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PASSCLAIM¹ – Diet-related cancer

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Summary Background The role of dietary factors in the aetiology of human cancer is an area, which has attracted intense interest in recent years. The suggestion that approximately one third of all cancers may be caused by an ‘inappropriate’ balance of food components has led to the attractive contention that we can significantly decrease cancer incidence through dietary recommendations and a change in dietary habits in populations. Thus, a key issue must be to establish clear criteria, which must be met in order to be able to make ‘cancer risk reduction’ claims for food components. In this area, the one true marker is the malignant human tumour, which for practical reasons is usually not accessible to claims. In its absence, we must rely on alternative markers – biomarkers to the endpoint tumour and their usefulness for making claims. Some claims have been made based on epidemiological studies. Aim Can we identify targets/biomarkers in the chain of events from initial ‘exposure’ to overt malignant tumour, whose modification can be used to make ‘anticancer’ claims for food components? Results We identified 18 targets/markers in the above chain of events whose modification ‘have the potential’ to be used for ‘reduction of cancer risk’ claims for food components. These targets/markers fall under 5 broad headings: tumours & preneoplastic changes; cellular targets/markers; gut luminal markers; angiogenesis & metastasis; carcinogen metabolising enzymes; genetic events. Conclusions The strongest markers presently available are precancerous lesions (e.g. polyps or aberrant crypt foci) in humans and precancerous lesions and tumours in animal models. The only marker that presently can be used for a ‘reduction of disease risk’ claim (type B) for food components is ‘polyp recurrence’. Type B claims cannot be made on the basis of results in animal models. All of the other biomarkers examined presently lack validation against the ‘true endpoint’, the tumour, and thus cannot be used for type B claims. ‘Reduction of disease risk’ claims in the area of ‘diet-related cancer’ should be based primarily on human intervention studies using relevant/acceptable endpoints. An important area for future research will be the validation of these surrogate endpoints.

Key words functional foods – diet-related cancer – colon cancer – biomarkers – surrogate endpoints

¹ Process for the Assessment of Scientific Support for Claims on Foods.
General introduction

Epidemiological studies indicate that the processes of carcinogenesis and tumourigenesis are mainly induced by environmental factors. An extensive review of the link between nutrition and cancer on a global scale has been recently published by the World Cancer Research Fund (WCRF) and the American Institute for Cancer Research (AICR). Some 35% of all cases of death by cancer are caused by high risk diet (e.g. high saturated fat/low fibre/low consumption of fruit and vegetables), 30% is due to tobacco smoking, together with reproductive behaviour (7%) and alcohol abuse (3%), thus about 75% of death by cancer is due to diet and lifestyle. Another 20% are due to an unhealthy environment (7%), infection (9%) and pollution (4%). Finally there are about 5% of death by cancer which are caused by hereditary, genetic factors.

Cancer incidence in Europe is highest for lung cancer in man and breast cancer in women, followed by colorectal cancer for both sexes and then prostate cancer in man and lung cancer in women. Smoking is the major cause for lung cancer and diet, but particularly a diet high in antioxidant-rich foods (fruits and vegetables) reduces the risk of disease even in heavy smokers.

The development of breast and prostate cancer seems to be linked to the hormonal status and both cancers are promoted by endogenous estrogens, respectively. However, phytooestrogens from dietary sources appear to counteract the tendency of the chromosomes to become shorter during a round of replication. Consequently, inhibiting telomerase decreases the potential of cancer cells to become immortal. It has been demonstrated in cell systems that certain flavonoids potently affect telomerase activity.

Besides being immortalised, tumour cells have infinite replicative potential. DNA replication takes place in the S-phase of the cell cycle. The process of replication essentially duplicates the genetic material with the help of the replication machinery (DNA polymerases, DNA ligases, topoisomerases). Inhibiting replication ensures that malignant cells do not progress in the cell cycle.

Healthy cells undergo apoptosis, programmed cell death. This process is initiated by means of death sensors and effectors. Evading apoptosis as tumours cells do, requires mechanisms that inhibit transmission of the death signals. Potentially any mechanisms which
specifically would increase the rate of apoptosis in tumour cells could be used in cancer therapy.

Fast growing tumours need oxygen and nutrients, which need to be provided by new blood vessels. Malignant cells can induce angiogenesis by secreting vascular endothelial growth factors (VEGFs) and fibroblast growth factors (FGF1/2). The signalling pathways leading to angiogenesis are activated when these growth factors bind to their receptors on the endothelial cell. The signalling leading to angiogenesis also can occur via integrin receptors (integrins are transmembrane proteins promoting adhesion of cells).

When primary cancer cells come loose out of the tumour mass, they can metastasise and invade healthy tissue anywhere in the body, where they can further proliferate, and destroy surrounding tissue, or hinder it in its physiological functioning. The loosening of cells is possibly due to altered expression of adhesion molecules, into less ‘sticky’ ones. Another retained possibility is through the activation of matrix metalloproteases (MMPs). These deteriorate extracellular matrix proteins (laminin and collagen). It was also observed that cell surface integrin receptors, which were isolated from metastatic cancer cells more efficiently bind to degraded matrix proteins.

The clinical therapy of existing cancers is still primarily based on surgery, radiation treatment and chemotherapy. However, for example advanced colon carcinoma are very refractive to any of these therapies. It is put forward that cancer more efficiently can be controlled by reducing the risk to get it, by preventing it. In order to make a healthy cell cancerous, several errors need to occur at the same time. Many of the external factors, which seem to cause death by cancer can be controlled. Nutrition entails some of the external factors which can cause errors in a cell to occur, and as such certainly has a potential to reduce the risk for cancer. The enzymes involved in the biotransformation of xenobiotics, e.g. phase I and II enzymes, are involved in toxification of chemical carcinogens like the aflatoxins; however, they also are involved in the metabolism of certain phytochemicals occurring in fruits and vegetables. The expression and activity of these enzymes has been shown to be affected by various phytochemicals. This may be a mechanistic link to the cancer risk reduction observed in groups with a high consumption of fruits and vegetables.

Nutritional studies have demonstrated effects in experimental models. Some mechanisms of interaction between nutrition and one or several steps described above have been postulated, and have to some extent been demonstrated in experimental models. Animal models, particularly rats and mice have been used extensively to study the processes of carcinogenesis. In these animal studies often times carcinoma are induced by chemical carcinogens. Recently gene knock-out mice have been used to investigate the involvement of certain specific genes, like APC, in the carcinogenic process. Certainly these model systems are important to understand the mechanisms involved in carcinogenesis; however, a direct link to the human situation is particularly questionable for the association between nutrition and cancer development.

In the present paper, it will be discussed which conditions should be met, in order to claim that food ingredients, or foods, have a potential to reduce the risk for cancer. The paper will focus on the numerically most important cancers. These are colon cancer, lung cancer, breast cancer, and prostate cancer. The following chapter deals with sporadic tumour formation, cancers with a strong genetic disposition will not be discussed in detail. Colon cancer receives much attention as evidence indicates that this is a tumour particularly amenable to dietary influence.

Prospective, dietary intervention studies are considered the gold standard in evidence-based nutrition for evaluating the relation between a disease and nutritional factors. However, especially for carcinogenesis this is exceedingly difficult, time-consuming and expensive to perform and also creates ethical problems if a ‘control’ group is included. For this reason, much of the chapter deals with early/intermediate ‘markers’ for cancer, their link to the endpoint tumour and their usefulness for making claims. It can also be mentioned that to date some claims have been made based on epidemiological studies.

Inflammatory bowel disease increases the risk of colorectal carcinomas; however, this issue will be dealt with in the ITG on Gut Health and Immunity.

Tumours and preneoplastic changes

Carcinoma incidence is the ultimate endpoint of cancer prevention intervention studies. In rodents, using a chemical carcinogen, this can mostly be achieved within a year. However, human trials with carcinoma as the primary endpoint will be looking at many years (> 10 yr) to prove a chemopreventive effect of a certain agent. Therefore, there is a strong need for sensitive and specific intermediate biomarkers that accurately reflect the multiple stages of carcinogenesis. The most promising biomarkers are discussed below.

- **Tumours and mortality in animal models**

- **Definition of target.** Macroscopically visible tumours in rodents can be induced by chemical carcinogens. Examples are 1,2-dimethylhydrazine (or its metabolite azoxymethane) for colon cancer, dimethylbenz(a)anthracene for breast cancer, and diethylntirosamine for...
lung cancer. In long-term studies, also the incidence of mortality, resulting from the progression of these tumours, can be analysed as a marker.

- **Relationship of target with cancer.** The presence of adenomas and adenocarcinomas, their size and multiplicity are directly linked to cancer. Mortality is a hard endpoint resulting from tumour progression.

- **Modulation of target by diet.** Macroscopic tumours can be modulated by diet in several ways: the size of tumours can vary, as well as the number of tumours per animal, and the number of tumour-bearing animals per group. Also, the incidence of mortality per group can be modulated by diet. As an example for colon cancer, several types of dietary fibre have been frequently shown to reduce the number of tumours in rodent models [reviewed in 1]. Antioxidants appear to have a high potential for inhibiting lung cancer. NSAIDs are effective in bladder cancer, and retinoids are efficacious in breast cancer models [reviewed in 2].

- **Methodological considerations.** The incidence of macroscopic tumours has been the 'gold standard' endpoint of chemoprevention studies in rodents for many years. There are however several practical drawbacks of these types of studies: the large groups of rodents required; the long time scale (up to 8 months) needed for tumours to develop; and the time-consuming histological procedures for confirmation of the tumours. For colon cancer, an alternative marker has been developed and validated: aberrant crypt foci (see below, section Precancerous lesions (aberrant crypt foci)). Therefore, the rodent tumour model for colon cancer has gradually lost attractiveness, due to the above-mentioned drawbacks.

More recently, other models have been introduced for cancer research, such as tumour-transplantation animal models, available for breast and prostate cancer, and mouse transgenic models. Indeed in 1990, the mutant Min mouse was found with multiple intestinal neoplasia [L50539]. It was shown to have a mutated Apc gene, similar to that in patients with familial adenomatous polyposis, and in many sporadic cancers. Then other mice have been genetically modified (with truncated Apc at different positions or mutated Msh2 or Mlh1). They have been used as models to evaluate the effect of diets and chemopreventive agents in more than 70 studies [3]. Compared with the carcinogen-treated rat model, the use of mutated mice avoids the hazard of carcinogen handling, and leads to shorter studies. Dietary treatments are initiated in mice by the age of 4–5 weeks, when tumours may be already present. This timing mimics human clinical trials, where dietary treatments are given to adults, likely to bear minute polyps, the visible ones having been removed before randomisation.

- **Conclusions.** Chemical carcinogen-induced rodent models are well accepted and widely used for the study of potential chemopreventive agents. New transgenic and implantation rodent models are interesting from a mechanistic point of view, but should be interpreted with great care. For instance, in the APC Min mouse, adenomas develop mainly in the small intestine and in the first weeks of life, which is very much unlike the human situation.

- **Precancerous lesions (aberrant crypt foci)**

- **Definition of target.** Aberrant crypt foci (ACF), first described in 1987 [4], are precancerous lesions of the colonic epithelium, which consist of a number of crypts that are larger than normal crypts, slightly elevated above the mucosal surface, with a thick epithelial lining.

- **Relationship of target with cancer.** Several lines of evidence strongly suggest that ACF with certain morphological, histological, cell kinetic and genetic features are precursor lesions of colon cancer in both rodents and humans [reviewed by 5]:
  - ACF are induced by all colon carcinogens in a dose-dependent manner [6];
  - In ACF, a shift of proliferation towards the luminal surface of the epithelium occurs, like in adenomas [7]. Apoptosis is far less defined in experimental ACF.
  - The number of ACF, as well as the number of crypts per ACF (so called crypt multiplicity), are affected by chemopreventive agents, and predict tumour incidence. A recent review by Corpet [1] reported a significant correlation between the potencies against ACF and tumours for 57 agents studied for both biomarkers. Using two different protocols, the interference of chemopreventive agents with the initiating phase and the post-initiative phase of carcinogenesis can be discriminated [8].
  - ACF correlate with colon cancer risk and adenoma size and number in humans. For instance, the number of ACF was higher in patients with Familial Adenomatous Polyposis, and dysplasia was shown in most cases [9]. Also, the number of ACF in resected sections of the normal mucosa of cancer patients was higher in regions with higher colon cancer mortality [10].

- **Modulation of target by diet.** The following parameters can be modified by diet: number of ACF in colon; number of crypts per ACF; proliferation rate in ACF. For instance, lycopene [8, 11] and fish oil [12–14] have been shown to reduce the number of ACF in rats. See also the review by Corpet [1].

- **Methodological considerations.** ACF can be easily visualised with methylene blue staining and scored with a...
low-magnitude microscope in resected colon samples of rodents and humans, without tissue sectioning. Several studies have described ACF visualisation in vivo during endoscopy in cancer patients, with variable results [15–17]. This technique is technically difficult, but promising, and has to be further developed and validated.

Although most data support the use of ACF as a surrogate end point for the screening of agents for chemoprevention, there is still some discussion whether ACF are true preneoplastic lesions. The azoxymethane dose that yields one cancer per rat, yield 100–200 ACF per colon, indicating that most ACF regress and never become cancers. In some ACF, hyperplastic or carcinoma tissue has been observed [18]. Also, crypt multiplicity in ACF was a predictor of tumour incidence in several studies [e.g. 19]. Most probably only larger foci, with altered morphology, dysplastic, with altered cell kinetics, and with mutations in some genes involved in carcinogenesis, will progress toward cancer.

Since, a few agents have shown opposite effects on ACF and on tumours (for example, dietary cholic acid, a known colon tumour promoter decreases ACF) other types of micro-lesions have been studied (that are enhanced by cholic acid).

β-Catenin-Accumulated Crypts (BCAC), were first described by Yamada et al. (2003) [20]. As they are only identified in the histological sections of en face preparations, BCAC are not yet ready to be used for routine chemoprevention studies.

Mucin Depleted Foci (MDF), first described by Caderni et al. (2003) [21]. Fifteen weeks after injections of azoxymethane to rats, six to ten MDF could be scored on the unsectioned colon stained with High-Iron Diamine Alcian Blue (HID-AB). Thus, in contrast with BCAC, MDF might be used for chemoprevention studies.

It is possible that MDF and BCAC are two views of the same lesion. Indeed, mucin production is usually absent in BCAC, and it is likely that MDF cells accumulate beta-catenin.

■ Definitions. From the above it can be concluded that ACF are a valuable biomarker in rodents, providing a quantitative assessment of the development of colon cancer. To judge the relevance in humans of ACF and a fortiori of MDF, not enough data are available at the moment.

■ Adenomatous polyps

■ Definition of target. Adenomatous polyps are well demarcated, circumscribed lumps of epithelial dysplasia. Adenomas are present in all segments of the large bowel, but their distribution tends to parallel that of colorectal malignancies, so that about 70% of adenomas are localised in the left colon and rectum. Endoscopic examination for the detection of adenomatous polyps is a common diagnostic procedure, in which single or multiple polyps can be visualised and removed surgically. Common clinical practice is that all polyps detected during endoscopy are resected.

■ Relationship of target with cancer. Adenomatous polyps in the colon and rectum are generally considered to represent the most likely precursor lesions for colorectal cancer in humans. They can vary in the number, size, histological type and degree of dysplasia [22]; all are important determinants of potential malignancy. Adenoma size and number have been shown to be associated with adenoma recurrence [23], and removal of polyps decreases the risk of colon cancer [24]. Furthermore, it was shown that invasive carcinomas developed at sites where large polyps (> 1 cm) were not excised [25]. FAP patients, who have hundreds of polyps in their large bowel, will almost all develop cancer if left untreated [26]. In addition, adenomatous polyps and cancer share the same aetiological factors as well as molecular genetic alterations.

■ Modulation of target by diet. The following parameters can be modified by diet: size, number, growth and recurrence of polyps; histological features. As an example, calcium has been shown to reduce the recurrence of colorectal polyps in a few studies [27, 28]. On the other hand, a beneficial effect of fibre supplementation on polyp recurrence could repeatedly not be demonstrated [28–30].

■ Methodological considerations. The major drawback for using polyp recurrence as a biomarker in human chemoprevention trials is the long time scale required to allow recurrence of the polyps: at least 3–5 years is needed, which makes these studies complex and very expensive. In addition, the cohort of patients studied should be quite large. An alternative is found in studying the growth (or regression) of non-resected polyps over time. This allows results to be obtained earlier. However the awareness that detected polyps are deliberately not being removed, sometimes encounters ethical objections, as it will increase the risk of developing malignant lesions.

A further limitation of the use of polyp recurrence as a biomarker of colon cancer risk is the fact that polyps occur only in part of the general population [31]. Therefore, these persons might already reflect a certain susceptibility to colon cancer and might not be representative of the general public.

Finally, the majority of adenomatous polyps do not progress into invasive cancer. Therefore, although many studies have indicated that polyp recurrence may be...
modulated by chemopreventive agents, it remains to be determined whether dietary or pharmacological modulation of polyp recurrence leads to a reduction in the incidence of colon cancer.

■ Conclusions. However, even taking into account the above-mentioned reservations, the recurrence of adenomatous polyps is still probably the most reliable surrogate end-point in human chemoprevention studies, since it measures the occurrence of a lesion in the pathway leading from normal mucosa to cancer. In addition, polyp size and their histological features (tubular/villous) are important biomarkers too.

However, caution should be taken in interpreting the results of studies which show no or even a negative effect, such as the large fibre intervention trials, mentioned above. Because of the overwhelming amount of available scientific evidence that dietary fibre is protective against colon cancer, it becomes clear that also other targets simultaneously.

So, for cellular targets, results that are mostly obtained from in vitro and animal studies are not sufficient to base a claim on but they could be used as supportive evidence.

■ Tumour markers

Tumour markers are molecules that indicate either the presence or the progress of malignancy. Tumour markers are directly secreted or exfoliated by tumour tissue. They are useful for diagnosis, progression of disease and efficacy of cancer therapy, rather than for screening of healthy subjects.

Widely used tumour markers are PSA (prostate-specific antigen) for prostate cancer, and oestrogen and progesterone receptors for breast cancer [32]. A change in concentration can easily be detected in a blood test. As an example of dietary modulation, PSA levels in blood can be decreased by supplementation with vitamin D [33].

For colon cancer, no such marker is widely used. Several biochemical markers have been identified as risk indicators of colonic neoplasia, such as insulin and insulin-like growth factor [34], and plasminogen activators and inhibitors [35]. Epidermal growth factor receptor has been detected in lung and colon cancer patients [36]. Very promising are recently developed molecular markers. These detectable mutations include 8p, 18q, DCC, p1, p53 and K-ras [reviewed by McLeod, 37].

In general, the currently available tumour markers lack sensitivity for early cancer diagnosis, and lack specificity for scoring malignancy. However, improvements in molecular and genetic techniques have permitted the detection of more sensitive and more specific markers, such as tumour derived DNA and RNA circulating in plasma, so that genetic mutations that occur during tumour progression can be detected in a blood sample. For colon cancer, although a large number of studies directed at molecular markers have been reported to provide prognostic or predictive indicators, none of the potential markers has yet come to widespread clinical application [37]. For developing health claims, only markers that have diagnostic value on the level of dietary intervention, such as PSA for prostate cancer, are important.

■ Cellular targets/markers

This section presents the main targets, at the cellular level, which have consequences for carcinogenesis during the post-initiation phase and can be modulated by food components.

These targets are known to have common regulatory pathways. Indeed, various endogenous or exogenous agents, including dietary compounds, may affect several of these targets simultaneously.

Cellular targets have two main limitations:

- Very often, these targets are studied using cellular models (spontaneously transformed rodent cell lines, or human tumour cell lines) that do not completely reflect the in vivo situation in a normal tissue.
- The choice of the cell type is also critical, since cell specificity of regulatory mechanisms could influence the outcome of the study.

So, for cellular targets, results that are mostly obtained from in vitro and animal studies are not sufficient to base a claim on but they could be used as supportive evidence.

■ Cell proliferation

■ Definition of target. In adults, most cell types (especially epithelia) undergo a number of cell divisions, before differentiating and dying. In each tissue, cell proliferation is induced and finely regulated by the binding of endogenous hormones and growth factors to their specific receptors expressed in the tissue.

Cell division itself is regulated by a complex interplay of many genes that control the cell cycle, with DNA replication and mitosis as major checkpoints. The cell cycle is tightly regulated to minimise transmission of genetic changes to subsequent cell generations. Progression through the cell cycle is under the control of different types of proteins: cyclins (A, B, D, E), cyclin-dependent kinases (CDKs: cdk2, cdk3, cdk4, cdk6, cdc2…) and inhibitors of cyclin-CDK complexes of the WAF1/CIP1 family (p21), the KIP family (p27, p57) and the INK4 family (p16, p15, p18). Tens of molecules have been identified as components of the signalling cascades which couple regulation of the cell cycle and detection of DNA damage. The product of the tumour
suppressor gene p53 is one of these key regulatory proteins [38].

**Relationship of target with cancer.** Cell proliferation plays a crucial role at different stages of multistage carcinogenesis [39]:
- Throughout the successive cell cycles, errors in DNA replication due to genetic instability, which are not or incorrectly repaired, can result in DNA sequence changes (gene mutations). Also, DNA replication converts DNA alterations (DNA adducts) due to stress (irradiation, exposure to carcinogens) to DNA sequence changes. Thus, in proliferating tissues, various genetic changes can progressively accumulate.
- Clonal proliferation of the genetically damaged cells leads to the formation of foci, nodules and then to tumours which can escape from the control of neighbouring cells.

Perturbation of cell proliferation is observed in all malignant tissues. For example, in colon, the mucosal cell proliferation, which is normally restricted to the stem cell zone, extends to the entire crypt during carcinogenesis and leads to the formation of aberrant crypt foci, polyps, adenomas or carcinomas. Patients with a high risk of colorectal cancer (e.g. FAP) have a corresponding high mucosal proliferation [40]. Refer also to section on ‘short chain fatty acids’ under Metabolites below.

**Modulation of target by diet.** Cell proliferation is inhibited by many chemopreventive agents, including retinoids, vitamin D, polyphenols, phytooestrogens, fatty acids, sodium butyrate, indole-3-carbinol. They act at the level of growth hormones and growth factors or their receptors or on the genes and proteins regulating the cell cycle [for review, see 41, 42].

**Methodological consideration.** To assess cell proliferation, different methods are available:
- Cell growth curves are done with cell cultures in vitro.
- DNA synthesis, determined by incorporation of precursors: tritiated thymidine or bromodeoxyuridine (BrdU) into cells or tumour fragments in vitro.
- Distribution of cells in different cell cycle phases, using flow cytometry.
- Measurement of proliferation biomarkers, detected using immunohistochemistry. Among them, Ki-67 and PCNA (proliferating cell nuclear antigen) are nuclear proteins whose level varies as a function of the cell cycle. Ki-67, whose function is unknown, is expressed throughout the cell cycle except G0 [43]. PCNA, which is an auxiliary protein to polymerase delta, is expressed during the G1 phase of the cell cycle [44]. Recently a monoclonal antibody to recombinant parts of Ki-67 antigen, named Mib-1, has been developed. It appears as a more reliable tool for measuring proliferation activity in human tissues [45].

In clinical trials, cell proliferation is frequently evaluated by the BrdU index or PCNA content. These methods have both advantages and disadvantages, which have been recently discussed by Renehan et al. [46].

**Conclusion.** Inhibition of cell proliferation could be a valuable surrogate marker. The possibility to use it as a basis for a claim may depend on the methodological approach (dynamic or static assessment, measurement in cellular or animal models, preferably in humans).

A nutritional inhibition of cell proliferation could be beneficial in the case of tumour cells (therapy), whereas it remains questionable in the case of normal cells (prevention).

**Apoptosis**

**Definition of target.** Apoptosis is a cell suicide mechanism that enables multi-cellular organisms to regulate cell number in tissues and to eliminate unneeded or ageing cells. Apoptosis is a programmed cell death. In can be distinguished both morphologically and functionally from necrosis, which is a pathological cell death, resulting from gross insults such as prolonged ischaemia that affects many adjacent cells simultaneously. In contrast, apoptosis typically occurs in single cells. Normally, it is initiated by endogenous stimuli, such as the absence of vital growth factors or hormones and the action of cytokines, like tumour necrosis factor α or Fas ligand [47, 48].

Apoptosis is characterised by membrane blebbing, cell shrinkage, chromatin condensation and DNA fragmentation followed by rapid engulfment by neighbouring cells or macrophages [49].

It involves a series of death sensors and effectors. Cellular death receptors (e.g. tumour necrosis factor receptor TNFR, death receptor CD95 Fas) are sensors that initiate the death pathway. Death signals typically are focused on the mitochondria where the release of cytochrome C (haeme-containing protein that transfers protons during cellular respiration) catalyses apoptosis. Caspases (death effectors) finally transmit the death signal: these proteases specifically cleave vital proteins of the nuclear lamina, such as poly (ADP-ribose) polymerase, and the cytoskeleton (actin, fodrin, lamin), which results in cell disassembly.

Endogenous molecules, which have other activities in the cell, regulate apoptosis. Some regulatory transmembrane proteins of the Bcl-2 family, such as Bax, Bak and Bad, are pro-apoptotic effectors. The tumour suppressor gene p53 (of which the corresponding p53 pro-
tein functions in the checkpoint control that arrests human cells with damaged DNA in G1) can induce expression of Bax, as well as death receptors. Antiapoptotic effectors in the Bcl-2 family include Bcl-2 and Bcl-XL. These are central in inhibiting signals addressed to the mitochondria, which leads to cell survival. Other genes preventing programmed cell death are for instance NF-kB, which expresses cell survival genes, which induce IAPs, which bind to and inhibit certain caspases.

■ **Relationship of target with cancer.** Apoptosis provides a protective mechanism against neoplasia by removing genetically damaged stem cells from the epithelium before they can undergo clonal expansion [50]. Loss of apoptosis may result in disordered cell growth. It contributes to tumour development but also to tumour initiation, progression and metastasis. Defects in apoptosis allow neoplastic cells to survive beyond senescence, thereby providing protection from hypoxia and oxidative stress as the tumour mass expands.

Evading apoptosis as tumour cells do, requires mechanisms that inhibit transmission of the death signals. Genetic alterations of regulators of apoptosis in tumours have been described: for example, mutations of p53 gene in more than 50% of tumours, overexpression of Bcl-2 due to chromosomal translocation in lymphomas [for review see 51].

■ **Modulation of target by diet.** Many chemopreventive agents have been found to induce cell death. They include retinoic acid, perillyl alcohol, curcumin, EGCG, apigenin, quercetin, chrysin, resveratrol and genistein [41].

In the case of the colorectal mucosa, different kinds of dietary components have been shown to modulate apoptosis both in vitro and in vivo: they include polyunsaturated fatty acids (PUFA), the short-chain fatty acid butyrate and some flavonoids and glucosinolate breakdown products [for review see: 50].

■ **Methodological consideration.** Various complementary techniques have been developed to monitor apoptosis. Since the common methods have their own set of limitations, it is recommended to confirm the results by using a combination of the following techniques [52]:

- Morphological analysis of apoptotic cells (electron microscopy) and counting of apoptotic bodies (membrane-bound structures containing nuclear fragments and condensed cell contents).
- Detection of DNA fragmentation in the nuclei of apoptotic cells in tissue sections, by the TUNEL (TdT-mediated dUTP nick end labelling) method [53]. One important weakness of the TUNEL method is that it may detect necrotic cells containing damaged DNA.
- Annexin V binding: annexin V binds to membrane-bound phosphatidyl serine, a constitutive anionic membrane phospholipid that is normally restricted to the inner leaflet of the plasma membrane lipid bilayer but is selectively exposed on the surfaces of cells as they undergo apoptosis. In vivo uptake of 99mTc-radiolabeled annexin V, assessed by SPECT imaging, allows non invasive monitoring of cell death dynamics [54, 55].
- Immunodetection of caspase activity, using antibodies directed against caspase-cleaved substrates, has been recently developed. Such a method would be very useful in future clinical trials [56].

■ **Conclusion.** Induction of apoptosis could be a valuable surrogate marker. The possibility to use it as a basis for a claim will depend mainly on the methodological approach (use of a combination of complementary assays to overcome artefacts and difficulties related to measuring apoptosis in humans and interpretation).

As previously mentioned for cell proliferation, a nutritional induction of apoptosis could be beneficial in the case of tumour cells (therapy), whereas it remains questionable in the case of normal cells (prevention). It must be kept in mind that it is the balance between proliferation and apoptosis that is important.

■ **Differentiation**

■ **Definition of target.** Normal cells do not live indefinitely due to senescence. Senescent cells cannot be stimulated to divide further, become resistant to programmed cell death and acquire differentiated functions. Differentiation is obviously associated with slowing of the cell cycle.

Differentiation can be described as the process by which, during development or tissue maturation, cells acquire functions that are specific of the tissue or cell type. It results from the induction of a differential expression of genes, some becoming activated and others repressed.

■ **Relationship of target with cancer.** It is generally agreed that the malignant neoplastic cell is less differentiated than the normal adult cell in the organ from which the cancer originates. However, for a long time, there has been a controversy on the link between transformation and de-differentiation. Nevertheless, malignant neoplastic transformation occurs only in cells that are capable of dividing. In general, it appears that the commitment to a given pathway of cellular differentiation is irreversible, but the steps of terminal differentiation may be reversible.

■ **Modulation of target by diet.** There are a number of examples of animal malignant tumours or human can-
Cancer cells in culture that can be induced to lose their malignant phenotype by treatment with certain differentiation-inducing agents: differentiation of murine embryonal carcinoma cells by exposure to retinoic acid or sodium butyrate; differentiation of human acute promyelocytic (HL-60) cells in culture by sodium butyrate, vitamin D3 and retinoic acid analogues. Among dietary compounds, curcumin and phytic acid have also been shown to induce differentiation of various tumour cell lines [57, 58].

The best examples of cancer treatment through induction of cellular differentiation is the treatment of acute promyelocytic leukaemia and oral leukoplakia with retinoids [59]. Since the 1980s, vitamin D3 has been recognised as a potent antiproliferative and prodifferentiation agent (more recently, it has also been shown to induce apoptosis and to inhibit tumour invasion and angiogenesis). Several epidemiological studies support the hypothesis that vitamin D3 is an important endogenous cancer protective agent. Trials of systemic 1,25 (OH)2D3 with various malignant diseases [for review, see 60].

**Methodological consideration.** Various morphological signs of differentiation are studied. Some of them are specific to the cell type taken into consideration:

- Increase of the nuclear/cytoplasmic ratio (for mouse embroyal carcinoma PCC4 cells, for example).
- Morphological differentiation of human teratocarcinoma NT2 cells in neurons, of epidermal precursors in keratinocytes, of myelopoietic progenitors/stem cells in monocytes-macrophages, and mononuclear precursor cells in osteoclasts [60, 61].
- Induction of tissue-specific molecular markers: for example in breast cancer cells, expression of milk components (lipid droplets, β-casein mRNA) and down-regulation of the Her2/neu membrane receptor [62].
- Expression of components of the cytoskeleton, like laminin A, actin, cytokeratin, which are considered as differentiation-specific markers [57].

**Conclusion.** Induction of cell differentiation could be a valuable surrogate marker. The possibility to use it as a basis for a claim may depend on the use of a set of reliable and complementary markers. The science base would be more solid if the results from different methods are consistent.

**Cyclo-oxygenase 2 (COX-2)**

**Definition of target.** Cyclo-oxygenase (COX) is an important modulator protein in the eicosanoid pathway. It catalyses the conversion of arachidonic acid to prostaglandins. It is a bifunctional enzyme that has both cyclo-oxygenase and peroxidase activities. There are two COX isofoms that differ both in their tissue distribution and their regulation. COX-1 is a constitutively expressed “housekeeping” gene involved in processes like gastric acid secretion, vascular homeostasis and water reabsorption in the kidney. COX-2 is inducible and thought to be involved in different processes such as inflammation and ovulation [63].

**Relationship of target with cancer.** COX-2 is overexpressed in many cancers including oesophagus, stomach, colon, lung, pancreas, head and neck, and prostate [for review, see 64]. Recent studies using animal models provide evidence that COX-2 is mechanistically linked to cancer development: 1) over-expression of COX-2 in transgenic mice induces mammary gland tumours [65]; 2) knocking out of the COX-2 gene in the ApcΔ716 mouse reduces the number and size of intestinal polyps and of skin papillomas [66, 67].

Both COX-2 and its substrates, the prostaglandins, play an active role in carcinogenesis, since they affect xenobiotic metabolism, angiogenesis, apoptosis, inflammation, immuno-suppression and invasiveness [for review, see 64]. Several population-based studies have shown that regular use of non-steroidal anti-inflammatory drugs (NSAIDs), which are COX inhibitors, decrease the relative risk of colorectal and prostate cancer [68, 69].

**Modulation of target by diet.** Inhibitors of the enzyme both reduce the formation of tumours in many tissues and suppress the growth of established tumours [64]. Many studies are currently performed with celecoxib, aspirin, curcumin, and genistein, for example.

It has been demonstrated that lipid components of the human faecal fraction in intimate contact with the epithelium (faecal water) and specific bile acids can alter the transcription of COX-2. Available data support an influence of diet on the levels of the faecal water components. In view of the knowledge that the amount of COX-2 is important, since there is a correlation between its level of expression and the size of the colorectal tumours and their propensity to invade underlying tissue, even small effects over time on the transcriptional regulation of this enzyme by dietary components may be important for tumour development in the colon.

**Methodological consideration.** COX-2 expression in cells or frozen sections of animal or human tissues is generally studied by immunohistochemistry or immunoblotting [for a detailed description of the techniques, see ref 70].

**Conclusion.** Inhibition of COX-2 activity is a valuable surrogate marker, since convincing data obtained in humans with NSAIDs are available. However the effect of diet is less explored.
The possibility to use it as a basis for a claim may depend on the availability of clinical data for the tissue taken into consideration: COX-2 data are mainly arising from colon (and prostate and breast) cancer, and it is not sure if it is also valid for other types of cancers. Definition of target groups may be particularly relevant for this marker.

### Potential targets

Several other potential targets (intercellular communication, gene expression, signal transduction, etc), for which numerous but incomplete data are available, are listed in Table 1. However at present, they are further away from the true endpoint and as such can only be used for mechanistic supporting evidence. This field will particularly benefit from a development of research and the use of high scale technologies for genomics and proteomics.

### Gut luminal markers

The gut microflora has been implicated in the aetiology of colorectal cancer by a number of studies [reviewed by Mallet and Rowland 71] and these observations form the theoretical basis for use of gut flora biomarkers (faecal biomarkers). They are composed of two main categories: those examining the activity of bacterial enzymes or bacterial metabolites and those based on bioassays on faecal water. A number of these have been used to investigate gut bacterial function [72, 73] and some have potential mechanistic links to colorectal cancer (CRC) aetiology.

### Bacterial enzymes

A wide range of enzyme activities capable of generating potentially carcinogenic metabolites in the colon is associated with the gut microflora, including β-glucuronidase, β-glucosidase, nitrate reductase and nitroreductase (Table 2). These are usually assayed in faecal suspensions and appear to be present in many bacterial types [74]. Of these enzymes, β-glucuronidase has been the most extensively investigated as a biomarker of CRC risk. It should be noted that these factors are associated with the generation of carcinogens and promoters and do not have a direct link with tumours.

#### β-Glucuronidase

**Definition of target.** Many carcinogenic compounds are metabolised in the liver and then conjugated to glucuronic acid before being excreted via the bile into the small intestine. In the colon bacterial β-glucuronidase can hydrolyse the conjugates, releasing the parent compound or its activated, hepatic metabolite.

**Relationship with cancer.** The activity of β-glucuronidase in the colon can alter the likelihood of tumour induction in animal models of CRC. The use of a β-glucuronidase inhibitor administered in conjunction with the carcinogen azoxymethane (which undergoes activation and conjugation in the liver) significantly reduces the number of tumours formed in the rat colon, indicating that microflora β-glucuronidase has a role in tumour induction [75]. Metabolic epidemiological studies have shown that populations at high risk of CRC have high levels of faecal β-glucuronidase activity. Furthermore, faecal β-glucuronidase activity in colon cancer patients is significantly higher than in healthy controls [76].

**Modulation by diet.** The activity of β-glucuronidase is influenced by diet. High-risk diets for colorectal cancer have consistently been shown to increase β-glucuronidase activity relative to low risk diets [77]. Furthermore, various types of fibre decrease the activity of β-glucuronidase in rats [78–80].

**Methodological considerations.** β-Glucuronidase can be assayed in fresh or frozen faecal samples by a simple reproducible colorimetric procedure. It is usually expressed as mmol p-nitrophenol released/min/g wet weight faeces. There is considerable interindividual variation in activity.

**Conclusions.** Although it represents a simple reproducible marker, evidence for a role for β-glucuronidase in human CRC is indirect and is remote from the final endpoint (tumours).

### Metabolites

A wide range of metabolites with potential genotoxic, tumour promoting and anti-carcinogenic activities have been identified in faeces. These are summarised in Table 3.

#### N-nitroso compounds

**Definition of target.** Nitrate, ingested via diet and drinking water, is reduced by gut bacterial nitrate reductase to its more reactive and toxic reduction product, nitrite. Nitrite reacts with nitrogenous compounds in the body to produce N-nitroso compounds (NOC). 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 in the colon [81].
Table 1  Cellular effects: other potential targets

<table>
<thead>
<tr>
<th>Target</th>
<th>Definition</th>
<th>Relationship with cancer</th>
<th>Modulation by diet</th>
<th>Methods</th>
<th>References</th>
</tr>
</thead>
</table>
| Gap junctional intercellular communication (GJIC) | - Gap junctions: channels between adjacent cells permitting direct cell-cell communication.  
  - Important role of GJIC in the control of tissue homeostasis and regulation of cell proliferation, differentiation and apoptosis.  
  - Modulation of GJIC at different levels of regulation: specific connexin gene expression, protein phosphorylation, connexon assembly, cell adhesion and channel gating. | - Dysruption of GJIC involved in carcinogenesis.  
  - Reduced level of GJIC in most human and animal tumours.  
  - Transformed cells (in vitro and in vivo) do not communicate with neighbouring normal cells.  
  - Many tumour promotors inhibit GJIC.  
  - Transfection of connexin genes into communication-deficient malignant cells reduces or eliminates the tumourigenicity of recipient cells. | - Retinoids, carotenoids, and flavonoids (apigenin, tangeretin) enhance GJIC in vitro. | - Dye transfer after microinjection in cell monolayers and tissues.  
  - Expression of tissue-specific connexins by immunoblotting or immunostaining.  
  - Electrophysiology.  
  - Metabolic cooperation.  
  - Photobleaching.  
  - Scrape loading. | [240–244] |
| Ornithine decarboxylase (ODC) | - ODC participates in carcinogenesis.  
  - Blocking ODC *in vitro* inhibits cell transformation.  
  - ODC is now considered a putative proto-oncogene. | - Retinoids, green-tea polyphenols and flavonoids inhibit ODC activity *in vitro*. | - ODC activity.  
  - Spermidine/spermine ratio. | | [245–247] |
| Senescence of human cells (after 50–70 divisions) due to telomere attrition. | - Telomerase prevents telomere erosion and is able to synthesise new telomeric repeats.  
  - Telomerase is detected in more than 85 % of cancers, and is usually undetectable in benign tumours and in normal tissues surrounding tumours.  
  - Telomerase inhibition leads to an immediate induction of apoptosis. | - Dietary restriction inhibits liver telomerase activity *in vivo*. | - Enzyme activity.  
  - Fiber optical biosensor for enzyme activity detection in tumours. | | [186, 248–250] |
| Lipoxigenases (5-LOX, 8-LOX, 12-LOX, 15-LOX) | - LOX products involved in development and progression of human tumours.  
  - Abnormal expression and activities of LOX in pancreatic cancer.  
  - LOX inhibitors reduce chemically-induced carcinogenesis, and induce apoptosis in human cancer cells. | - Inhibition of LOX by curcumin, phenolic compounds of olive oil. | - Enzyme activity.  
  - LOH products. | | [251–254] |
**Relationship to cancer.** The term NOC covers a wide range of compounds including N-nitrosamines, N-nitrosamides, N-nitrosoguanidines and N-nitrosoureas, the majority of which are highly carcinogenic, DNA alkylating agents. However, the genotoxic or carcinogenic activity of the NOC produced by the bacterial N-nitrosation process in the large intestine has not yet been established.

**Modulation by diet.** Faecal apparent total NOC (ATNC) excretion is increased by red meat consumption [82–84]. In conjunction with high meat intakes, wheat
bran, resistant starch and vegetable consumption had no effect on faecal ATNC excretion or concentration [82, 84, 85].

**Methodological considerations.** N-nitroso compounds in faeces can be monitored using a group-selective method that determines ATNC in faeces and biological fluids by a procedure employing a thermal energy analyser [81, 86].

### Table 1  Continued

**Table 1**  **Signal transduction**

<table>
<thead>
<tr>
<th>Target</th>
<th>Definition</th>
<th>Relationship with cancer</th>
<th>Modulation by diet</th>
<th>Methods</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Farnesyl protein transferase</td>
<td>Prenylation: a type of lipid modification involving covalent addition of either farnesyl (15-carbon) or more commonly geranylgeranyl (20-carbon) isoprenoids via thioether linkages to cysteine residues at or near the C terminus of intracellular proteins.</td>
<td>• Prenylation is catalysed by the farnesyl protein transferase (FPT): the first step in the processing of the oncogenic Ras proteins. • Ras is often overexpressed in tumours.</td>
<td>• Several monoterpenes, including limonene and perillyl alcohol inhibit farnesyl protein transferase.</td>
<td>• Measure of the enzymatic activity.</td>
<td>[265, 266]</td>
</tr>
<tr>
<td>Protein kinases</td>
<td>Many signal transduction events involve phosphorylation steps. Activation of protein kinases is a key mechanism in regulating signals for cell proliferation.</td>
<td>• The substrates of protein kinases include transcriptional regulatory factors, for example, the proteins encoded by the jun, fos, myb, rel and ets proto-oncogenes.</td>
<td>Various polyphenolic compounds (EGCC, flavonoids, resveratrol) inhibit MAPK pathways.</td>
<td>Enzyme activities.</td>
<td>Western blotting.</td>
</tr>
<tr>
<td>P27 Tumour suppressor function</td>
<td>Central role in proliferation, differentiation and cell death. It inhibits cyclin/cyclin-dependent kinase complexes, thereby blocking cell cycle progression.</td>
<td>• In contrast to most tumour suppressor genes (Rb, p53), which are recessive at the cellular level, p27 is haploinsufficient for tumour suppression. • Skp2, the specific recognition factor for p27 ubiquitination has oncogenic properties.</td>
<td>• Apigenin (and NSAIDs) upregulates p27 in vitro.</td>
<td>Western blotting.</td>
<td>[268–270]</td>
</tr>
</tbody>
</table>

### Secondary bile acids

**Definition of target.** The primary bile acids, chenodeoxycholic acid and cholic acid, are subject to extensive metabolism, predominantly 7-α-dehydroxylation, by the intestinal microflora [87], which converts cholic to deoxycholic acid (DCA) and chenodeoxycholic to lithocholic acid (LCA). These are termed secondary bile acids.

**Relationship to cancer.** The secondary bile acids exert a range of biological and metabolic effects *in vitro* and in animal models including cell necrosis, hyperplasia.
### Table 2  Faecal bacterial enzymes

<table>
<thead>
<tr>
<th>Target</th>
<th>Definition of target</th>
<th>Relationship with cancer</th>
<th>Modulation by diet</th>
<th>Methodology</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Glucuronidase (GN)*</td>
<td>Hydrolyzes biliary conjugates in the colon releasing potential carcinogens.</td>
<td>Populations at high risk of CRC have high GN activity.</td>
<td>High risk diets for CRC increase GN in human and animal studies.</td>
<td>Analysed by chromogenic substrate. Simple, rapid. Can be assayed in frozen samples.</td>
<td>[77, 271]</td>
</tr>
<tr>
<td>β-Glycosidase</td>
<td>Hydrolyzes glycoside conjugates of plant compounds.</td>
<td>Some plant aglycones have mutagenic and carcinogenic activity. Others act as anti-genotoxins.</td>
<td>Poor relationship to high risk diets.</td>
<td>Analysed by chromogenic substrate. Simple, rapid. Can be assayed in frozen samples.</td>
<td>[77, 271]</td>
</tr>
<tr>
<td>Nitrate reductase</td>
<td>Reduces nitrate to more reactive nitrile.</td>
<td>Nitrite is precursor of faecal NOC.</td>
<td>No consistent response to dietary change.</td>
<td>Spectrophotometric analysis, needs fresh faecal sample and strict anaerobic conditions.</td>
<td>[272]</td>
</tr>
<tr>
<td>Nitroreductase</td>
<td>Reduces nitro compounds to amines.</td>
<td>Some amine products are carcinogenic.</td>
<td>No consistent response to dietary change.</td>
<td>Spectrophotometric analysis, needs fresh faecal sample and strict anaerobic conditions.</td>
<td>[272, 273]</td>
</tr>
<tr>
<td>IQ oxidoreductase</td>
<td>Converts IQ to 7-OHIQ.</td>
<td>IQ is a known dietary carcinogen requiring activation. The bacterial enzyme converts IQ to a direct acting genotoxin.</td>
<td>Increased by high risk CRC diets in rats.</td>
<td>Radiolabel chromatography assay, anaerobic conditions needed.</td>
<td>[274]</td>
</tr>
</tbody>
</table>

* For further details see main text

### Table 3  Faecal metabolites

<table>
<thead>
<tr>
<th>Target</th>
<th>Definition of target</th>
<th>Relationship with cancer</th>
<th>Modulation by diet</th>
<th>Methodology</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonia</td>
<td>Formed by bacterial catabolism of protein and urea in colon.</td>
<td>Tumour promoter in rats; induces colon cell proliferation.</td>
<td>High-risk diets for CRC increase faecal ammonia in rats.</td>
<td>Simple chromogenic assay.</td>
<td>[271, 275]</td>
</tr>
<tr>
<td>N-Nitroso compounds (NOC)*</td>
<td>Formed by bacterial action in colon from nitrite and amino compounds.</td>
<td>Many NOC are potent carcinogens (but activity of faecal NOC not known).</td>
<td>Increased by high red meat diets.</td>
<td>Difficult, expensive analysis (Thermal Energy Analyser).</td>
<td>[83, 86]</td>
</tr>
<tr>
<td>Diacylglycerols (DAG)</td>
<td>Produced by gut bacteria from phospholipids and triglycerides.</td>
<td>Activate protein kinase C. Humans with high fat content in faeces have high DAG in gut and epithelial hyperproliferation.</td>
<td>Faecal DAG increased by high fat diets in rats and mice. DAG decreased by wheat bran in humans.</td>
<td>Lipid extraction, then commercial kit.</td>
<td>[88, 276, 277]</td>
</tr>
<tr>
<td>Fecapentaenes</td>
<td>Glycerol ethers containing a pentaene moiety. Produced by gut bacteria in vitro.</td>
<td>Direct acting mutagens in Ames test. Associated with increased cell proliferation, tumour promotion in some but not all studies. Inconsistent associations with CRC.</td>
<td>Faecal excretion of fecapentaenes is lower in vegetarians.</td>
<td>Difficult, hplc method</td>
<td>[88, 278–280]</td>
</tr>
<tr>
<td>Secondary Bile acids*</td>
<td>Formed by bacterial metabolism of cholic and chenodeoxycholic acids.</td>
<td>Tumour promoters, inducers of cell proliferation, genotoxic. Concentration of faecal bile acids higher in populations and individuals at high CRC risk.</td>
<td>High fat intake increases faecal bile acid concentrations.</td>
<td>Analysis by GLC or HPLC, time consuming extraction required.</td>
<td>[88, 89, 96]</td>
</tr>
<tr>
<td>Short chain fatty acids (SCFA)*</td>
<td>Major SCFA are acetic, propionic and n-butyric acids. Produced by fermentation of carbohydrates in colon.</td>
<td>Butyric acid has trophic effect on colonic epithelium. In vitro butyric acid induces apoptosis and differentiation of colon cancer cell lines, but increases cell proliferation in normal colon in vivo.</td>
<td>SCFA increased (usually) in subjects consuming diets high in fibre, resistant starch or non-digestible oligosaccharides.</td>
<td>Analysis by GLC. Simple, rapid.</td>
<td>[281]</td>
</tr>
</tbody>
</table>

* For further details see main text
and tumour promoting activity in the colon, induction of DNA damage, and apoptosis [reviewed by 88]. It has also been suggested that secondary bile acids influence colorectal cancer by selecting for apoptosis-resistant cells or by interacting with various secondary messenger signalling systems. Epidemiological studies indicate that concentrations of secondary bile acids are higher in populations at high risk of CRC and in case control studies 7-α-dehydroxylase activity is higher in cases than controls. A number of human observational studies in patients with adenomas or CRC have reported a correlation between faecal bile acid (FBA) concentrations and CRC risk [89–92]. Some studies [93, 94] have also suggested that high DCA concentration and the DCA to LCA ratio are associated with increased CRC risk. It should be noted however that not all studies have confirmed these relationships between bile acids and cancer risk [95].

**Modulation by diet.** In human studies, high fat intake, which correlates with CRC risk, increases FBA concentrations [96], whereas increased consumption of wheat bran (negatively correlated with CRC risk) reduces FBA concentration.

**Methodological considerations.** Measurement of FBA is usually performed by soxhlet extraction of faecal samples followed by gas chromatographic analysis.

### Short chain fatty acids

**Definition of target.** The short chain fatty acids (SCFA) acetate, propionate and butyrate are the principal end products of carbohydrate fermentation. These are absorbed from the colonic lumen and metabolised by various body tissues [97]. Butyrate is preferentially metabolised by colonocytes [98].

**Relationship to cancer.** Butyrate is the most intensively studied SFCA. Interest in butyrate’s role as a possible antineoplastic agent has arisen from its potential to inhibit proliferation of cells in vitro, including colon tumour cell lines [99]. Moreover, at physiological concentrations, sodium butyrate induces apoptosis in human colon carcinoma cell lines [100].

In addition, reduced pH as a consequence of SCFA production, has also been implicated as a protective factor against colon cancer [101, 102]. This may be as a result of altering the solubility of bile acids, or altering the metabolic activity of colonic microflora.

**Modulation by diet.** There is evidence from in vitro studies and animal models (where caecal SCFA concentrations can be measured) that the type of carbohydrate has an important influence on the amount and proportions of SCFA produced, with starch and wheat bran being particularly associated with elevated butyrate production [103]. In human studies, inulin has been shown to enhance excretion of total SCFA in human faeces, whereas wheat bran increased absolute or relative proportions of butyrate in faeces [104]. Where the butyrate is produced relative to proximal and distal regions of the colon is important and should be a methodological consideration.

**Methodological considerations.** Measurement of SCFA in faeces is by a simple gas chromatographic method, but the value obtained represents the balance of bacterial production and the extensive colonic absorption, so may not reflect accurately the concentrations of SCFA in the colonic lumen [97].

**Conclusions for sections on Bacterial enzymes and Metabolites.** Gut bacterial enzymes and faecal metabolites are relatively simple to measure routinely and in general may be of use in assessing effects of diet on modulating exposure of the colon to potential carcinogens, rather than reflecting cancer risk.

### Faecal water activities

**Faecal water cytotoxicity**

**Target.** 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 [105, 106], since this will contain most of the free reactive species. For assessment of faecal water cytotoxicity, the effect on proliferation of human colon carcinoma cells in culture is used.

**Relationship to cancer.** Proliferative zone expansion in the colonic crypts and an increased rate of epithelial proliferation are considered to be an early step in carcinogenesis. Stimulation of proliferative activity in colonic epithelium may in part be mediated via cytotoxic mechanisms, resulting in increased cell loss at the epithelial surface and a compensatory rise in mitotic activity of the crypts. Such considerations led to the development of assays to assess cytotoxic activity in faecal water towards colon cells in vitro [105]. It is thought that bile acids, especially secondary bile acids, make a major contribution to faecal water cytotoxicity [105]. In a comparison of faecal water cytotoxicity in patients at low (no colon adenomas), medium (small colorectal adenomas)
and high (large tubular adenomas) risk of CRC, no significant differences between the groups were observed [95].

**Modulation by diet.** Interventions using dietary regimes associated with increased or decreased CRC risk have been shown to modulate appropriately faecal water cytotoxicity. For example, dietary calcium has frequently been shown to reduce the cytotoxicity of faecal water presumably by precipitating soluble bile acids [106, 107]. Faecal water cytotoxicity was higher in subjects on a high fat, low calcium, low fibre diet compared with those on a low fat, high calcium, high fibre regime [105]. In rats, high red meat consumption increases the cytotoxicity of faecal water. This effect was independent of the fat and bile acid content of the faecal water and may be related to dietary haeme [108, 109].

**Methodological considerations.** Human colon cells (e.g. Caco-2 or HT29) are exposed to faecal water fractions (prepared by high speed centrifugation of fresh faecal samples) and inhibition of cell proliferation is measured usually using a dye such as MTT [110].

**Faecal water genotoxicity**

**Definition of target.** Venturi et al [111] demonstrated the presence of DNA damage activity towards human cultured colon cells in samples of faecal water from healthy human subjects. A wide variation was found ranging from negligible to high activity. The presence of genotoxic activity in faecal water can be considered to reflect exposure of the colonic mucosa to carcinogens.

**Relationship to cancer.** There is now convincing evidence that CRC is induced by a series of mutational events in a number of critical genes [112]. Sporadic colorectal tumours have been shown to contain mutations and deletions in oncogenes and tumour suppressor genes such as Apc, K-ras and p53. DNA damage has been detected in biopsies of colon tissue derived from laboratory animals and human subjects. Thus, the presence in the colonic lumen of DNA damaging agents could represent an important risk factor for CRC. There are as yet no reports of validation studies for the endpoint in patients at different risk of colorectal cancer.

**Modulation by diet.** In healthy subjects, a diet high in fat and meat, but low in dietary fibre (hence considered to be of high CRC risk) was associated with a significantly increased faecal water genotoxicity by comparison to a diet low in fat and meat [113].

**Methodological considerations.** Human colon carcinoma cell lines (e.g. Caco-2 and HT29) are exposed to faecal water fractions and genotoxicity is usually measured using the single cell gel electrophoresis (Comet) assay, which provides a simple, rapid and sensitive method for measuring single strand breaks in DNA. The intra- and interindividual variation in faecal water genotoxicity has been studied in healthy individuals [114].

**Conclusions.** Cytotoxicity and particularly genotoxicity of faecal water have a good mechanistic link with colon carcinogenesis and hence provide potentially valuable, non-invasive methods for assessing colorectal cancer risk in human subjects. However, there is a need for more extensive validation of these endpoints.

**Diagnostic markers in faeces**

There are several tumour markers that can be detected in faeces. Haemoglobin is currently used for mass screening for colon cancer (faecal occult blood test). Haemoglobin is not continuously but intermittently leaking through the intestinal wall, thereby compromising the sensitivity of the test. Other stool tumour markers, such as abnormally glycosylated mucins, are continuously secreted. However immunochemical methods to detect these mucins in stool samples have failed to accurately detect neoplasia, probably due to luminal instability [115]. Most promising are exfoliated markers, such as whole colonocytes and mutant DNA. Tumours exfoliate large amounts of epithelial cells that can be recovered from stool. Using RT-PCR, genes that are overexpressed in tumours can be demonstrated in these cells. Even more promising are neoplasm-specific DNA alterations, as DNA appears to be stable in the gut lumen. Several investigators have recovered mutant DNA in stools from colon cancer patients, mostly using K-ras mutations as marker [reviewed by 116]. More recently, the specificity of this test was improved by targeting multiple DNA mutations (including K-ras, APC and p53), to minimise false-positives and false-negatives [117]. Long DNA (> 200 base pairs) appeared to be the most informative stool marker in this study. Faecal DNA testing holds a promise for the future as a non-invasive tumour marker, as a single stool specimen will be sufficient to accurately detect both precursor adenomas and carcinomas.

**Conclusions.** Of the considerable number of potential prognostic or predictive indicators of colon cancer based on faecal markers, only faecal occult blood has achieved widespread clinical application. However, it has limited usefulness as an indicator of cancer risk for assessment of functional foods. Validation of the newer techniques should be the goal for future research.
Angiogenesis and metastasis

Inhibition of angiogenesis

Definition of target. Tumours require blood supply to develop and grow. They take over existing blood vessels and stimulate production of new vessels from these – a process termed angiogenesis.

Relationship to cancer. Angiogenesis is a crucial early event in tumour progression, beginning in premalignant lesions [see review by Ruoslahti 118]. The key initiators of endothelial cell growth are the vascular endothelial growth factors (VEGFs), which act through a family of tyrosine kinase receptors (VEGFRs) expressed by angiogenic vessels. Angiopoietins and platelet-derived growth factors are also involved and subsequently the vessels acquire supporting cells such as pericytes, smooth muscle cells and extracellular matrix.

Another group of receptors, integrins, expressed by endothelial cells in angiogenic vessels, also play an important role in the process of angiogenesis by mediating cell adhesion and transmitting signals from the extracellular matrix to cell types that regulate cell growth and survival. It has been found in neuroblastoma that the level of expression of the integrins reflects the grade of malignancy.

Given that in the absence of a blood supply, a tumour will become hypoxic and die, angiogenesis provides a logical target for therapeutic and preventative agents. A number of potential anti-angiogenic agents are currently undergoing clinical trials. These substances include drugs, peptides and antibodies that target vascular growth factors and integrins and also compounds such as endostatin, identified in activity-based assays.

Modulation by diet. In vitro assays have been used to demonstrate potential anti-angiogenic effects of NSAIDs [119] and of various dietary components such as genistein [120] and conjugated linoleic acids (CLA [121]). In vivo, dietary CLA was shown to suppress angiogenesis in a mouse matrigel model [121]. In the CLA fed mice, the injected gel pellets contained fewer infiltrating cells forming only limited branching networks. Dietary CLA also decreased serum levels of VEGF. In a study of a novel oestrogen metabolite, 2-methoxyoestradiol, Fotsis et al. [122] demonstrated that, when administered orally in mice, it strongly inhibited the neovascularisation of solid tumours and suppressed their growth.

Methodological considerations. A range of in vitro assays for assessing angiogenesis inhibition have been used including

• Inhibition of endothelial differentiation into capillary-like structures on Matrigel.
• Suppression of bFGF or VEGF-stimulated invasion of collagen by endothelial cells.

There are a number of in vivo mouse models based on modulation of angiogenesis after stimulation with various growth factors such as fibroblast growth factor –2 [119] or subcutaneous injection with Matrigel followed by measuring infiltration of the gel with blood vessels [121].

Potential biomarkers of angiogenesis for human studies: There is considerable interest in the use of plasma VEGF and bFGF as indicators for prognosis in cancer patients with a range of tumour types, including colorectal, myeloma and lung. If substantiated, this could provide a biomarker for intervention studies, although severe ethical problems could arise. At present the results are variable, with some studies indicating a good correlation with other prognostic indicators and others showing poor associations [123–125].

Conclusions. Inhibition of angiogenesis represents a potentially useful target for dietary compounds and there is a wide range of in vitro and in vivo assays that can be used to screen potential candidates. However, the efficacy of such substances in patients may be questioned on the basis of current experience with drugs. Furthermore, given the importance of angiogenesis for normal tissues, there is likely to be little or no use for functional foods with anti-angiogenic activity for the general population. In other words, target groups must be carefully specified in order to avoid anti-angiogenic diets being deleterious.

Inhibition of metastasis

Definition of target. The metastatic escape and spread of cells from a tumour to distant organs is a great barrier to cancer cure. The process involves a complex series of interactions between the cancer and its environment.

Relationship to cancer. Epithelial cells normally lie between thin sheets of basement membranes and are surrounded by an extracellular matrix, which they secrete themselves. In order to become successfully invasive, tumour cells must adhere to the basement membrane (BM), produce and secrete proteolytic enzymes that are capable of degrading the extracellular matrix (ECM) and BM, adhere to and degrade the ECM components of host cells and regulate genes encoding for proteolytic enzymes for ECM degradation and adhesion. Degradation is achieved by enzymes termed Matrix Metalloproteinases (MMPs). MMP activity is inhibited by natural tissue inhibitors of MMPs (TIMPS) and the
balance between MMP and TIMP activity is a strong indicator of metastasis. MMP’s are also involved in some of the later events in the metastatic process, namely invasiveness (in which the cancer cells enter the blood or lymphatic vessels), extravasation (passage of the cell from the vessels into tissues) and proliferation of the cells at the secondary tissue [126].

- **Modulation by diet.** N-3 fatty acids have been shown to inhibit invasion of kidney tumour cells using the Matrigel invasion assay [127]. Animal models have been used to evaluate effects of food components on metastasis. For example retinoids suppress the development of bone metastasis of prostate cancer cells [128] and modified pectin inhibited metastasis of human colorectal and breast carcinoma cells [129]. Thus they provide a method for assessing functional foods for potential anti-metastatic activity, although the relevance to the human situation remains to be established. There is some evidence from less specialised animal models that dietary change can modulate late events in cancer. For example, probiotic treatment of rats given dimethylhydrazine to induce colon tumours, resulted in a reduction in tumour size and a lower proportion of malignant tumours [130].

- **Methodological considerations.** The Matrigel Invasion assay (MIA) is an established in vitro assay to assess cell invasion. Matrigel contains components of the basement membrane and only cells with invasive properties will pass through this layer. In this assay, cells from a metastatic tumour line are seeded onto matrigel-coated tissue culture inserts containing an 8-micron pore size membrane. The matrigel occludes the pores of the membrane blocking non-invasive cells from migrating through the membrane. In contrast, invasive cells are able to detach themselves from and migrate through the matrigel matrix treated membrane in response to a chemoattractant present in the underlying medium.

There are also a number of animal models (usually based on athymic mice) that are used to study the whole process of metastasis, in which the tumour cells are inoculated subcutaneously or orthotopically and the number of spontaneous metastases to secondary sites such as lung liver and bone are assessed [131]. A wide range of tumour cell lines have been used as the source of the inoculum including colorectal tumour cells, melanoma, prostate cell lines, T cell lymphomas and mammary adenocarcinomas [126, 132–135].

In humans, detection of circulating tumour cells in bone marrow and lymph nodes by immunocytochemistry is a well-defined prognostic marker for colorectal cancer progression, especially metastasis [136]. The presence of micrometastases in bone marrow appeared as an independent indicator of an aggressive tumour with a poor outcome [137]. More recently, the detection of tumour cells in the blood circulation is proving a promising biomarker of tumour presence and progression in humans. Longitudinal studies have shown that the levels of circulating epithelial cells in peripheral blood are directly linked to the tumour burden and response to therapy in e.g. prostate cancer [138]. In animals, clusters of tumour cells were found to be a more relevant prognostic marker than single cells [139]. A new technique, called immunomagnetic cell separation, was recently used to show that cell clusters were also present in peripheral blood samples of colon carcinoma patients [140]. It is believed that the vast majority of these cells originate from the primary tumour [141].

- **Conclusions.** Inhibition of metastasis represents a potentially useful target for dietary intervention and there is a wide range of in vitro and in vivo assays that can be used to screen potential candidates.

- **Tumour cells in circulation**

As blood samples can be obtained easily and repeatedly, this makes the detection of tumour cells in blood circulation a very promising biomarker of tumour presence and progression.

**Carcinogen metabolising enzymes**

Metabolism is often the major process responsible for the elimination of a chemical, and hence is of key importance as a target of functional effects. Metabolism includes phases of biotransformation and of toxicokinetics. Biotransformation is the process whereby a substance is changed from one chemical to another (transformed) by a chemical reaction within the body [142]. During this process a large number of phase I and phase II enzymes metabolically convert lipophilic xenobiotics to hydrophilic metabolites. This facilitates their cellular elimination (phase III) and excretion, thus minimising exposure to them.

- **Biotransformation of carcinogens**

**Definition of target.** Phase I enzymes (e.g. cytochrome P450, flavin-dependent monoxygenases, oxidoreductases) convert hydrophobic compounds to reactive electrophiles by oxidation, hydroxylation and reduction reactions to prepare them for reaction with water-soluble moieties. Phase II enzymes (e.g., glutathione S-transferases [GST], UDP-glucuronosyltransferases [UGT], sulphotransferases, aryl amine acetyltransferases) primarily catalyse conjugation reactions.
**Relationship with cancer.** Biotransformation enzymes play a major role in regulating the toxic, mutagenic and neoplastic effects of chemical carcinogens (Table 4). Phase I enzymes, particularly the cytochrome P450s, have the propensity to result in bioactivation, compared with the inactivation that often results from Phase II reactions. This has led to the concept that selective induction of phase II enzymes over phase I will contribute to cancer chemoprevention. An example is the agent [4-methyl-5-(2-pyrazinyl)-3H–1, 2-dithiole-3-thione] (oltipraz) which may protect against liver cancer caused by high aflatoxin B1 exposure in Qidong, People’s Republic of China [143, 144]. The studies have shown that intermittent, high-dose oltipraz inhibited phase I activation of aflatoxins, whereas sustained low-dose oltipraz increased phase II conjugation of aflatoxin B1, yielding higher urinary levels of aflatoxin-mercapturic acid [145]. There are numerous other reports indicating that the balance of metabolising enzymes are directly associated with the magnitude of toxic response for selected chemicals [146]. Genetic enzyme polymorphisms for genes encoding phase I and phase II metabolism are

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**Table 4** Carcinogen metabolism

<table>
<thead>
<tr>
<th>Target</th>
<th>Relationship with cancer</th>
<th>Modulation by diet</th>
<th>Methodology</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phase I</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytochrome P450 (CYP)</td>
<td>Activate carcinogens</td>
<td>Broccoli induces CYPs in rat tissues; dietary fibers differently modulate CYPs in rat tissues; oltipraz inhibits CYPs that activate aflatoxin; flavonoids modulate CYPs, fried meat induces CYP1A2</td>
<td></td>
<td>[147, 282–292]</td>
</tr>
<tr>
<td>CYP1A1</td>
<td>polycyclic, aromatic hydrocarbons</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP1A2</td>
<td>heterocyclic amines, nitroamines</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2A6</td>
<td>cyclopentamidine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2B6</td>
<td>tobacco-specific nitroamines, vinil chloride, aflatoxin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2D6</td>
<td>flavin-containing monooxygenases (FMO)</td>
<td>OXIDISE xenobiotics with broad substrate specificity. Metabolic conversion may result in detoxication. Substrates are tertiary, secondary and primary amines.</td>
<td>Gaps in knowledge, reviewed in [293]</td>
<td>[293–296]</td>
</tr>
<tr>
<td>CYP2E2</td>
<td>EC 1.14.13.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP3A4</td>
<td>flavin-containing monooxygenases (FMO)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EC 1.14.13.8</td>
<td>Flavine-containing monooxygenases (FMO)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EC 1.6.99.2</td>
<td>Flavine-containing monooxygenases (FMO)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAD(P)H Quinone oxidoreductases</td>
<td>Oxidise xenobiotics with broad substrate specificity. Metabolic conversion may result in detoxication. Substrates are tertiary, secondary and primary amines.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(NQO, QR) EC 1.6.99.2</td>
<td>Flavine-containing monooxygenases (FMO)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAD(P)H Quinone oxidoreductases (NQO, QR) EC 1.6.99.2</td>
<td>Can either bioactivate or detoxify quinones; contributes to reducing superoxide radical formation. Lower NQO1 activity may make individuals vulnerable to leukemia secondary to benzene exposure</td>
<td>Numerous plant food extracts or ingredients induce QR in cell models</td>
<td>Both bioactivation and detoxification of substrates is possible.</td>
<td>[297–304]</td>
</tr>
</tbody>
</table>

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**Phase II**

| Glutathione S-transferases (GST) | Catalyse conjugation of reduced glutathione to activated substrates. Metabolic conversion usually results in detoxication | Induction of GST has been shown for a high number of food ingredients, metabolites in animal experiments, cell cultures and in humans. | Species differences in gene regulation. Bioactivation of selected xenobiotics by GSTs is possible. Genetic polymorphisms (deletions, sequence alterations) are common | [169, 178, 191, 305–308] |
| EC 2.5.1.18. GSTA | | | | |
| GSTM | | | | |
| GSTP | | | | |
| GSTT | | | | |
| UDP-Glucuronosyltransferases (UGTs) | Glucuronidation of endo-, xenobiotics usually results in termination or reduction of biological activity. Induction of UGTs is associated with decreased cancer related activities in some animal experiments | Green tea induces UGTs and enhances glucuronidation of 2-amino-1-methyl-6-phenyl imidazo-[4,5b]pyridine (PhIP). Feeding green tea lowers incidence of preneoplastic lesions in animal experiments. | Glucuronide formation can also result in bioactivation. Allelic variants of UGT1A1 may cause specific clinical conditions. Putative phytoprotectants (e. g. isoflavones) are UGT substrates | [309–314] |
| EC 2.4.1.17. UGT1A | | | | |
| UGT2A | | | | |
| UGT2B | | | | |
| Arylamine acetyltransferases (NAT) EC 2.3.1.5. | Catalyse three reactions involving the transfer of an acetyl moiety, resulting in N-acetylation (NAT), O-acetylation (OAT), N,O-acetylation (AHAT). Substrates include carcinogenic aromatic amines and heterocyclic amines (food contaminants) | Glucuronidation of endo-, xenobiotics usually results in termination or reduction of biological activity. | Depending on structure of the substrate, the reactions can lead to detoxification or bioactivation, e. g. of heterocyclic amines. Polymorphisms affect toxicological responses under specific exposure situations | [181, 184, 290, 315, 316] |
| NAT2 | | | | |
| NAT1 | | | | |
well recognised susceptibility factors for sporadic tumours, depending on the exposure situation and functional consequences of the polymorphisms [147–150]. The induction of specific enzymes involved in the metabolism of known risk factors can result in an enhancement or reduction of exposure and accordingly will modulate cancer related endpoints in model or animal systems [151–154] and in humans [155].

**Modulation of target by diet.** A claim by which phytotoxics from plant foods could be of benefit is that they modulate biotransformation in such a manner that carcinogens are less active and thus exert less harm in the target cells of cancer. This is considered to be one of the mechanisms by which fruits and vegetables act chemoprotectively [156]. One example is chemoprotection conferred by cruciferous vegetables, due to their high glucosinolate content and the capacity of glucosinolates to modulate biotransformation enzyme systems (e.g. cytochrome P450 and conjugating enzymes) [157]. Glucosinolates (β-thioglycoside-N-hydroxysulfates) are hydrolysed by the plant enzyme myrosinase releasing the biologically active isothiocyanates (ITCs). ITCs induce expression of phase I and phase II enzymes and also directly inhibit CYP450 [120]. Broccoli and Brussels sprouts increase metabolism of cooked meat-derived heterocyclic aromatic amines, which implies that they induce both CYP1A2 and phase II enzymes involved in heterocyclic amine metabolism [158]. The results of six cohort studies have shown inverse associations between the consumption of Brassica vegetables and risk of lung cancer, stomach cancer, and all cancers taken together (reviewed in [159]). Two thirds of 74 case-control studies have also shown an inverse association for risks of several cancer sites including the colon (reviewed in [159]). In a recent study [160] dietary ITC intake from cruciferous vegetables was assessed for 213 incident cases of colorectal cancer and compared to 1194 controls. The additional analysis of GSTM1, T1 or P1 genotypes revealed that there were no overall associations between genotype and colorectal cancer risk. However, among individuals with both GSTM1 and T1 null genotypes, a 57% reduction in risk among high versus low consumers of ITC was observed for colon cancer. The results are compatible with the hypothesis that ITCs from cruciferous vegetables modify risk of colorectal cancer in individuals with low GST activity. In the gut lumen the glucosinolate breakdown product sulforaphane can inhibit DNA adduct formation induced by a heterocyclic amine in a dose-dependent manner, possibly acting through the induction of phase II detoxification enzymes such as glutathione transferases and UDP-glucuronosyltransferases [161].

**Methodological considerations.** Some plant food ingredients (ITCs) induce phase I enzymes, others induce only phase II enzymes, and some induce both [158–162]. The situation is uniquely complex because each plant food contains a variety of glucosinolates and other phytochemicals [163], which modulate biotransformation systems in multiple ways. Therefore it is not easily predictable, in which manner modulated biotransformation results in risk reduction. More accurate knowledge is needed on whether carcinogens cause specific types of tumours and how these carcinogens are metabolically transformed in the relevant human tissues. It is also necessary to increase our understanding on how diet can modulate the biotransformation in a favourable manner, and how individual susceptibilities (genetic polymorphisms, regulation of the target genes, baseline expression levels) affect biotransformation and exposure. The elucidation of these complex situations will require experimental approaches of equal complexity, e. g. to elucidate multiple genes and pathways of induction arising from the impact of diet. Promising approaches to achieve the analysis are the employment of new techniques of genomics to reveal patterns of gene expression (transcriptomics), revealing complete metabolic pathways (metabonomics) and assessing the functional consequences on cellular and organism levels. The criteria for this approach, which are being developed in the field of toxicogenomics [164] and are already applied in pharmacogenetics [165] will also be an important scientific basis for better understanding dietary chemoprevention.

**Conclusion.** It is very feasible, from a theoretical point of view, to provide humans with foods, which enhance the capacity of tissues to metabolise chemical carcinogens in such a way that these are eliminated from the body without being transformed to reactive intermediates. The selective induction of Phase II over Phase I could mediate such protection, provided that the resulting conjugates are not more reactive than the substrates. Taken together carcinogen metabolising enzymes could be considered as potential "susceptibility markers".

**Glutathione S-transferases**

The glutathione S-transferases (GSTs) are dealt with in more detail below as they represent one of the more interesting specific targets within the general target “biotransformation of carcinogens”.

**Definition of target.** The glutathione S-transferases (GSTs) are a multigene family of enzymes largely involved in the detoxification of chemicals. They catalyse the conjugation of the tripeptide glutathione via its sulphur atom to many toxins containing an electrophilic functional group, allowing these compounds to be excreted from the body [166]. The best-characterised GST isoenzymes in mammals have been grouped into four
major cytosolic classes, termed Alpha (A), Pi (P), Mu (M), Theta (T), and three membrane-bound GSTs [167].

- **Relationship with cancer.** Oxidative molecular damage in cells is linked to degenerative diseases including cancer [168]. Glutathione S-transferases contribute to resistance against oxidative stress [169] by inactivating toxic and mutagenic alkene products of oxidative processes and thus protect cells and tissues [170]. An important factor is that GSTs are inducible and that in rat and mouse induction appears to be mediated through the antioxidant response element (ARE), a transcription enhancer [171, 172]. It contains sequences required for basal expression of, for example rGSTA2, and its induction is mediated by phenolic antioxidants, for example green tea phenols [173]. These compounds also activate genes via the AP-1 family of transcription factors, which include Jun, fos, Maf, Nrl and Fra proteins [174]. AP-1-binding sites have been identified in the promoter regions of the hGSTA1, hGSTA4 and hGSTP1 genes [175, 176]. Other transcription factor binding sites (NF-κB, SP-1, AP-2, GRE) have been described for GSTP1 and/or GSTA1. Whether or not an enhanced expression of GSTs contributes to cancer prevention has not been demonstrated in humans conclusively. But there are several lines of evidence which support this assumption: 1) A large number of animal experiments have shown that cancer reducing activities of chemicals can be associated with increases in phase II metabolism [177]; 2) In human cells an enhanced GST-level is associated with less damage by 4-hydroxynonenal [178] whereas lower GST-levels are associated with more damage [179]; 3) Individuals with null polymorphisms [180] for GSTM1, GSTT1 or other types of genetic predispositions [181] are at higher risks for developing tumours, especially in the case of specific exposure situations [182–184]. Alternatively, sequence polymorphisms for GSTP1 protect from benzo(a)pyrene mediated genotoxic damage [185].

- **Modulation by diet.** Numerous diets, food ingredients and related endogenous metabolites induce GSTs in animal experiments [166, 186–190]. In humans, evidence is also available on dietary or intervention mediated regulation of GSTs [191–193]. Both inducing and suppressing activities have been reported. One example is the effects in human colon cells of butyrate, an abundant fermentation product present in the human gut [178, 194]. Also, genetic polymorphisms alter the preventive effects of cruciferous vegetables, which are inducers of GST [157].

- **Methodological considerations.**
  - **Species differences in gene regulation:** Different mechanisms of transcriptional and posttranscriptional events are important in controlling levels of expression and these are to be expected in different genes, cell types and tissues. For instance most of our knowledge on the regulation of GSTs has been derived from studies in rodents. However, the sequences of the 5'-flanking regions of the human genes are different from the rodents', suggesting species-differences in the regulation [195].
  - **Activation by phase II reactions:** Not all phase II reactions yield products that are less reactive than the substrate prior to conjugation. For certain haloalkanes (ethylene dibromide, methylene chloride) the conjugate is instable and rapidly decomposes to reactive intermediates with mutagenic potential [196].
  - **Shift in organotrophy:** The modulation of biotransformation does not necessarily provide the same protection in all tissues. In some cases there may only be a shift of metabolism, which can result in a shift of tumour localisation. For example, in animal experiments I-3-C decreased formation of aberrant crypt foci in the colon, but enhanced the formation of GSTP foci in the liver [197].
  - **Genetic polymorphisms of GSTs:** Confounding situations may be encountered on the basis of individual susceptibilities. Null genotypes for GSTM1 and GSTT1 occur in frequencies of approximately 50 % and 20 % of the population and result in absence of the respective enzymes. The primary hypothesis has been that individuals with the GST-null genotypes are at higher risk for cancer because of a reduced capacity to eliminate activated carcinogens. Therefore epidemiological studies have focused on interactions between these polymorphisms and carcinogen exposure, whereas only few human dietary interventions have tested the effects of diet on biotransformation enzymes in relation to the genetic polymorphisms of the individuals. One was a controlled feeding study to test if GSTM1genotype affects response to a diet high in cruciferous vegetables [163]. Serum GSTT1 concentration, an enzyme induced by isothiocyanates, increased significantly in response to cruciferous vegetable feeding, but only in GSTM1-null individuals, indicating that the relationships between cruciferous vegetable intake and cancer risk are influenced by genetic polymorphisms of GSTs [157].

- **Conclusion.** It seems likely, that an induced expression of the GSTs will result in the protection of cells from genotoxic insult by specific chemicals, especially since these particular enzymes are more involved in deactivation rather than activation and they are inducible. Moreover, many putative carcinogens are relevant substrates for GSTs. However, the limitations in claiming such functions from foods are based on the need for the experimental verification of the hypothesis. To date there are...
no data available showing that in humans the final consequence of an induction of GST coupled to a favourable overall modulation of biotransformation is the prevention of tumours.

**Genetic events**

Genetic events, that is mainly damage to the genome of a cell, constitute various types of lesions such as DNA damage, DNA adducts, gene mutations, and cytogenetic alterations. Associated with these is a deficiency of DNA repair leading to genetic instability and to an even higher incidence of genetic lesions (see Table 5).

There is substantial evidence that genetic events are involved in the initiation, promotion and progression phases of carcinogenesis. The selected endpoints described in the following sections are usually measured in surrogate tissues (e.g. peripheral lymphocytes). The endpoints largely serve as biomarkers of exposure, but there are some probable lines of evidence showing that causative associations are involved as well. Therefore, several larger scale validation studies are presently underway to assess causal relationships for individual endpoints of genetic damage.

Guidelines considering several genetic endpoints are available to provide concise guidance on the planning, performing and interpretation of studies to monitor groups or individuals exposed to genotoxic agents [198]. The guidelines contain important methodological considerations, including ethical aspects, details on sampling procedures, size and characteristics of studied populations, how to assess individual exposures, and potential confounders or effect modifiers, aspects of performing the statistical analysis, as well as procedures for publishing and archiving data, and ensuring sufficient high quality of data. They provide a basis for studies to monitor subjects of dietary intervention trials. Requirements for reporting antioxidant, antimutagenic or anticarcinogenic potential of test substances in in vitro experiments and animal studies in vivo have been identified as well [199].

Optimising vitamin and mineral intake by encouraging dietary change, multivitamin and mineral supplements, and fortifying foods might prevent genetic lesions and therefore cancer and other chronic diseases [200].

**DNA strand breaks**

**Definition of target.** Breaks of the DNA backbones (single strand breaks). If two breaks occur on opposite strands in close vicinity, these are called double strand breaks.

**Relationship of target with cancer.** Many cancer-causing compounds are genotoxic and some of these have the potential to induce DNA strand breaks. However, strand breaks can also result from the enzymatic activity of endonucleases in apoptosis or during DNA repair. So, the significance of strand breaks is complex and reflects DNA damage as well as DNA repair.

**Modulation of target by diet.** The first studies showing that dietary intervention in humans alters (lowers DNA strand breaks) in lymphocytes were performed with vegetable [201] and fruit juices [202]. Meanwhile several other studies are showing that other types of dietary intervention have similar effects. Faecal water obtained from humans consuming a diet high in fibre causes fewer DNA strand breaks in cultured human colon cells than a diet high in meat and animal fat, but low in dietary fibre [113]. Moderate wine consumption protects against hydrogen peroxide-induced DNA strand breaks [203]. Instead of using only the surrogate lymphocytes, studies using target tissues of cancer occurrence are considered to be more relevant. To date, DNA strand breaks in cells of potential target tissues have been shown in animal studies, e.g. in the colon of rats after oral ingestion of probiotics [204, 205], but methods are now available to investigate effects of diet also in colon cells of humans [206–209].

**Methodological considerations.** Guidelines considering this endpoint are available to provide concise guidance on the planning, performing and interpretation of studies to monitor groups or individuals exposed to genotoxic agents. They provide a basis for studies to monitor subjects of dietary intervention trials [198]. The most frequently applied assay to detect strand breaks and related events is the alkaline single cell gel electrophoresis (Comet) assay [209] which has largely replaced the technique of alkaline elution [210, 211]. Recent recommendations for performing the in vivo Comet assay are available [212]. For a review of the clinical applications of the Comet assay in different cell types (lymphocytes, buccal and nasal cells, and sperm) see also reference [213].

**Conclusion.** The determination of DNA damage, as is now being performed with the comet assay, provides a useful tool to investigate the effects of diet in a panel of different human tissues, including tissues relevant for diet-associated tumour occurrence. The modulation of DNA damage reasonably well reflects also the modulated exposure to genotoxic compounds that cause the damage. Therefore the reduction of DNA damage will indicate a reduced exposure, which in turn is related to decrease of risk.
■ DNA adducts

■ Definition of target. Modified DNA bases resulting from the addition of DNA with an electrophile or free radical are called DNA adducts. Adducts can also be generated by the effect of ultraviolet radiation creating thymine dimers. Depending on the type of chemical involved, alkylated bases, oxidised bases, bulky adducts, etheno- and propano-adducts can result as adducts. Sometimes, the effect of an adduct can result in the formation of DNA adducts from polycyclic aromatic hydrocarbons. A recent meta-analysis has shown that current smokers with high levels of bulky adducts have an increased risk of lung and bladder cancers, which also suggested that similar (aromatic) compounds may be involved in the aetiology of both types of cancer. 

Table 5 Genetic events

<table>
<thead>
<tr>
<th>Target</th>
<th>Relationship with cancer</th>
<th>Modulation by diet</th>
<th>Methodology</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA strand breaks (e.g. Comet assay)</td>
<td>Reduction of damage indicates a reduction of exposure to genotoxic compounds. Since a high proportion of cancers is most likely due to exogenous compounds, reduced exposure is equivalent to reducing risks.</td>
<td>Vegetable and fruit juices decrease damage in peripheral lymphocytes of humans. In animals DNA damage is decreased in colon cells of rats consuming probiotics. Faecal water genotoxicity can be modulated by diet.</td>
<td>Can be measured in any tissue and in exfoliated cells; body fluids can be monitored for damaging capacity, reflecting exposure; multiple classes of damage can be detected; analysis proceeds on level of single cell</td>
<td>[201, 202, 205, 317]</td>
</tr>
<tr>
<td>DNA adducts</td>
<td>Bulky DNA adducts from polycyclic aromatic hydrocarbons</td>
<td>A recent meta-analysis has shown that current smokers with high levels of bulky adducts have an increased risk of lung and bladder cancers, which also suggested that similar (aromatic) compounds may be involved in the aetiology of both types of cancer.</td>
<td>A number of different EU projects are being performed to associate DNA adducts, exposure and cancer in molecular epidemiological studies. Methodological limitations are that the level of measurement error for bulky adducts is high. More standardised measurements are needed in future investigations.</td>
<td>[155, 214, 318, 319]</td>
</tr>
<tr>
<td>Oxidised DNA bases (and oxidative stress)</td>
<td>Oxidised DNA bases cause mutations that are commonly observed in mutated oncogenes and tumour suppressor genes. Reduction indicates a reduction of exposure. An EU-funded Concerted Action (QLK1–1999–00568; ESCODD) has been set up to establish a European standards committee on oxidative DNA damage. The project aims ultimately to establish the level of DNA damage in white blood cells from representative groups of men and women. One objective is to resolve the problems in measuring this marker and to reach a consensus on the level of damage in normal cells. This is important from the point of view of estimating cancer risk and cancer-preventive effects of dietary antioxidants.</td>
<td>Carotenoid-rich juices and supplementation with antioxidants have reduced oxidised bases measured with the comet assay. Oxidative DNA-damage in humans is reduced by Brussels sprouts consumption. In a recent epidemiological study, individual dietary and lifestyle habits only modestly affected lymphocyte DNA oxidation (measured in the modified comet assay) and suggested that specific dietary patterns, rich in fresh fruit and vegetables, are not clearly related to decreased oxidative damage in peripheral lymphocytes in a Mediterranean population.</td>
<td>Different methods are available to measure oxidised DNA bases, namely oxidised pyrimidine and purine bases, using the comet assay with repair specific enzymes, 8-oxo-7,8-dihydro-8-oxoguanosine, using HPLC with electrochemical detection (HPLC/ECD), GC/MS methods, enzyme-linked immunosorbent assay (ELISA) techniques and 5-hydroxymethyl-2-deoxyuridine (HMdU), a product of thymine oxidation, using serum auto antibodies. Recent studies are showing that the comet assay and HPLC are equally efficient for detecting induced damage. Standards are being evaluated in the EU-funded Concerted Action (QLK1–1999–00568; ESCODD).</td>
<td>[318, 201, 202, 220, 320–322]</td>
</tr>
<tr>
<td>Exocyclic DNA adducts (etheno- and propano-adducts)</td>
<td>Oxygen radicals attack lipids to generate reactive intermediates that can couple to DNA resulting in exocyclic bases. The promutagenic lesions can be related to increased probability of cancer risks if induced in oncogenes and tumour suppressor genes. For example the major lipid peroxidation product, trans-4-hydroxy-2-nonenal, preferentially forms DNA adducts at codon 249 of human p53 gene, a unique mutational hotspot in hepatocellular carcinoma.</td>
<td>High dietary omega-6 polyunsaturated fatty acids drastically increase the formation of etheno-DNA base adducts in white blood cells.</td>
<td>Presently an EU-funded project is being performed (QLK4CT-2000–00286) with the objective to analyse the repair functions, the persistence and the mutational specificity of e-DNA adducts at the gene level, so to better define the mutagenic and carcinogenic potential of these miscoding lesions in humans. An immunohistochemical method has been developed to detect malondialdehyde (MDA)-modified DNA adducts in single human oral mucosa cells.</td>
<td>[320, 323–325]</td>
</tr>
</tbody>
</table>
Table 5  Continued

<table>
<thead>
<tr>
<th>Target</th>
<th>Relationship with cancer</th>
<th>Modulation by diet</th>
<th>Methodology</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutations</td>
<td>The accumulation of multiple mutations, especially in protooncogenes, tumour suppressor genes and DNA repair genes is associated with cancer initiation, promotion and progression. In fact tumour progression selects for cells with specific mutations including those which maintain genetic stability.</td>
<td>Using the HPRT (hypoxanthine – guanine phosphoribosyl transferase) gene assay with T-lymphocytes from 312 individuals, it was found that HPRT mutant frequency (MF) was significantly decreased in relation to intake of vegetables, citrus fruits and berries, respectively, as well as calculated vitamin C intake from diet. Only a borderline significant association was observed for beta-carotene</td>
<td>The tissue-specific mutagenicity of IQ was studied at the lacI locus in the liver, colon and kidney of Big Blue(R) transgenic rats. This is probably the most efficient system to analyse mutations in vivo. However, the mutations identified were different to the mutations identified in IQ-induced tumours, which indicates the responses to be non-specific in relation to the effects in humans.</td>
<td>[326, 327]</td>
</tr>
<tr>
<td>Chromosomal aberrations</td>
<td>Reduction in occurrence should indicate a reduction of exposure, and possibly of cancer risk. A present EU-funded concerted action (BIOMED 2) of 13 European institutes, is characterising cytogenetic lesions, occupational exposure and cancer incidence in large cohorts, with the aim to analyse the predictivity of chromosome changes as indicators of cancer risk</td>
<td>Food mutagens can induce cytogenetic lesions. Probiotics have been shown to reduce CA in animal experiments, but otherwise there are gaps of knowledge on how diet may reduce CA in humans</td>
<td>Putative anticarcinogens, namely the phytoestrogens coumoestrol and genistein induce structural chromosomal aberrations in cultured human peripheral blood lymphocytes</td>
<td>[328–331]</td>
</tr>
<tr>
<td>Micronuclei</td>
<td>Reduction of occurrence indicates a reduction of exposure, and possible of risk. “The International Collaborative Project on Micronucleus Frequency in Human populations (HUMNI)” is collecting an international data base to determine associations with cancer and ageing <a href="http://ehs.sph.berkeley.edu/holland/humn/">http://ehs.sph.berkeley.edu/holland/humn/</a></td>
<td>Different levels of micronuclei incidence were reported with age, gender and vegetarian, versus non-vegetarian diet. Moderate wine consumption protects against hydrogen peroxide-induced micronuclei. Micronuclei are modulated in lymphocytes and exfoliated buccal cells of postmenopausal women with dietary changes in folate. Cigarette smoking, intracellular vitamin deficiency, are associated with micronuclei in epithelial cells of the buccal mucosa</td>
<td>Micronuclei are easier to score (less time consuming) than chromosomal aberrations. Different types of staining methods can reveal the nature of origin of micronuclei. Determinations of micronuclei in exfoliated cells of buccal and bladder mucosa are a promising alternative to lymphocytes in human studies. Presently larger scale international validation and endpoint assessments are taking place.</td>
<td>[203, 234, 332–337]</td>
</tr>
<tr>
<td>DNA Repair BER</td>
<td>Significantly increased risk for orolaryngeal cancer was observed for polymorph hOGG1 genotypes, in smokers but not in non-smokers, suggesting that hOGG1 may play an important role in the repair of 8-OH-DG adducts in the aerodigestive tract and that the hOGG1 (Ser326Cys) polymorphism plays an important role in risk for smoking- and alcohol-related orolaryngeal cancer</td>
<td>Significantly increased risk for orolaryngeal cancer was observed for polymorph hOGG1 genotypes, in alcohol drinkers but not in never drinkers smokers.</td>
<td>Until recently, only two mammalian DNA glycosylases specific for oxidised bases had been characterised, the E.coli endonuclease III (Nth) homolog NTH1, and 8-oxoguanine-DNA glycosylase (OGG1), pathophysiological effects. However, mouse mutants lacking either NTH1 or OGG1 have no obvious phenotype, in spite of accumulation of mutagenic and toxic base lesions in their genomes. An explanation is that repair is mediated by additional DNA glycosylases.</td>
<td>[338, 339]</td>
</tr>
</tbody>
</table>

breaking of a base into small residues or its removal from DNA creating an abasic site.

■ **Relationship of target with cancer.** A recent meta-analysis testing the hypothesis that the presence of a
high level of bulky DNA adducts in tissues is associated with an increased risk of cancer in humans reports that current smokers with cancer have statistically significant higher levels of adducts than controls, whereas results were negative or contradictory in ex-smokers and non-smokers. The authors conclude that current smokers with high levels of adducts have an increased risk of lung and bladder cancers [214].

Modulation of target by diet. DNA adducts of acetaldehyde can be detected in peripheral white blood cells of alcohol abusers [215]. Plasma levels of a combination of antioxidant vitamins (vitamins C and E and β-carotene) rose significantly during a randomised clinical trial among heavy smokers, but the measurement of DNA damage (immunological determination of polycyclic aromatic hydrocarbon-DNA adducts) and oxidative DNA damage (8-oxo- or hydroxydeoxyguanosine) in mononuclear and oral cells was not statistically significantly different in the group with antioxidant vitamin intake than in the controls, thus providing no evidence that vitamins prevent this type of DNA damage in smokers [216]. The studies confirm earlier studies measuring oxidative DNA damage in male smokers receiving β-carotene supplementation [217]. In another study, antioxidant supplementation decreases oxidative DNA damage in male smokers receiving β-carotene supplementation [217]. In another study, antioxidant supplementation decreases oxidative DNA damage in human lymphocytes [218]. Oxidised DNA bases are reduced in lymphocyte-DNA of healthy, non-smoking males after consumption of vegetable juices [201] and of a soy milk supplement [219]. However when considering epidemiological studies instead of intervention trials the associations are not as apparent. In a recent epidemiological study [220], comparing dietary and lifestyle determinants of oxidative DNA damage (modified comet assay) basal levels of DNA oxidation were positively associated with coffee (P = 0.01) and tomato consumption (P = 0.05). Instead, the consumption of cruciferous vegetables tended to be negatively associated with oxidative damage. A positive non-significant association between the consumption of total vegetables and fresh fruit and DNA damage was noted (P = 0.08 and P = 0.10, respectively). In contrast, the estimated intake of simple sugars was positively associated with oxidative DNA damage, while vitamin E showed a borderline positive association. The plasma levels of several micronutrients did not appear to influence DNA damage. Thus, the results indicated that individual dietary and lifestyle habits only modestly affected the levels of lymphocyte DNA oxidation and suggested that specific dietary patterns, rich in fresh fruit and vegetables, are not clearly related to decreased oxidative damage in peripheral lymphocytes in a Mediterranean population [221].

Methodological considerations. Guidelines considering this endpoint are available to provide concise guidance on the planning, performing and interpretation of studies to monitor groups or individuals exposed to genotoxic agents. They provide a basis for studies to monitor subjects of dietary intervention trials [198]. The best established methods to detect DNA adducts are sensitive chemical analytical detection methods, involving 32P post labelling of the DNA [222, 223]. Biological determinations (e.g. using modifications of the comet assay) are increasingly becoming available, which may facilitate larger scale monitoring studies in the future [220].

Conclusion. Adducts are specific indicators of DNA damage caused by individual chemical groups (e.g. “Bulky” DNA adducts by polycyclic aromatic hydrocarbons, etheno-adducts by reactive aldehydes, oxidised bases by reactive oxygen species). They therefore represent an integrated marker of exposure to the specific compounds, and of the ability of the individual to metabolically activate carcinogens and to repair DNA damage.
age. A reduction of adducts therefore reflects a lower level of exposure and with that a lower risk. A number of international studies are being performed to determine in which manner specific types of DNA adducts are causally related to cancer risks. The outcome of these studies will be of high value for using the parameters as a basis for possible claims.

### DNA repair

**Definition of target.** Base excision repair (BER) is primarily responsible for repairing small base modifications and abasic sites caused by endogenous damage and environmental insult. Nucleotide excision repair (NER) is the repair process which incorporates the excision of damaged and altered bases (e.g. bulky adducts, cyclobutane pyrimidine dimers) as part of an oligonucleotide fragment. Mismatch repair (MMR) is the DNA repair process by which mispaired bases are excised as single nucleotides [224]. Interstrand cross-links and DNA double strand breaks are repaired by homologous recombination and/or end-joining repair. O6-methylguanine methyltransferase, a single repair protein, reverses damage by removing the non-native methyl group from O6-methyl guanine [225].

**Relationship of target with cancer.** Inherited defects in any of these pathways, in general, predispose to malignancy [225]. Examples are defective NER, which is the cause for Xeroderma pigmentosum (XP), a syndrome characterised by a severe predisposition to skin cancers of various types (squamous cell carcinomas and basal cell carcinomas). Defects in MMR in humans predispose to cancer of the colon, but also to uterine, ovarian and gastric cancer (reviewed in [224]). No human disorders caused by inherited BER deficiencies have been identified, but according to mouse models, the knockout of BER enzymes (glycosylases) show only mild phenotypes, although mutagenesis and cancer susceptibility are probably increased [225]. X-ray-sensitive persons afflicted with ataxia telangiectasia have a defective ataxia telangiectasia (ATM) protein kinase, which predisposes to lymphoma [reviewed in 224]. Latent genetic instability has been associated with an increased risk for several cancers. Genetic instability, as measured by the comet assay is associated with an increased estimated relative risk of bladder cancer [216].

**Modulation of target by diet.** Repair enzymes have not been well characterised for regulation via transcriptional activity. There are few data available related to exogenous factors involved in regulation of these different proteins, with perhaps the exception of alkyl methyltransferase albeit the physiological consequences are not well elucidated [226]. There are large gaps in knowledge pertaining to the potential regulation of repair enzymes by diet, and hardly any information is available.

**Methodological considerations.** DNA repair deficiency is related not only to cancer, but also probably to ageing and exposure to oxidative stress. New knock-out mouse models reflecting specific repair deficiencies are becoming more and more available to study these associations. There are hardly any studies on modulation of transcriptional activity of DNA repair genes (e.g. potential induction by dietary factors), although they would be highly needed as a tool to study the possibility of repair induction.

**Conclusion.** Large gaps in knowledge exist. Whether or not it would be possible to make the potential claim that DNA repair can be activated by enhanced transcription of the respective genes has hardly been investigated so far.

### Cytogenetic effects

**Definition of target.** Chromosomal structural aberrations are deletions, insertions, translocations, inversions, ring structures and abnormalities of chromosome number. Micronuclei originate from chromosome deletions and chromosome loss. Nucleoplasmic bridges originate from dicentric chromatids and chromosomes.

**Relationship of target with cancer.** A large number of studies are available which demonstrate that deletions in tumour suppressor genes and DNA repair genes [227], resulting in the loss of a functional gene product, are involved in the transformation of normal, dividing cells into tumour cells. The alterations and the sequences by which these alterations occur as well as the subsequent functional responses have been reasonably well investigated in, for example colon cancer [228]. Chromosomal aberrations and micronuclei in peripheral lymphocytes are used as surrogate markers, although the long-term effects of an increased frequency of chromosomal aberrations in individuals are still uncertain. In a recent study, new support for the associations between cytogenetic damage and cancer was obtained [229]. An increased risk of cancer in healthy individuals with high levels of chromosomal aberrations (CAs) in peripheral blood lymphocytes has been described in recent epidemiological studies [230]. This association did not appear to be modified by sex, age, country, or time. The study evaluated whether CAs predicted cancer because they were the result of past exposure to carcinogens or because they were an intermediate endpoint in the pathway leading to disease. A nested case-control study was performed on 93 incident cancer cases and 62 deceased cancer cases coming from two
prospective cohort studies performed in Nordic countries (Denmark, Finland, Norway, and Sweden) and Italy. For each case, four controls matched by country, sex, year of birth, and year of CA test were randomly selected. Occupational exposure and smoking habit were assessed by a collaborative group of occupational hygienists. Logistic regression models indicated a statistically significant increase in risk for subjects with a high level of CAs compared to those with a low level in the Nordic cohort (odds ratio, 2.35; 95% confidence interval, 1.31–4.23) and in the Italian cohort (odds ratio, 2.66; 95% confidence interval, 1.26–5.62). These estimates were not affected by the inclusion of occupational exposure level and smoking habit in the regression model. The risk for high versus low levels of CAs was similar in subjects heavily exposed to carcinogens and in those who had never, to their knowledge, been exposed to any major carcinogenic agent during their lifetime, supporting the idea that chromosome damage itself is involved in the pathway to cancer. The results have important ramifications for the understanding of the role played by sporadic chromosome damage for the origin of neoplasia-associated CAs.

Modulation of target by diet. One study has addressed the effects of age and lifestyle factors on the accumulation of cytogenetic damage as measured by chromosome painting, and has found that stable chromosome aberrations show a greater accumulation with age than do unstable aberrations, suggesting that lifestyle factors contribute to the accumulation of cytogenetic damage [231]. Hardly any studies in humans exist showing that diet may contribute to reducing the occurrence of chromosomal aberrations. Micronuclei have been used in this context more frequently, for example intervention with vitamin E had no significant impact on spontaneous genetic damage in human lymphocytes [232]. Moderate wine consumption protects against hydrogen peroxide-induced DNA damage, measured as micronuclei [203]. The micronuclei index is enhanced in older men, deficient in homocysteine, folate and vitamin B12 [233]. A highly significant (p = 0.001) positive association between plasma levels of vitamin B12 and frequencies of SCE was reported in smokers [234]. After correction for age, gender and GSTM1 genotype, a significant association (p = 0.026) between the MTRR 66GG variant genotype and higher micronucleus rates was observed [234], whereas another study has shown that folate and vitamin B12 supplementation reduces genome damage in young adults [235]. In vitro studies have shown that genomic instability in human cells is minimised when folic acid concentration in culture medium is > 227 nmol/l. Intervention studies in humans show among other effects that micronucleus formation is minimised when plasma concentration of vitamin B12 is > 300 pmol/l and plasma homocysteine is < 7.5 micro-

Methodological considerations. Cytogenetic lesions are determined mainly in surrogate tissues such as peripheral blood lymphocytes, and rarely in the target tissues of dietary related cancers, mainly since it is technically not easy to isolate dividing cells from the tissues [237]. Alternative approaches include the determinations of micronuclei in exfoliated epithelial cells of the buccal cavity [238] or of the urinary bladder excreted in the urine. Guidelines considering this end point are available to provide concise guidance on the planning, performing and interpretation of studies to monitor groups or individuals exposed to genotoxic agents. They provide a basis for studies to monitor subjects of dietary intervention trials [198].

Conclusion. Cytogenetic lesions are indicators of DNA damage caused by exposure. Also, they occur as the consequence of genetic instability in cancer patients. They therefore represent an integrated marker of exposure and of disease. A reduction of cytogenetic lesions reflects a lower level of exposure and with that a lower risk, although systematic studies for these associations are not available. A reduction of cytogenetic lesions as a consequence of retardation of disease has also not been reported. A number of international studies are being performed to determine in which manner specific types of cytogenetic lesions are causally related to cancer risks. The outcome of these studies will be of high value for using the parameters as a basis for possible claims.

Overall concluding remarks

This working group has identified 18 targets/markers in the chain of events from initial exposure to overt malignant tumour whose modification 'have the potential' to be used for 'anticancer' claims for food components (Table 6). In this area, the one true marker is the malignant human tumour, which for practical reasons is usually not accessible to claims. In its absence, we must rely on alternative markers (late and intermediate markers of disease). At present, the strongest markers available are precancerous lesions (e.g. polyps) in humans and precancerous lesions and tumours in animal models. The only marker that presently can be used for a type B claim for food components is 'polyp recurrence'. However, even this marker has several limitations (e.g. not all polyps progress to cancer) and the claim should specify only 'reduces risk of polyps'. Type B claims cannot be made on the basis of results in animal models.
All of the other markers (intermediate markers) presently lack validation against the ‘true endpoint’, the tumour, and thus cannot be used for type B claims. However, the majority of these intermediate markers, which reflect events earlier in the chain of events leading to the tumour and are mechanistically well linked to the disease process, have varying degrees of support from in vitro/animal studies. They may, thus, be used for type A claims, such as ‘reduces carcinogen exposure’, ‘inactivates carcinogens’, ‘reduces DNA damage’, ‘modulates cellular events important for cancer prevention’, ‘inhibits COX-2’.

‘Reduction of risk of disease’ claims in the area of ‘diet-related cancer’ should be based primarily on human intervention studies using relevant/acceptable endpoints. In the absence of the true endpoint, the tumour, which is not always practical to use, precancerous lesions (e.g. polyps, aberrant crypt foci) are to be preferred. However, in this field, the availability of additional intermediate markers is particularly important in order to make dietary intervention studies more attractive/accessible to those wishing to make claims. An important area for future research will be the validation of these surrogate markers [239]. It should also be mentioned that in certain instances, claims might be made based on results from large epidemiological studies. For further information, the reader is referred to the “set of interim criteria for the scientific substantiation of health claims on foods and food components” [see 345].

The working group emphasises that in the area of ‘diet-related cancer’ when a claim is made, it should be specified who may benefit from the effect, e.g. the healthy population, high-risk persons or patients and evidence should be presented from the target group. Susceptibility to the claimed effect is of particular importance in this area. We believe that a scientifically substantiated mechanism is essential for any claim, especially if evidence is presented using intermediate markers as endpoints. We would also emphasise that in human dietary intervention trials, an appropriate duration and sample size to demonstrate the intended effect be employed.

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### Table 6 Concluding remarks

<table>
<thead>
<tr>
<th>Possible claim</th>
<th>Markers/targets</th>
<th>Supportive data from animal/in vitro studies</th>
<th>Evidence from human studies</th>
<th>Target group</th>
<th>Potential application of marker for claims</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduction in exposure to carcinogens</td>
<td>Gut enzymes, Gut metabolites, Faecal cytotoxicity, Faecal genotoxicity, DNA adducts (DNA strand breaks) (Cytogenetic effects)</td>
<td>+++</td>
<td>++</td>
<td>Healthy population</td>
<td>A</td>
</tr>
<tr>
<td>Inactivation of carcinogens</td>
<td>Biotransformation of carcinogens (e.g. GSTs)</td>
<td>++</td>
<td>+</td>
<td>Healthy population</td>
<td>A</td>
</tr>
<tr>
<td>Reduction of DNA damage</td>
<td>DNA strand breaks, DNA repair, Cytogenetic effects (Apoptosis)</td>
<td>+</td>
<td>+</td>
<td>Healthy population</td>
<td>A</td>
</tr>
<tr>
<td>Modulation of cellular events</td>
<td>Proliferation, Differentiation, Apoptosis, COX-2</td>
<td>+++</td>
<td>++</td>
<td>Healthy population, Patients</td>
<td>A</td>
</tr>
<tr>
<td>Prevention of precancerous lesions</td>
<td>Aberrant crypt foci, Polyps</td>
<td>+++</td>
<td>++</td>
<td>Healthy population, High risk persons, Patients</td>
<td>B, B*</td>
</tr>
<tr>
<td>Prevention of tumours</td>
<td>Tumours in animals</td>
<td>+++</td>
<td>–</td>
<td>Healthy population, High risk persons</td>
<td>B*</td>
</tr>
<tr>
<td>Inhibition of tumour progression</td>
<td>Angiogenesis, Metastasis</td>
<td>++</td>
<td>+</td>
<td>Patients</td>
<td>A</td>
</tr>
</tbody>
</table>

* Animal basis for claim but must have additional data linking animal data to human
References


299. Smith MT (1999) Benzene, NQO1, and the structure of FMOs. Drug Metabolism Reviews 34:532


