Diet and cancer: assessing the risk

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Globally, colorectal cancer (CRC) is a leading cause of mortality from malignant disease. Case–control and cohort studies provide strong support for a role of diet in the aetiology of CRC. However to establish causal relationships and to identify more precisely the dietary components involved, intervention studies in human subjects are required. Cancer is an impractical endpoint in terms of numbers, cost, study duration and ethical considerations. Consequently, intermediate biomarkers of the disease are required. This review aims to provide an overview of the intermediate endpoints available for the study of CRC, particularly non-invasive faecal biomarkers. Examples of their use in dietary intervention studies are given.

Colorectal cancer: Biomarkers: Diet: Gut microflora

Cancer epidemiology

Cancer is an important global public health problem. Annually 10·1 million new cancer cases are diagnosed with a further 6·2 million people losing their lives worldwide. This disease accounts for a quarter of all deaths in countries with a Westernised lifestyle (International Agency for Research on Cancer (IARC), 2000). Colorectal cancer (CRC) is the fourth most common cause of cancer-related mortality in the world. Approximately 944 000 new cases were diagnosed globally in 2000 and this accounts for 9·2 % of all new cancer cases (IARC, 2000). Within Europe, North America, Australia and New Zealand, it is the second most common cancer after lung or breast (Boyle & Langman, 2000). In 2000, 363 000 new cases were reported in Europe. CRC affects 6 % of men and women by the age of 75, in almost equal proportions. Pronounced differences appear to exist in 5-year survival following cancer diagnosis between the UK (30–35 %) and the USA (50–55 %; Sant *et al.* 1995). The reasons for such a discrepancy remain unclear, but are likely to be attributed to the progression at diagnosis or treatment delivery. Generally the incidence and mortality of the disease are increasing (Cummings *et al.* 1996; Boyle & Langman, 2000). The Modena colorectal registry (Italy) reported a 12·2 % increase in incident rates from 1985 to 1997 and other European studies have reported similar trends (Johansen *et al.* 1993; Kemppainen *et al.* 1997).

Globally, incidence rates show an approximate 20-fold variation, with the developed world suffering the highest rates and India one of the lowest (IARC, 2000). Even within countries the rates may vary, as in India where the Westernised Parsi population have a higher rate of CRC than the strictly vegetarian Janists (Indian Cancer Society, 1985). Such fluctuations are generally attributed to both genetic factors and environmental factors, especially diet. Migrant studies (Japan to USA, Eastern Europe to North America) give further support to the role of environmental factors in the aetiology of colorectal malignancies, with reported incidence rates of migrants and their descendants reaching those of the host country, sometimes within one generation (World Cancer Research Fund (WCRF), 1997). The highest rates of the disease are currently seen within Hawaiian Japanese men with an incidence of 53·5 per 100 000 (IARC, 1997).

Evidence suggests that diet plays a significant role in the aetiology of CRC. Identifying conclusively which constituents (e.g. vegetables, meat, fibre, fat, micronutrients) exert an effect on risk has been more problematic however, due to inconsistent data (Potter, 1999). The 1997 WCRF report concluded that the evidence (mainly from case–control studies) for diets rich in vegetables protecting against CRC was convincing, but that the effect of fruits could

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Abbreviations: APC, adenomatous polyposis coli; ATNC, apparent total NOC; CCPS, crypt cell proliferative state; CRC, colorectal cancer; DAG, diacylglycerol; DCA, deoxycholic acid; DMH, 1,2-dimethylhydrazine; FAP, familial adenomatous polyposis; GST, glutathione S-transferase; HCA, heterocyclic amines; IARC, International Agency for Research on Cancer; HFA, human flora associated; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; LCA, lithocholic acid; MAM, methylazoxymethanol; MNNG, *N*-methyl-*N*-nitro-*N*-nitroguanidine; MSI, microsatellite instability; NOC, *N*-nitroso compounds; O6-med-G, O(6)-methyldeoxyguanosine; PKC, protein kinase C; SCFA, short-chain fatty acids; WCRF, World Cancer Research Fund.

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not be judged due to limited and contradictory data. Data from prospective studies are less convincing than case–control studies (Bingham, 2000). High fibre diets were reported to possibly decrease the risk of CRC, with suggested protective mechanisms including toxin dilution or adsorption (WCRF, 1997; American Gastroenterological Association, 2000). Additionally several micronutrients including carotenoids, ascorbate and folate have been examined epidemiologically in relation to the protective effect associated with vegetables, but the results have frequently been discordant, and coupled with the paucity of data, no strong associations can be observed (Giovannucci et al. 1993; Slattery et al. 1997). Collectively, studies examining the effect of meat consumption (especially red and processed meats) on CRC have produced neither strong nor consistent findings, but it is believed that the weight of evidence points towards a slighty elevated risk (WCRF, 1997; Norat & Riboli, 2001), although the mechanisms remain unclear. A high intake of saturated or animal fat is possibly related to increased risk (Potter, 1999; Zock, 2001) and does not appear to contribute to the risk associated with meat consumption (Giovannucci & Goldin, 1997).

High calcium intake has been suggested to offer a protective effect from fat-induced promotion of carcinogenesis by binding cytotoxic bile and fatty acids (Kleibeuker et al. 1996a) or reducing proliferation in the upper part of the colonic crypt (Bostick et al. 1995). Although most evidence suggests a reduced risk or no association, the results of both cohort and case studies are inconsistent (Potter et al. 1993). Alcohol has long been suspected as a risk factor for colorectal neoplasms, since Stocks first reported a slightly elevated risk in beer drinkers compared to abstainers (Stocks, 1957). Further studies on the topic have provided controversial results as detailed in a review by Potter et al. (1993). The WCRF reported that elevated alcohol consumption probably increases the risk of cancers of the colon and rectum and that this association is related to total ethanol intake rather than the type of alcoholic drink (WCRF, 1997).

Despite the weight of the epidemiological evidence for diet playing an important role in CRC risk, definitive evidence for causal association is lacking due to difficulties of conducting dietary intervention studies. Surrogate endpoints (biomarkers) are therefore required. Before considering biomarkers that can be used it is necessary to provide a brief summary of the processes of carcinogenesis in the colon.

Colon carcinogenesis

Approximately 70 % of colorectal malignancies appear to be localised in the left (descending) large bowel between the lower rectum and the splenic fissure, though curiously this subsite distribution appears to be undergoing a proximal shift towards the right (ascending) large bowel, for reasons unknown (Faivre et al. 1989; Ponz de Leon & Roncucci, 2000). Colonic microarchitecture is characterised by crypts, which are approximately fifty cells in depth. The normal structure and replicative dynamics of the crypts ensure that both stem cells and immediate

daughter cells replicate in the lowest region. When the immediate daughter cells divide and migrate they give rise to all the cells that line the crypt. Eventually these cells will reach the surface by which stage they are fully differentiated columnar epithelial cells, covered with microvilli, intimately connected via numerous tight junctions and involved in water and electrolyte transport. The constant outward movement of cells from the crypts should ensure that no interaction occurs between replicating cells and the luminal environment, as such, any mutagens should then only affect the already differentiated colonocytes and effectively have no impact upon the integrity of the crypt cell population (Potter, 1999).

The nature of the microarchitecture was used by Potter to argue that the first mutagenic event occurring to a progenitor cell would have to be a blood-borne rather than a luminal agent. It was suggested that for luminal constituents to have any role in carcinogenesis a polyp must already exist and be in contact with the faecal stream. Other authors offer the contention that a luminal agent could provide the 'first hit' (mutation), if a focal failure in the epithelial barrier occurred due to chemical/physical insult or as a result of failure in terminal differentiation. An agent could then directly or indirectly affect cells in the crypt and lead to the formation of a polyp (Bruce et al. 2000). Mutation of the adenomatous polyposis coli (APC) gene permits an adenomatous polyp to develop and such a formation is considered an important predisposing risk factor for CRC. However, this does not mean that all polyps become malignancies (only approximately 5 % do) nor does it prohibit the possibility that de novo colorectal tumourigenesis may occur (Owen, 1996). The adenomas are well demarcated clumps of epithelial dysplasia, classified into three histological types (tubular, villous and tubulovillous), which increase in prevalence with age, being present in 24–40 % of people over 50 years old (Ponz de Leon & Roncucci, 2000).

It is estimated that up to 15 % of all CRC are due to genetic predisposition, with a further 60 % due to sporadic CRC that appear to develop from adenomatous polyps. Adenomas and carcinomas develop through a stepwise accumulation of somatic mutations (Fig. 1). Although the precise sequence of genetic events is not completely understood, it involves inactivation of various tumour-suppressing genes (e.g. APC, p53), activation mutations in proto-oncogenes (e.g. k-ras, c-myc) and loss of function in DNA repair genes (e.g. hMLH1, hMSH2). This classic multistep model has been termed the 'adenoma–carcinoma sequence' (Vogelstein et al. 1988; Fearon & Vogelstein, 1990). For detailed information on the genetic events and pathways to CRC the reader is referred to Potter (1999), Chung (2000), Ponz de Leon & Percesepe (2000) and Souza (2001).

The potential role of luminal factors in the development of colonic tumours has led to the theory that the large bacterial population in the colon is involved in the formation of carcinogens, tumour promotors and anticarcinogens in the gut (Hill, 1975). This in turn has encouraged the search for dietary components that modulate the gut microflora and its activities and thus may influence CRC, e.g. probiotics, prebiotics and fibre (Table 1).
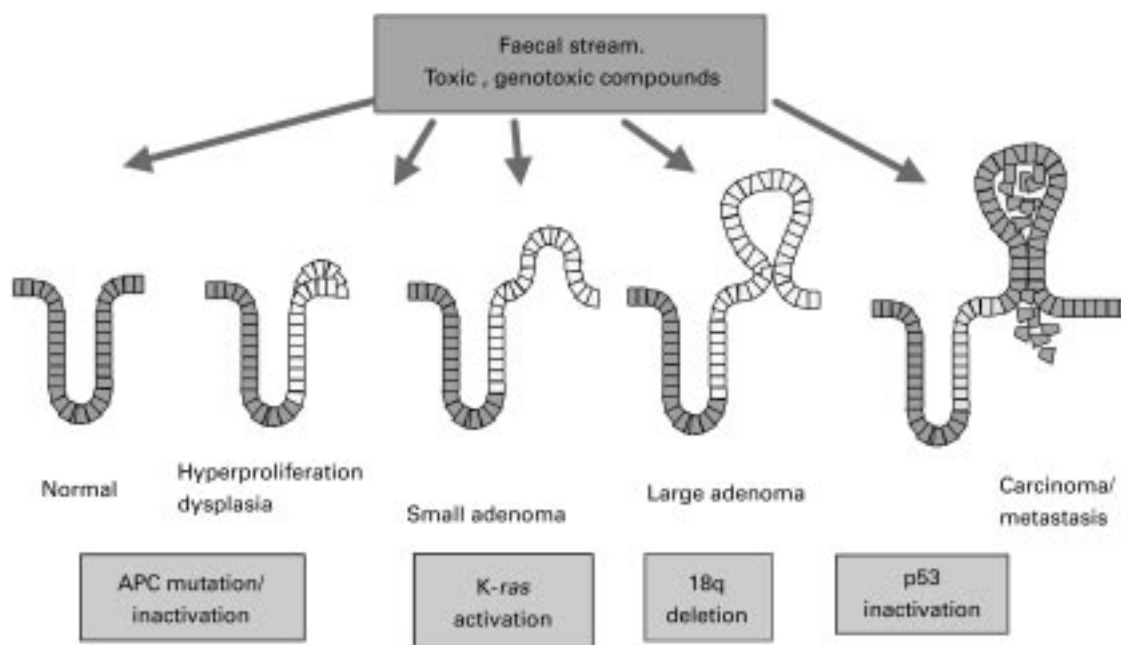
Fig. 1. The adenoma–carcinoma sequence. Inactivation of the adenomatous polyposis coli (APC) gene facilitates the development of a hyperplastic epithelium. Activation of oncogenic *ras* occurs in early adenomas. Deletion of 18q is found in dyplastic late adenomas. Finally with the inactivation of p53 colorectal cancer develops. Components of the faecal stream are believed to modulate colorectal carcinogenesis.

Biomarkers for colorectal cancer

To establish causal relationships between diet and CRC risk and to identify more precisely the dietary components involved, human intervention trials are required. The problem with human intervention studies is that cancer is an impractical endpoint in terms of numbers, cost, study duration and ethical considerations. The long lag phase (up to 20 years) between exposure to a carcinogenic event and appearance of tumours is a particular problem. An alternative strategy is the use of intermediate endpoint biomarkers of cancer, which may be biochemical, molecular, cellular or rooted in pathologic change (e.g. recurrence of polyps, faecal water, epithelial markers).

Biomarkers have been developed from an understanding of the sequence of events leading to colonic cancer, the biology of normal mucosa and the factors associated with changes symptomatic of progression toward cancer and the manifestation of cancer itself. The particular advantages of biomarkers are that they represent short-/intermediate-term endpoints, which allow intervention on a reasonable time scale. Ethical approval is readily obtainable for biomarker studies as they are minimally invasive, with measurements occurring on accessible material (e.g. faeces and small biopsies, FAP). Ideally biomarkers should be sensitive, reproducible and rigorously validated, although this is not the case with all biomarkers in the cancer field. Biomarkers should be causally linked, or correlated with cancer and hence of biological significance. Thus validation of a biomarker is critical to its application as a research tool, as an appropriate response from the marker is required when assayed in cancer patients (e.g.

familial adenomatous polyposis, FAP) or in healthy individuals on low-risk and high- risk diets for CRC. The biomarkers available for the study of CRC are composed of two main types: (1) tissue, (2) biochemical. Both categories possess distinct advantages.

Tissue biomarkers

These endpoints are analysed from a tissue biopsy and as such necessitate invasive procedures of varying complexities to retrieve samples of rectal/colonic mucosa. Use of biopsies increases the technical complexity of studies, but reduces the degree of inference required to interpret the results when compared to biochemical markers. Tissue can be directly observed, manipulated and analysed. Tissue biomarkers, therefore, provide the scope to examine a range of cellular aspects intimately linked to CRC.

Adenoma growth and recurrence

Adenomas (or polyps) are considered to represent the most likely precursor lesions for colonic cancer in humans. Surveillance and removal generally offer a protective effect, although adenoma recurrence is common (Wehrmann & Fruhmorgen, 2000). Polyps appear to be the most reliable premalignant biomarkers for CRC, but consequently studies are long (1–5 years) and expensive. The adenoma size and the extent of the villous component are major risk factors associated with high grade dysplasia and as such the malignancy potential of a colorectal polyp increases with size (O'Brien *et al.* 1990; Simons *et al.* 1992; Hofstad & Vatn, 1997). In individuals with a large colon polyp (>1 cm) the rate of development of CRC is four times faster than expected (Otchy *et al.* 1996). Larger polyps

Table 1. Potential anticancer properties of foods/nutrients

Food/nutrient	Potential anticancer activity	References
Prebiotics (e.g. inulin, fructo-oligosaccridies)	Selective effects for *Bifidobacterium*, *Lactobacillus* spp. Fermentation influences bowel function (e.g. motility)	Cummings *et al.* (2001)
Probiotics (e.g. *Lactobacillus casei* Shirota)	Beneficially modulate gut physiology (e.g. mucosal integrity, pH) and microflora content. Increase carbohydrate fermentation, decrease cytotoxicity	Holzapfel *et al.* (1998) Burns & Rowland (2000) Hirayama & Rafter (2000)
Fermented dairy products	Increase gut microflora population and carbohydrate fermentation	St-Onge (2000)
Fibre and resistant starch	Increase stool bulk, colonic fermentation and SCFA synthesis, decrease faecal pH and faecal water cytotoxicity	Cummings *et al.* (1996) Phillips *et al.* (1995) Hylla *et al.* (1998)
Folate	Prevents DNA hypomethylation, possible early step in carcinogenesis	Su (2001)
Phytoestrogens	Inhibit proliferation of colon cancer cells, poor association with risk of CRC	Serraino & Thompson (1992) Yanagihara *et al.* (1993) Jenab & Thompson (1996)

CRC, colorectal cancer; SCFA, short-chain fatty acids.

therefore tend to be more predictive of CRC than small polyps. Dietary effects on cancer risk are assessed through examination of small adenoma recurrence or growth of large polyps. The study groups usually consist of patients with sporadic polyps. Intervention studies completed on such groups have provided conflicting information as to the protective effects of low fat, high fibre and high calcium intakes. Several studies have indicated no effect on recurrence or polyp growth rates (McKeown-Eyssen *et al.* 1994; Hofstad *et al.* 1998; Schatzkin *et al.* 2000), whilst others have indicated an inhibitory effect (MacLennan *et al.* 1995; Baron *et al.* 1999). Further polyp studies are currently being undertaken which may resolve these issues including the Concerted Action Polyp Prevention trial and UK Colonic Adenoma Prevention trial. Although polyps are considered a reliable biomarker, the studies can be complex, invasive and time consuming. Simpler, less invasive biomarkers that can be used on healthy subjects are therefore desirable.

Mucosal proliferation

Colon cancer arises out of perturbation of the normally ordered and balanced proliferation and deletion mechanisms of the cell crypt. This results in hyperproliferation and a shift in the proliferation zone from a restricted band to the entire crypt (Wilson *et al.* 1990). Generally increased cell proliferation results in an increased tumour load, with carcinogens (e.g. azoxymethane) in animal models often inducing cell proliferation. Patients with a high risk of CRC (e.g. FAP) have a correspondingly high mucosal cell proliferation (Bostick *et al.* 1997). To determine the extent of mucosal proliferation, tissue biopsy specimens are examined using immunolabelling techniques including proliferating cell nucleus antigen and bromodeoxyuridine or by isolation of individual crypts and determination of mitotic cells (Goodlad *et al.* 1983). Such studies have provided conflicting data on the effect of high calcium

and fibre intakes on hyperproliferation. (Van Munster *et al.* 1994; Alberts *et al.* 1997; Macrae *et al.* 1997; Holt *et al.* 1998). Although whole crypt microdissection and mitosis counting has been shown to be a reliable, reproducible and robust technique for assessing crypt cell proliferative state (CCPS) in the human colon, the precise relation of an increased CCPS to the neoplastic process remains uncertain (Mills *et al.* 2001). Furthermore for some methods, noticeable assay variability exists, which may ultimately compromise their usefulness as biomarkers (McShane *et al.* 1998; Kulldorff *et al.* 2000). Thus at present, reliance solely on cell proliferation as a marker of diet-related CRC risk would be incautious. The use of mucosal proliferation as a biomarker can be strengthened with the incorporation of apoptosis and differentiation indicators (e.g. *in situ* terminal transferase nick end labelling, *Dolichos biflorus* agglutinin), so as to provide a more integrated view of crypt function and architecture (Chang *et al.* 1997). It has been demonstrated that the non-steroidal anti-inflammatory drug mesalazine significantly increases the rate of apoptosis while concurrently decreasing cell proliferation (Reinacher-Schick *et al.* 2000).

DNA adducts

Exposure to carcinogens and mutagens can occur from the diet, environment or in some instances even from endogenous pathways. The covalent binding of such compounds (or their metabolites) to DNA results in the formation of adducts, which are believed to contribute to initiation and/or progression of carcinogenesis (Schmid *et al.* 2000). ^{32}P-postlabelling is used as a biomarker to examine the exposure of DNA to carcinogens. The presence of numerous DNA adducts in colonic mucosa is associated with increased risk of CRC (Hamada *et al.* 1994; Pfohl-Leszkowicz *et al.* 1995). Polycyclic aromatic hydrocarbons are environmental pollutants (present in cigarette smoke). They have been demonstrated to form stable DNA adducts

in various tissues including human colon mucosa and may be responsible for tumour initiation (Alexandrov *et al.* 1996; Melendez-Colon *et al.* 1999). Alkylating agents result in O(6)-methyldeoxyguanosine (O6-med-G)–DNA adducts which are believed to represent a marker of exposure to *N*-nitroso compounds (NOC). These adducts are in part suggested to account for the risk associated with red meat consumption. DNA damage induced by NOC has been demonstrated to vary within the large bowel, being highest in areas with high incidences of bowel tumours (Povey *et al.* 2000). Most O6-med-G–DNA adducts have been found in the proliferating zone of colonic crypts rather than the differentiated cell compartment (Hong *et al.* 1999). Whilst DNA adducts appear an excellent choice of biomarker for CRC risk, it must be remembered that adduct formation does not always correlate with tumour formation (Alldrick & Lutz, 1989). DNA in cells isolated from the colon can be rapidly and conveniently collected and damage can be assessed using the COMET assay. Hambly *et al.* (1997) demonstrated that the DNA damage in colonocytes isolated from rats fed various diets (i.e. low fat, high fat) could be characterised as low or high risk for CRC with the carcinogen (1,2-dimethylhydrazine DMH). Studies with lactic acid bacteria have shown that some (not all) can strongly inhibit (*N*-methyl-*N*-nitro-*N*-nitroguanidine MNNG) and DMH-induced gentoxicity in rat colon cells. It was postulated that thiol-containing breakdown products created via catalysis by bacterial proteases could be one mechanism by which MNNG and other carcinogens are deactivated (Pool-Zobel *et al.* 1996; Wollowski *et al.* 1999). The cytotoxic and genotoxic effects of numerous known carcinogens were studied in freshly derived human colon tumour cells and in the rodent equivalent, with some disparate results (Pool-Zobel & Leucht, 1997). Although as yet the technique has not been employed in colon biopsy specimens from human intervention trials, it has been used successfully to demonstrate a protective effect against DNA damage in human lymphocytes for carotenoid-rich foods such as tomato, carrot or spinach. Mechanisms suggested to account for the effect of some vegetables include enhancement of cytosolic (glutathione S-transferase pi GST pi) and DNA repair proteins (Pool-Zobel *et al.* 1997, 1998).

DNA repair

Colon mucosal cells are under constant, but low, genotoxic stress. Such damage is normally repaired, but any factors influencing the integrity of the repair process are important in determining the risk of cancer. DNA mismatch repair genes, if inactivated, tend to result in the colorectal mucosal cells accumulating additional mutations at a higher rate, potentially enhancing their capacity for malignant transformation (Boland, 1996). The inactivation of this critical system results in genomic instability, which is particularly evident at microsatellite loci. These are highly repetitive sequences scattered throughout the human genome, most commonly CA, GT, A, T sequences. Currently a panel of five marker genes exists to determine the degree of microsatellite instability (MSI). Tumours can be characterised

as high frequency (MSI-H) if two or more markers show instability or low frequency (MSI-L) if only one marker is affected (Boland *et al.* 1998). The majority of hereditary non-polyposis CRC malignancies show MSI, as do approximately 10–15 % of all sporadic colorectal tumours. Therefore the presence of MSI indicates a failure in the DNA repair mechanisms and an increased susceptibility to genotoxic dietary agents, thus elevating the risk of CRC. No dietary intervention studies have yet been performed with this endpoint.

Oncogene and tumour-suppressor gene mutation

Mutations in specific oncogenes and tumour-suppressor genes (e.g. K-*ras*, p53) are involved in tumour development in the colon, as they occur at high frequencies in human colon cancers (Erdman *et al.* 1997). The expression levels of these genes can be measured and used to demonstrate the effect of various dietary components on tumour formation in colonic mucosa. Addition of the probiotic *Bifidobacterium longum* to the diet of rats was shown to exert a strong antitumour activity on colonic mucosa by reducing the expression level of *ras*-p21 expression and cell proliferation. (Singh *et al.* 1997*b*; Reddy, 1998). Similarly, a diet high in fish oil (*n*-3 fatty acids) has also been demonstrated to decrease expression of *ras*-p21 in colonic mucosa, whilst a diet high in corn oil (*n*-6 fatty acids) appears to promote tumourigenesis as it increased the expression of this gene (Singh *et al.* 1997*a*). Chronic use of sennoside laxatives increases the risk of CRC in rats; a dose of highly purified senna extract has been demonstrated to increase cell proliferation rates in colonic mucosa, although expression of *ras*-p53 was unaffected (Van Gorkom *et al.* 2000).

In conclusion, the use of novel tissue biomarkers (genotoxicity, DNA repair capacity, oncogene mutations) offer great potential as judged by numerous animal studies and some limited human data, but they are yet to be exploited widely for dietary intervention trials with colonic biopsies.

Biochemical markers

In contrast to the invasive techniques needed to obtain colonic tissue, the majority of biochemical markers available can be readily measured in blood, urine or faeces, and thus are minimally or non invasive. Biochemical markers are composed of two main groups, mammalian enzymes and gut microflora associated biomarkers. The former are measures of specific endogenous enzyme activity in blood, urine or biopsy specimens, e.g. CYP1A2, GST, whilst the latter include bacterial enzyme activities and faecal metabolites.

Mammalian enzyme biomarkers

A common biomarker is the hepatic enzyme CYP1A2, a member of the cytochrome P450 superfamily. It is involved in the metabolic activation of several carcinogens including aromatic and heterocyclic amines. Wide inter-individual variation has been described in CYP1A2 activity and this variation may relate to susceptibility to cancer.

Individuals with high CYP1A2 activity are suggested to have an increased risk of CRC (Lang *et al.* 1994). Induction of CYP1A2 activity has been reported as a consequence of several factors including diet (reviewed Landi *et al.* 1999). Ingestion of a diet rich in chargrilled meat has been shown to induce CYP1A2 activity (Fontana *et al.* 1999), while associations have also been described between cooked food, increased CYP1A2 activity and increased CRC risk (Lang *et al.* 1994; Badawi *et al.* 1996). Vegetables appear to have a diverse affect on CYP1A2 with brassica (cruciferous) vegetables increasing and apiaceous (carrot and parsley-like) vegetables reducing activity levels (Lampe *et al.* 2000*b*). These effects may in part be the result of the presence or absence of various chemopreventive flavonoids, which are suggested to inhibit activity of CYP isoforms (Sousa & Marletta, 1985; Bear & Teel, 2000; Shih *et al.* 2000).

GST (alpha, pi, mu) are an important family of phase II detoxification enzymes, which play a crucial role in protecting the colon mucosa from dietary carcinogens. Reduction in GST activity is associated with an increased risk for CRC (Szarka *et al.* 1995). Dietary components have been demonstrated to modulate GST activity in the intestine of rats (O'Neill *et al.* 1997) and man (Nijhoff *et al.* 1995). Interestingly, consumption of Brussels sprouts was found to increase GST in rectal biopsies and lymphocytes from blood, suggesting that this marker could be used as a less invasive method. Supplementation of the diet with resistant starch elevates GST (pi) levels in the rat colon probably through alteration of the colonic fermentation profile and microflora population (Treptow van Lishaut *et al.* 1999). Wheat bran and soya also increase GST activity in the rat colon, with the effects attributed to micronutrients or phytochemicals rather than dietary fibre (Appelt & Reicks, 1997; Helsby *et al.* 2000). The use of lactulose separately or in conjunction with *B. longum* has been demonstrated to significantly increase GST levels in rat colon (Challa *et al.* 1997). Apiaceous vegetables (celery, carrot and parsley-like vegetables) appear to decrease human GST activity in specific groups, whereas brassica vegetables have consistently been shown to increase GST activity levels (Nijhoff *et al.* 1995; Lampe *et al.* 2000*a*).

Gut flora-associated biomarkers

The relationship between diet, gut microflora and CRC is complex and intimate. Substances entering the colon from the ileum and the resident microflora are major determinants of colonic physiology. These, together with the innate biology of the colon, are pertinent to the initiation and promotion of CRC. The concentration of bacteria resident in the colon increases distally with an estimated 300–400 different cultivatable species (belonging to 190 genera) resident within a healthy adult colon (Holzapfel *et al.* 1998). Approximately 100 of these species are present at concentrations around 10^{11} CFU/g. The anaerobes, *Bacteroides*, *Bifidobacterium* and *Eubacterium* spp., represent greater than 99 % of those species present in the colon. Once the microflora is established, the composition shows little qualitative variation over time (Kleibeuker *et al.* 1996*b*), although there is considerable evidence that the metabolic activity of the microflora can be modulated by diet especially non-digestible carbohydrates (fibre, oligosaccharides; Rowland *et al.* 1985; Rowland, 1988). With the capacity for the microflora to modulate colonic conditions, it becomes obvious why analysing their dynamic interaction with the colonic environment and mucosa is of such interest. The microflora has been implicated in the aetiology of CRC in a number of studies (reviewed in Mallett & Rowland, 1990) and these observations form the theoretical basis for use of gut flora biomarkers (faecal biomarkers). These biomarkers are advantageous as they can be assessed in faeces, which is readily accessible and non-invasive. They are composed of two main categories; those examining the activity of bacterial enzymes or bacterial metabolites and those based on bioassays of faecal water and metabolites. A number have been used to investigate gut bacterial function (Rowland *et al.* 1991; 1998) and some have potential mechanistic links to CRC aetiology — for example, 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) activation, β-glucuronidase and ammonia production.

Bacterial enzymes/metabolites

IQ activation

Heterocyclic amines (HCA) are formed during cooking of protein-rich foods. Normally, these molecules require activation by the liver to become mutagens and carcinogens capable of producing tumours in various tissues in rodents and non-human primates (Nagao & Sugimura, 1993). Certain isoflavones potentially offer a protective effect against HCA-induced carcinogenesis, by reducing DNA adduct formation (Agus *et al.* 2000). The HCA IQ is a compound that can also be activated in the colon by *Eubacterium* and *Clostridium* species to its genotoxic derivative 7-hydroxy-IQ (Van Tassell *et al.* 1990), which is genotoxic to colon cells in a dose responsive manner (IR Rowland, unpublished results). Diet and the human gut flora interact to modulate the activation rates of IQ (Rumney *et al.* 1993*b*). A high-risk diet (high fat and sugar, low calcium) increases activation levels relative to low-risk diets (low fat, high starch) in human flora associated (HFA) rats (Hambly *et al.* 1997). Diets high in animal fat or fibre also elevate activation levels (Rumney *et al.* 1993*a*). Transgalactosylated oligosaccharides supplementation has also been shown to decrease IQ activation in HFA rats (Rowland & Tanaka, 1993).

β-Glucuronidase

Many toxic and carcinogenic compounds and also endogenously produced compounds such as steroids are metabolised in the liver and then conjugated (with glucuronic acid) by phase II enzymes before being excreted via the bile into the small intestine. In the colon, the bacterial enzyme activities of β-glucuronidase can hydrolyse the conjugates, releasing the parent compound or its hepatic metabolite. In the case of carcinogens and mutagens, the activity of β-glucuronidase in the colon may increase

the likelihood of tumour induction. The colon carcinogen DMH is metabolised in the liver and small amounts of the procarcinogenic conjugate of the activated metabolite methylazoxymethanol (MAM) are excreted in the bile. Hydrolysis of the conjugate by colonic bacteria releases MAM into the colon. Germ-free animals treated with DMH or MAM have fewer colon tumours than do conventional animals (Reddy *et al.* 1974), and the use of a β-glucuronidase inhibitor administered in conjunction with the carcinogen (azoxymethane) significantly reduces the number of tumours in the rat colon, indicating that microflora-derived β-glucuronidase has an important role to play in the aetiology of colon cancer (Takada *et al.* 1982). The activity of β-glucuronidase is influenced by diet, with high-risk diets consistently shown to increase β-glucuronidase activity relative to low-risk diets (Eriyamremu & Adamson, 1995; Reddy *et al.* 1977). Recently Hambly and colleagues demonstrated a 2·5-fold increase in β-glucuronidase activity on a high-risk diet (Hambly *et al.* 1997). Alternatively various types of fibre including coffee fibre, resistant starch and rice bran decrease the activity of β-glucuronidase in rats, with the extent of the effect apparently dependent on the nature of the fibre (Gestel *et al.* 1994; Maziere *et al.* 1998; Rao *et al.* 1998). In human studies, wheat bran, oat bran and wholemeal rye have been demonstrated to reduce β-glucuronidase activity (Reddy *et al.* 1992; Grasten *et al.* 2000). Inhibitory effects on enzyme activity have also been observed using lemon grass extract, cumin and black pepper (Suaeyun *et al.* 1997; Nalini *et al.* 1998). Dietary supplementation with *B. longum* has proved successful in both rats and man, by decreasing β-glucuronidase activity, suggesting that the probiotic influences the metabolic activity of certain types of intestinal microflora.

Faecal ammonia

Epidemiological studies indicate a link between a high intake of protein and the incidence of colon cancer. These studies, together with investigations *in vitro* and in laboratory animals, have led to the hypothesis that colonic ammonia, a toxic substance produced by bacterial catabolism of protein and urea (Clinton, 1992), may be a potential tumour promoter in the colon. Bacteria assimilate nitrogen to create bacterial protein during carbohydrate fermentation. The colonic ammonia concentration at any given moment therefore depends upon the balance between bacterial protein synthesis and amino acid deamination. As such, increasing the protein content of the diet increases the colonic luminal concentration of protein breakdown products. Ammonia exhibits a number of effects that suggest it may be involved in tumour promotion, including increasing colonic epithelial cell proliferation (Ichikawa & Sakata, 1998), altering morphology and DNA synthesis and reducing the lifespan of cells (Visek, 1978; Lin & Visek, 1991). More definitively, it has been shown to increase the incidence of colon carcinomas induced by MNNG in rats (Clinton *et al.* 1988). A low level of ammonia production in the gut is associated with low protein, high fibre diets, which appear to protect against cancer of the colon. Ammonia levels have been shown to be elevated

in rats consuming a diet containing high risk factors for CRC (Hambly *et al.* 1997). Supplementation of the diet with resistant starch decreased ammonia concentration in HFA rats, whilst wholemeal rye had no effect on human colonic ammonia concentration (Silvi *et al.* 1999; Grasten *et al.* 2000). Modification of bacterial metabolism was successful using the probiotic *B. longum* in rats as it significantly decreased caecal ammonia concentration (Rowland *et al.* 1998). Inulin, a chicory fraction containing β(2-1) fructans, also reduces ammonia in the gut, although not always significantly (Rowland *et al.* 1998; Hughes & Rowland, 2001). Transgalacto-oligosaccharides, which are completely fermented in the human colon, also have been shown to have no effect on ammonia concentration (Alles *et al.* 1999).

N-*nitroso compounds*

Nitrate, ingested via diet and drinking water, is readily converted by the nitrate reductase activity of the intestinal microflora to its more reactive and toxic reduction product, nitrite. Nitrite reacts with nitrogenous compounds such as amines, amides and methylureas in the body to produce NOC, many of which are highly carcinogenic, DNA-alkylating agents (Hughes *et al.* 2001). The reaction can occur chemically in the acidic conditions prevalent in the human stomach and can also be catalysed at neutral pH by gut bacteria. Bacterial N-nitrosation occurs in the large intestine and can be analysed using a method that determines apparent total NOC (ATNC) in the faeces and several biological fluids. Such an approach was used to demonstrate that N-nitrosation in the large intestine of rats is dependent on the presence of gut microflora (Massey *et al.* 1988). Although the mechanism for bacterial N-nitrosation is still unknown, concentrations of ATNC positively correlate with intestinal transit time and inversely with faecal output (Hughes *et al.* 2001). In faecal samples from healthy human subjects, ATNC was detected and its excretion related to both dietary nitrate (Rowland *et al.* 1991) and red meat consumption (Silvester *et al.* 1997; Hughes, 1999; Hughes *et al.* 2001). However a similar association was not found for white meat or fish (Cummings *et al.* 1996). In conjunction with high meat intakes, wheat bran, resistant starch and vegetable consumption had no effect on faecal ATNC excretion or concentration (Cummings *et al.* 1996; Silvester *et al.* 1997; Hughes, 1999).

Diacylglycerols

Protein kinase C (PKC) activation is a critical step in the stimulation of cell proliferation. The enzyme can be directly activated *in vivo* by the secondary messenger diacylglycerol (DAG), which is a lipid produced by phospholipase C catalysed hydrolysis of phosphatidyinositol and polyphosphoinositides. DAG activates PKC by decreasing the enzyme's requirement for calcium ions. The sustained activation of PKC is believed to be a critical event in tumour promotion as it affects regulation of long-term cellular events such as proliferation and differentiation. Evidence supporting this hypothesis is provided

by phorbol ester tumour promoters which potently activate PKC by a mechanism that appears analogous to that of DAG. Bacterial microflora can also produce DAG using phospholipids and triglycerides from the colonic contents. The bacterial production of DAG can be enhanced by bile acid (Morotomi *et al.* 1990). Unsaturated fatty acids and bile acids activate PKC and increased cell proliferation in rat colons, either through a direct method or through an action on the cell membrane (Craven & DeRubertis, 1988). Hence human subjects with grossly increased fat content of the faeces, e.g. jejunoileal bypass patients, have extremely high luminal DAG concentrations and hyperproliferation of the colonic epithelium (Steinbach *et al.* 1994). For a detailed review of PKC and its implications in cancer the reader is referred to reviews by Blobe *et al.* (1994) and O'Brian & Ward (1989).

Fecapentaenes

These potent and direct-acting mutagens are glycerol ether compounds containing a pentaene moiety with a chain length of twelve or fourteen (fecapentaenes twelve or fourteen). Both types are found in faeces, although the ratio varies considerably (Baptista *et al.* 1985). Although fecapentaenes occur in faeces of the majority of Western populations, more detailed epidemiological studies have revealed some anomalies. For example, lower fecapentaene levels have been found in faeces from colorectal patients than in controls (Schiffman *et al.* 1989) and faecal excretion of fecapentaenes is higher in vegetarians, who are at lower risk from colon cancer (de Kok *et al.* 1992). These and other studies suggest that the more fecapentaene excreted the lower the risk of CRC. This reasoning has a certain logic since fecapentaenes are very potent, direct-acting mutagens which would be expected to react extremely rapidly with DNA and other macromolecules in the colonic mucosa. Thus increased faecal excretion may reflect lower endogenous exposure to the genotoxin. The gut microflora has been implicated in fecapentaenes synthesis by the demonstration of this process *in vitro* by faecal suspensions, under anaerobic conditions, and the inhibition of synthesis by antibiotics and heat sterilisation (Wilkins *et al.* 1980; Hirai *et al.* 1982). Further work has established that *Bacteroides* species are the organisms mainly responsible for fecapentaene production (Van Tassell *et al.* 1982*a*). Production of mutagens *in vitro* is stimulated by bile acids (Van Tassell *et al.* 1982*b*; Nair *et al.* 2000) although they are not thought to be precursors. However, *in vivo* studies have yielded more equivocal results, as some studies have demonstrated mucosal proliferation, DNA damage and tumour-promoting activity (Hinzman *et al.* 1987; Vaughan *et al.* 1987; Zarkovic *et al.* 1993), whilst others have reported no carcinogenic properties (Ward *et al.* 1988; Weisburger *et al.* 1990; Shamsuddin *et al.* 1991).

Secondary bile acids

The bile acids consist of a number of related amphiphilic acidic steroids. The primary bile acids, chenodeoxycholic acid and cholic acid, are synthesised from cholesterol in the liver (Hofmann, 1984), conjugated with taurine or glycine and released into the bile to solubilise fats and cholesterol for uptake in the small intestine. The primary bile acids are subject to extensive metabolism by the intestinal microflora (MacDonald *et al.* 1993), predominantly 7-α-dehydroxylation, which converts cholic to deoxycholic acid (DCA) and chenodeoxycholic to lithocholic acid (LCA), thereby increasing their hydrophilicity. These secondary bile acids, which comprise over 80 % of the faecal bile acids, are postulated to play an important role in the aetiology of colon cancer by acting as promoters of the tumourigenic process (Nagengast *et al.* 1995). The capacity of DCA to enhance colon tumour development in the rat colon could be attenuated by all-trans retinoic acid (Narahara *et al.* 2000). It is also postulated that a high DCA concentration and DCA to LCA ratio may be a risk indicator of CRC (Kamano *et al.* 1999). However a recent study by de Kok found no significant correlations with either bile acid concentrations or ratios (de Kok *et al.* 1999). Although there is no definite proof that bile acids are the cause of CRC, there is considerable evidence to indicate that acid steroids, in particular secondary bile acids, can exert a range of biological and metabolic effects. They induce cell necrosis, hyperplasia, metabolic alteration and DNA synthesis in intestinal mucosal cells, enhance the genotoxicity of a number of mutagens in *in vitro* assays, and exhibit tumour-promoting activity in the colon (reviewed by Rowland *et al.* 1985; Radley *et al.* 1996; Chaplin, 1998; Hofmann, 1999). Secondary bile acids can also induce DNA damage in colon cells (Venturi *et al.* 1997), leading to apoptosis, as DCA-induced DNA damage triggers calcium ion dependent apoptosis, in a manner independent of p53 (Marchetti *et al.* 1997; Powolny *et al.* 2001). It has also been suggested that secondary bile acids influence CRC by selecting for apoptosis-resistant cells (Payne *et al.* 1995) or potentially through bile acid interactions with important secondary messenger signalling systems known to be activated in CRC (arachidonic acid–prostaglandin E2 and PKC; reviewed in Radley *et al.* 1996). Serum levels of DCA are correlated with increased rates of mucosal proliferation, which is a known factor in CRC causation (Ochsenkuhn *et al.* 1999). Diet has an obvious and pronounced effect on bile acids as high levels of animal fat and protein increase both secretion and flow (Thompson *et al.* 1985; Villalon *et al.* 1992), but by itself diet cannot be considered harmful, unless in the absence of balancing amounts of carbohydrate (Chaplin, 1998). Fibre (ispaghula husk) has been shown to lower faecal levels of LCA and decrease DCA to LCA ratio (Anderson *et al.* 1988; Chaplin *et al.* 2000).

Faecal water activity

There is considerable evidence that colon tumours are a result of gut luminal factors damaging the mucosa. Furthermore, free reactive and soluble factors are more likely to affect the epithelium than substances bound to the insoluble matrix such as fibre. Therefore, an alternative approach to assaying enzymes or metabolites in faeces is to assess toxicological activity of fractions using short-term tests

for toxicity, genotoxicity and mutagenicity. Usually the aqueous phase of the human faeces (faecal water) is used (Rafter *et al.* 1987), since this will contain most of the free reactive species. This approach provides a direct estimate of the potential of the faecal sample to damage the colonic mucosa and has been used to provide insights into the possible processes involved in colon cancer including cytotoxicity and genotoxicity. Despite considerable intra- and interindividual variability in faecal water activity, effective experimental protocols can enable detection of dietary modulations of the level of toxic and genotoxic effects (Osswald *et al.* 2000).

Faecal water cytotoxicity

Proliferative zone expansion in the colonic crypts and an increased rate of epithelial proliferation are often viewed as an early step in carcinogenesis. Stimulation of proliferative activity in colonic epithelial may in part be mediated by chemical or physical cytotoxic mechanisms, such that epithelial damage induced by these sources would increase cell loss at the epithelial surface. This would result in a compensatory increase in the mitotic activity of the crypts, thus elevating the risk for CRC. Such considerations led to the development of assays to assess cytotoxic activity in faecal water towards colon cells *in vitro* (Rafter *et al.* 1987). It is thought that bile acids, especially secondary bile acids, make a major contribution to faecal water cytotoxicity (Rafter *et al.* 1987). This technique has been used in a number of dietary intervention studies. Dietary calcium has frequently been shown to reduce the cytotoxicity of faecal water presumably by precipitating soluble bile acids (Van der Meer *et al.* 1991, 1997; Govers *et al.* 1993; Lapre *et al.* 1993). Interestingly a recent study showed that a shift from a dairy product rich diet (high fat) to a dairy product free diet showed an increase in cytotoxicity of the faecal water, possibly as a result of decreased calcium (Glinghammar *et al.* 1997). Conflicting data exist regarding the effect of resistant starch supplementation on human faecal water activity, with outcomes indicating a decrease or no change in faecal water cytotoxicity (Van Munster *et al.* 1994; Heijnen *et al.* 1998). High red meat consumption, which is associated with an increased risk of colon cancer, increases the cytotoxicity of faecal water in rats. This effect was independent of the fat and bile acids content of the faecal water and may be related to dietary haem (Sesink *et al.* 1999, 2000).

Faecal water genotoxicity

There is now convincing evidence that CRC is induced by a series of mutational events in a number of critical genes (Vogelstein *et al.* 1988). How these mutations arise and what induces them is still not fully understood, as there are a variety of causes. Dietary factors are believed to have a role in the carcinogenic process, as the potential for components of food (e.g. HCA, wheat bran) or their metabolites to influence genetic damage has been examined with mutagenic assays (Venitt *et al.* 1986; Knize *et al.* 1995; Johansson *et al.* 1998; Sinha *et al.* 2001). Venturi *et al.* (1997) pioneered the use of faecal water

genotoxicity testing as a means of assessing the ability of the colonic environment to induce DNA damage in epithelial cells. A wide variation in the genotoxicity of faecal water samples obtained from thirty-five healthy subjects was found, ranging from negligible to high activity (Venturi *et al.* 1997). Furthermore a human intervention study demonstrated that a shift to a dairy product poor diet did not affect faecal water genotoxicity even though the cytotoxicity did increase (Glinghammar *et al.* 1997). Most recently it was shown that a diet high in fat and meat but low in dietary fibre increased faecal water genotoxicity, which may contribute to an enhanced risk of CRC (Rieger *et al.* 1999).

Conclusions

There is no doubt that in the last few decades we have greatly expanded our knowledge about the causes and risk factors associated with CRC. Currently, a wide range of potential biomarkers is available to augment investigations into the activity of specific foods, compounds and metabolites in CRC. Most of these assays still require further validation before they can be used with complete confidence and compromises must be made in terms of study complexity and the strength of the causal information provided. It is apparent, however, that a sound theoretical basis exists for the use of biomarkers in the study of CRC. The validation of a biomarker is critical to its application as a research tool, as an appropriate response from the indicator is required when used in cancer patients (e.g. FAP) or in healthy individuals on low-risk and high-risk diets for CRC.

Acknowledgements

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