IN HUMAN FAECAL WATER (paper IV-V)

Study population

In view of the considerable interest in COX-2 inhibitors as cancer preventive agents, we considered it of interest to investigate the presence of natural COX-2 inhibitors in human colonic contents (faecal water). We choose to initiate our screen with a pilot group of vegetarians (Table 2) as a diet rich in fruit and vegetables would be more likely to give rise to high levels of phytochemicals in the gut lumen.

A 24 hour stool sample was collected from 20 healthy vegetarians (16 female and 4 male Caucasians) and frozen below -20°C. The study population included vegans, vegetarians, and vegetarians with a small intake of fish. Subjects with a regular intake of non-steroidal anti-inflammatory drugs (NSAIDs) or other anti-inflammatory drugs were excluded from the study.

Faecal water preparation

Faecal water from the volunteers was prepared according to a general procedure with minor modifications (Venturi, Hambly et al. 1997). Briefly, each faecal sample was homogenized for 2 min and faecal water was prepared by centrifugation of 25 g faeces for 2 hour at 30,000 x g at 10°C. The supernatant was decanted, and further ultracentrifuged at 90,000 x g for 2 hours. The water phase was decanted and diluted 1:4 in PBS prior to sterile filtration (0.8/0.2 μm pore size), and stored at -20 °C until later analysis. Noticeably, the colour of the faecal waters markedly differed between the samples (Fig. 14).

Figure 14. High variability in the colour of 5 different faecal waters (diluted 1:4).
Results & discussion

The inducible enzyme cyclooxygenase-2 (COX-2) plays a major role in regulation of inflammation and possibly in the development of colon cancer. Compounds of dietary origin can influence COX-2 in the colon, and the water phase of faeces (faecal water) is one factor that may mediate the effect. Today, little is known about the actual composition of faecal water, but it is believed that it roughly reflects the food intake, and that there is a mixture of compounds that prevent and promote cancer development (Bruce, Giacca et al. 2000). 20 faecal waters were prepared as described above, and analyzed by gas chromatography-mass spectrometry for their content of phenolic compounds, and by NMR to further characterize the composition of faecal water. Intact faecal waters were also fractionated into a lipid and aqueous phase on C18 columns, and the latter phase was further fractionated into 5 new fractions using sephadex gel chromatography (Fig. 15). These samples and fractions thereof were evaluated for their effects on COX-2 protein levels (western blot) and prostaglandin E2 production in tumour necrosis alpha stimulated HT29 cells and pure enzymatic activity in a COX-2 catalyzed prostaglandin biosynthesis in vitro assay.

The major phenolic compounds identified by GC-MS were phenylpropionic acid, phenylacetic acid-, cinnamic acid-, and benzoic acid-derivatives. NMR analysis of the intact and aqueous phase of faecal waters confirmed the presence of significant quantities of phenolics, and indicated the presence of saturated and unsaturated fatty acids. NMR data can also be used for metabolic fingerprinting. As far as we know metabolic fingerprinting (metabolomics) of faecal waters is a new approach. In this case, the NMR profiles from the individual samples were very similar (Fig. 16). However, this was expected, since we studied samples from a homogenous group of vegetarians. In the aqueous phase of faecal water the same phenolic compounds as identified in intact faecal water were found; phenylalanine, 3-phenylpropionic acid, hydroxyl phenyl acetic acid, 3-hydroxy phenyl acetic acid, fumaric acid. Other major metabolites in both phases were; propylmethyl ketone, alanine, threonine, valine, leucine, isoleucine. Small amounts of glycine were also detected.

The intact faecal waters of 20 volunteers were fractionated into lipid and aqueous phase with C18 column. The aqueous phase was further separated into 5 fractions (0-100% methanol) using sephadex gel chromatography.

![Figure 15](image-url)
Figure 16. 1H-NMR spectra of the aqueous phase of faecal waters dissolved in D$_2$O (KH$_2$PO$_4$ buffer, pH 6.0) in the range of d 6.0 – d 8.0 (10mg/mL). 1; H-2, H-3, H-4, H-5, and H-6 of phenylalanine, 2; H-2, H-3, H-4, H-5, and H-6 of 3-phenylpropionic acid, 3; H-2 and H-6 of tyrosine, 4; H-5 of 3-hydroxy phenyl acetic acid, 5; H-3 and H-5 of tyrosine, 6; H-2, H-4, and H-6 of 3-hydroxy phenyl acetic acid, 7; fumaric acid.

The majority of the 14 tested faecal water samples evaluated for their effects on COX-2 in stimulated HT29 cells, decreased COX-2 protein levels (mean 38.8 ± 4.4, range 19-63% inhibition). The results for four faecal waters are presented in Fig. 17. Two of the 20 faecal waters also weakly inhibited enzymatic activity of purified COX-2 (22-24% inhibition). All the tested faecal waters (13 analyzed) were found to significantly decrease PGE$_2$ production (range 5.4-39.7 % inhibition, p-value<0.05) compared to control cells. Three compounds identified in faecal water, 3-phenylpropionic acid, 3-hydroxyphenylacetic acid and 3-(4-hydroxyphenyl)-propionic acid, were also found to decrease the protein level at 250 μM (15-62 % inhibition). In order to further characterise the COX-2 inhibitory effects of faecal water, a number of samples were fractionated into lipid and aqueous phase. The majority of the tested aqueous phases (derived from faecal water) showed a significant ability to inhibit prostaglandin production in cells.
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(13.8±1.34, p=0.01), while none of the lipid phases (0.5±1.59, p>0.05) had an effect on COX-2 activity, indicating that the active components are polar in nature. However, no COX-2 inhibitory effect was detected after sephadex gel fractionation of the aqueous phase of faecal water, which may indicate that the polar COX-2 inhibitors may be acting in synergy. To address synergistic effects, various combinations of phenolic compounds previously reported to be found in faecal water, were also investigated for their ability to inhibit PG production in cells. However, none of the phenolic compounds tested or any combination thereof exhibited any inhibitory effect. Thus, while the inhibitory effects cannot be attributed to any of the tested phenolics, hitherto untested phenolic analogues/minor metabolites may be responsible.

![Western blot and graph](image)

**Figure 17.** Upper panel shows a western blot membrane, with untreated control cells (1) to the left, cells treated with TNF-α (50 ng/ml) (2) and HT-29 cells simultaneously treated with TNF-α and faecal water treated cells (fw17-20). The lower panel shows the corresponding graph. TNF-α treated cells were put to zero inhibition of COX-2 for an easy comparison between different experiments).

However, it cannot be ruled out that the inhibitory activity in faecal water is due to the presence of other metabolites identified by NMR, e.g. the metabolic active fumaric acid or glycine. These will be tested in future studies. A summary of all results is presented in Table 2.

This study shows for the first time that human faecal water contains components that can affect both the COX-2 protein level and enzymatic activity. Further characterization of faecal water showed that the faecal COX-2 inhibitors are of polar nature and that they may be acting in synergy. GC-MS and NMR analysis identified several components potentially responsible for the COX-2 inhibitory activity of faecal water. NMR analysis indicated the presence of phenolic compounds, fatty acids, amino acids and the major metabolite propylmethyl ketone. We believe that the concept of metabolomics can be a useful tool in the field of colon cancer research. Collecting,
analyzing, and comparing faecal samples from humans with different health status, diet and genetic characteristics can provide useful information about the importance of the colonic contents. One target would be to identify COX-2 inhibitors in faecal water, which originate from the diet and to be able to design new dietary recommendations to decrease colon cancer risk.
Table 2. Summary of COX-2 vegetarian study. Results are presented as mean ± SEM.

<table>
<thead>
<tr>
<th>ID in paper</th>
<th>Gender</th>
<th>Age</th>
<th>Diet</th>
<th>Duration (years)</th>
<th>NSAIDs</th>
<th>Other info</th>
<th>Faecal water µl/g faeces</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>22</td>
<td>Vegan</td>
<td>5</td>
<td>Yes</td>
<td>No NSAIDs prior to sample collection</td>
<td>118</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>34</td>
<td>Lacto-veg</td>
<td>&gt;5</td>
<td>No</td>
<td>No</td>
<td>80</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
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<td>Vegan</td>
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<td>No</td>
<td>279</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>33</td>
<td>Lacto-ovo</td>
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<td>No</td>
<td>673</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>25</td>
<td>Vegan</td>
<td>&gt;5</td>
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<td>No</td>
<td>138</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>26</td>
<td>Semi (fish)</td>
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<td>No</td>
<td>39</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>35</td>
<td>Lacto-ovo</td>
<td>&gt;5</td>
<td>No</td>
<td>Pregnant</td>
<td>173</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>23</td>
<td>Vegan</td>
<td>1-5</td>
<td>No</td>
<td>No</td>
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</tr>
<tr>
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<td>F</td>
<td>31</td>
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<td>No</td>
<td>No</td>
<td>697</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>25</td>
<td>Vegan</td>
<td>&lt;1</td>
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<td>No</td>
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<tr>
<td>11</td>
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<td>42</td>
<td>Lacto-ovo</td>
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<td>History with colonic polyps</td>
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</tr>
<tr>
<td>12</td>
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<td>22</td>
<td>Lacto-ovo</td>
<td>&lt;5</td>
<td>No</td>
<td>No</td>
<td>76</td>
</tr>
<tr>
<td>13</td>
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<td>22</td>
<td>Vegan</td>
<td>&gt;5</td>
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<td>No</td>
<td>284</td>
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<tr>
<td>14</td>
<td>F</td>
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<td>Lacto-ovo</td>
<td>1-5</td>
<td>No</td>
<td>No</td>
<td>252</td>
</tr>
<tr>
<td>15</td>
<td>M</td>
<td>35</td>
<td>Semi (fish)</td>
<td>1-5</td>
<td>No</td>
<td>No</td>
<td>234</td>
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<tr>
<td>16</td>
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<td>20</td>
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**These samples were too toxic to the cells, and gave rise to detachment of the cells from the culture plate surface.
CONCLUDING REMARKS

PEG and synbiotics may be useful as preventative agents for human colon cancer. PEG has been used in animal studies for a long period of time, but the protective mechanism is still somewhat unclear. For the first time, we report that a reduction of inflammation may be involved in the protective effect of PEG. In the literature, there is a wealth of evidence indicating that probiotic bacteria can efficiently decrease the risk of colon cancer in animals, but the effects of probiotics/synbiotics are not well characterized in man. The SYNCAN study was one of the first human trials evaluating the effect of synbiotics in man. Synbiotic supplementation was reported to change the gut flora in a favourable way (a decrease in the numbers of coliforms and bacteroides, and an increase in the numbers of lactobacilli). The most evident effect of synbiotics was seen on the biomarkers colorectal proliferation and DNA damage.

Perhaps the most interesting finding in this thesis is the detection of natural COX-2 inhibitors in human faecal water. In view of the large interest in COX-2 inhibitors in recent years, the finding of natural COX-2 inhibitors in faeces opens up a new field. We believe that these natural inhibitors may have fewer side effects compared to the available specific COX-2 inhibitors due to the presence of low levels of COX-1 inhibitors. Work must continue to further elucidate which compounds are responsible for this effect. Finally, the concept of metabolomics was for the first time used on faecal water. Metabolomics is a powerful tool which can be used to compare profiles of faecal water metabolomes from different groups on different diets. The ultimate goal would be to identify a faecal water profile which represents a healthy state at low risk for colon cancer. If the main components are characterized, these can be tested in a multi-biomarker model to identify “high risk” and “low risk” metabolomes. In the future, it may be possible to design new dietary recommendations on the basis of this new approach, to decrease the risk of colon cancer in humans.
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Biomarkers for colon cancer: Applications in human and rat studies


Department of medicinal chemistry. Uppsala, Uppsala university.


Pernilla C. Karlsson


Biomarkers for colon cancer: Applications in human and rat studies


ORIGINAL ARTICLES I-V