Biomarkers in Cancer Chemoprevention
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Biomarkers in colorectal cancer

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Epidemiological studies have revealed that the major dietary constituents implicated in colorectal carcinogenesis are fat/red meat (causative) and calcium/fibre (protective). Biomarkers have been used in both animal studies and clinical trials to investigate the effect of dietary factors and chemotherapeutic agents on colon carcinogenesis. They can be used as short-term end-points when investigations based on the development of cancer are not feasible. Although they can help in elucidating dietary or pharmacological effects, important results should be confirmed with longer-term studies.

Colon cancer develops through an adenoma–carcinoma sequence. The appearance of colonic polyps in individuals at risk for colon cancer has been used as an end-point in clinical trials to assess diets and pharmacological agents for their effect on colon carcinogenesis. Normal-appearing mucosa can contain small foci of aberrant crypts, which can be dysplastic and thought of as microadenomas. The appearance and growth of such foci have been used to assess the effect of dietary factors and chemopreventive agents in experimental animals. Increased proliferation both increases the sensitivity of the colon to carcinogenesis and may represent an early step in colon carcinogenesis. Etheno-DNA adducts are an end-product of lipid peroxidation processes, and are strongly pro-mutagenic lesions. High dietary levels of *n*–6 fatty acids appear to be important here and may also increase eicosanoid or isoprostane exposure and provide a selective growth stimulus for tumour precursor cells. Low dietary calcium may lead to inhibition of apoptosis and possibly to an increase in cell proliferation. In three recently completed intervention trials, calcium moderately reduced the recurrence of adenomas, but in one study fibre increased recurrence dramatically.

Introduction

The incidence of colorectal cancer is second only to that of lung cancer in both the United States and Europe; rates are generally low in Africa, Asia and South America. Epidemiological studies provide strong support for environmental factors, especially diet, in its etiology (Armstrong & Doll, 1975; Giovannucci *et al.*, 1994; Willett *et al.*, 1990). Migrants from an area of low incidence to a region of higher incidence generally assume the colorectal cancer risk of the host population within a generation. For example, the mortality rate due to colorectal cancer in Japanese immigrants to the United States is 3–4-fold greater than that of Japanese in Japan. Similarly, colorectal cancer incidence is much higher among Puerto Ricans in New York City than in natives in Puerto Rico.

Many dietary components have been examined in relation to colorectal cancer. Dietary fat/meat, protein, alcohol and sugar as well as smoking have been linked to increased risk of colorectal cancer, whereas fibre, calcium, vitamins and selenium have been implicated as protective. Only dietary fat, fibre and calcium (Owen, 2000) have been extensively studied; there is, in general, insufficient evidence to support or refute the role of the other dietary factors.

Identification of people with susceptibility to cancer of the colorectum is an important clinical need. Five-year survival after curative surgery (Enker *et al.*, 1979) decreases markedly with severity of the disease. Early detection correspondingly offers significantly improved prognosis. A five-year survival rate of close to 100% is associated with surgical resection of Dukes A tumours, the spread of which is limited to the colonic mucosa.

Biomarkers

The American Cancer Society (2000) recommends annual digital rectal and faecal occult blood testing after the ages of 40 years and 50 years respectively for all the population. It also recommends a proctosigmoidal examination of the rectum and colon every 3–5 years after the age of 50 years. If

any of these tests reveals a possible problem, more extensive tests (colonoscopy and X-ray barium enemas) are usually required. The American Cancer Society estimates that 27 000 additional lives could be saved annually in the United States alone through the use of early detection tests. However, implementation of the recommendations mentioned above would probably be too expensive. Therefore there is a need for surrogate markers (intermediate and frank biomarkers) of susceptibility to colorectal cancer; a brief review of biomarkers that have been suggested and tested (Figure 1) is presented below.

Bile acids

A role for bile acids in the causation of colorectal cancer was first proposed by Aries *et al.* (1969). Initially it was suggested that certain bile acids and neutral steroids might be transformed into carcinogens or co-carcinogens by anaerobic gut bacteria such as *Clostridia* (Aries & Hill, 1970a, b; Goddard & Hill, 1973), but due to a lack of evidence for the formation of such carcinogens *in vivo*, a different slant was proposed that perhaps constitutes the first implication of biomarkers in colorectal cancer, namely, that elevated levels of total bile acids in the stool would lead to increased susceptibility to colorectal cancer (Hill *et al.*, 1971). Many subsequent studies have shown that the major secondary bile acid metabolites (deoxycholic and lithocholic acid) formed in the bowel are either co-carcinogenic in animal model systems or co-mutagenic in mutagenicity testing systems (Bull *et al.*, 1983; Kawasumi & Shigemasa, 1988; Kelsey & Pienta, 1981; Narisawa *et al.*, 1974; Silverman *et al.*, 1977; Wilpart *et al.*, 1983). Animal and tissue-culture studies demonstrating the toxic and dysplastic effects of secondary bile acids have been supported by cohort studies correlating bile acid concentrations with adenoma size and dysplasia (Hill *et al.*, 1983) and with dysplasia in ulcerative colitis (Hill *et al.*, 1987).

However, in contrast to this extensive support from population, animal and *in-vitro* studies,

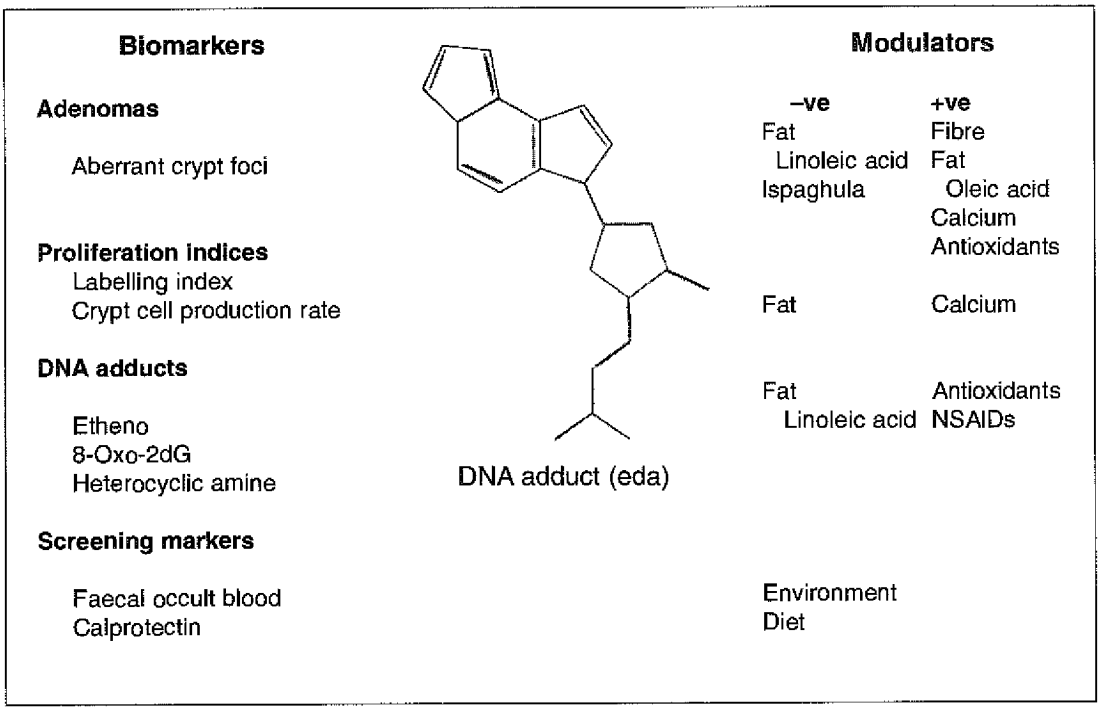

Figure 1. Biomarkers relevant to colorectal carcinogenesis

case–control studies have yielded equivocal results. Two showed positive differences between cases and controls (Hill *et al.*, 1975; Reddy & Wynder, 1977), but many other studies did not (Kaibara *et al.*, 1983; Moskowitz *et al.*, 1979; Mudd *et al.*, 1980; Murray *et al.*, 1980; Owen *et al.*, 1987; Roy *et al.*, 1999). Further studies were directed at identifying the most discriminating faecal bile acid marker. The *in-vitro* studies had highlighted the importance of the secondary bile acids lithocholic acid and deoxycholic acid, which between them account for over 90% of the total faecal bile acid concentration. Owen *et al.* (1987, 1992) and Owen (1997) showed that the ratio of lithocholic acid to deoxycholic acid was a better risk marker than deoxycholic acid, lithocholic acid or the total concentration of faecal bile acids in several case–control studies involving both adenoma and colorectal cancer patients. However, despite initial promise that this biomarker would have application in screening procedures, this was not upheld in several later case–control (Roy *et al.*, 1999) and prospective studies (Haines *et al.*, 2000).

Although studies on bile acids have tended to concentrate on faecal concentrations, some attempts have been made to correlate serum bile acid profiles with the incidence of colorectal cancer. van der Werf *et al.* (1982) and Bayerdorffer *et al.* (1993) demonstrated increased concentrations of deoxycholic acid in serum of adenoma patients compared with controls, but, as with the studies of the faecal matrix, overlap between cases and controls was too extensive to allow sensitive discrimination between high-risk individuals and healthy controls.

Cell proliferation

Hyperproliferation of the intestinal mucosa is regarded as an intermediate biomarker of colorectal cancer. It has been reported in patients with sporadic colorectal adenomas (Bleiberg *et al.*, 1985), in first-degree relatives of colorectal cancer patients (Rozen *et al.*, 1990), in patients suffering from ulcerative colitis (Biasco *et al.*, 1990), in familial adenomatous polyposis (FAP) patients (Lipkin *et al.*, 1984) as well as colorectal cancer patients (Deschner & Maskens, 1982). It appears that these events are not localized to any particular part of the colorectum, but amount to a field effect involving the whole organ. Adenomas arise with greater frequency from this hyperproliferative field, presumably because the rate of cell proliferation exceeds that of DNA repair. Risio *et al.* (1991) have reported that removal of adenomas by polypectomy restores proliferation to normal levels and this may explain why recurrence occurs in only about 40% of patients. However, the situation is very complex, involving both genetic polymorphisms and epigenetic factors, of which the relative importance is hard to determine. Despite this, hyperproliferation has been used as a surrogate end-point biomarker of colorectal neoplasia in a number of studies, especially those evaluating the effect of potential chemopreventive agents. The effect of calcium on hyperproliferation has been studied extensively and is described in more detail below in the section on Intervention Studies.

Aberrant crypt foci

Aberrant crypt foci (ACF), which are microscopic lesions (Roncucci *et al.*, 2000), are possible precursors of adenomas in the colon and therefore may be useful markers of susceptibility to neoplasia. The major advantage of ACF over adenomas as a biomarker of colorectal cancer is that animal experiments can be conducted over a very short time period (weeks rather than months). A drawback, however, is that ACF are so minute that they have to be observed by staining with methylene blue and low-power microscopic examination. In the rat model, removal of the colon for observation is of course not problematic and human resection specimens can also be obtained during surgery. Recent developments in methodology (Dolara *et al.*, 2000) also allow ACF to be visualized *in situ* in humans during colonoscopy, but since all individuals are likely to have ACF (and they cannot be removed), it is difficult to see what advantage the detection of ACF in humans would have over location of an adenoma and polypectomy. Nevertheless, they will continue to have utility in animal model systems for testing the efficacy of chemopreventive agents in short-term carcinogenicity tests (Wargovich *et al.*, 1996).

DNA adducts

Various DNA lesions such as etheno and heterocyclic amine adducts and 8-oxodeoxyguanosine may have relevance as biomarkers of colorectal

cancer. However, little research in this area has been conducted in relation to the colorectal mucosa. Friesen *et al.* (1994) have shown that DNA adducts of the heterocyclic amine 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) can be detected in colon biopsies of humans using gas chromatography/electron capture mass spectrometry. Such adducts are preneoplastic lesions in the rat and therefore may serve as biomarkers of exposure to charred red meat which is the source of heterocyclic amines in the diet.

Schmid *et al.* (2000) used an ultrasensitive and specific immunoaffinity/^{32}P-postlabelling method to detect etheno adducts in colonic tissue of familial adenomatous polyposis (FAP) and sporadic cancer patients. The levels of 1,N^6-ethenodeoxyadenosine (εdA) and 3,N^4-ethenodeoxycytidine (εdC) in polyps removed from the FAP patients were 2–3 times higher than in unaffected colon tissue. In the patients with sporadic carcinoma, no difference in the level of etheno adducts between tumours and adjacent tissue was detected. This suggests that etheno DNA adducts may act as initiators of preneoplastic lesions. The formation of etheno adducts is inherently linked to the metabolism of polyunsaturated (ω–6) long-chain fatty acids (especially arachidonic and linoleic acids) and lipid peroxidation processes and thus these adducts may represent biomarkers of exposure to fats containing high levels of such lipids (Bartsch *et al.*, 1999).

Although 8-oxodeoxyguanosine has been inferred to be a preneoplastic lesion in many cancers, no reports have yet implicated it in the etiology of colorectal cancer.

DNA adducts have potential for use as biomarkers of colorectal cancer, but as yet no convincing strategy has been formulated as to how they could fit into screening procedures. As with many biomarkers, tissue is required for analysis and therefore application would be invasive. Future research may show a specific correlation between levels of certain DNA adducts in either blood or urine and those in tissues such as the colorectum, obviating the need for biopsy material.

Screening markers

Faecal occult blood

The simplest and most dominant diagnostic aid available for colorectal screening at present is the Haemoccult test. This is a rapid colorimetric test based on a guaic–peroxide-catalysed reaction with faecal occult blood. The concept is now more than 100 years old and the test has been applied in cancer screening for over 30 years.

Faecal occult blood testing of the general population shows a rate of slide positivity from 1 to 5%, with a predictive value for neoplastic lesions of 20–50%. The percentage of patients with positive tests who are ultimately found to have adenomas or cancer increases with the age of the population, ranging from 15% in the 40–49-year age group to 60% for individuals over 69 years (Schein & Levin, 1986; Winawer & Sherlock, 1982).

However, all faecal occult blood tests suffer from a number of crucial drawbacks and over the years the test has been extensively criticized. It is not specific for human haemoglobin, also reacting with animal haem, and peroxidases and catalases ingested as part of the normal diet massively interfere with the test. Furthermore, vitamin C, by virtue of its antioxidant properties, produces false negative results with Haemoccult.

The majority of colorectal adenomas have intact epithelium and do not bleed. Thus, faecal occult blood tests are currently of little value in accurately screening the asymptomatic general population for adenomas and predisposition to colorectal cancer. In addition, faecal occult blood testing has little diagnostic value.

Calprotectin

Calprotectin is a prominent cytosol protein in neutrophil granulocytes and is regarded as a marker of inflammatory and neoplastic disease in the lower gastrointestinal tract. Kristinsson *et al.* (1998) studied the faecal concentration of calprotectin in patients with colorectal carcinoma. The median faecal calprotectin concentration in 119 colorectal cancer patients was 50 mg/L (range 2–950), significantly higher than in 125 control patients (median 5.2 mg/L) ($p < 0.0001$). In 23 patients studied after resection, excretion fell dramatically. However, a correlation was not found between plasma and faecal levels. The authors suggested that the measurement of faecal calprotectin might become a diagnostic tool for detecting colorectal carcinoma. This has gained support from a recent study by Kronborg *et al.* (2000), who studied the sensitivity and specificity of faecal calprotectin for

detection of adenomas in high-risk individuals undergoing colonoscopy. Patients with confirmed adenomas had significantly higher calprotectin levels than those without (median 9.1 mg/L, 95% CI 7.5–10.1 versus 6.6 mg/L, 95% CI 5.6–7.4). Levels in patients with confirmed cancer were significantly higher than those in the adenoma and control groups (median 17.6 mg/L, 95% CI 11.5–31.0). Using a cut-off limit of 10 mg/L, the sensitivity for cancer was 74% and for adenoma 43%. The authors conclude that the measurement of faecal calprotectin as a marker of colorectal cancer would be of utility in high-risk groups but is not specific enough for use in the general population. It may be hoped that improvements in the assay of faecal calprotectin (Ton *et al.*, 2000) will lead to more generalized use, because it has the advantage over the faecal occult blood test that it does not suffer from interference by food, pharmaceuticals or nutraceuticals.

Intervention studies

Both calcium and fibre regimens have been utilized in human chemoprevention trials in an attempt to reduce recurrent preneoplasia and to verify mechanistic aspects.

In several small calcium intervention trials in humans, the results overall have been equivocal. Several studies (Lipkin *et al.*, 1989; Rozen *et al.*, 1989; Steinbach *et al.*, 1994) have shown that supplementing the human diet with calcium can reduce intestinal cell proliferation, an intermediate biomarker of colorectal cancer, but others (Gregoire *et al.*, 1989; Stern *et al.*, 1990) have shown no effect, whilst one (Kleibeuker *et al.*, 1993) has shown the opposite. In view of these equivocal clinical data, a long-term, double-blind intervention trial was undertaken by Weisgerber *et al.* (1996) in polypectomized, sporadic adenoma patients in which the putative role of calcium (2 g per day) as a protective factor in colon carcinogenesis was studied. Despite differences in stool biochemistry elicited by supplementary calcium after nine months' intervention, a similar non-significant decrease of total cell proliferation rate in sigmoidal mucosa was evident in both the calcium (13.5 down to 11.4) and placebo groups (13.7 down to 10.7). An increase in the concentration and daily excretion of total bile acids, primary bile acids, long-chain fatty acids and long-chain fatty acid soaps was observed in the calcium group, while there was no significant reduction in the concentration of the potentially toxic free bile acids and long-chain fatty acids. This indicated that even if calcium was beneficial, it did not mediate its effects via chelation of intestinal lipid. Baron *et al.* (1995) conducted a multicentre, randomized placebo-controlled, double-blinded trial in polypectomized, sporadic adenoma patients. Subjects received either 3 g of calcium carbonate (providing 1.2 g elemental calcium) or an identical-looking placebo tablet. In rectal mucosal samples obtained six to nine months later from 333 patients (intervention, $n = 173$, placebo, $n = 160$), no decrease in rectal mucosal proliferation due to calcium supplementation was detected.

A number of larger intervention studies designed to assess the efficacy of calcium and antioxidants (Hofstad *et al.*, 1998), calcium (Baron *et al.*, 1999), calcium and fibre (Bonithon-Kopp *et al.*, 2000; Faivre *et al.*, 1997) and fibre (Alberts *et al.*, 2000; Schatzkin *et al.*, 2000) on the growth and/or prevention of adenoma recurrence have been completed recently. In a placebo-controlled study in which 832 polypectomized adenoma patients received supplemental calcium (1.2 g per day) for three years, a significant reduction (RR = 0.81, 95% CI 0.67–0.99) ($p = 0.04$) in recurrence was observed in the calcium ($n = 127$) compared to the placebo arm ($n = 159$) (Baron *et al.*, 1999).

Hofstad *et al.* (1998) studied the effects of both calcium (1.6 g per day) and a cocktail of antioxidants and vitamins (β-carotene 15 mg, vitamin C 150 mg, vitamin E 75 mg, selenium 101 µg per day) in a placebo-controlled intervention trial. All adenomas up to 9 mm in diameter were left *in situ* and the effect on both adenoma growth and the appearance of new adenomas was evaluated. While intervention had no significant effect on adenoma growth, the appearance of new adenomas was significantly lower (log-rank test, $p = 0.035$) in the active group ($n = 58$) than in the placebo group.

Bonithon-Kopp *et al.* (2000), representing the European Agency for Cancer Prevention (ECP), conducted a placebo-controlled pan-European calcium/fibre intervention study in polypectomized sporadic adenoma patients ($n = 625$). In this three-arm clinical trial, 178, 176 and 198 patients were randomized (Table 1) to intervention with placebo (sucrose), calcium (2 g per day) and

Table 1. Baseline characteristics of patients in the ECP calcium fibre intervention study

	Calcium	Fibre	Placebo
Age (years)	58.8 [8.8]	59.1 [8.9]	59.3 [8.4]
Sex			
Male	116 (65.9)	128 (64.6)	107 (60.1)
Female	60 (34.1)	70 (35.4)	71 (39.9)
History of adenoma			
No	145 (82.4)	175 (88.4)	148 (83.1)
Yes	31 (16.6)	23 (11.6)	30 (16.9)
Geographical area			
S. Europe and Israel	34 (19.3)	40 (20.2)	37 (20.8)
W. Europe	72 (40.9)	86 (43.4)	70 (39.3)
N. Europe	70 (39.8)	72 (36.4)	71 (39.9)
No. of adenomas			
Single	104 (49.1)	138 (69.7)	118 (66.3)
Multiple	72 (40.9)	60 (30.3)	60 (33.7)
Adenoma size			
At least 1 > 10 mm	99 (56.3)	105 (53.0)	105 (59.0)
At least 1 > 20 mm	36 (20.5)	25 (12.6)	30 (16.8)

Mean values [SD] or number of subjects (%)
Data from Bonithon-Kopp *et al.* (2000)

Table 2. Risk of adenoma recurrence associated with fibre or calcium treatment in the ECP calcium fibre intervention study

	Calcium treatment		Fibre treatment	
	OR (95% CI)	*p* value	OR* (95% CI)	*p* value
Crude	0.75 (0.43–1.29)	0.29	1.63 (1.01–2.64)	0.042
Adjusted[a]	0.66 (0.38–1.17)	0.16	1.67 (1.01–2.76)	0.042

OR, odds ratio; CI, confidence interval
[a] Adjustment for age, sex, past adenoma history, number and location of adenomas at inclusion
Data from Bonithon-Kopp *et al.* (2000)

fibre (Ispaghula, 3.5 g per day) respectively for three years. The risk of adenoma recurrence (Table 2) associated with calcium supplementation was non-significantly reduced (OR = 0.75, 95% CI 0.43–1.29). In contrast, supplementation with Ispaghula increased the risk of adenoma recurrence significantly (OR = 1.63, 95% CI 1.01–2.64; p = 0.042) and this association was even stronger (Table 3) in those patients with > 80% compliance to the fibre supplement (OR = 1.91, 95% CI 1.08–3.35; p = 0.023). Furthermore the risk of adenoma recurrence associated with fibre treatment according to baseline dietary intake of calcium above the median (Table 4) was highly

Table 3. Risk of adenoma recurrence associated with fibre or calcium treatment according to compliance

	Calcium treatment		Fibre treatment	
	OR (95% CI)[a]	*p* value	OR (95% CI)[a]	*p* value
Compliance <80%	0.53 (0.17–1.72)	0.29	1.06 (0.34–3.33)	0.93
Compliance >80%	0.70 (0.36–1.36)	0.20	1.91(1.08–3.35)	0.023

[a] Odds ratio and 95% confidence interval adjusted for age, sex, past adenoma history, number and location of adenomas at inclusion

Data from Bonithon-Kopp *et al.* (2000)

Table 4. Risk of adenoma recurrence associated with fibre or calcium treatment according to baseline intake of calcium

Baseline dietary calcium[a]	Calcium treatment		Fibre treatment	
	OR (95% CI)[b]	*p* value	OR (95% CI)[b]	*p* value
Below the median	0.51 (0.22–1.18)	0.11	1.04 (0.49–2.18)	0.92
Above the median	0.65 (0.43–2.30)	0.99	2.81 (1.33–5.92)	0.005
Interaction test	$p = 0.12$		$p = 0.028$	

[a] Baseline dietary assessment missing for 29 patients; median value of dietary calcium intake = 918 mg/day

[b] Odds ratio and 95% confidence interval adjusted for age, sex, past adenoma history, number and location of adenomas at inclusion

Data from Bonithon-Kopp *et al.* (2000)

significantly elevated in those patients (OR = 2.81, 95% CI 1.33–5.92; $p = 0.005$).

Alberts *et al.* (2000) recently reported on a two-arm intervention study (1429 men) with high (13.5 g per day) or low (2 g per day) wheat bran supplementation for three years. Of the 1303 men who completed the study, 719 were randomly assigned to high supplementation and 584 to low supplementation. At the end of the study, at least one adenoma was detected in 47% of the high-fibre group as opposed to 51.2% in the low-fibre group, showing conclusively that intervention with high-fibre dietary regimes confers no protection against recurrent colorectal adenomas. This conclusion was supported by a similar study in which 2079 men and women were randomized either to a diet that was low in fat (20% of total calories) and high in fibre (18 g of dietary fibre per 1000 kcal) and fruits and vegetables (3.5 servings per 1000 kcal) or to a healthy eating non-supplemented usual diet for four years (Schatzkin *et al.*, 2000). Of the 1905 who completed the study (958 in the intervention group; 947 in the control group), 39.7% and 39.5% respectively had at least one recurrent adenoma. Therefore adopting a diet that is low in fat and high in fibre, fruits and vegetables does not influence the risk of adenoma recurrence.

A mechanism has been put forward by Nair *et al.* (1997) to explain the link between diet and

cancer. In a dietary intervention study with either sunflower oil (high in polyunsaturated fatty acids, especially linoleic acid) or rapeseed oil (high in monounsaturated fatty acids, especially oleic acid), a dramatic increase in DNA bridged etheno adducts was detected in whole white blood cells in females on the sunflower oil diet. Etheno adducts result from lipid peroxidation processes, initiated and propagated by reactive oxygen species and the results indicate that diets which are rich in linoleic acid may be non-beneficial to health. These observations show for the first time a plausible and tangible link between an unhealthy diet and carcinogenesis.

Conclusions

It is evident that the mechanisms related to nutritional factors which lead to colorectal cancer are still somewhat of a mystery. The best and most relevant biomarker of colorectal cancer is adenoma formation and using recurrence as an endpoint is really useful in assessing the efficacy of chemopreventive agents, as exemplified by the consistent moderate beneficial effect of supplemental calcium. It should be noted that detection of adenomas in the general population in countries where screening procedures are not practised (the vast majority) is usually too late to permit hope of improved prognosis, because a large proportion of adenomas are asymptomatic and in general patients present only when it is too late due to blood in the stool, severe pain or irritable bowel syndrome. However, clinical trials are labour-intensive, time-consuming and expensive and therefore a vast range of potential health-promoting substances will never be tested by a sound method. There is a dire need for clinically relevant surrogate biomarkers which give reliable results and correlate significantly with either adenoma formation or recurrence. Preferably these should be measurable in easily accessible material such as blood, faeces or urine.

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