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Emerging diet-related surrogate end points for colorectal cancer: UK Food Standards Agency diet and colonic health workshop report

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The UK Food Standards Agency convened a group of expert scientists to review current research investigating emerging diet-related surrogate end points for colorectal cancer (CRC). The workshop aimed to overview current research and establish priorities for future research. The workshop considered that the validation of current putative diet-related surrogate end points for CRC and the development of novel ones, particularly in the emerging fields of proteomics, genomics and epigenomics, should be a high priority for future research.

Colorectal cancer: Diet: Food Standards Agency: Biomarkers: Surrogate end points

On 14 February 2003, the UK Food Standards Agency (FSA) convened a workshop on emerging diet-related surrogate end points for colorectal cancer (CRC). The results from recently completed studies and details from ongoing studies were presented (both FSA- and non-FSA-funded). The workshop was chaired by Professor Joseph Rafter (Karolinska Institute, Sweden). The aim of the workshop was to determine where further work should be concentrated, as well as acting as a vehicle for dissemination. The research recommendations will feed into the future direction of FSA-funded nutrition research, and may also be of value in guiding other funding agencies.

CRC is the second most common cancer in men and women in the UK, only surpassed by lung and breast cancers respectively. While a small proportion (<5 %) of CRC is attributable to familial cancer syndromes (familial adenomatous polyposis and hereditary non-polyposis CRC), the majority appear to arise sporadically. There is strong epidemiological evidence for environmental factors in the development of sporadic CRC. There are substantial numbers of dietary factors, and factors related to the diet, which may modify the risk of CRC, e.g. vegetable-rich diets are thought to be protective (Baron, 2001). The mechanisms by which dietary factors can alter the risk, however, and a clear causal link between diet and the risk of CRC, are yet to be fully established.

Intervention studies or prospective observational epidemiological studies that use incident cancer as an end point are large, lengthy and costly. For this reason, studies with surrogate end points, biomarkers of preclinical carcinogenesis, are attractive: they are potentially smaller, shorter and less costly. Studies based on such surrogate end points are, however, inherently less informative than studies with the ‘true’ end point (e.g. incident cancer, cancer recurrence or cancer mortality).

The UK FSA Diet and Colonic Health research programme’s initial focus is the development of validated diet-related surrogate end points for CRC risk; once these

Abbreviations: CRC, colorectal cancer; FISH, fluorescence *in situ* hybridisation; FSA, Food Standards Agency; GST, glutathione S-transferase; HNE, 4-hydroxy-2-nonenal.

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have been established they will be used in dietary intervention trials.

Background

Most cases of sporadic CRC are believed to develop via the adenoma–carcinoma sequence: although not proved directly, this is supported by a considerable body of indirect evidence: epidemiological, clinical, histopathological and genetic studies (Leslie *et al.* 2002). This underpins the use of adenomatous polyp (adenoma) recurrence and regression in high-risk populations as a surrogate outcome for CRC. Only a small proportion of adenomas, however, progress to cancer (Leslie *et al.* 2002). It is unlikely that there would be a definitive clinical trial of dietary factors for the primary or secondary prevention of CRC, because a large sample and long follow-up would be required, and adenoma occurrence is generally regarded as a valid surrogate end point (Baron, 2001).

Germline mutations in *APC* and DNA mismatch repair genes (e.g. *hHLM*) are responsible for familial adenomatous polyposis and hereditary non-polyposis CRC respectively (Leslie *et al.* 2002); somatic mutations occur in these genes in a high proportion of sporadic CRC. A multistep model for the genetic events in the progression of sporadic CRC has been proposed (Vogelstein *et al.* 1988). Underlying genetic alterations have been described in three classes of gene: tumour suppressor (e.g. *p53*, *APC*) and DNA repair genes (e.g. *hMLH*, *MYH*) and oncogenes (e.g. *K-ras*) (Vogelstein *et al.* 2000). It has been suggested that the accumulation, rather than the specific nature and temporal order, of mutations is responsible for tumour development (Kinzler & Vogelstein, 1996); a recent study revealed the heterogenous nature of *APC*, *K-ras* and *p53* tumour mutation patterns, suggesting multiple genetic pathways to CRC (Smith *et al.* 2002).

Mucosal homeostasis requires a balance between proliferation and apoptosis (Bedi *et al.* 1995), and it is thought that an imbalance in the mucosa gives rise to adenomas. Field effects (an imbalance in mucosal homeostasis affecting the whole colorectal mucosa) may long predate the emergence of identifiable focal lesions. Apoptosis activity in the normal rectal mucosa has been shown to be negatively associated with adenomas elsewhere in the colon, consistent with the existence of a field effect (Martin *et al.* 2002). Normal mucosal proliferation rates, however, have not been shown to be associated with adenoma development (Keku *et al.* 1998; Sandler *et al.* 2000; Martin *et al.* 2002). In another recent study, apoptotic activity in the flat mucosa was lower in patients with colonic neoplasia than in individuals without (Bernstein *et al.* 2002). Dietary factors may modulate the vulnerability of the colorectal mucosa at these early stages (Johnson, 2002); however, the mechanisms underlying pre-neoplastic changes in the mucosa are poorly defined.

Epigenetic indicators

Epigenetic mechanisms do not involve alterations to the DNA sequence, but cause somatic cells to acquire changes in gene expression that are transmissible through mitosis. A variety of regulatory proteins including DNA methyltransferases, methyl-CpG (the covalent binding of a methyl group to the 5′ position of a cytosine nucleotide adjacent to a guanine nucleotide) binding proteins, histone-modifying enzymes, and chromatin remodelling factors and their multi-molecular complexes are involved in the overall epigenetic process. Epigenetic events are susceptible to change and may be involved in the mechanisms by which environmental factors modify cancer risk.

DNA methylation (CpG) is an important epigenetic feature of DNA, shown to regulate gene expression (Jones & Takai, 2001) and chromosomal stability (Chen *et al.* 1998). The supply of methyl groups for the formation of *S*-adenosylmethionine, DNA methyltransferase activity and DNA demethylation activity could all affect the extent of DNA methylation.

Abnormal DNA methylation patterns are evident in most cancers, including colon, lung, prostate and breast cancer; global DNA hypomethylation, accompanied by gene-specific hypermethylation, is a common characteristic among tumour cells (Baylin *et al.* 1998). Global DNA hypomethylation has been associated with genetic instability (Chen *et al.* 1998). Interestingly, gene-specific hypermethylation in normal colorectal mucosa has been shown to be positively associated with age (Ahuja *et al.* 1998), although considerable individual variation was also observed.

Gene-specific hypermethylation of CpG islands (CpG-rich sequences located in the promoter region or first exon of genes) is associated with the inactivation of virtually all pathways involved with the cancer process, including DNA repair (e.g. *hMLH1*, *MGMT*), cell cycle regulation (e.g. $p16^{INK4a}$), inflammatory and/or stress response (e.g. *COX-2*) and apoptosis (e.g. *DAPK*, *APAF-1*) (Jubb *et al.* 2001). Unlike the cytosines elsewhere in the genome, CpG islands are normally completely unmethylated in expressed genes.

Alterations in the degree of CpG-island methylation regulate gene promoter regions by modifying the binding of transcription factors and methyl-DNA binding proteins (Jubb *et al.* 2001). Aberrant methylation of CpG islands in the promoter region has been shown to contribute to the genetic dysfunction associated with CRC, e.g. in the *HPP1* (Sato *et al.* 2002), *hMLH1* (Herman *et al.* 1998; Ricciardiello *et al.* 2003) and *APC* genes (Esteller *et al.* 2000). Silencing of the DNA repair gene *hMLH1* in adenomas by promoter methylation is strongly associated with microsatellite instability in sporadic CRC (Ricciardiello *et al.* 2003). In colorectal tumours, promoter hypermethylation of the DNA repair gene O^6-methylguanine-DNA methyltransferase (*MGMT*) is associated with the presence of G:G to A:T transition mutations in *p53* and *K-ras* (Esteller *et al.* 2000, 2001).

Aberrant CpG methylation has also been demonstrated in adenomas (Rashid *et al.* 2001), suggesting the early role of methylation in colorectal tumourigenesis. Whether aberrant CpG methylation underlies field effects remains to be determined; however, it has been shown that age-related methylation of the CpG islands of the hMLH1 promoter does occur in the apparently normal mucosa of patients with CRC, and this abnormality is associated with microsatellite instability (Nakagawa *et al.* 2001). Cancer has, therefore, become to be understood as both a genetic and epigenetic disease with complex connections between the pathways.

Various nutrients appear to affect DNA methylation status, including Se (Davis *et al.* 2000), folate, vitamin B_{12} and choline (Johanning *et al.* 2002), methionine (Rowling *et al.* 2002*a*), retinoic acid (Rowling *et al.* 2002*b*) and isoflavones (Day *et al.* 2002). Interestingly, histone acetylation, another related epigenetic event, is potently inhibited *in vitro* by butyrate (Hinnebusch *et al.* 2002), modified by dietary fibre *in vivo* (Boffa *et al.* 1992) and associated with marked changes in gene expression (Williams *et al.* 2003).

Professor Ian Johnson presented results from an FSA-funded pilot project in collaboration with Newcastle University (NJ Belshaw, GO Elliott, EA Williams, DM Bradburn, SJ Mills and JC Mathers, unpublished results), performed using mucosal biopsies, tumour tissue and faecal samples obtained from consenting gastrointestinal cancer patients. Methods were developed for extraction of faecal DNA and application of methylation-specific PCR (highly sensitive) and combined bisulfite restriction analysis (quantitative). Analysis was performed on six genes known to be involved in adenoma–carcinoma sequence, and/or previously reported to be partially methylated in the flat mucosa of older patients (*ESR1*, *APC*, *hMLH1*, *HPP1*, *P16*, *MGMT*). The results and conclusions were:

- faeces are a practical source of DNA for methylation studies;
- refined combined bisulfite restriction analysis assay provides quantitative estimates of the extent of methylation at specific sites in genes even at low levels of methylation;
- methylation varied significantly and consistently between genes;
- methylation of most, but not all, target genes was enriched in the faecal DNA;
- *ESR1* and *MGMT* both showed substantial levels of methylation in the promoter regions, detectable in DNA in stool. For *ESR1* there was a statistically significant correlation between the levels of methylation measured in the faecal DNA, and those in the morphologically normal mucosa.

The use of faecal DNA samples to detect and quantify DNA methylation in a range of target genes might provide a valuable non-invasive marker of pre-cancerous changes; this marker could be applied in epidemiological studies at the population level. These preliminary observations are now being followed up in a further FSA-funded project that will define CpG-island methylation patterns in a much larger set of genes. Faecal samples are being obtained from both healthy volunteers with widely differing ages, and from patients with well-defined gastrointestinal abnormalities.

In a parallel project, workers the Institute of Food Research (Norwich, Norfolk, UK) and Newcastle University are using two-dimensional gel electrophoresis and MS to characterise the patterns of protein expression in the apparently normal mucosa of human volunteers, with and without evidence of colorectal neoplasia. The objective is to identify consistent differences in gene translation associated with mucosal field changes, to search for possible epigenetic mechanisms underlying such differences and to provide further novel biomarkers of early neoplasia.

Professor John Mathers and Dr Hilary Powers presented details of an ongoing FSA-funded double-blind randomised controlled trial in subjects with no evidence of bowel pathology and in those at enhanced risk of CRC because they carry one or more adenomatous polyps. The aim is to recruit 120 subjects per group. Subjects are randomised to one of four interventions: placebo, 400 μg folic acid/d, 1200 μg folic acid/d, 400 μg folic acid plus 5 mg riboflavin/d. Blood and colonic mucosal biopsies will be collected at baseline and after 6–8 weeks of intervention for assay of:

- biochemical indices of folate status in blood and in mucosa;
- uracil mis-incorporation (a form of damage to DNA);
- whole genome methylation and methylation at specific sites in a panel of CRC-related genes e.g. *hMLH1*, *APC* and *HPP1*.

Because of the interaction between methyl donor status and the common C677T polymorphism in the folate-metabolising gene *MTHFR* on risk of CRC, volunteers will be stratified for this polymorphism. For comparative purposes, baseline measurements are being made on an additional cohort of 120 CRC patients.

Dr Hilary Powers presented preliminary results (MH Hill, HJ Powers, EA Williams, W Bal & M Welfare, unpublished results) from twenty-eight subjects, examining both the responses to intervention in various biochemical measures of folate and riboflavin status and associations between responses in mucosal tissue and plasma. 5-Methyl tetrahydrofolate was determined in small amounts of flat mucosal colonic biopsy material, by homogenisation in ascorbic acid followed by protein precipitation, and analysis by reverse-phase HPLC with fluorescence detection. The main results were:

- plasma 5-methyltetrahydrofolate concentrations increased from an average of 41·4 (SD 23·7) to 74·7 (SD 38·8) nmol/l over the period of intervention, and mucosal 5-methyltetrahydrofolate increased from 0·44 (SD 0·33) to 0·80 (SD 0·39) nmol/g tissue. Riboflavin status also improved, showing a change in erythrocyte glutathione reductase activation coefficient from a mean value of 1·41 (SD 0·12) to 1·30 (SD 0·13);
- at baseline there was a significant association between plasma homocysteine and plasma 5-methyl tetrahydrofolate ($P=0{\cdot}028$), and also between plasma homocysteine and mucosal 5-methyltetrahydrofolate ($P=0{\cdot}001$). A strong positive association was evident between the change in mucosal and plasma 5-methyl tetrahydrofolate ($P=0{\cdot}025$).

The concentration of 5-methyltetrahydrofolate determined in colonic mucosa was comparable with total folates reported in patients with colorectal polyps (Kim *et al.* 1998). Results at this early stage in the intervention (before breaking the randomisation code) support the use of plasma measurements of folate status as a surrogate of responsiveness of colonic mucosa to folate supplementation. Other workers have suggested that in the face of very-high-folate supplements, neither erythrocyte nor plasma folate will accurately reflect colonic mucosal folate (Kim *et al.* 2001).

Angela McGlynn and Rae Dare presented details of FSA-funded projects from Professor Stephen Downes' laboratory, employing comet assays and microarray techniques to quantify global and gene-specific DNA methylation status of colon cells, and to determine whether these patterns of methylation can be modified by folate supplementation.

The alkaline comet assay, originally used to measure DNA damage, was developed, by the use of methylation specific endonucleases *Hpa* II and *Hha* I, to determine the global DNA methylation status of single colonic cells derived from small human biopsies. The endonucleases specifically cleave unmethylated DNA, giving rise to DNA strand breaks and a comet tail after electrophoresis. The level of DNA in the comet tail is therefore indicative of the degree to which the DNA in that particular cell is hypomethylated. The methylation–comet analysis is currently being performed on cells derived from three separate sites of the colon, including tumour or polyp sites and sites adjacent and distal to either, in an ongoing folate intervention study with human subjects.

The combination of fluorescence *in situ* hybridisation (FISH) of DNA sequences with the methylation–comet assay has been developed to measure *p53* gene-specific methylation. Comparison of the number of FISH hybridisation spots in the comet head (unbroken methylated DNA) compared with those in the comet tail (digested hypomethylated DNA) allows some measure of the degree of hypomethylation in the *p53* region, specific to the FISH probe. Folate-depleted colonic cell lines showed increased hypomethylation in the *p53* region. This comet–FISH method was also applied successfully to a small number of human biopsy samples. *p53* gene comet–FISH is currently being further optimised with the design of FISH probes that are entirely specific to the *p53* gene.

Several molecular techniques have been developed to analyse the methylation status of DNA. While sodium bisulfite sequencing is seen as the 'gold standard', there are also a number of other related PCR-based techniques. These approaches usually involve the investigation of a single CpG island, and thus studies using these approaches are limited due to the complex epigenetic nature of cancers.

Microarrays are commonly used to provide information on the expression of tens of thousands of genes. Alternatively, microarrays can also be used to provide information on the methylation status of DNA. Microarray analysis enables investigation of methylation on a global scale, and allows for high-throughput analysis. There are currently two main techniques for microarray methylation analysis: methylation-specific oligonucleotide (Gitan *et al.* 2002) and differential methylation hybridisation microarrays (Yan *et al.* 2002). Methylation-specific oligonucleotide arrays are capable of differentiating methylated and unmethylated CpG sites at specific locations of a promoter, while differential methylation hybridisation arrays allow the methylation status of multiple CpG islands to be determined.

Diet and genetic susceptibility

Dr Ellen Kampman presented preliminary results from an ongoing Dutch case–control study conducted at Wageningen University and Research Centre in collaboration with University Medical Centre Nijmegen (Dr Fokko Nagengast) and regional hospitals to investigate the interplay between diet and genetic susceptibility on risk of colorectal adenomatous polyps. All cases with adenomatous polyps and polyp-free controls filled out the EPIC food-frequency questionnaire, and blood samples were collected for evaluation of biomarkers of exposure (e.g. folate status, fatty acid profiles) and genotyping. In a subgroup of the participants, fat aspirates were collected to assess internal exposure, e.g. to fatty acids and antioxidants.

Genetic variants in metabolising enzymes (e.g. coding region polymorphisms in alcohol dehydrogenases, *N*-acetyl-transferases, glutathione S-transferases (GST), sulfotransferases, microsomal epoxide hydrolase, methyltetrahydrofolatereductase and cyclooxygenases) are being determined. DNA extracted from paraffin-embedded adenoma tissue will be assessed for somatic mutations in tumour suppressor genes and oncogenes as well as for the methylation status of specific genes in collaboration with University Medical Centre Nijmegen and Maastricht University respectively.

- Preliminary results of this study are consistent with the findings of others showing an increased risk of adenomas with smoking, alcohol consumption, and, although not statistically significant, meat consumption (Tiemersma *et al.* 2002). Alcohol consumption especially increased risk among those with the *ADH3*1/*1* genotype (Tiemersma *et al.* 2003). Inverse associations were observed for aspirin use and consumption of dairy foods. Vegetable and fruit consumption do not appear to markedly influence overall adenoma risk in this study. Preliminary analyses including 495 cases and 510 controls also showed no significant associations with folate intake, stratifying for the MTHFR 677 polymorphism and adjusting for age, sex, indication for endoscopy, and intake of total energy and dietary fibre. Analyses will be finalised with data from more than 600 cases and 600 controls. Sub-site analyses, and analyses taking the size, number and histology of the adenomas into account will be conducted.

Among the adenoma cases, an intervention trial is ongoing to assess whether supplementation with folic acid (5 mg/d) and cyanocobalamin (1·25 mg/d) alters gene-specific DNA methylation in the colon differently for those with the *MTHFR-TT* and *MTHFR-CC* 677 genotype (Van den Donk *et al.* 2002). Blood samples as well as colonic biopsies were collected at the beginning and after 6 months supplementation. In a subgroup, mucosal proliferation, apoptosis and the expression of selective response genes, as identified by *in vitro* experiments, will be evaluated. Ongoing experimental research at Wageningen University also focuses on polymorphisms in the antioxidant-response–electrophile-response elements in the promoter region of GST and NAD(P)H:quinone oxidoreductases and inducibility by plant foods.

The antioxidant-response–electrophile-response elements-signalling pathway appears to upregulate expression of several phase II detoxifying enzymes and affects p53 stabilisation in response to stress. Induction of phase II detoxifying enzymes, such as GST and NAD(P)H:quinone oxidoreductases, leads in general to protection of cells and tissues against exogenous and/or endogenous carcinogenic intermediates. Compounds found in plant foods, e.g. flavonoids and isothiocyanates (a gut breakdown product of glucosinolates found in cruciferous vegetables) have been shown to induce phase II detoxifying enzymes (see Johnson, 2002; Lampe & Peterson, 2002).

GST are a super-family of phase II enzymes that may contribute to resistance against oxidative stress (Hayes & Strange, 1995). The best-characterised GST isoenzymes in mammals have been grouped into four major classes, termed alpha (α), pi (π), mu (μ), and theta (θ), but additional forms exist. Null genotypes for *GSTM1* and *GSTT1* occur in frequencies of approximately 50 and 20 % of the population respectively and result in absence of the respective enzymes. Although no overall associations between *GSTM1*, *T1* or *P1* genotypes and CRC risk have been observed, a recent nested case–control study (Seow *et al.* 2002) found a significant reduction in CRC risk among individuals with both *GSTM1* and *T1* null genotypes with a high *v.* low dietary intake of isothiocyanates. Lampe *et al.* (2000) observed that serum GSTα concentration increased significantly in response to cruciferous vegetable feeding, but only in *GSTM1*-null individuals (Lampe *et al.* 2000). Overall, this suggests that GST genetic polymorphisms influence the relationship between cruciferous vegetable intake and cancer risk (Lampe & Peterson, 2002).

Professor Beatrice Pool-Zobel presented results from studies investigating the effects of nutritional components on expression of GST, and on damage to DNA, in human primary colon cells, human colonic adenoma cells (LT97) and a human colon tumour cell line (HT29) (Schäferhenrich *et al.* 2003*a*,*b*). The pre-neoplastic lesions that occur in LT97 are also observed at increasing frequency with increasing age in the general population.

Oxidative stress and resulting lipid peroxidation are possible risk factors for diet-related colon cancer (Pool-Zobel *et al.* 1999; Liegibel *et al.* 2000); however, the risk potential needs to be characterised. The genotoxic effects of 4-hydroxy-2-nonenal (HNE; a lipid peroxidation product), H_2O_2, the induction of GST (Ebert *et al.* 2001) by butyrate (a gut bacteria carbohydrate-fermentation product), complex fermentation products (e.g. products produced *in vitro* after incubating dietary fibres and faecal slurries) and selected phytoprotectants (catechins of green tea, isoflavonoids) were investigated. Butyrate is found in mmol concentrations in the gut lumen and serves as a principal energy source for colon epithelial cells (Roediger, 1989).

Genotoxicity was determined using the comet assay. Sensitivity of *p53*, a crucial target gene for transition of adenoma to carcinoma, was studied with FISH. Expressions of GST isoenzymes, some of which deactivate HNE, were determined as GST-activity and GSTP1 protein levels. Genotoxic impact of HNE was compared in butyrate-treated and non-treated cells using the comet assay. Responses were compared with primary human colon cells and to a differentiated clone of HT29.

The main results were:

- both HNE and H_2O_2 were clearly genotoxic in human colon cells. HNE was more genotoxic in LT97 than in HT29 clone19A and primary human colon cells. DNA regions that were labelled with the *p53*-specific probe migrated more efficiently in to the comet tail than the majority of the global DNA;
- butyrate (4 mM) induced ERK1/2 phosphorylation after 5–30 min. After 24–72 h incubation with butyrate, and some selected mixtures from fermentation samples, GST mRNA, GSTP1 protein, GSTP1 activity and total protein were increased (1·2- to 2·5-fold) and glutathione levels were maintained. Moreover, a marked reduction of HNE-induced genotoxicity was caused by pre-incubation with butyrate and fermentation samples from selected dietary fibres.

HNE was more genotoxic in human adenoma cells than in tumour cells, and this was partly due to the different GST expression-levels. *P53*-labelled DNA regions were more sensitive to HNE than global DNA (Schäferhenrich *et al.* 2003*a*,*b*). Recent studies show that butyrate could play a role in the early and later stages of cancer prevention by reducing exposure to this and other relevant risk factors via induction of different GST in transformed and non-transformed human colon cells (Ebert *et al.* 2003).

DNA adducts

The accumulation of DNA damage (breaks, oxidative damage, adduct formation) may be indicative of increased CRC risk. DNA adducts are formed by genotoxic compounds binding covalently to DNA, and if not repaired, they can lead to mutations, e.g. G:C → T:A transversions. They appear to indicate internal dose exposure to genotoxic agents and levels are affected by diet (Palli *et al.* 2000) and genotype (Matullo *et al.* 2003).

Professor Sheila Bingham presented the results from several FSA-funded projects investigating both the effect of meat consumption on endogenous *N*-nitrosation in the colon, and the relationship between diet and the formation of 1,N2 malondialdehyde–deoxyguanosine adducts and N7-methyldeoxyguanosine and O^6-carboxymethylguanine adducts in the colon. *N*-nitroso compounds are found in the colon and are formed endogenously, because the amines and amides produced primarily by bacterial decarboxylation of amino acids can be *N*-nitrosated in the presence of a nitrosating agent. A number of facultative and anaerobic colonic bacteria are able to catalyse the formation of *N*-nitroso compounds at an optimum pH of 7·5. In the anaerobic large bowel, nitrate is reduced to nitrite during dissimilatory nitrate metabolism by the colonic flora. Supplements of nitrate have therefore been shown to elevate faecal *N*-nitroso compound levels.

Epidemiological studies consistently suggest that high red meat consumption (e.g. 120 g/d) is associated with an increased risk of CRC (Norat *et al.* 2002). Whether increased nitrogenous residues from red meat increase endogenous *N*-nitrosation was examined by feeding increased levels of red meat (0–420 g/d) and measuring apparent total *N*-nitroso compounds in faecal samples in a series of studies of volunteers maintained under controlled conditions. A consistent dose–response was observed to red meat consumption, but not to white meat consumption (Bingham *et al.* 1996, 2002; Silvester *et al.* 1997; Hughes *et al.* 2001, 2002). While red meat diets increased faecal apparent total *N*-nitroso compounds, the equivalent amount of protein from eggs, milk, cheese and vegetable protein has no effect; furthermore, there appears to be a specific effect of haem-Fe whereas inorganic Fe has no effect (Cross *et al.* 2003). Under certain conditions, haems are known to be nitrosated, and act as nitrosating agents. The formation of *N*-nitrosoarginine by haem enzymes under anaerobic conditions has also been demonstrated by Hirst & Goodin (2000).

To determine whether faecal *N*-nitroso compounds are candidates in explaining the association between meat and colon cancer risk, the genotoxic effects of increased *N*-nitrosation is presently being investigated: the effects of faecal water from low *v.* high meat diets on strand breaks (comet test), mouse lymphoma cell line L5178Y mutations and *p53* mutations are being investigated in a yeast mutation assay. The chemical composition of *N*-nitroso compounds is being investigated by liquid chromatography–MS. A method for isolating exfoliated cells from faeces has been developed (Bandaletova *et al.* 2002; Davies *et al.* 2002) and the link between diet and the presence of 1,N2 malondialdehyde–deoxyguanosine adducts in colonic biopsy samples investigated, but no relationship was found (Leuratti *et al.* 2002). This was probably due to the low 1,N2 malondialdehyde–deoxyguanosine adduct levels found in blood that cannot be reliably measured. In previous and ongoing work the presence of N7-methyldeoxyguanosine and O^6-carboxymethylguanine adducts in blood, colonic mucosa biopsy samples and exfoliated cells is being investigated in order to develop exposure and/or intermediate risk markers of meat exposure and large-bowel cancer risk.

Discussion

'Biomarkers' can be explicitly biomarkers of exposure (to dietary components) or biomarkers of risk (surrogate end points of disease), but are not necessarily both. The objective is to identify measurements that can be validated as both biomarkers of exposure and disease risk. The development of appropriate, feasible markers of exposure, e.g. for vegetable consumption, remains important, however, as evidence of the relationship between diet and CRC from epidemiological studies is not always consistent.

For the development of diet-related surrogate end points of CRC, it is important to ascertain whether the test of an association between an exposure and a surrogate end point will reliably indicate whether there is an association between the exposure and cancer. Three statistical conditions are needed to establish this: (1) the surrogate end point is associated with cancer; (2) the exposure is associated with the surrogate end point; (3) the surrogate end point 'mediates' the association between exposure and cancer. An important consideration, as well as reproducibility and variability, is whether the magnitude of the association between exposure and the surrogate end point predicts the magnitude of the association between exposure and cancer.

Relevant changes will probably occur in 'flat' colonic mucosa, i.e. in advance of the development of lesions such as aberrant crypt foci or polyps; these early changes may be apparent as 'field effects' that may be amenable to detection. Events hypothesised to precede clinical lesions may be reversible by appropriate dietary or other manoeuvres. Emphasis, therefore, should be given to early stage indicators that are underpinned by a mechanistic understanding of the carcinogenesis process. Cancer is a genetic disease and surrogate end points need to be related to fundamental processes, e.g. the switching off of tumour suppresser genes by mutation or silencing; equally, modulation by diet must also be demonstrated.

The DNA alterations that underlie CRC are heterogeneous. Case–control studies that have investigated associations between specific mutations and/or alterations in CRC patients and dietary factors point to different pathways in colorectal carcinogenesis in which dietary factors may be involved, e.g. different dietary factors are associated with mutations in k-ras (Slattery *et al.* 2000), p53 (Slattery *et al.* 2002) and adenomatous polyposis coli (Diergaarde *et al.* 2003). There may be, therefore, need to detect alterations in a number of genes, and to consider several biomarkers in combination.

The importance of using human colonic mucosal biopsies was noted. It will be important to investigate parallels with surrogate tissues to determine the extent to which more accessible tissues can be used. The development of non-invasive surrogate end points was also highlighted, and the use of exfoliated mucosal cells in stool (e.g. for the extraction and amplification of colorectal epithelial DNA) for determining biomarkers may have great potential.

Last, the workshop highlighted the need to clarify the exposure of colonic mucosa to food components, i.e. tissue specificity in response to food components. A better understanding of the mechanisms by which nutrients can affect the tissue could underpin biomarker development.

Recommendations

- The validation of current putative diet-related surrogate end points for CRC and the development of novel ones, particularly in the emerging fields of proteomics, genomics and epigenomics.
- To introduce into CRC-screening protocols measures of dietary exposure and the collection and validation of putative diet-related surrogate end points.

Attendees

Professor Joseph Rafter, Karolinska University, Stockholm, Sweden; Professor John Mathers, Professor Doug Turnbull and Dr Elizabeth Williams, Newcastle University, Newcastle, UK; Professor Ian Johnson and Dr Nigel Belshaw, Institute of Food Research, Norwich, UK; Professor Stephen Downes, Dr Rae Dare, Dr Angela McGlynn and Professor Ian Rowland, Ulster University, Coleraine, UK; Professor F. Charles Campbell, Queen's University of Belfast, Belfast, UK; Professor Fokko Nagengast, University Medical Centre Nijmegen, The Netherlands; Professor Beatrice Pool-Zobel, Friedrich Schiller University, Jena, Germany; Dr Ellen Kampman, Wageningen University, Wageningen, The Netherlands; Professor Sheila Bingham and Ms Joanne Lunn MRC Dunn Human Nutrition Unit, Cambridge, UK; Dr Hilary Powers and Dr Bernard Corfe, University of Sheffield; Sheffield, UK; Dr Sharon Moore, Open University, Milton Keynes, UK; Dr Andrew Povey, University of Manchester, Manchester, UK; Dr Judy Buttriss, British Nutrition Foundation, London, UK; Dr Elaine Stone, World Cancer Research Fund International, London, UK; Dr Leigh Henderson, Henderson Scientific Consultancy, Cuddington, UK; Mr Ben Walters, Ms Lynn Burns, Dr Diane Benford, Dr Caroline Tahourdin and Dr Peter Sanderson, FSA, London, UK.

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