Green vegetables and colon cancer:

the mechanism of a protective effect by chlorophyll

Johan de Vogel

Promotor: **Prof. dr. M.B. Katan**

Persoonlijk hoogleraar bij de afdeling Humane Voeding,
Wageningen Universiteit

Co-promotor: **Dr. R. van der Meer**

Projectleider Wageningen Centre for Food Sciences
NIZO food reseach, Ede

Promotiecommissie: **Prof. dr. ir. P van 't Veer**

Wageningen Universiteit

Prof. dr. J.H. Kleibeuker

Universitair Medisch Centrum Groningen

Prof. dr. ir. I.M.C.M. Rietjens

Wageningen Universiteit

Dr. E.K. Lund

Institute of Food Research, Norwich, UK

Green vegetables and colon cancer:

the mechanism of a protective effect by chlorophyll

Johan de Vogel

Abstract

Consumption of green leafy vegetables is probably associated with a decreased risk of colon cancer. The objective of this thesis was to find what molecular mechanism explains the modulating effect of green vegetables on the risk of colon cancer.

Red meat consumption is associated with an increased risk of colon cancer. Feeding rats heme, which is the iron porphyrin pigment of red meat, increased cytotoxicity of the gut contents, injured colonocytes, increased colon epithelial cell death, and impaired normal physiological function of the colon. Furthermore, heme in the diet resulted in epithelial hyperproliferation and inhibited apoptosis to compensate for increased colonic cell loss. The ubiquitous pigment in green leafy vegetables is chlorophyll. Chlorophyll is a phytol-esterified magnesium porphyrin and thus structurally analogous to heme. Mixing spinach or equimolar amounts of chlorophyll through the heme diet prevented all heme-induced effects. In contrast, addition of chlorophyllin, the hydrophilic analogue of chlorophyll, to the heme diet did not prevent the heme-induced detrimental effects on the colon mucosa, indicating that intact chlorophyll is required. Studies *in vitro* showed that heme and chlorophyll preferentially accumulate in the lipid phase and that heme and chlorophyll have a high affinity for each other. This high affinity was confirmed in a chlorophyll dose-response study, which showed that even low amounts of dietary chlorophyll (equimolar to heme) prevented all heme-induced detrimental effects in the rat colon. Finally, we studied the relevance of our *in vitro* and animal experimental studies in the Netherlands Cohort Study on diet and cancer. A weak trend was observed for an association between the increased intake of heme and the risk of colon cancer in men but this trend became more significant when chlorophyll intake was considered. Furthermore, we demonstrated that increasing the molar ratio of heme over chlorophyll is associated with an increased risk of colon cancer in men. No consistent associations were observed for women.

In conclusion, hydrophobic binding of chlorophyll to heme in the gut lumen of the rat prevents the heme-induced disrupted balance in colon epithelial cell turnover. This offers a molecular explanation for the preventive effect of green leafy vegetables on colon carcinogenesis.

Contents

Chapter 1

General introduction

- Introduction
- The colon and the crypt
- Colorectal cancer
- Mutagen vs. irritant and the risk of colon cancer
- Meat and colon cancer
- Green vegetables and colon cancer
- What might be the magic bullet in green leafy vegetables?
- Scope and Outline of thesis

Introduction

In The Netherlands almost 9000 cases of colorectal cancer are diagnosed and 4000 people die from this disease every year (1). Worldwide, more than 945 000 people develop colorectal cancer and around 492 000 patients die each year (2). This makes colorectal cancer the third most common cancer and the second cause of cancer deaths in Western countries (2). In developed countries the life-time incidence is around 5% (3) and incidence rates of colorectal cancer vary widely between countries and regions (**Fig. 1**). The highest rates are observed in westernized areas (including North America, Western Europe, Australia, New Zealand, and Japan) and relatively low rates in Africa, Asia, parts of Latin America, and the Caribbean (4,5). Migrant and other epidemiological studies indicate that this variation is mainly due to environmental factors (6,7), with special emphasis on diet (8).

Vegetable consumption is associated with a decreased risk of colon cancer (8). Especially the consumption of green leafy vegetables is related to a protective effect (9-11). **We aimed to find what molecular mechanism explains the protective effect of green leafy vegetables on the risk of colon cancer.**
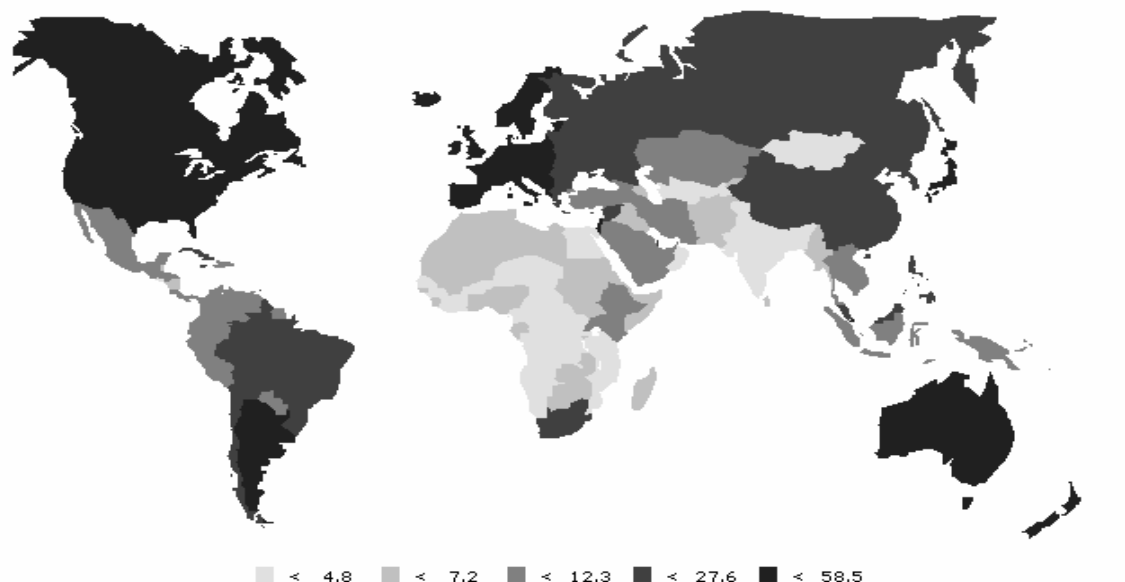
Figure 1. Age-standardized incidence rates of male colon and rectum cancer per 100.000 (~ similar rates for females) (8).

The colon and the crypt

To understand colon cancer, we first need to understand the function and physiological characteristics of the colon and the colonic epithelium. Basically, the colon in a living human adult is an approximately 1.5 meter long tube in which water, sodium, and other minerals are absorbed. This tube is composed of three layers (**Fig. 2**). The outer layer is smooth muscle that facilitates peristalsis. The middle layer, or submucosa, consists of stromal tissue and the inner layer of this tube is an epithelial layer, like the epidermal cells of the skin, which forms a barrier between the body and the outside world. The epithelial layer is folded to form invaginations, or crypts, which are embedded in the stromal tissue (12).
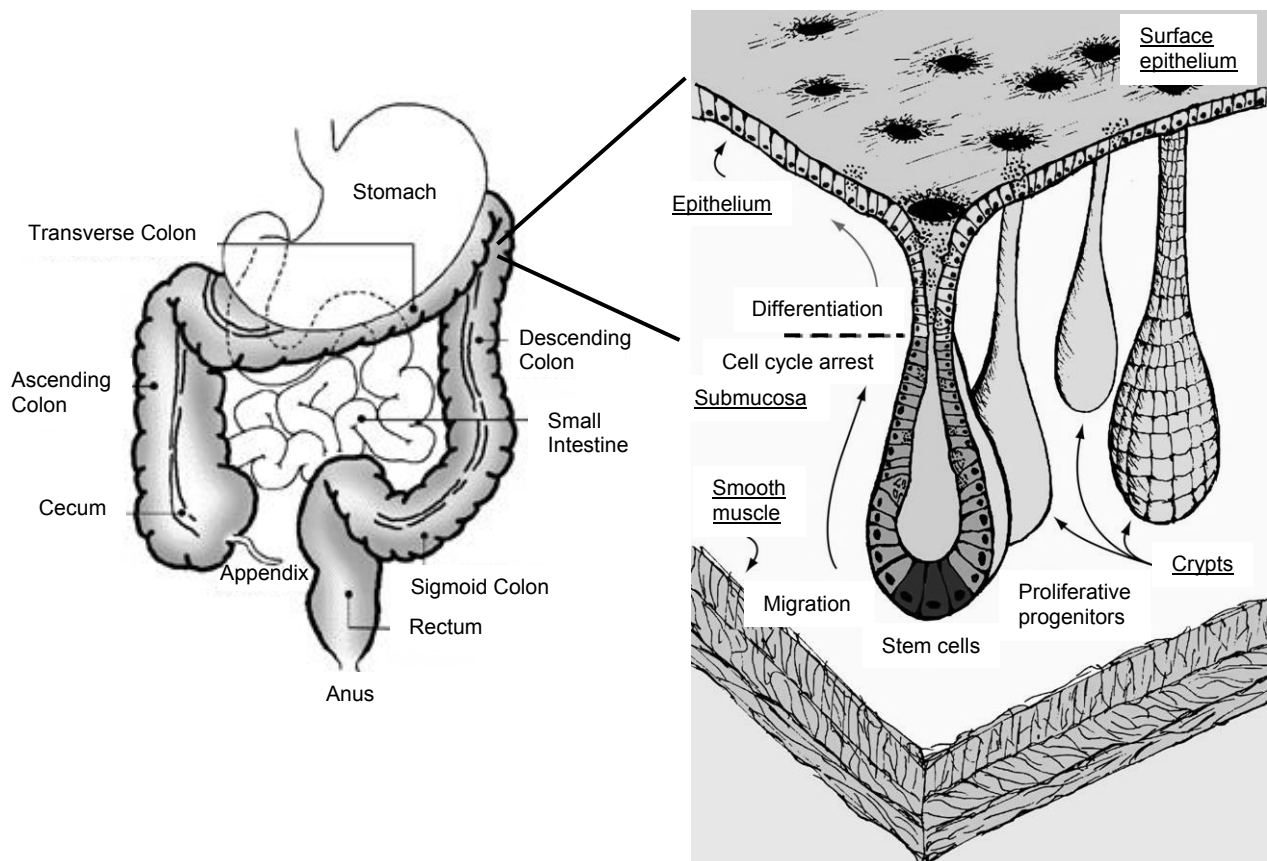
Figure 2. Schematic overview of the human intestinal system and the structure of the large intestine. "reprinted with permission, from the Annual Review of Cell and Developmental Biology, Volume 20 © 2004 by Annual Reviews".

All cells in the crypt arise from stem cells, which are located at the base of the crypt (13). The lower half of the crypt is occupied by proliferative cells. These cells actively migrate in

a continuous pattern from the bottom compartment upward to the luminal surface of the colon. After the cells exit the proliferative compartment they differentiate into enterocytes (absorptive cell lineage) or secretory cells. Secretory cells in the colon encompass mainly goblet cells secreting protective mucins or enteroendocrine cells involved in the secretion of different hormones (14). These processes imply that only fully differentiated epithelial cells are found at the top of the crypts and the surface epithelium of the colon. In time, these terminally differentiated surface epithelial cells are detached from the basal membrane and shed intact into the luminal contents. An important characteristic of the epithelial cells is their capacity to renew themselves. Normal homeostasis in the crypt implies that the rate of crypt cell regeneration is tightly coupled with loss of surface epithelial cells (13,15). A consequence of the high self-renewing rate of epithelial cells might be a high susceptibility to malignant transformation, as this can only occur in dividing cells.

Colorectal cancer

Approximately 5-10% of all colorectal cancers occur as part of a hereditary cancer syndrome. Two of the main forms are familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC) (16). FAP is caused by a germline mutation in the adenomatous polyposis gene (APC) and HNPCC is associated with germline mutations in DNA mismatch repair (MMR) genes. More than 90% of the colorectal cancers occur sporadically, which means that affected patients do not have a family history of colon cancer (2).

Fortunately, a normal epithelial cell has to undergo at least 5-7 mutations before it is transformed to a carcinoma cell (the so-called carcinogenesis). Of all cancers, colorectal cancer is probably the best-understood malignancy, because of easy accessibility of the tumors and the fact that different stages of the same malignancy can coexist within one patient. Histological and molecular analyses of these tumors have led to the definition of the adenoma-carcinoma sequence of tumor progression (17). According to this definition colorectal cancers result from mutational activation of oncogenes combined with inactivation of tumor suppressor genes (**Fig. 3**). These genes are involved in the regulation of key cellular processes like cell proliferation, cell-cycle control, apoptosis, and DNA repair. Furthermore, multiple mutated genes are required to develop a tumor and the

mutations may occur in a preferred sequence, yet the accumulation of changes rather than their order determines the characteristics of the colorectal tumor (18).

In the adenoma-carcinoma sequence the earliest identifiable lesion is an aberrant crypt focus (ACF), which is a small dysplastic lesion in the colonic epithelium. Two different models propose the origin and growth of these dysplastic aberrant crypt foci.
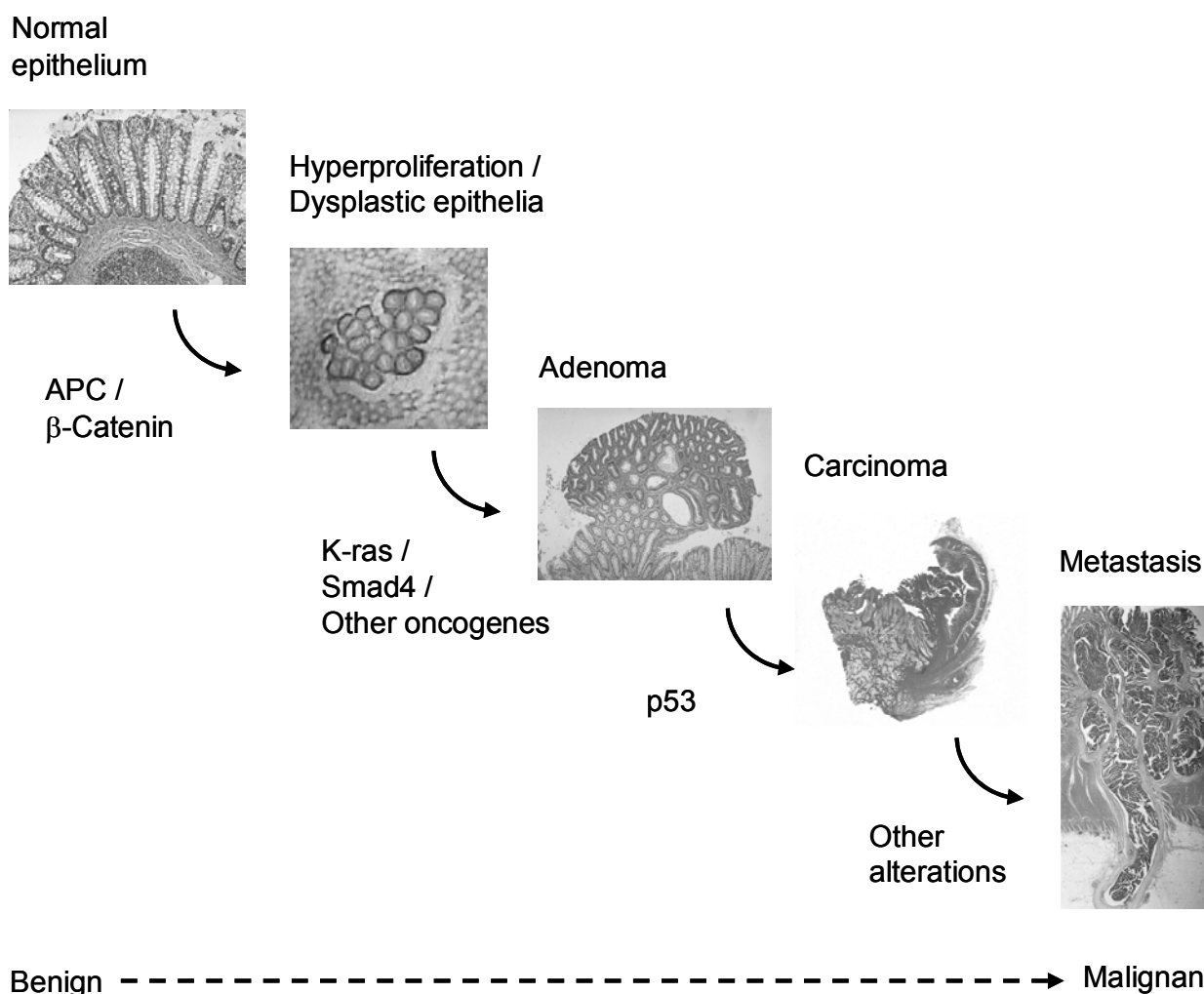
Figure 3. A genetic model for colorectal tumorigenesis, according to Fearon and Vogelstein (17).

Vogelstein and co-workers suggested a top-down morphogenesis model where mutant cells from the surface epithelium spread laterally and downward to form new crypts (19). However, Preston et al. (20) proposed an alternative model where adenomas grow initially in a bottom-up pattern. A very small fraction of aberrant crypt foci transforms to larger (benign) adenomas of several centimeters. The adenomas progress into carcinomas after

acquisition of more mutations in situ (21). At a later stage these carcinomas show malignant features and metastasize by invasion through the colonic wall (Fig. 3) (21,22).

Mutagen vs. irritant and the risk of colon cancer

Important for the prevention of the risk of colon cancer is the discovery of agents that cause this disease. Diet is one of the most important environmental factors identified in the etiology of colon cancer (2). It was assumed that dietary mutagens were responsible for an increased risk of colon cancer. However, comparison of mutational fingerprints of human colon tumors with fingerprints of rat tumors caused by mutagens provided little evidence for a role of mutagens as causative agent for colon cancer. Studies with dietary mutagens *in vitro* or in animal experiments showed a preference of mutagens to form adducts with guanine and adenine, primarily leading to G-to-T or G-to-A mutations (23,24). Typical mutation patterns observed in human lung and liver tumors also have G-to-T or G-to-A transversions and might therefore be characteristic of DNA damage associated with environmental/dietary mutagens (25). In contrast, characteristic mutations in human colon tumors observed in the genes p53 and APC are caused by C-to-T transitions at CpG dinucleotides (25). Such mutations are characteristic of endogenous processes during DNA replication leading to hydrolytic deamination of 5-methylcytosine. This might indicate that not dietary mutagens lead to colon cancer but rather luminal irritants that cause epithelial hyperproliferation to compensate for damage-induced cell death (26-29). Under these conditions, i.e. faster DNA replication, it is more likely that a mutation might occur in a gene critical to the carcinogenic process (30). Indeed, increased proliferation is considered an important risk factor for the development of cancers including colon cancer (31,32). This is corroborated by results of clinical studies demonstrating that subjects at high risk of colon cancer have a higher proliferative activity than healthy controls (33,34).

Meat and colon cancer

Most components from the diet are absorbed in the small intestine. However, non-absorbed components can modify the composition of the intestinal contents that reach the colon. Some of these unabsorbed nutrients dissolve in the water phase of the gut contents to which the colonic mucosa is most exposed. These unabsorbed nutrients may irritate the

mucosa and eventually injure and kill epithelial cells of the colon mucosa. This must be followed by a compensatory hyperproliferation to restore the mucosa and increases the risk of endogenous mutations in tumor suppressor and oncogenes as described above (26).

Diets high in red meat and processed meat are especially associated with a moderately increased risk for colon cancer (35-41). In contrast, diets with a high content of white meat (poultry or fish) are not associated with an increased risk (35,42). The mechanism explaining the risk-enhancing effect of red meat is not precisely known. Results of several animal studies on the effects of dietary beef compared with different protein sources in experimentally induced colonic tumor animal models are inconsistent (43-50) and evidence for an association between fat intake and colon cancer risk is also not supported by recent literature (51,52).

An important difference between red meat and white meat is their heme content. Heme, an iron porphyrin pigment (**Fig. 4A**), is the prosthetic group in hemoglobin and myoglobin, which are proteins involved in oxygen transport. Sesink et al. (53) hypothesized that heme explains the differences in colon cancer risk promotion between red meat and white meat. **Figure 4B** shows that the heme content varies significantly between several types of meat, with for example ~0.45 µmol/g of heme in beef and only 0.03 µmol/g of heme in chicken (54). Dietary heme enhanced cytotoxicity of the luminal contents in rat studies, which was not mediated by well-known surfactants such as bile acids or fatty acids (53), suggesting involvement of a heme-induced cytotoxic metabolite. This metabolite is a lipid-soluble covalently modified porphyrin formed in the gastrointestinal tract of the rat (53,55). The mechanism of the heme-induced cytotoxic effects is not fully known. However, heme is poorly absorbed in the small intestine (56) and therefore available in an environment with oxygen as a catalyst in the formation of oxygen radicals. We speculate that these radicals are involved in the formation of the cytotoxic metabolite, which probably damages the colon epithelial cells (53,57). Unfortunately, the exact chemical structure of this metabolite is at present unknown, because it was not possible to ionize the metabolite for mass spectrometric analysis (55). Nevertheless, an enhanced cytotoxicity implies an increased exposure of the colonic mucosa to luminal irritants, resulting in compensatory epithelial hyperproliferation (53). In line with these results Pierre et al. (58,59) showed that dietary heme or meat supplemented to the diet promoted luminal cytotoxicity and increased the number and size of aberrant crypt foci in rat colon. Aberrant crypt foci are

preneoplastic lesions which correlate with tumor incidence in most studies (60). Recent results from two large epidemiological studies also support the hypothesis that heme from red meat could be a causal factor related to the risk of colon cancer (39,40).

One could argue that not heme but the iron content of the meat is responsible for an association between red meat consumption and the risk of colon cancer, since the iron content is higher in red meat than in white meat.

A

B
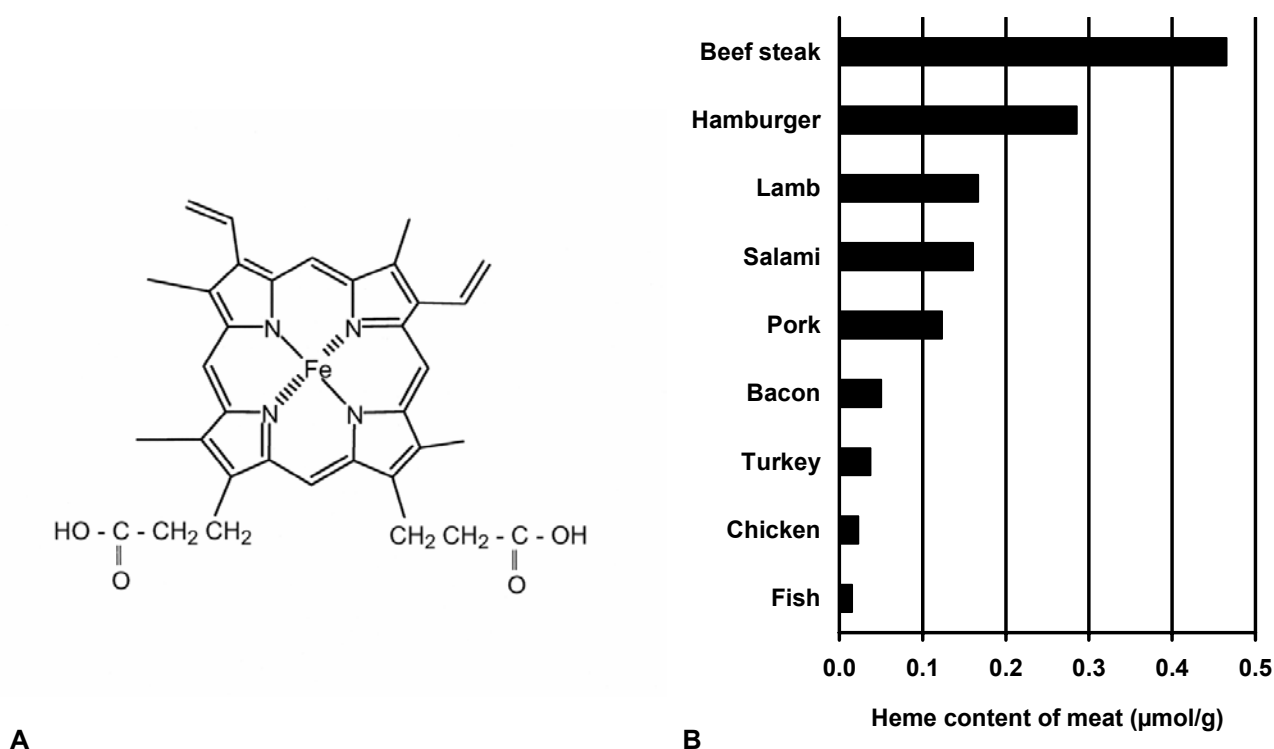
Figure 4. (**A**) Molecular structure of heme, (**B**) heme content in several types of meat (54).

However, dietary iron did not enhance colonic epithelial hyperproliferation (61,62) or formation of aberrant crypt foci (63). Furthermore, Sesink et al. (53) compared the effects of dietary heme with an equimolar amount of iron or protoporphyrin IV in the rat diets and only found increased luminal cytotoxicity and colon epithelial hyperproliferation in the heme-fed rats. Thus, not iron but heme could explain the effect of red meat on the risk of colon cancer.

Green vegetables and colon cancer

Several epidemiological studies have shown that consumption of vegetables is associated with a decreased risk of colorectal cancer (reviewed in (8)). In contrast, recent cohort studies (64-66) have obtained inconsistent results. However, a meta-analysis by Riboli and Norat (67) combining 17 case-control and 10 cohort studies showed a significantly decreased risk of colon cancer with high intakes of vegetables.

Unfortunately, the association of specific types of vegetables with decreased risk of colon cancer is not clear. Most of the epidemiological studies investigated only an association between total vegetable consumption (grams) or total servings of all vegetables and the risk of colon cancer. However, vegetables are extremely heterogeneous and the concentration of a protective ingredient may vary substantially between different types of vegetables. For example, one person consumed 100 g tomatoes and 100 g cauliflower on a day and another person consumed 150 g carrots and 50 g spinach. In these studies it is summarized that both persons consumed 200 g of vegetables but the total intake of specific ingredients between these two individuals varies enormously.

Case-control studies showed a consistent association between a decreased risk of colon cancer and consumption of raw vegetables and green vegetables (2,9). The role of green leafy vegetable consumption was also assessed in cohort studies (10,11,65). Michels et al. (64) did not find an association between intake of green leafy vegetables and the risk of colon cancer. However, the consumption of vegetables was higher in this health-conscious cohort than in the average U.S. population (66). Consequently, the participants in this cohort might already consume adequate amounts of these foods. The cohort study of Voorrips et al. (10) showed that consumption of cooked green leafy vegetables was associated with a decreased risk of colon cancer among men and women. Furthermore, Bueno-de-Mesquita et al. (11) investigated the association between vegetable consumption and colon cancer risk in the European Prospective Investigation into Nutrition and Cancer (EPIC study). This is an excellent population to study because consumption of vegetables varies greatly across the EPIC study population (68). Their preliminary conclusions were that total vegetable consumption showed a weak inverse association with the risk of colorectal cancer (11). However, among five categories of vegetables studied only leafy vegetables (excluding cabbages) conferred significant protection against the development of colorectal cancer (11).

**What might be the magic bullet in green leafy vegetables?**

Having established green leafy vegetables as being important for modulation of the risk of colon cancer it is interesting to examine specific components of these foods.

Green leafy vegetables contain non-digestible/low-fermentable fibers. Fibers could increase stool bulk and speed the transit of food through the colon, diluting the gut contents and decreasing the interaction time of surfactants with the epithelial cells (69). Numerous case-control studies observed a moderately lower risk in association with high consumption of dietary fiber (70), but results of recent large prospective cohort studies have been inconsistent (71,72). Furthermore, a four year intervention with a high fiber diet did not prevent recurrence of colorectal cancer adenomas (73). At present, the role of fiber from vegetables in protection against the risk of colon cancer is at best questionable.

Another characteristic of green leafy vegetables is their high content of folic acid (74). Folate is hypothesized to have a preventive effect on the development of colon adenomas and carcinomas. It is an essential cofactor for the *de novo* biosynthesis of purines and thymidine needed for DNA synthesis and plays a crucial role in DNA methylation, stability and integrity, and repair (75). A deficiency in folate intake may cause incorporation of uracil instead of thymidine into DNA of dividing cells, ultimately leading to an increased risk of colon cancer (76,77). Furthermore, aberrant genomic methylation caused by folate deficiency might be associated with colon cancer risk. However, available evidence indicates that genomic DNA-hypomethylation in the colon is not a probable mechanism by which folate deficiency enhances risk of colon cancer (75) and the relation of blood folate status to sequence-specific alterations of critical cancer-related genes is at the moment also not clear (78). Moreover, results of studies that investigated an association between folate status and colorectal cancer risk are inconsistent (79,80).

Besides folic acid, green vegetables also contain high amounts of vitamin E and some vitamin C (74), both of which have potential antioxidant properties (81). However, recent data do not support a role of antioxidant vitamins in reducing the risk of colorectal cancer (82).

The color of green vegetables is due to the pigment chlorophyll (**Fig. 5A**), which absorbs light for photosynthetic purposes. Color per se might be deceptive, because only green leafy vegetables have a high chlorophyll content and not, for example, broccoli or cabbage (**Fig. 5B**).
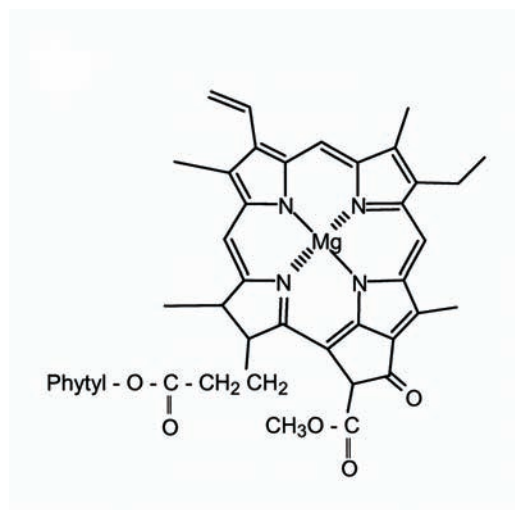
A
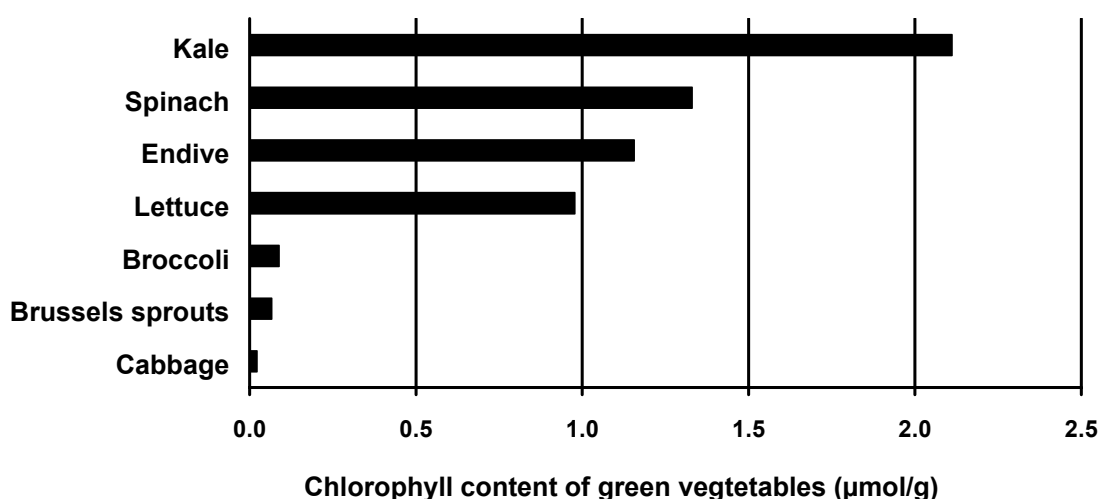
B

Figure 5. (**A**) Molecular structure of chlorophyll a, phytyl: $CH_2CHC_2H_3(CH_2CH_2CH_2C_2H_4)_3CH_3$, (**B**) chlorophyll content of several green vegetables (83).

In plants, chlorophyll is found in the chloroplasts of the photosynthetic tissues (mainly the leaves) and located in the thylakoids, which are the photosynthetically active biomembranes within the chloroplast (84). Chlorophyll, like heme, also has a planar porphyrin backbone but differs from heme mainly by having non-reactive magnesium instead of the highly-reactive transition metal iron in the center of the porphyrin. In addition, chlorophyll has an esterified phytol tail instead of one of the propionic side chains in heme. The other propionic side chain is integrated in a fifth ring and esterified with a methyl group, which leaves chlorophyll without charge. Furthermore, 90-95% of dietary

chlorophyll was recovered in the feces of rats and humans, indicating poor absorption and metabolism of chlorophyll during passage in the intestinal tract (85,86). These high amounts of chlorophyll might compete with heme for solubilization in the intestinal tract and thus prevent heme-induced detrimental effects on the colonic epithelial cells.

Scope and outline of thesis

Vegetable consumption is associated with a decreased risk of colon cancer. Especially the consumption of green leafy vegetables is related to a protective effect. Furthermore, there is now considerable evidence, epidemiological as well as animal experimental, that consumption of red meat, and thus of heme, increases the risk of colon cancer. The objective of this study was to find what molecular mechanism explains the protective effect of green vegetables on the dietary heme-induced risk of colon cancer.

In **chapter 2** we describe the effects of dietary heme on the modulation of colonic epithelial cell turnover in rats. In this study we used three methods to study the effect of diet on cellular proliferation and measured caspase-3 activity as an indicator of apoptosis in the colon mucosa. **Chapter 3** addresses the question whether green leafy vegetables could inhibit the heme-induced increased cytotoxicity of the luminal contents and hyperproliferation of the colonic epithelial cells. Furthermore, we studied whether this effect could be mimicked by an equimolar dose of chlorophyll. The purpose of the study in **chapter 4** was to investigate whether chlorophyllins, which are water-soluble analogues of chlorophyll, could inhibit the heme-induced luminal cytotoxicity and colonic hyperproliferation as natural chlorophyll does. In **chapter 5**, our study on the minimal molar ratio of chlorophyll to heme required to inhibit all the detrimental heme-induced effects is described. A dose-dependent protective effect of chlorophyll was studied by feeding rats a diet containing a constant amount of heme with increasing concentrations of dietary chlorophyll. A possible interaction of chlorophyll with heme was also investigated *in vitro*. To ascertain the human relevance of this work, we studied whether intake of heme was associated with the risk of colon cancer in humans and whether chlorophyll could modify this association. This hypothesis was tested in the Netherlands Cohort Study on diet and cancer and the results are described in **chapter 6**. Finally, **chapter 7** provides a general discussion of the studies described.

References

1. KWFKankerbestrijding (2005) Cijfers over dikkedarmkanker. http://www.kwfkankerbestrijding.nl/stat/statOpen.jsp?id=4139.
2. Stewart, B.W. and Kleihues, P. (eds.) (2003) *World Cancer Report*. IARC*Press*, Lyon.
3. Weitz, J., Koch, M., Debus, J., Hohler, T., Galle, P.R. and Buchler, M.W. (2005) Colorectal cancer. *Lancet*, **365**, 153-65.
4. Pisani, P., Bray, F. and Parkin, D.M. (2002) Estimates of the world-wide prevalence of cancer for 25 sites in the adult population. *Int J Cancer*, **97**, 72-81.
5. Potter, J.D. (1999) Colorectal cancer: molecules and populations. *J Natl Cancer Inst*, **91**, 916-932.
6. Armstrong, B. and Doll, R. (1975) Environmental factors and cancer incidence and mortality in different countries, with special reference to dietary practices. *Int J Cancer*, **15**, 617-631.
7. McMichael, A.J. and Giles, G.G. (1988) Cancer in migrants to Australia: extending the descriptive epidemiological data. *Cancer Res*, **48**, 751-6.
8. Ferlay, J., Bray, F., Pisani, P. and Parkin, D.M. (2004) GLOBOCAN 2002: Cancer Incidence, Mortality and Prevalence Worldwide. *IARC CancerBase No. 5. version 2.0*.
9. Steinmetz, K.A. and Potter, J.D. (1996) Vegetables, fruit, and cancer prevention: a review. *J Am Diet Assoc*, **96**, 1027-1039.
10. Voorrips, L.E., Goldbohm, R.A., van Poppel, G., Sturmans, F., Hermus, R.J. and van den Brandt, P.A. (2000) Vegetable and fruit consumption and risks of colon and rectal cancer in a prospective cohort study: The Netherlands Cohort Study on Diet and Cancer. *Am J Epidemiol*, **152**, 1081-92.
11. Bueno-de-Mesquita, H.B., Ferrari, P. and Riboli, E. (2002) Plant foods and the risk of colorectal cancer in Europe: preliminary findings. *IARC Sci Publ*, **156**, 89-95.
12. Booth, C. and Potten, C.S. (2000) Gut instincts: thoughts on intestinal epithelial stem cells. *J Clin Invest*, **105**, 1493-9.
13. Potten, C.S. (1998) Stem cells in gastrointestinal epithelium: numbers, characteristics and death. *Philos Trans R Soc Lond B Biol Sci*, **353**, 821-30.
14. Potten, C.S., Booth, C. and Pritchard, D.M. (1997) The intestinal epithelial stem cell: the mucosal governor. *Int J Exp Pathol*, **78**, 219-43.
15. Podolsky, D.K. (1993) Regulation of intestinal epithelial proliferation: a few answers, many questions. *Am J Physiol*, **264**, G179-86.
16. Lynch, H.T. and de la Chapelle, A. (2003) Hereditary colorectal cancer. *N Engl J Med*, **348**, 919-32.
17. Fearon, E.R. and Vogelstein, B. (1990) A genetic model for colorectal tumorigenesis. *Cell*, **61**, 759-67.
18. Fearon, E.R. and Jones, P.A. (1992) Progressing toward a molecular description of colorectal cancer development. *Faseb J*, **6**, 2783-90.
19. Shih, I.M., Wang, T.L., Traverso, G., Romans, K., Hamilton, S.R., Ben-Sasson, S., Kinzler, K.W. and Vogelstein, B. (2001) Top-down morphogenesis of colorectal tumors. *Proc Natl Acad Sci U S A*, **98**, 2640-5.
20. Preston, S.L., Wong, W.M., Chan, A.O., Poulsom, R., Jeffery, R., Goodlad, R.A., Mandir, N., Elia, G., Novelli, M., Bodmer, W.F., Tomlinson, I.P. and Wright, N.A. (2003) Bottom-up histogenesis of colorectal adenomas: origin in the monocryptal adenoma and initial expansion by crypt fission. *Cancer Res*, **63**, 3819-25.
21. Sancho, E., Batlle, E. and Clevers, H. (2004) Signaling pathways in intestinal development and cancer. *Annual Review of Cell and Developmental Biology*, **20**, 695-723.
22. Radtke, F. and Clevers, H. (2005) Self-renewal and cancer of the gut: two sides of a coin. *Science*, **307**, 1904-9.
23. Schut, H.A. and Snyderwine, E.G. (1999) DNA adducts of heterocyclic amine food mutagens: implications for mutagenesis and carcinogenesis. *Carcinogenesis*, **20**, 353-68.
24. Weisburger, J.H. (1993) Heterocyclic amines in cooked foods: possible human carcinogens. *Cancer Res*, **53**, 2422-4.
25. Harris, C.C. and Hollstein, M. (1993) Clinical implications of the p53 tumor-suppressor gene. *N Engl J Med*, **329**, 1318-27.
26. Kinzler, K.W. and Vogelstein, B. (1996) Lessons from hereditary colorectal cancer. *Cell*, **87**, 159-170.
27. Ehrlich, M. and Wang, R.Y. (1981) 5-Methylcytosine in eukaryotic DNA. *Science*, **212**, 1350-7.

28. Cooper, D.N. and Youssoufian, H. (1988) The CpG dinucleotide and human genetic disease. *Hum Genet*, **78**, 151-5.

29. Thilly, W.G. (2003) Have environmental mutagens caused oncomutations in people? *Nat Genet*, **34**, 255-259.

30. Preston-Martin, S., Pike, M.C., Ross, R.K., Jones, P.A. and Henderson, B.E. (1990) Increased cell division as a cause of human cancer. *Cancer Res*, **50**, 7415-21.

31. Cohen, S.M. and Ellwein, L.B. (1990) Cell proliferation in carcinogenesis. *Science*, **249**, 1007-11.

32. Baron, J.A. (2001) Intermediate effect markers for colorectal cancer. *IARC Sci Publ*, **154**, 113-129.

33. Lipkin, M. (1988) Biomarkers of increased susceptibility to gastrointestinal cancer: new application to studies of cancer prevention in human subjects. *Cancer Res*, **48**, 235-245.

34. Terpstra, O.T., van Blankenstein, M., Dees, J. and Eilers, G.A. (1987) Abnormal pattern of cell proliferation in the entire colonic mucosa of patients with colon adenoma or cancer. *Gastroenterology*, **92**, 704-8.

35. Norat, T., Bingham, S., Ferrari, P., Slimani, N., Jenab, M., Mazuir, M., Overvad, K., Olsen, A., Tjonneland, A., Clavel, F., Boutron-Ruault, M.C., Kesse, E., Boeing, H., Bergmann, M.M., Nieters, A., Linseisen, J., Trichopoulou, A., Trichopoulos, D., Tountas, Y., Berrino, F., Palli, D., Panico, S., Tumino, R., Vineis, P., Bueno-de-Mesquita, H.B., Peeters, P.H., Engeset, D., Lund, E., Skeie, G., Ardanaz, E., Gonzalez, C., Navarro, C., Quiros, J.R., Sanchez, M.J., Berglund, G., Mattisson, I., Hallmans, G., Palmqvist, R., Day, N.E., Khaw, K.T., Key, T.J., San Joaquin, M., Hemon, B., Saracci, R., Kaaks, R. and Riboli, E. (2005) Meat, fish, and colorectal cancer risk: the European Prospective Investigation into cancer and nutrition. *J Natl Cancer Inst*, **97**, 906-16.

36. Norat, T., Lukanova, A., Ferrari, P. and Riboli, E. (2002) Meat consumption and colorectal cancer risk: dose-response meta- analysis of epidemiological studies. *Int J Cancer*, **98**, 241-256.

37. Chao, A., Thun, M.J., Connell, C.J., McCullough, M.L., Jacobs, E.J., Flanders, W.D., Rodriguez, C., Sinha, R. and Calle, E.E. (2005) Meat consumption and risk of colorectal cancer. *Jama*, **293**, 172-82.

38. Larsson, S.C., Rafter, J., Holmberg, L., Bergkvist, L. and Wolk, A. (2005) Red meat consumption and risk of cancers of the proximal colon, distal colon and rectum: the Swedish Mammography Cohort. *Int J Cancer*, **113**, 829-34.

39. Lee, D.H., Anderson, K.E., Harnack, L.J., Folsom, A.R. and Jacobs, D.R., Jr. (2004) Heme iron, zinc, alcohol consumption, and colon cancer: Iowa Women's Health Study. *J Natl Cancer Inst*, **96**, 403-7.

40. Larsson, S.C., Adami, H.O., Giovannucci, E. and Wolk, A. (2005) Re: Heme iron, zinc, alcohol consumption, and risk of colon cancer. *J Natl Cancer Inst*, **97**, 232-3; author reply 233-4.

41. Sandhu, M.S., White, I.R. and McPherson, K. (2001) Systematic review of the prospective cohort studies on meat consumption and colorectal cancer risk: a meta-analytical approach. *Cancer Epidemiol Biomarkers Prev*, **10**, 439-46.

42. Giovannucci, E., Rimm, E.B., Stampfer, M.J., Colditz, G.A., Ascherio, A. and Willett, W.C. (1994) Intake of fat, meat, and fiber in relation to risk of colon cancer in men. *Cancer Res*, **54**, 2390-2397.

43. Belobrajdic, D.P., McIntosh, G.H. and Owens, J.A. (2003) Whey proteins protect more than red meat against azoxymethane induced ACF in Wistar rats. *Cancer Lett*, **198**, 43-51.

44. Nutter, R.L., Gridley, D.S., Kettering, J.D., Goude, A.G. and Slater, J.M. (1983) BALB/c mice fed milk or beef protein: differences in response to 1,2-dimethylhydrazine carcinogenesis. *J Natl Cancer Inst*, **71**, 867-74.

45. McIntosh, G.H., Regester, G.O., Le Leu, R.K., Royle, P.J. and Smithers, G.W. (1995) Dairy proteins protect against dimethylhydrazine-induced intestinal cancers in rats. *J Nutr*, **125**, 809-16.

46. Goldin, B.R. and Gorbach, S.L. (1980) Effect of Lactobacillus acidophilus dietary supplements on 1,2-dimethylhydrazine dihydrochloride-induced intestinal cancer in rats. *J Natl Cancer Inst*, **64**, 263-5.

47. Khil, J. and Gallaher, D.D. (2004) Beef tallow increases apoptosis and decreases aberrant crypt foci formation relative to soybean oil in rat colon. *Nutrition and Cancer - an International Journal*, **50**, 55-62.

48. Pence, B.C., Butler, M.J., Dunn, D.M., Miller, M.F., Zhao, C. and Landers, M. (1995) Non-promoting effects of lean beef in the rat colon carcinogenesis model. *Carcinogenesis*, **16**, 1157-60.

49. Parnaud, G., Peiffer, G., Tache, S. and Corpet, D.E. (1998) Effect of meat (beef, chicken, and bacon) on rat colon carcinogenesis. *Nutr Cancer*, **32**, 165-73.

50. Mutanen, M., Pajari, A.M. and Oikarinen, S.I. (2000) Beef induces and rye bran prevents the formation of intestinal polyps in Apc(Min) mice: relation to beta-catenin and PKC isozymes. *Carcinogenesis*, **21**, 1167-73.

51. Martinez, M.E. (2005) Primary prevention of colorectal cancer: lifestyle, nutrition, exercise. *Recent Results Cancer Res*, **166**, 177-211.

52. Willett, W.C. (2000) Diet and cancer. *Oncologist*, **5**, 393-404.
53. Sesink, A.L.A., Termont, D.S., Kleibeuker, J.H. and Van der Meer, R. (1999) Red meat and colon cancer: the cytotoxic and hyperproliferative effects of dietary heme. *Cancer Res*, **59**, 5704-5709.
54. Schwartz, S. and Ellefson, M. (1985) Quantitative fecal recovery of ingested hemoglobin-heme in blood: comparisons by HemoQuant assay with ingested meat and fish. *Gastroenterology*, **89**, 19-26.
55. Sesink, A.L.A., Termont, D.S., Kleibeuker, J.H. and Van Der Meer, R. (2000) Red meat and colon cancer: dietary haem, but not fat, has cytotoxic and hyperproliferative effects on rat colonic epithelium. *Carcinogenesis*, **21**, 1909-1915.
56. Young, G.P., Rose, I.S. and St John, D.J. (1989) Haem in the gut. I. Fate of haemoproteins and the absorption of haem. *J Gastroenterol Hepatol*, **4**, 537-45.
57. Sawa, T., Akaike, T., Kida, K., Fukushima, Y., Takagi, K. and Maeda, H. (1998) Lipid peroxyl radicals from oxidized oils and heme-iron: implication of a high-fat diet in colon carcinogenesis. *Cancer Epidemiol Biomarkers Prev*, **7**, 1007-12.
58. Pierre, F., Tache, S., Petit, C.R., Van Der Meer, R. and Corpet, D.E. (2003) Meat and cancer: haemoglobin and haemin in a low-calcium diet promote colorectal carcinogenesis at the aberrant crypt stage in rats. *Carcinogenesis*, **24**, 1683-1690.
59. Pierre, F., Freeman, A., Tache, S., Van der Meer, R. and Corpet, D.E. (2004) Beef meat and blood sausage promote the formation of azoxymethane-induced mucin-depleted foci and aberrant crypt foci in rat colons. *J Nutr*, **134**, 2711-6.
60. Corpet, D.E. and Tache, S. (2002) Most effective colon cancer chemopreventive agents in rats: a systematic review of aberrant crypt foci and tumor data, ranked by potency. *Nutr Cancer*, **43**, 1-21.
61. Chin, J. and Carpenter, C. (1997) Effects of dietary fat type and iron level on in vivo lipid peroxidation in rat colon. *Nutrition Research*, **17**, 1381-1389.
62. Soyars, K.E. and Fischer, J.G. (1998) Iron supplementation does not affect cell proliferation or aberrant crypt foci development in the colon of sprague-dawley rats. *J Nutr*, **128**, 764-70.
63. Wright, M.O., Burden, V., Lee, J., Eitenmiller, R.R. and Fischer, J.G. (1999) High dietary iron enhances oxidative stress in liver but does not increase aberrant crypt foci development in rats with low vitamin E status. *Nutr Cancer*, **35**, 143-52.
64. Michels, K.B., Giovannucci, E., Joshipura, K.J., Rosner, B.A., Stampfer, M.J., Fuchs, C.S., Colditz, G.A., Speizer, F.E. and Willett, W.C. (2002) Fruit and vegetable consumption and colorectal cancer incidence. *IARC Sci Publ*, **156**, 139-40.
65. Michels, K.B., Edward, G., Joshipura, K.J., Rosner, B.A., Stampfer, M.J., Fuchs, C.S., Colditz, G.A., Speizer, F.E. and Willett, W.C. (2000) Prospective study of fruit and vegetable consumption and incidence of colon and rectal cancers. *J Natl Cancer Inst*, **92**, 1740-52.
66. Terry, P., Giovannucci, E., Michels, K.B., Bergkvist, L., Hansen, H., Holmberg, L. and Wolk, A. (2001) Fruit, vegetables, dietary fiber, and risk of colorectal cancer. *J Natl Cancer Inst*, **93**, 525-33.
67. Riboli, E. and Norat, T. (2003) Epidemiologic evidence of the protective effect of fruit and vegetables on cancer risk. *Am J Clin Nutr*, **78**, 559S-569S.
68. Agudo, A., Slimani, N., Ocke, M.C., Naska, A., Miller, A.B., Kroke, A., Bamia, C., Karalis, D., Vineis, P., Palli, D., Bueno-de-Mesquita, H.B., Peeters, P.H., Engeset, D., Hjartaker, A., Navarro, C., Martinez Garcia, C., Wallstrom, P., Zhang, J.X., Welch, A.A., Spencer, E., Stripp, C., Overvad, K., Clavel-Chapelon, F., Casagrande, C. and Riboli, E. (2002) Consumption of vegetables, fruit and other plant foods in the European Prospective Investigation into Cancer and Nutrition (EPIC) cohorts from 10 European countries. *Public Health Nutr*, **5**, 1179-96.
69. Burkitt, D.P. (1969) Related disease--related cause? *Lancet*, **2**, 1229-31.
70. Trock, B., Lanza, E. and Greenwald, P. (1990) Dietary fiber, vegetables, and colon cancer: critical review and meta- analyses of the epidemiologic evidence. *J Natl Cancer Inst*, **82**, 650-661.
71. Michels, K.B., Fuchs, C.S., Giovannucci, E., Colditz, G.A., Hunter, D.J., Stampfer, M.J. and Willett, W.C. (2005) Fiber intake and incidence of colorectal cancer among 76,947 women and 47,279 men. *Cancer Epidemiol Biomarkers Prev*, **14**, 842-9.
72. Bingham, S.A., Day, N.E., Luben, R., Ferrari, P., Slimani, N., Norat, T., Clavel-Chapelon, F., Kesse, E., Nieters, A., Boeing, H., Tjonneland, A., Overvad, K., Martinez, C., Dorronsoro, M., Gonzalez, C.A., Key, T.J., Trichopoulou, A., Naska, A., Vineis, P., Tumino, R., Krogh, V., Bueno-de-Mesquita, H.B., Peeters, P.H., Berglund, G., Hallmans, G., Lund, E., Skeie, G., Kaaks, R. and Riboli, E. (2003) Dietary fibre in food and protection against colorectal cancer in the European Prospective Investigation into Cancer and Nutrition (EPIC): an observational study. *Lancet*, **361**, 1496-501.
73. Schatzkin, A., Lanza, E., Corle, D., Lance, P., Iber, F., Caan, B., Shike, M., Weissfeld, J., Burt, R., Cooper, M.R., Kikendall, J.W. and Cahill, J. (2000) Lack of effect of a low-fat, high-fiber diet on the

recurrence of colorectal adenomas. Polyp Prevention Trial Study Group. *N Engl J Med*, **342**, 1149-55.

74. NEVO (2001) *NEVO-tabel*. The Netherlands Nutrition Centre, Den Haag.
75. Kim, Y.I. (2004) Folate and DNA methylation: a mechanistic link between folate deficiency and colorectal cancer? *Cancer Epidemiol Biomarkers Prev*, **13**, 511-9.
76. Choi, S.W. and Mason, J.B. (2002) Folate status: effects on pathways of colorectal carcinogenesis. *J Nutr*, **132**, 2413S-2418S.
77. Blount, B.C., Mack, M.M., Wehr, C.M., MacGregor, J.T., Hiatt, R.A., Wang, G., Wickramasinghe, S.N., Everson, R.B. and Ames, B.N. (1997) Folate deficiency causes uracil misincorporation into human DNA and chromosome breakage: implications for cancer and neuronal damage. *Proc Natl Acad Sci U S A*, **94**, 3290-5.
78. McCabe, D.C. and Caudill, M.A. (2005) DNA methylation, genomic silencing, and links to nutrition and cancer. *Nutr Rev*, **63**, 183-95.
79. Giovannucci, E., Stampfer, M.J., Colditz, G.A., Rimm, E.B., Trichopoulos, D., Rosner, B.A., Speizer, F.E. and Willett, W.C. (1993) Folate, methionine, and alcohol intake and risk of colorectal adenoma [see comments]. *J Natl Cancer Inst*, **85**, 875-84.
80. van den Donk, M., Buijsse, B., van den Berg, S.W., Ocke, M.C., Harryvan, J.L., Nagengast, F.M., Kok, F.J. and Kampman, E. (2005) Dietary intake of folate and riboflavin, MTHFR C677T genotype, and colorectal adenoma risk: a Dutch case-control study. *Cancer Epidemiol Biomarkers Prev*, **14**, 1562-6.
81. Turini, M.E. and DuBois, R.N. (2002) Primary prevention: phytoprevention and chemoprevention of colorectal cancer. *Hematol Oncol Clin North Am*, **16**, 811-40.
82. Bjelakovic, G., Nikolova, D., Simonetti, R.G. and Gluud, C. (2004) Antioxidant supplements for prevention of gastrointestinal cancers: a systematic review and meta-analysis. *Lancet*, **364**, 1219-28.
83. Khachik, F., Beecher, G.R. and Whittaker, N.F. (1986) Separation, Identification, and Quantification of the Major Carotenoid and Chlorophyll Constituents in Extracts of Several green Vegetables by Liquid Chromatography. *Jounal of Agricultural Food Chemistry*, **34**, 603-616.
84. Schwartz, S.J. and Lorenzo, T.V. (1990) Chlorophylls in foods. *Crit Rev Food Sci Nutr*, **29**, 1-17.
85. Baxter, J.H. and Steinberg, D. (1967) Absorption of phytol from dietary chlorophyll in the rat. *J Lipid Res*, **8**, 615-20.
86. Baxter, J.H. (1968) Absorbtion of chlorophyll phytol in normal man and in patients with Refsum's disease. *Journal of lipid research*, **9**, 636-641.

Chapter 2

Dietary heme injures surface epithelium and consequently disturbs epithelial cell turnover in rat colon mucosa

Johan de Vogel, Wytske Boersma van-Eck, Aloys L.A. Sesink, Denise S.M.L. Jonker-Termont, Jan H. Kleibeuker and Roelof van der Meer

Abstract

Epidemiological and animal model studies suggest that a high intake of heme, present in red meat, is associated with an increased risk of colon cancer. The aim of this study was to elucidate the effects of dietary heme on colonic cell homeostasis in rats. Rats were fed a purified, humanized, control diet or a similar diet supplemented with 0.5 mmol heme/kg for 14 days. Colons were excised and subjected to histological examination to score intercryptal colonic surface epithelial damage. Fecal water cytotoxicity was determined with a bioassay and colon epithelial cell proliferation was evaluated with ^{3}H-thymidine or 5-bromo-2'-deoxyuridine (BrdU) incorporation into DNA, both administered 2 h before sacrifice, or by Ki-67 immunohistochemistry. Exfoliation of colonocytes was measured as the amount of rat DNA in feces and caspase-3 expression and activity were measured to study colonic mucosal apoptosis. Dietary heme induced a more than ten-fold increased cytotoxicity of the fecal water and a hundred-fold lower excretion of host DNA. Colons of heme fed rats showed injured surface epithelium and an approximately 25% increase in crypt length. Finally, dietary heme doubled colonocyte proliferation, shown by all three markers, but inhibited colonic mucosal apoptosis. In conclusion, our results demonstrate that dietary heme injures colonic surface epithelium, which is overcompensated by inhibition of apoptosis and hyperproliferation of cells in the crypts, resulting in crypt hyperplasia. This disturbed epithelial cell homeostasis might explain why a high intake of dietary heme is associated with an increased risk of colon cancer.

Introduction

Colorectal cancer is a leading cause of cancer death in Western countries (1). Diet is one of the important environmental factors associated with increased colorectal cancer risk (2). Epidemiological and experimental studies suggest that high consumption of red meat and processed meat is associated with an increased risk of colorectal cancer (3-6). In contrast, consumption of white meat, such as poultry or fish, is not associated with increased risk (7). We and others (8,9) hypothesized that this differential effect of red and white meat is explained by the difference in heme content. Heme is the iron porphyrin pigment of red meat and the heme content of red meat is ten times higher than that of white meat (10). We demonstrated previously that in rats a heme-supplemented diet increased fecal water cytotoxicity and colonic epithelial proliferation (8,11). The latter is probably due to a compensatory reaction to damage and premature death of colonic epithelial cells. Furthermore, supplementation of heme or meat to a diet increased the number and size of aberrant crypt foci in rat colon after AOM treatment (5,12). Aberrant crypt foci are the earliest morphologically recognizable preneoplastic lesions that correlate with tumor incidence in most studies (13).

The surface of the colon is lined by an epithelial layer which is a permeability barrier that prevents undesirable solutes entering the body. The intestinal epithelium also reabsorbs water and minerals and is involved in secretion of protective compounds. The surface epithelium is in constant contact with contents from the colonic lumen. Therefore, dietary components that are not absorbed and modulate the composition of the intestinal contents might damage colonic epithelial cells causing early cell death. This initiates compensatory proliferation to maintain crypt cell homeostasis. Hyperproliferation in colonic crypts is considered as a risk marker for colon cancer (14,15). However, maintenance of crypt cell homeostasis not only depends on cell proliferation but also on cell loss (16). In the colon, cell loss is due to exfoliation/anoikis and intraepithelial apoptosis (17). Prolonged cell survival by inhibition of apoptosis is also associated with tumorigenesis (18).

Deregulation of the balance between cell death and proliferation could increase the risk of endogenous mutations in tumor suppressor genes and oncogenes and as a consequence transform epithelial cells in the end to tumor cells (19,20). As recent cohort studies showed that colon cancer risk is indeed associated with heme intake (21,22), we now address the question whether and how heme can disturb this balance between cell death and proliferation. Therefore we studied whether dietary heme-induced cytotoxicity of the colonic contents indeed injured colonic epithelial cells and modulated exfoliation, proliferation and apoptosis of cells in rat colon.

Materials and Methods

Animals and diets.

The experimental protocol was approved by the animal welfare committee of Wageningen University and Research Centre. Eight week old outbred male SPF Wistar rats (WU, Harlan, Horst, The Netherlands) were housed individually in metabolic cages in a room with controlled temperature (20-22°C), relative humidity (50-60%) and a 12 h light/dark cycle (lights on 06.00-18.00h). Animals were acclimatized to housing conditions for 5 days before the start of the experiment.

To study the effects of heme on the colonic epithelium 2 groups of 16 rats were fed purified humanized diets (40 energy % fat, 20 mmol/kg of calcium) during 2 weeks as described in detail previously (11). One group was fed a control diet and the other group was fed this diet supplemented with 0.5 mmol heme/kg diet (Sigma-Aldrich Chemie, St Louis, USA).

Food was administered to the rats just before dark to prevent possible degradation of the heme. Food and demineralized drinking water were supplied ad libitum. Food intake and body weights were recorded every 2-4 days. Feces were quantitatively collected during days 11-14 of the experiment, frozen at -20°C, and subsequently freeze-dried.

Cytotoxicity of fecal water.

Fecal water was prepared by reconstituting a small amount of freeze-dried feces with double-distilled water to obtain a physiologic osmolarity of 300 mOsmol/L, as described earlier (8). After preparation, the fecal waters were stored at -20°C until further analysis. Cytotoxicity of fecal water was quantified by potassium release of human erythrocytes after incubation with fecal water as described previously (8) and validated earlier with human colon carcinoma-derived Caco-2 cells (23). The potassium content of the erythrocytes was measured with an Inductive Coupled Plasma Absorption Emission Spectrophotometer (ICP-AES) (Varian, Mulgrave, Australia) and the cytotoxicity of fecal water was calculated and expressed as percentage of maximal lysis.

Total feces analysis.

One of the important physiological functions of colonocytes is the reabsorption of minerals and water. Therefore, we measured the fecal cations sodium and potassium as markers for colon epithelial cell function. Feces was treated with 5% trichloroacetic acid for 1h at room temperature and centrifuged for 2 min at 14 000 *g*. The supernatants were diluted with 0.05% CsCl, and sodium and potassium were analyzed with an ICP-AES.

We quantified host DNA in feces as a marker for epithelial exfoliation, as described earlier (11,24). Briefly, fecal host DNA was extracted from freeze-dried feces and the DNA in all isolates was of good purity (A_{260}/A_{280} approximately 1.8). The standard DNA used for quantification was isolated

from rat spleen. Quantification was based on real-time PCR, performed with a rat-specific probe and rat specific primers targeted to the β-globin gene sequence (24).

DNA-synthesis of epithelial cells.

After 14 days of experimental feeding, colon mucosal proliferation was quantified by measuring DNA-replication *in vivo*, using 5-bromo-2'-deoxyuridine (BrdU) or ^{3}H-thymidine incorporation into DNA. Eight rats fed a control diet and 8 rats fed a heme diet were non-fasted injected intraperitoneally (i.p.) with BrdU (Sigma) at a dosage of 100 mg/kg body weight. The 16 remaining rats (8 control and 8 heme) were injected i.p. with [methyl-^{3}H]thymidine (specific activity 925 GBq/mmol; dose 3.7 MBq/kg body weight; Amersham International, Amersham, UK) in 154 mM NaCl. After 2 h, rats were killed by CO_2 inhalation.

The entire colons of the ^{3}H-thymidine injected rats were excised and opened longitudinally. The colonic contents were removed, and the mucosa was scraped, homogenized in buffer, and analyzed for ^{3}H-thymidine incorporation into DNA as described previously (11).

The entire colons of the BrdU injected rats were excised and the middle 3 cm part of the proximal (first half) and the middle 3 cm of the distal colon were sampled and fixed in 10% neutral-buffered formalin. After fixation the colon tissues were embedded in paraffin and serially cut in 3 μm sections, which were used for all immunohistochemical analysis described below. The paraffin embedded rat tissue sections were deparaffinized, hydrated, and placed in antigen retrieval solution (10 mMol/l citrate buffer, pH 6.0) for 15 min in a microwave oven at 350 W. Endogenous peroxidase was inhibited by incubation with 3% H_2O_2 in PBS for 30 min. The sections were then incubated with anti-BrdU mouse monoclonal antibody (25) for 1 h at room temperature. Next, tissue sections were sequentially incubated with mixture one (peroxidase conjugated rabbit anti-mouse (DakoCytomation, Heverlee, Belgium) at a dilution of 1:50 in 5% normal rat serum (Sigma) and 1% BSA (SERVA, Heidelberg, Germany) in PBS) and with mixture two (peroxidase conjugated goat anti-rabbit (DakoCytomation) at a dilution of 1:50 in 5% normal rat serum and 1% BSA in PBS) for 30 min. Sections were developed in staining solution (25 mg diaminobenzidine (DAB) (Sigma) substrate, 50 mg imidazol, and 50 μl 30% H_2O_2 in 50 mL PBS) for 10 min and counterstained with hematoxylin. Both in proximal and distal colon colonocytes from 15 well-oriented crypts (longitudinal section) were counted from each animal. A cell was scored positive for BrdU when the nucleus of the cell was distinctively brown. The number of positive cells per crypt column, labeling index, and total number of cells per full length hemi-crypt were determined. Labeling index was calculated as the ratio of the number of positive BrdU-labelled cells to the total number of crypt cells.

Immunohistochemistry Ki-67.

Ki-67 is a protein ubiquitously expressed in G1-, S-, and G2-phase of the cell cycle but not in the G0-phase (26). To visualize Ki-67 antigen, tissue sections were treated for optimal antigen retrieval

and to quench endogenous peroxidase activity as described above. The sections were then incubated with MIB-5 (DakoCytomation) at a dilution of 1:50 in combination with 1% BSA for 1 h at room temperature. After incubation with primary antibody, tissue sections were sequentially incubated with biotinylated rabbit anti-mouse antibody (DakoCytomation) at a dilution of 1:50 (in 5% normal rat serum and 1% BSA in PBS) and Streptavidin/HRP (DakoCytomation) at a dilution of 1:300 (in 5% normal rat serum and 1% BSA in PBS) for 30 min. Sections were developed with DAB, counterstained with hematoxylin, dehydrated, and mounted. For negative controls PBS and 1% BSA replaced the primairy antibodies. Colonocytes from 15 well oriented crypts (longitudinal section) were counted as described above for BrdU. A cell was scored positive for Ki-67 when the nucleus of the cell was distinctively brown.

Histological analysis.

Intercryptal surface epithelial damage was identified in paraffin embedded colon sections (3μm). Sections were stained with hematoxylin and eosin. Only surface epithelium between two intact U-shaped crypts that extended from the basal lamina to the gut lumen were accepted as satisfactory for evaluation. We developed a three grade classification score for injury of the surface epithelium. These grades were: 0, continuous surface epithelium with normal epithelial architecture; 1, ruffled surface epithelium with disturbed epithelial architecture, i.e. decreased apical cell volume; and 2, grade 1 plus injured epithelial cells with necrotic appearance in the surface epithelium. Surface epithelial injury was calculated as the sum of the scores of 25 surface intercryptal surface areas per rat and averaged per group. Thus this score varies between 0 and 50. All histological observations were done by an observer who was blinded to the origin of each tissue section.

Apoptosis.

We studied colonic apoptosis by measuring caspase-3 expression. Mucosal scrapings of colonic tissues were snap frozen and homogenized in N_2. A volume of 0.5 mL buffer containing: 0.2 M sucrose, 20 mM Tris and 1 mM dithiothreitol, pH 7.4, combined with a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) was added to half of the lyophilized and powdered colon scraping. This homogenate was shaken for 5 min and centrifuged at 20 000 *g* for 1 min. The supernatant was saved for further processing and stored at -20°C. Protein concentration in the supernatants was determined by BCA reagent (Pierce Chemical Company, Rockford, Illinois, USA).

Protein aliquots from colonic mucosal extracts (15 μg protein per lane) were subjected to 15% SDS-PAGE and electro-transferred to nitrocellulose membrane (Amersham). The membranes were stained with Ponceau S to confirm equal protein loading and effective transfer. No variability in transfer was observed. De-stained membranes were blocked with 5% nonfat milk powder in Tris-buffered saline-Tween (TBS-T, 20 mM Tris-base, pH 7.6, 137 mM NaCl, 0.3% (w/v) Tween 20) for 1 h at room temperature. Blots were incubated with 1:1000 diluted caspase-3 primary antibody

(clone H277, Santa Cruz Biotechnology, Heidelberg, Germany) in TBS-T with gentle agitation at 4°C overnight. Membranes were washed twice with TBS-T, blocked again with 5% nonfat milk powder in TBS-T for 30 min, and then washed twice in TBS-T at room temperature. Blots were then incubated with secondary antibody horseradish peroxidase-conjugated goat anti-rabbit (Sigma) at 1:5000 in TBS-T for 1 h at room temperature. After washing twice with TBS-T, peroxidase activity was visualized using SuperSignal enhanced chemiluminescence detection reagents (Pierce) according to the manufacturer's instructions. The detection of the cleaved p-17 form of caspase-3 on the blots represents the active form, whereas the uncleaved p-32 form of caspase-3 represents inactive caspase-3 (27).

Caspase-3 activity was measured simultaneously in the absence and presence of caspase-3 inhibitor (DEVD-CHO) (Calbiochem, Darmstadt, Germany). A volume of 10 µL of colonic mucosal extract was added to 973 µL of buffer containing 50 mM HEPES, 0.1 M NaCl, 0.1% (3--1-propanesulphonate) (CHAPS), 10 mM DTT, 0.1 mM EDTA, and 10% glycerol, pH 7.4. For incubations with the inhibitor, 7 µL of a stock solution (0.075 mM) of caspase-3 inhibitor in DMSO was added. For the other incubation, 7 µL of dimethyl sulfoxide (DMSO) was added. Both vials were pre-incubated for 10 min at room temperature in the dark. The reaction was started by adding 7 µL of caspase-3 substrate (3.75 mM Acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin (Ac-DEVD-AMC, Calbiochem) in 10 mM DMSO) and activity was measured on a fluorescence detector (Luminiscence Spectrometer, Perkin Elmer, Groningen, The Netherlands) every 10 min for 30 min with an excitation wavelength of 380 nm and an emission wavelength of 460 nm. The slope of the reaction was determined and the caspase-3 activity calculated. Pure AMC (Calbiochem) was diluted in DMSO and used as standard in a concentration range of 0-4 µM.

Statistical analysis.

All results are expressed as mean ± SEM. A commercially available package (Statistica 6.1, Statsoft Inc., Tulsa, USA) was used. Statistical significance was calculated by Student's *t*-test. Differences were considered significant at $P < 0.05$. Associations between colonic crypt length and labeling index, BrdU immunohistochemistry and Ki-67 immunohistochemistry were determined using the Pearson r correlation test.

Results

After two weeks feeding, food intake was higher among rats fed the heme-supplemented diets. However, this did not result in different growth rates compared with control diet fed rats (**Table 1**).

Fecal water cytotoxicity reflects the exposure of the colonic mucosa to luminal irritants. In line with previous studies dietary heme induces a greater than ten-fold increase in fecal water cytotoxicity (Table 1).

We studied whether this increased cytotoxicity had any implications for the physiology of the colon mucosa. To examine this function, we measured the concentration of the cations sodium and potassium in the feces. In rats fed heme both the sodium and potassium concentration increased in feces more than ten-fold and more than four-fold respectively, indicating an impaired reabsorption capacity of the surface epithelium (Table 1).

The increased luminal cytotoxicity might affect the viability of colon epithelial cells. We quantified the amount of host DNA in the feces as a marker of exfoliated intestinal epithelial cells. Table 1 shows that dietary heme reduced the amount of host DNA 100-fold compared with the non-heme fed rats.

Table 1. Effect of dietary heme on: food intake, animal growth, cytotoxicity of the luminal contents, fecal sodium and potassium concentrations, and fecal host DNA.

Analysis	Control	Heme
Food intake (g/d)	18.2 ± 0.4	19.2 ± 0.4 *
Growth (g/d)	3.2 ± 0.3	3.4 ± 0.3
Luminal cytotoxicity (% lysis)	9 ± 5	97 ± 1 *
Fecal sodium (µmol/d)	48 ± 6	487 ± 18 *
Fecal potassium (µmol/d)	66 ± 6	280 ± 14 *
Fecal host DNA (µg/d)	19 ± 3	0.1 ± 0.1 *

NOTE: All data represent mean ± SEM (n = 16). * Significantly different from their respective controls at $P < 0.05$ to $P < 0.001$ by Student's t test.

Histological examination showed that heme in the diet indeed resulted in increased injury of the surface epithelium (**Table 2**). Colonic samples of the heme-fed rats revealed severe disruption of surface epithelium architecture and presence of cells with a necrotic appearance compared with colon samples of control rats (**Fig. 1AB**). We observed a similar differential morphology in distal colon, but could not score injury because of the much smaller intercryptal surface epithelium in the distal colon.

Table 2. Effects of dietary heme on morphology and proliferation of colonic epithelium.

	Control	Heme
Proximal colon		
Injury of intercryptal surface epithelium	10 ± 3	35 ± 4 *
Crypt length (cells/half crypt)	43 ± 1	55 ± 2 *
^{3}H-thymidine (dpm ^{3}H/µg DNA)	43 ± 3	85 ± 4 *
BrdU IHC (LI)	7 ± 1	15± 1 *
Ki-67 IHC (LI)	10 ± 1	20 ± 2 *
Distal colon		
Crypt length (cells/half crypt)	55 ± 3	70 ± 3 *
^{3}H-thymidine (dpm ^{3}H/µg DNA)	46 ± 6	92 ± 5 *
BrdU IHC (LI)	10 ± 3	22 ± 2 *
Ki-67 IHC (LI)	16 ± 2	23 ± 2 *

NOTE: Injury score from was assigned to intercryptal surface epithelium according the criteria described in the material and methods. All data represents mean ± SEM (n = 8). IHC, immunohistochemistry. * Significantly different from control at $P < 0.05$ to $P < 0.001$ by Student's *t* test.

One of the mechanisms to compensate for cell loss and maintenance of crypt cell number is by increasing proliferation. In line with previous experiments we studied if dietary heme modified incorporation of ^{3}H-thymidine into DNA of cells, to quantify total DNA-synthesis as a marker for proliferating cells. Rats fed heme doubled colonic proliferation compared with controls (Table 2). Furthermore, proliferating colon epithelial cells in the S-phase of the cell cycle were visualized by incorporation of BrdU into DNA (28). The colonic crypts of rats fed a control diet showed a normal label distribution in the bottom third of the crypt (**Fig. 2A**). Colons of rats fed a heme supplemented diet showed a larger proliferation compartment sometimes extending over half of the crypt axis (**Fig. 2B**). Quantitatively this resulted in a doubled labeling index in the proximal and distal colon of heme-fed rats in line with the ^{3}H-thymidine results (Table 2).

Dietary treatment with heme also induced hyperplasia with an approximately 25% increase in number of cells in the crypts of proximal and distal colon (Table 2). In addition, colonic crypt length and LI correlated highly (0.82, P < 0.005).
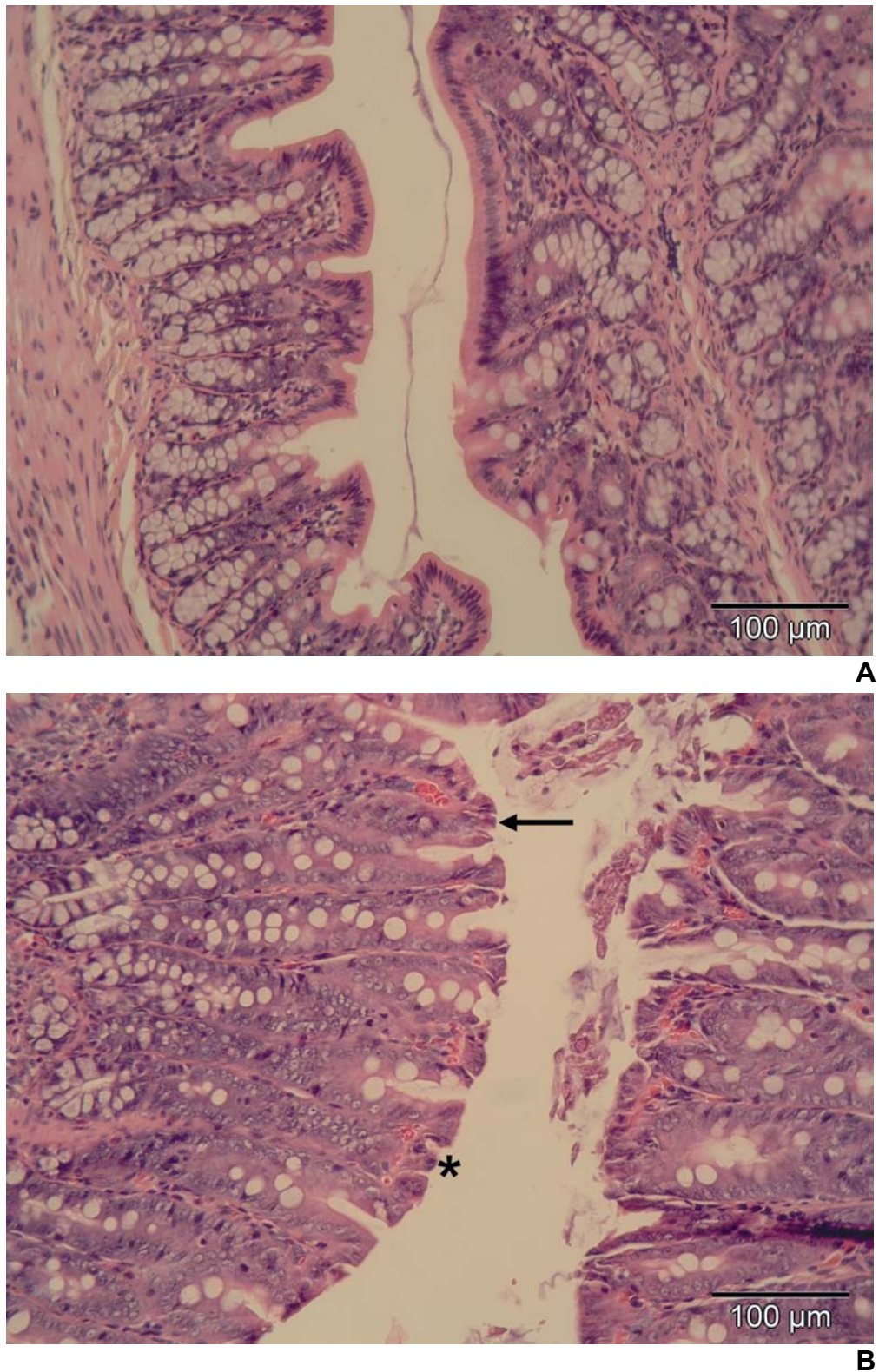
Figure 1. Representative sections of rat colonic mucosa stained with hematoxylin and eosin after 14 days feeding of control or heme diet, (**A**) Proximal colon of control rat showing normal colon epithelium, (**B**) Proximal colon of heme-fed rat showing a ruffled surface epithelium (arrow) and cells with a necrotic appearance (asterisk).
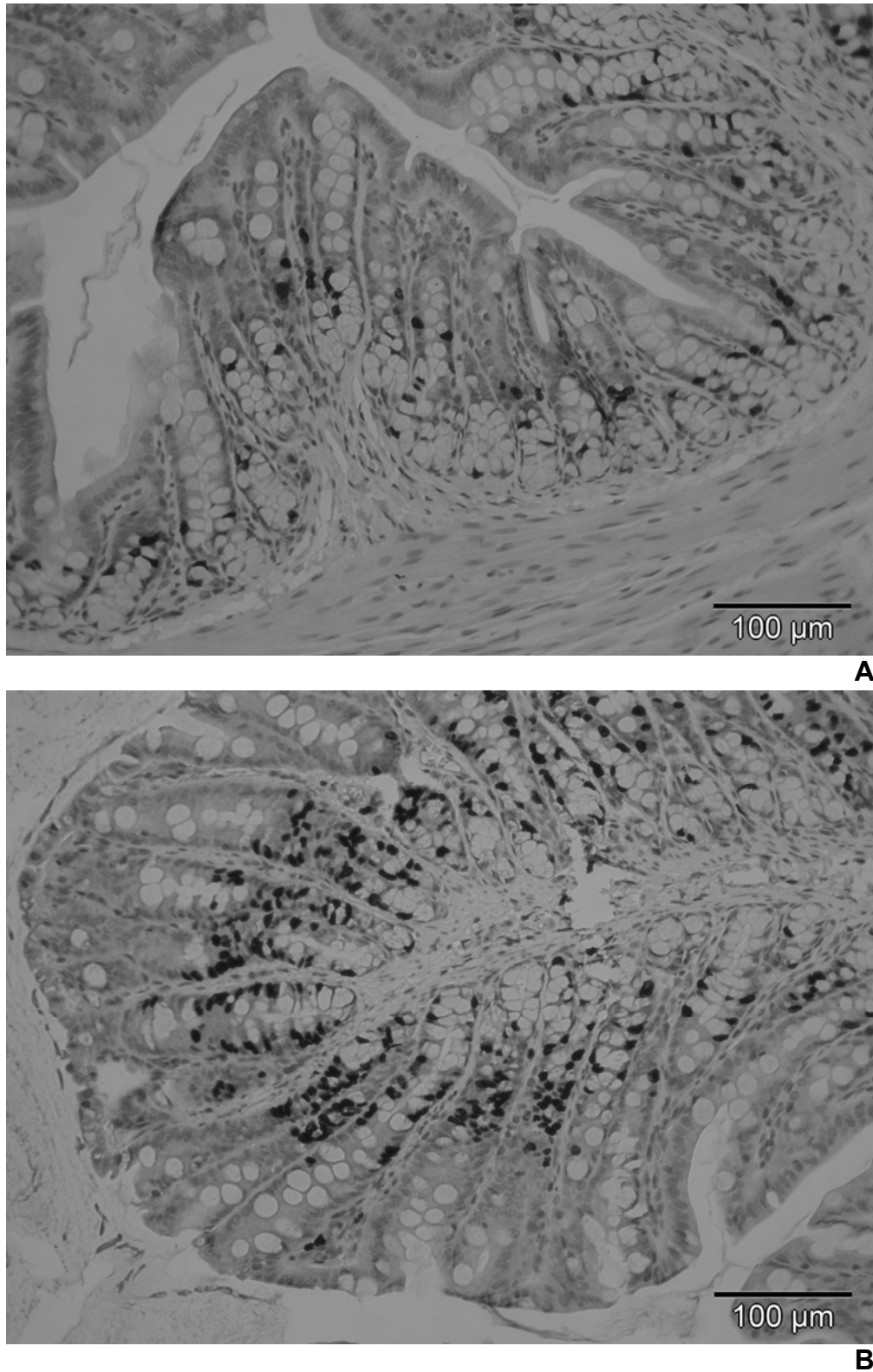
**Figure 2.** Representative sections of rat colonic mucosa stained with BrdU antibody after 14 days feeding of control or heme diet, (**A**) Proximal colon of control rat showing normal label distribution at the bottom third of the crypt, (**B**) Proximal colon of heme rat showing increased label distribution covering half the crypt.

Finally, colonocyte proliferation was also measured by demonstration of Ki-67 expression, which is a protein involved in the cell-cycle (26). The effect of dietary heme on the proximal and distal colonic labeling index measured with Ki-67 immunohistochemistry correlated with the results of the BrdU immunohistochemistry (r = 0.74, P < 0.005) and corresponded with results from the ^{3}H-thymidine assay (Table 2).

Another mechanism that maintains crypt cell number is modulation of intraepithelial apoptosis. We studied apoptosis by measuring caspase-3 in colon mucosal scrapings. Caspase-3 is one of the last downstream effector caspases, which are final caspases activated in the apoptotic process (29,30). Western blotting of homogenates of control colon mucosa demonstrated expression of active caspase-3 (P17), whereas expression of inactive caspase-3 (P32) was low (**Fig. 3, inset**).
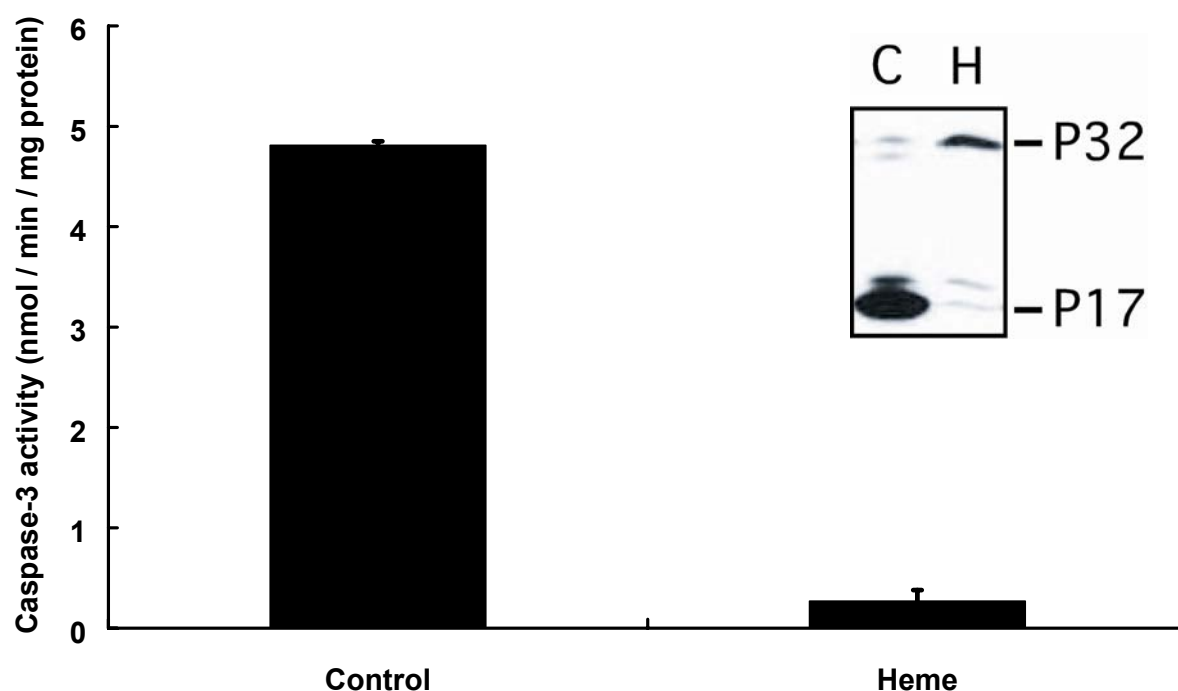
Figure 3. Effect of dietary heme on colonic mucosal apoptosis. The caspase-3 activity of rats fed the control or heme diet for 2 weeks. Colonic mucosa were scraped, homogenized, and centrifuged. Caspase-3 activity in the supernatant was determined using ac-DEVD-pNA as substrate. Results from n = 3, mean ± SE. Inset: Western blot of caspase-3 in mucosal scraping. C represents colon mucosa of rats fed a control diet, H represents colon mucosa of a heme-fed rat. P17: active caspase-3, P32: inactive caspase-3. *Significantly different from control at $P < 0.001$ by Student's t test.

Colon mucosa of the heme-fed rats expressed higher levels of inactive caspase-3 and the expression of active caspase-3 was much lower compared with mucosa of control diet fed rats. In addition to these results we also measured the activity of caspase-3 in colon mucosal scrapings. Dietary heme reduced caspase-3 activity almost completely compared with non-heme fed rats (**Fig. 3**). We verified that the observed activities were specific for caspase-3 by preincubation of the samples with caspase-3 inhibitor. Almost no caspase-3 activity was detected in colon mucosa of control or heme-fed rats in the presence of the inhibitor indicating the specificity of the activity method (data not shown). To test whether colon homogenates of heme-fed rats contained inhibitors of caspase-3, equal amounts of homogenates of control and heme colon were mixed and assayed for caspase-3 activity. No decrease in activity was observed in the mixture compared with the activity of control homogenate only, showing the absence of inhibiting compounds.

Discussion

This study shows that dietary heme disturbs epithelial cell homeostasis in the colon of the rat. Cytotoxic heme and/or its metabolites injure colonic surface epithelial cells and increase cell death. This heme-induced loss of cells triggers a compensatory epithelial hyperproliferation and inhibits mucosal apoptosis resulting in crypt hyperplasia.

The mechanisms underlying the association between colon cancer risk and consumption of red meat are not clear. However, recent epidemiological studies showed that the intake of heme might explain this association (21,22). In addition, human intervention studies suggested that endogenous nitrosation arising from ingestion of heme may account for the increased risk associated with red meat consumption (9). Here, we show an alternative explanation why intake of heme might be associated with increased colon cancer risk. In line with our previous studies, dietary heme increased cytotoxicity of fecal water (11). This may well be an initiating event by which heme affects cell turnover. Histological examination of the colonic tissues demonstrated that the much higher concentration of luminal irritants in rats fed heme indeed increased injury to the colonic surface epithelium, as indicated by a morphologically interrupted surface epithelium and presence of cells with a necrotic appearance. Microscopic analyses of the colon samples did not show any sign of heme-induced inflammation or macrophage infiltration, indicating that the permeability of the barrier is not disturbed. An important function of the colonocytes at the luminal surface is reabsorption of water and minerals. The ten-fold and four-fold increased concentrations of fecal sodium and potassium in rats fed heme indicate that reabsorption was drastically inhibited, which implies severe damage to the colonic surface epithelium thus supporting our histological findings.

The heme-induced cytotoxicity of the colonic contents may also explain the more than hundred-fold decrease in fecal excretion of host DNA. Senescent enterocytes are normally shed (exfoliated) from the epithelial surface into the fecal stream after their life-cycle of 3-5 days (17,31,32). As these cells go into apoptosis, their DNA becomes fragmented because a caspase-activated DNAse only cuts internucleosomal linker DNA (33). The resulting fragments are sufficiently large to be detected by the small-size target sequence (85 bp) of the rat β-globin gene in our real-time PCR (24). In contrast, heme-induced lyses of exfoliated cells and of surface colonocytes exposes host DNA to endo- and exo-nucleases in the fecal stream, resulting in its complete hydrolysis. Therefore, we propose that a diet-induced increase in cytolytic activity of the colonic contents causes necrosis of exfoliated cells and of surface colonocytes, thus reducing recovery of host DNA in the feces. Quantification of host DNA levels is also proposed as a tool to diagnose colorectal cancer (34). Our results, however, indicate that diet-dependent changes in cytolytic activity of colonic contents may hamper the diagnostic value of such a test.

Heme-induced loss of surface epithelium must be compensated by either a decreased apoptosis or an increased proliferation of colonocytes. We studied apoptosis by examination of caspase-3 expression and corresponding caspase-3 activity in colonic mucosa (35). Western blotting showed that colon mucosa of rats fed the control diet expressed the active form of caspase-3 and this corresponded with caspase-3 activity measured in the colon mucosa. This activity is similar to that reported earlier (36,37). In contrast, active caspase-3 expression in colons of heme fed rats was low, which correlated with the very low activity in the colon mucosal scrapings. This indicates that heme-induced luminal cytotoxicity inhibits colonic mucosal apoptosis. We do not know whether this inhibition of apoptosis occurs in surface or in crypt epithelial cells and this requires further study. But for the heme effects it is of relevance that inhibition of apoptosis is an important step in the carcinogenesis cascade (38).

In steady state, epithelial cell death and cell proliferation are balanced to maintain tissue homeostasis. Compared to rats fed the control diet, dietary heme increased colonic epithelial proliferation and thus cell death approximately two-fold. In previous studies we showed already that heme induced colonic proliferation in rats, using incorporation of ^{3}H-thymidine into DNA of cells as a marker of proliferation (8,11). Although this provided a quick and reproducible answer on the question whether diet modulates colonic proliferation it did not give information on proliferation of non-epithelial versus epithelial cells or changes of the proliferation compartment within the crypt. Here, we demonstrated with BrdU and Ki-67 immunohistochemistry that heme-induced hyperproliferation is indeed limited to the epithelial cells in the crypt proliferation compartment. This supports our previous conclusions on dietary heme-induced effects on colonic epithelial proliferation. Thus, measurement of ^{3}H-thymidine incorporation into DNA provides a fast and reproducible screening method for the effects of diet on proliferation in the colonic epithelium.

Finally, the heme-induced hyperproliferation and decreased apoptosis of colonic epithelial cells resulted in hyperplasia in the colonic crypts. In line with our results Newmark and colleagues (39,40) showed in mice and rats that feeding a Western diet (low calcium and vitamin D, high fat and phosphate) without carcinogen treatment resulted in hyperproliferation of colonic epithelial cells and hyperplasia of colonic crypts. Long-term treatment of mice with this Western diet even depleted apoptotic cells in the colonic crypts and resulted in crypt dysplasias and the formation of tumors (40,41). This corroborates our finding that diet-induced modifications of the composition of the luminal contents can disturb the balance in colonic epithelial cell turnover and thus induce crypt hyperplasia. Hyperplasia and a disturbed cell turnover are considered early risk markers for colon cancer (15,42).

In conclusion, we demonstrated that dietary heme disturbs colonic epithelial homeostasis in rats. This was due to a heme-induced injury and loss of surface colonocytes resulting in hyperproliferation, hyperplasia in colonic crypts, and in inhibition of apoptosis and fecal excretion of host DNA. Further mechanistic studies of the dietary modulation of this heme-induced aberrant cell turnover in colonic crypts may provide new leads for prevention of colon cancer.

Acknowledgments

References

1. Shibuya, K., Mathers, C.D., Boschi-Pinto, C., Lopez, A.D. and Murray, C.J. (2003) Correction: Global and regional estimates of cancer mortality and incidence by site: II. results for the global burden of disease 2000. *BMC Cancer*, **3**, 20.
2. *World Cancer Research Fund. Food, Nutrition and the prevention of cancer: a global perspective, 1997*, Washington, DC: American Institute for Cancer Research.
3. Chao, A., Thun, M.J., Connell, C.J., McCullough, M.L., Jacobs, E.J., Flanders, W.D., Rodriguez, C., Sinha, R. and Calle, E.E. (2005) Meat consumption and risk of colorectal cancer. *Jama*, **293**, 172-82.
4. Norat, T., Lukanova, A., Ferrari, P. and Riboli, E. (2002) Meat consumption and colorectal cancer risk: dose-response meta- analysis of epidemiological studies. *Int J Cancer*, **98**, 241-256.
5. Pierre, F., Freeman, A., Tache, S., Van der Meer, R. and Corpet, D.E. (2004) Beef meat and blood sausage promote the formation of azoxymethane-induced mucin-depleted foci and aberrant crypt foci in rat colons. *J Nutr*, **134**, 2711-6.
6. Norat, T., Bingham, S., Ferrari, P., Slimani, N., Jenab, M., Mazuir, M., Overvad, K., Olsen, A., Tjonneland, A., Clavel, F., Boutron-Ruault, M.C., Kesse, E., Boeing, H., Bergmann, M.M., Nieters, A., Linseisen, J., Trichopoulou, A., Trichopoulos, D., Tountas, Y., Berrino, F., Palli, D., Panico, S., Tumino, R., Vineis, P., Bueno-de-Mesquita, H.B., Peeters, P.H., Engeset, D., Lund, E., Skeie, G., Ardanaz, E., Gonzalez, C., Navarro, C., Quiros, J.R., Sanchez, M.J., Berglund, G., Mattisson, I., Hallmans, G., Palmqvist, R., Day, N.E., Khaw, K.T., Key, T.J., San Joaquin, M., Hemon, B., Saracci,

R., Kaaks, R. and Riboli, E. (2005) Meat, fish, and colorectal cancer risk: the European Prospective Investigation into cancer and nutrition. *J Natl Cancer Inst*, **97**, 906-16.

7. Giovannucci, E., Rimm, E.B., Stampfer, M.J., Colditz, G.A., Ascherio, A. and Willett, W.C. (1994) Intake of fat, meat, and fiber in relation to risk of colon cancer in men. *Cancer Res*, **54**, 2390-2397.

8. Sesink, A.L.A., Termont, D.S., Kleibeuker, J.H. and Van der Meer, R. (1999) Red meat and colon cancer: the cytotoxic and hyperproliferative effects of dietary heme. *Cancer Res*, **59**, 5704-5709.

9. Cross, A.J., Pollock, J.R. and Bingham, S.A. (2003) Haem, not protein or inorganic iron, is responsible for endogenous intestinal N-nitrosation arising from red meat. *Cancer Res*, **63**, 2358-2360.

10. Schwartz, S. and Ellefson, M. (1985) Quantitative fecal recovery of ingested hemoglobin-heme in blood: comparisons by HemoQuant assay with ingested meat and fish. *Gastroenterology*, **89**, 19-26.

11. de Vogel, J., Jonker-Termont, D.S., van Lieshout, E.M., Katan, M.B. and van der Meer, R. (2005) Green vegetables, red meat and colon cancer: chlorophyll prevents the cytotoxic and hyperproliferative effects of haem in rat colon. *Carcinogenesis*, **26**, 387-93.

12. Pierre, F., Tache, S., Petit, C.R., Van Der Meer, R. and Corpet, D.E. (2003) Meat and cancer: haemoglobin and haemin in a low-calcium diet promote colorectal carcinogenesis at the aberrant crypt stage in rats. *Carcinogenesis*, **24**, 1683-1690.

13. Corpet, D.E. and Tache, S. (2002) Most effective colon cancer chemopreventive agents in rats: a systematic review of aberrant crypt foci and tumor data, ranked by potency. *Nutr Cancer*, **43**, 1-21.

14. Terpstra, O.T., van Blankenstein, M., Dees, J. and Eilers, G.A. (1987) Abnormal pattern of cell proliferation in the entire colonic mucosa of patients with colon adenoma or cancer. *Gastroenterology*, **92**, 704-8.

15. Lipkin, M. (1988) Biomarkers of increased susceptibility to gastrointestinal cancer: new application to studies of cancer prevention in human subjects. *Cancer Res*, **48**, 235-245.

16. Shanmugathasan, M. and Jothy, S. (2000) Apoptosis, anoikis and their relevance to the pathobiology of colon cancer. *Pathol Int*, **50**, 273-9.

17. Gibson, P.R. (2004) Apoptosis or necrosis--colonic epithelial cell survival. *Novartis Found Symp*, **263**, 133-45; discussion 145-50, 211-8.

18. Hickman, J.A. (2002) Apoptosis and tumourigenesis. *Curr Opin Genet Dev*, **12**, 67-72.

19. Kinzler, K.W. and Vogelstein, B. (1996) Lessons from hereditary colorectal cancer. *Cell*, **87**, 159-170.

20. Thilly, W.G. (2003) Have environmental mutagens caused oncomutations in people? *Nat Genet*, **34**, 255-259.

21. Lee, D.H., Anderson, K.E., Harnack, L.J., Folsom, A.R. and Jacobs, D.R., Jr. (2004) Heme iron, zinc, alcohol consumption, and colon cancer: Iowa Women's Health Study. *J Natl Cancer Inst*, **96**, 403-7.

22. Larsson, S.C., Adami, H.O., Giovannucci, E. and Wolk, A. (2005) Re: Heme iron, zinc, alcohol consumption, and risk of colon cancer. *J Natl Cancer Inst*, **97**, 232-3; author reply 233-4.

23. Lapre, J.A. and Van der Meer, R. (1992) Diet-induced increase of colonic bile acids stimulates lytic activity of fecal water and proliferation of colonic cells. *Carcinogenesis*, **13**, 41-44.

24. Van Lieshout, E.M., Van Doesburg, W. and Van der Meer, R. (2004) Real-time PCR of host DNA in feces to study differential exfoliation of colonocytes between rats and humans. *Scand J Gastroenterol*, **39**, 852-7.

25. Harms, G., van Goor, H., Koudstaal, J., de Ley, L. and Hardonk, M.J. (1986) Immunohistochemical demonstration of DNA-incorporated 5-bromodeoxyuridine in frozen and plastic embedded sections. *Histochemistry*, **85**, 139-43.

26. Muskhelishvili, L., Latendresse, J.R., Kodell, R.L. and Henderson, E.B. (2003) Evaluation of cell proliferation in rat tissues with BrdU, PCNA, Ki-67(MIB-5) immunohistochemistry and in situ hybridization for histone mRNA. *J Histochem Cytochem*, **51**, 1681-8.

27. Cohen, G.M. (1997) Caspases: the executioners of apoptosis. *Biochem J*, **326 (Pt 1)**, 1-16.

28. Lacy, E.R., Kuwayama, H., Cowart, K.S., King, J.S., Deutz, A.H. and Sistrunk, S. (1991) A rapid, accurate, immunohistochemical method to label proliferating cells in the digestive tract. A comparison with tritiated thymidine. *Gastroenterology*, **100**, 259-62.

29. Watson, A.J.M. (2004) Apoptosis and colorectal cancer. *Gut*, **53**, 1701-1709.

30. Hengartner, M.O. (2000) The biochemistry of apoptosis. *Nature*, **407**, 770-6.

31. Mayhew, T.M., Myklebust, R., Whybrow, A. and Jenkins, R. (1999) Epithelial integrity, cell death and cell loss in mammalian small intestine. *Histol Histopathol*, **14**, 257-267.

32. Watson, A.J., Chu, S., Sieck, L., Gerasimenko, O., Bullen, T., Campbell, F., McKenna, M., Rose, T. and Montrose, M.H. (2005) Epithelial barrier function in vivo is sustained despite gaps in epithelial layers. *Gastroenterology*, **129**, 902-12.

33. Sen, S. (1992) Programmed cell death: concept, mechanism and control. *Biol Rev Camb Philos Soc*, **67**, 287-319.

34. Osborn, N.K. and Ahlquist, D.A. (2005) Stool screening for colorectal cancer: Molecular approaches. *Gastroenterology*, **128**, 192-206.

35. Porter, A.G. and Janicke, R.U. (1999) Emerging roles of caspase-3 in apoptosis. *Cell Death Differ*, **6**, 99-104.

36. Yang, L., Mutanen, M., Cheng, Y. and Duan, R. (2002) Effects of red meat and fiber in high fat diet on activities of sphingomyelinase, ceramidase and caspase-3 in rat colonic mucosa. *J Nutr Biochem*, **13**, 499.

37. Yang, L., Mutanen, M., Cheng, Y. and Duan, R.D. (2002) Effects of fat, beef and fiber in diets on activities of sphingomyelinase, ceramidase and caspase-3 in rat colonic mucosa. *Med Princ Pract*, **11**, 150-6.

38. Lengauer, C., Kinzler, K.W. and Vogelstein, B. (1998) Genetic instabilities in human cancers. *Nature*, **396**, 643-9.

39. Newmark, H.L., Lipkin, M. and Maheshwari, N. (1990) Colonic hyperplasia and hyperproliferation induced by a nutritional stress diet with four components of Western-style diet. *J Natl Cancer Inst*, **82**, 491-6.

40. Risio, M., Lipkin, M., Newmark, H., Yang, K., Rossini, F.P., Steele, V.E., Boone, C.W. and Kelloff, G.J. (1996) Apoptosis, cell replication, and Western-style diet-induced tumorigenesis in mouse colon. *Cancer Res*, **56**, 4910-6.

41. Newmark, H.L., Yang, K., Lipkin, M., Kopelovich, L., Liu, Y., Fan, K. and Shinozaki, H. (2001) A Western-style diet induces benign and malignant neoplasms in the colon of normal C57Bl/6 mice. *Carcinogenesis*, **22**, 1871-5.

42. Baron, J.A. (2001) Intermediate effect markers for colorectal cancer. *IARC Sci Publ*, **154**, 113-129.

Chapter 3

Green vegetables, red meat and colon cancer: chlorophyll prevents the cytotoxic and hyperproliferative effects of heme in rat colon

Johan de Vogel, Denise S.M.L. Jonker-Termont, Esther M.M. van Lieshout, Martijn B. Katan and Roelof van der Meer

Abstract

Diets high in red meat and low in green vegetables are associated with increased colon cancer risk. This association might be partly due to the heme content of red meat. In rats, dietary heme is metabolized in the gut to a cytotoxic factor that increases colonic cytotoxicity and epithelial proliferation. Green vegetables contain chlorophyll, a magnesium porphyrin structurally analogous to heme. We studied whether green vegetables inhibit the unfavorable colonic effects of heme. First, rats were fed a purified control diet or purified diets supplemented with 0.5 mmol heme/kg, spinach (chlorophyll concentration 1.2 mmol/kg) or heme plus spinach (n = 8/group) for 14 days. In a second experiment we also studied a group that received heme plus purified chlorophyll (1.2 mmol/kg). Cytotoxicity of fecal water was determined with a bioassay and colonic epithelial cell proliferation was quantified *in vivo* by [methyl-^{3}H]thymidine incorporation into newly synthesized DNA. Exfoliation of colonocytes was measured as the amount of rat DNA in feces. In both studies heme increased cytotoxicity of the colonic contents 8-fold and proliferation of the colonocytes almost 2-fold. Spinach or an equimolar amount of chlorophyll supplement in the heme diet inhibited these heme effects completely. Heme clearly inhibited exfoliation of colonocytes, an effect counteracted by spinach and chlorophyll. Finally, size exclusion chromatography showed that chlorophyll prevented formation of the cytotoxic heme metabolite. We conclude that green vegetables may decrease colon cancer risk because chlorophyll prevents the detrimental, cytotoxic and hyperproliferative colonic effects of dietary heme.

Introduction

Colon cancer is one of the leading causes of cancer death in Western societies. For the USA in 2003 nearly 110 000 new cases and almost 48 000 deaths were estimated (1). Incidence rates of colon cancer vary 20-fold between high and low risk countries (2). Migrant and other epidemiological studies indicate that this variation is due to environmental factors, with diet as a major determinant (3). Diets high in red and processed meat are especially associated with a moderately increased risk for colon cancer (4). In contrast, diets high in white meat (poultry, fish) are not associated with an increased risk (5). The mechanism explaining the specific risk-enhancing effect of red meat is not precisely known. Based on mutational analysis of colon cancers, Kinzler and Vogelstein (6) argued that dietary factors that lead to colon cancer are probably not mutagens but rather luminal irritants that damage colonic epithelial cells (7). This damage triggers a compensatory epithelial hyperproliferation, which increases the risk of endogenous mutations in tumor suppressor and oncogenes. Clonal accumulation of these endogenous mutations may eventually result in the adenoma–carcinoma sequence of colon carcinogenesis (8). For these reasons, Sesink et al. (9) hypothesized that heme, the iron porphyrin pigment of red meat, might be an important dietary risk factor. They argued that heme may better explain the differential effects of red versus white meat on colon cancer risk, instead of earlier proposed meat-associated mutagens such as heterocyclic amines (10). Sesink et al. (9) showed in rat studies that dietary heme enhanced cytotoxicity of the fecal water. This was not mediated by well-known surfactants like bile acids or fatty acids, suggesting involvement of a heme-induced cytotoxic metabolite. This enhanced cytotoxicity implies an increased exposure of the colonic epithelial cells to luminal irritants, resulting in colonic epithelial hyperproliferation. As mentioned above, hyperproliferation could increase the risk of endogenous mutations in cell turnover genes and is therefore considered an early risk marker for colon cancer (11). This is supported by the results of clinical studies where subjects at high risk of colon cancer showed a higher proliferative activity compared with controls (12).

A large number of epidemiological studies indicate that vegetables protect against colon cancer, especially green and raw vegetables (13,14). The aim of this study was to investigate whether the protective effect of vegetables is due to inhibition of heme-induced colonic cytotoxicity and hyperproliferation of epithelial cells. We hypothesized that this

protection could be due to the high chlorophyll content of green vegetables. Chlorophyll is a phytol-esterified magnesium porphyrin and thus a structural analogue of heme. Therefore, it might compete with heme for solubilization in the gastrointestinal tract and thus prevent the formation of cytotoxic heme metabolites. Subsequently, this may prevent heme-induced hyperproliferative effects. We first examined the effect of the green vegetable spinach and, second, whether the effect of spinach can be mimicked by an equimolar concentration of chlorophyll.

Materials and Methods

Animals and diets

The experimental protocols were approved by the animal welfare committee of Wageningen University and Research Centre. Approximately 8-week-old outbred male Wistar rats (WU, Harlan, Horst, The Netherlands) were housed individually in metabolic cages in a room with controlled temperature (±20°C), relative humidity (50–60%) and light/dark cycle (lights on 6 a.m. to 6 p.m.). Animals were acclimatized to housing conditions for 5 days before the start of the experiment.

In the first study we tested whether spinach prevents the cytotoxic and hyperproliferative effects of dietary heme. During 2 weeks four groups of eight rats were fed purified diets. The compositions of the diets are given in **Table 1**. The heme and heme plus spinach diets were supplemented with 0.5 mmol heme/kg diet (Sigma-Aldrich, St Louis, MO). The iron content of the heme was analyzed with an inductive coupled plasma absorption emission spectrophotometer (ICP-AES) (Varian, Mulgrave, Australia), giving a purity of >90%.

The spinach diets contained 82 g/kg powdered freeze-dried spinach at the expense of acid casein, dextrose and cellulose, with a final chlorophyll concentration of 1.2 mmol chlorophyll/kg diet. We measured the chlorophyll concentration of spinach (Iglo, 's-Hertogenbosch, The Netherlands) after five successive extractions with acetone/water (80:20 v/v) by comparing the absorption spectra with standard chlorophyll solutions (Sigma) in a spectrophotometer (Lambda 2; Perkin Elmer, Norwalk, CT). Calcium was added as calcium phosphate ($CaHPO_4 \cdot 2H_2O$) (Fluka Chemie, Buchs, Switzerland). Vitamins and minerals other than calcium were added to all diets in similar concentrations according to the recommendations of the American Institute of Nutrition 1993 (15).

Table 1. Composition of the experimental diets (g/kg).

	Study	1			Study	2		
	Control	Heme	Spinach	Heme plus spinach	Control	Heme	Heme plus spinach	Heme plus chlorophyll
Acid casein*	200	200	176	176	200	200	176	200
Dextrose*	532	532	506	506	532	532	506	532
Palm fat	160	160	160	160	148	148	148	148
Palm oil	--	--	--	--	12	12	12	--
Corn oil	40	40	40	40	40	40	40	40
Cellulose*	20	20	--	--	20	20	--	20
Heme	--	0.326	--	0.326	--	0.326	0.326	0.326
Spinach	--	--	82	82	--	--	82	--
Natural Chlorophyll	--	--	--	--	--	--	--	12
CaHPO$_4$·2H$_2$O	3.44	3.44	--	--	3.44	3.44	--	3.44
Mineral mix*	35	35	26.3	26.3	35	35	26.3	35
Vitamin mix	10	10	10	10	10	10	10	10

The composition of the vitamin and mineral mixtures is according to the recommendation of the American Institute of Nutrition 1993, except that calcium was omitted. In addition tri-potassium citrate was added instead of KH$_2$PO$_4$, and choline was added as choline chloride. * Protein, carbohydrate, cellulose, calcium, and potassium were adjusted for their content in spinach.

In a second study, using a similar experimental design as the first, we tested whether the protection by spinach against heme-induced hyperproliferation might be due to its chlorophyll content. The chlorophyll plus heme diet was prepared as the heme diet (Table 1) with the addition of 12 g natural chlorophyll in palm oil (final concentration 1.2 mmol chlorophyll/kg diet) (Japan Chlorophyll Co., Chiyoda-ku, Tokyo) exchanged for 12 g palm fat. The control, heme and heme plus spinach diets were composed as described in Table 1. An aliquot of 12 g palm oil (Japan Chlorophyll Co.) was added instead of 12 g palm fat/kg diet as a control for the chlorophyll solution.

In a third study we compared the effects of a control and heme-supplemented diet on colon histology, using a similar study design and diets as described for the first study.

Food was administered to the rats just before dark, to prevent possible degradation of the supplements. Food and demineralized drinking water were supplied ad libitum. Food intake and body weights were recorded every 2–4 days. Feces were quantitatively collected during days 11–14 of the experiment and frozen at –20°C.

In vivo colonic epithelial cell proliferation

After 14 days of experimental feeding colon epithelial cell proliferation was quantified *in vivo* by measuring DNA replication, using [methyl-^{3}H]thymidine incorporation into DNA. We chose this method because it is a rapid, specific and highly quantitative marker of cell replication *in vivo*. Moreover, we have shown earlier that DNA replication correlates highly with fecal water cytotoxicity (16). This substantiates a cause and effect relationship that we want to address in the present study. Non-fasted rats were injected intraperitoneally (i.p.) with [methyl-^{3}H]thymidine (specific activity 925 GBq/mmol, dose 3.7 MBq/kg body wt; Amersham International, Amersham, UK) in 154 mM NaCl. After 2 h the rats were killed by CO_2 inhalation. The colon was excised and opened longitudinally. Colonic contents were removed by rinsing with 154 mM KCl. The mucosa was scraped using a spatula and homogenized (Ultraturrax Pro200; Pro Scientific Inc., Monroe, CT) in 1 ml of buffer containing 200 mM sucrose, 20 mM Tris and 1 mM dithiothreitol, pH 7.4, combined with a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). These homogenates were analyzed for ^{3}H-thymidine incorporation and for total DNA content. The macromolecular fraction was precipitated with 5% trichloroacetic acid (w/v), followed by 10 min centrifugation at 10 000 *g*. The supernatant was discarded and the pellet resuspended in 1 M perchloric acid and hydrolyzed at 70°C for 20 min. Part of the final hydrolyzate (0.5 ml) was dissolved in 15 ml of Aqua Luma (Lumac-LSC BV, Groningen, The Netherlands) and its radioactivity measured in a Beckman LS-7500 liquid scintillation counter with correction for quenching. DNA content of the scrapings was determined using the diphenylamine reaction with calf thymus DNA (Sigma) as standard (17).

Quantification of epithelial DNA in feces

Fecal host DNA content was quantified with real-time PCR, using a rat-specific probe and primers with the ß-globin gene as target sequence (18). DNA was isolated from 20 mg freeze-dried feces using a QIAamp DNA stool mini kit (Westburg, Leusden, The Netherlands). All isolates were of good purity (A_{260}/A_{280} 1.8) and were kept at 4°C or at -20°C for long-term storage. The standard DNA used for quantification of DNA in feces was isolated from rat spleen, using a Wizard Genomic DNA purification kit (Promega Corp., Madison, WI). Real-time PCR was performed using an ABI Prism 7700 sequence detection system (PE Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). Universal Taqman Master Mix and Taqman Exogenous Internal Positive Control (IPC)

Reagent (VicTM probe) were purchased from Applied Biosystems and the Taqman probe containing a FAM 5'-labelled fluorescent reporter dye (6-carboxyfluorescein) and a TAMRA 3'-labelled quencher dye (6-carboxytetramethylrhodamine) was purchased from Proligo (Paris, France). PCR primers were synthesized by the Amersham Pharmacia Biotech Custom DNA Synthesis Service (Roosendaal, The Netherlands). For amplification of host DNA, 5 µl of the purified DNA (100–200 ng) was used in a volume of 50 µl, containing 1x Master Mix, 200 nM forward primer (5'-TGATGGCCTGAAACACTTGG-3'), 200 nM reverse primer (5'-TCAGGATCCACATGCAGCTT-3'), 100 nM probe (5'-CAACCTCAAGGGCACCTTTGCTCA-3'), 1x IPC mix and 1x IPC DNA. The PCR protocol consisted of 2 min at 50°C, 10 min at 95°C and 40 cycles of 15 s at 95°C and 1 min at 62°C.

Histological measurements

After 14 days on the control or heme diet, the rats in the third study were killed by CO_2 inhalation and their colons excised for histological evaluation. A sample of the colon was stored in 10% neutral buffered formalin. The samples were embedded in paraffin and sectioned to 4 µm slides, which were stained with haematoxylin and eosin. The slides were examined by light microscopy (Reichert-Jung, Polyvar, Austria) and the image was captured with a color video camera (Leica DC 200, Switzerland) coupled to a desktop computer. Calibrated software (Leica DC Image Acquisition, Leica Microsystems, Switzerland) was used to measure crypt column height. A minimum of five crypts per slide were recorded by an observer who had no knowledge of their identity. We also studied apoptosis on the paraffin embedded slides with terminal deoxynucleotidyl transferase d-UTP nick end labeling (TUNEL) (DNA Fragmentation Detection Kit, TdT-FragELTM; Oncogene Research Products).

Preparation of fecal water

Fecal water was prepared by reconstituting a small amount of freeze-dried feces with double-distilled water to obtain a physiological osmolarity of 300 mOsmol/l, as described earlier (9). After centrifugation, the fecal waters were stored at –20°C until further analysis.

Determination of heme in feces

Total amount of heme excreted in the feces was determined by a previously described HemoQuant assay (19). To quantify heme in feces an acidified chloroform–methanol extract (20) was obtained from 30 mg freeze-dried feces (final HCl concentration 1 M). The chloroform phase of the extracted samples was dried under nitrogen and solubilized in 0.45 ml of 250 mM KOH. Subsequently, 0.45 ml of double-distilled water, 3.75 ml of 2-propanol and 0.75 ml of 1.15 M HCl was added to the samples and they were assayed for their heme content as described (19).

Cytotoxicity of fecal water

Cytotoxicity of fecal water was quantified by potassium release of a human erythrocyte suspension after incubation with fecal water as described previously (9) and validated earlier with human colon carcinoma-derived Caco-2 cells (21). The potassium content of the erythrocytes was measured with an ICP-AES and the cytotoxicity of fecal water was calculated and expressed as a percentage of maximal lyses.

Purification of the cytotoxic heme metabolite from fecal water

The cytotoxic heme metabolite (heme factor) was purified using lipid extraction and size exclusion chromatography. To a volume of 100 µl of fecal water pooled per treatment group was added 560 µl of water and 2.5 ml of diethyl ether (ether) and, after vigorous mixing, the mixture was centrifuged at 1500 g for 5 min. The ether phase containing chlorophyll but not heme was discarded, and the procedure was repeated twice (22). The remaining 660 µl sample was acidified with 60 µl of HCl (final HCl concentration 1 M) and further extracted with chloroform/methanol as described earlier (20). The lipid-containing chloroform phase was obtained after centrifugation (10 min, 1500 g) and evaporated under nitrogen. This extract was purified by size exclusion chromatography with chloroform/methanol/acetonitrile/triethylamine (55:30:10:5, v/v/v/v) as elution solvent. Dried lipid extracts of 100 µl fecal water were solubilized in 250 µl of this solvent. Then, 200 µl were injected. We used a Waters 501 Pumping System (Waters Corp., Milford, MA), a Gilson 231 auto sampler (Gilson Medical Electronics Inc., Villiers le Bel, France) fitted with a 500 µl loop, a Jordi GPC/divinylbenzene column (300 x 7.8 mm, 5 µm, 500 Å; Jordi Associates, Bellingham, MA), a photodiode array detector (Shimadzu, Kyoto, Japan), to determine the absorption spectrum of the eluted compounds, and a fraction collector

(Ultrorac II; LKB, Brommen, Sweden). Absorption at 400 nm was used to detect porphyrin structures. The data were processed and analyzed using Shimadzu software. Fractions were collected at 1 min intervals between 0 and 12 min after injection and evaporated under a nitrogen stream. Cytotoxicity of these fractions was determined as described above for fecal water, after resolubilizing the samples in 50 mM NaOH and adjustment to neutral pH and 300 mOsm/l with 3-(N-morpholino)-propanesulfonic acid.

Statistical analysis

All results are expressed as mean ± SEM (n = 8 per group). A commercially available package (Statistica 6.1; Statsoft Inc., Tulsa, OK) was used for all statistics. Normality of the data was tested with the Shapiro Wilk test and homogeneity of variances was tested using Levene's test. In the case of normal distribution and equal homogeneity, one-way analysis of variance (ANOVA) was performed to test for significant treatment effects, followed by Student's t-test. In the case of a non-Gaussian distribution of data, Kruskall–Wallis ANOVA was performed and in addition the non-parametric Mann–Whitney U-test was used as a post hoc test. Bonferroni correction was made for the number of comparisons (n = 3). Differences were considered statistically significant at $P < 0.05$.

Results

Spinach inhibits the detrimental colonic effects of heme

First, we tested whether spinach could inhibit heme-induced colonic cytotoxicity and hyperproliferation of the epithelial cells. Rats were fed purified control and heme diets in the presence and absence of spinach. Addition of heme or spinach to the diets did not cause differences in food intake or growth rate between the groups; average food intake was 17.0 ± 1.2 g/day and growth rate 2.0 ± 0.6 g/day.

Proliferation of the epithelial cells was measured to determine if changes in diet resulted in different responses of the colonic epithelium. As shown in **Figure 1A**, the group fed heme had a 50% increase in rate of DNA replication compared with the control group. Supplementation of the heme diet with spinach resulted in total inhibition of this heme-induced colonic epithelial hyperproliferation. No differences in epithelial proliferation were observed between the control group and the spinach without heme group. In addition, rats fed the heme diet had 4.3 ± 0.15 mg DNA/g scraping, which was significantly lower than

the 5.3 ± 0.3 mg DNA/g scraping of the rats fed the other diets. However, rats fed the heme diet had a significantly higher total scraping weight of 0.54 ± 0.04 g than the 0.30 ± 0.03 g scraping for the rats fed the other diets. This heme-specific increase in scraping weight may reflect an increased fragility and/or increased thickness of the colon mucosa. Histological observation in a third study of rats fed a control and heme-supplemented diet showed that the heme diet disrupted the structure of the surface epithelium, indicating necrosis (data not shown). In addition, heme significantly increased the crypt column height: control diet 255 ± 8 µm versus the heme diet 324 ± 10 µm. We also investigated a possible differential effect of heme versus control on apoptosis and/or necrosis more directly by end-labeling of DNA fragments in paraffin-embedded mucosa slides. In control rats we found positive staining of apoptotic bodies in a few crypt cells. In slides from heme-fed rats we always found a diffuse brown staining of the cytosol, indicating necrosis in surface cells and hardly any apoptotic crypt cells. Unfortunately, we could not further quantify these effects, because the day-to-day reproducibility of the TUNEL kit was insufficient. This requires further investigation. However, these qualitative effects at least indicate, in our opinion, that heme induces damage to surface cells and inhibition of apoptosis in crypt cells.

Subsequently we determined whether these differences in cell turnover were due to differences in exposure of the colonocytes to luminal irritants. **Figure 1B** shows that the cytotoxicity of fecal water of the heme group increased 7-fold compared with the control group. The addition of spinach to the heme diet abolished this cytotoxic effect of heme. Supplementation of the non-heme control diet with spinach did not affect cytotoxicity.

We determined the amount of native heme excreted in feces, to study whether spinach affects intestinal heme metabolism. As expected, fecal output of heme was low on the control and the spinach diets and supplementation of the control diet with heme resulted in an increased heme excretion (**Fig. 1C**). The amount of native heme excreted on the heme plus spinach diet was 3-fold higher than on the heme diet, even though the amounts of heme fed were equal. This indicates that spinach inhibits intestinal catabolism of heme.

These data show that spinach inhibits the heme-induced detrimental colonic effects. This raised the question which component in spinach is responsible for its protective effect.
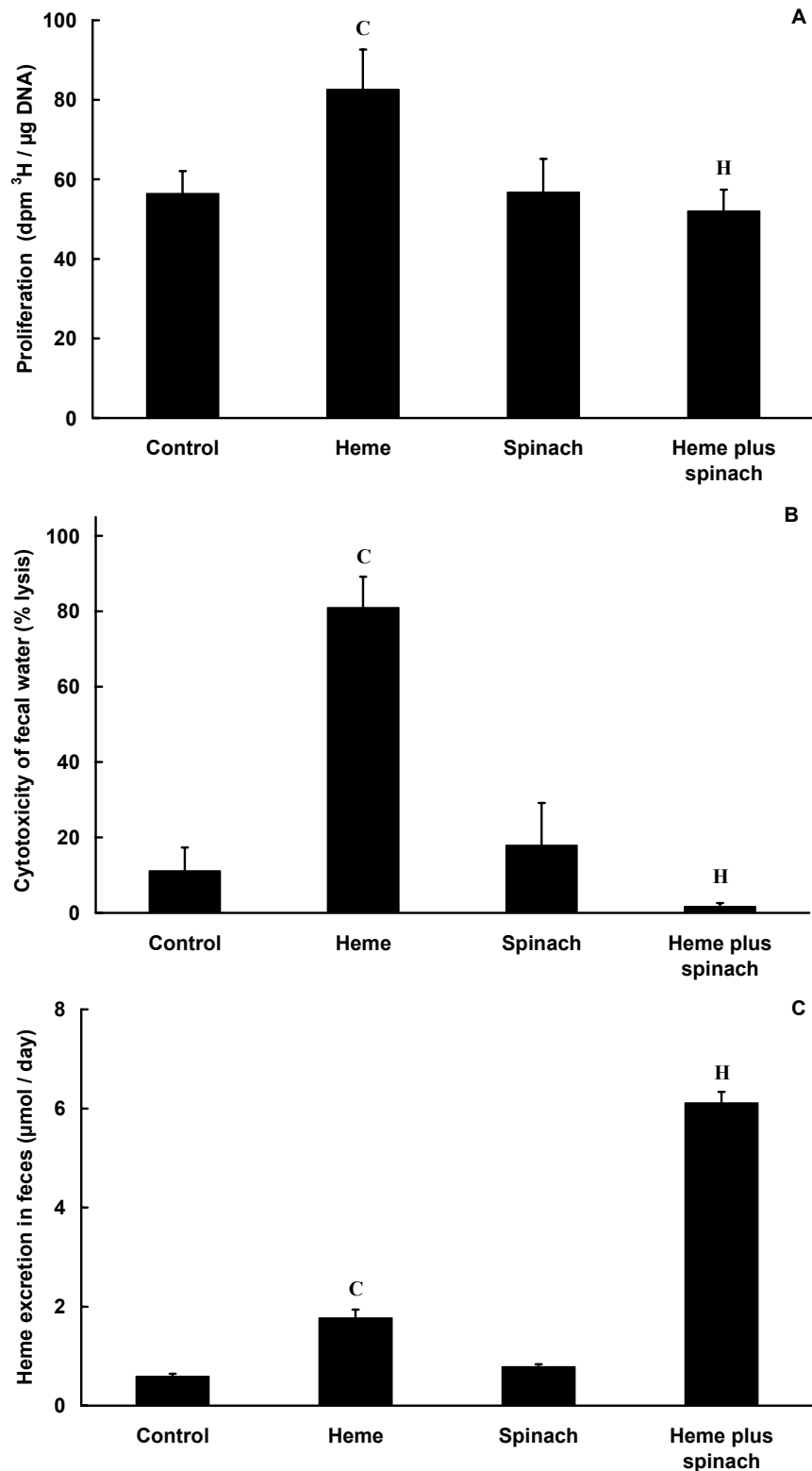
Figure 1. Effect of dietary heme, spinach and heme plus spinach on: (**A**) colonic epithelial cell proliferation, determined by *in vivo* [methyl-^{3}H]thymidine incorporation into colonic mucosa; (**B**) cytotoxicity of fecal water, determined with an erythrocyte bioassay; (**C**) heme excretion in the feces, determined by HemoQuant. Results are mean ± SEM (n = 8). C Significantly different from control group; H significantly different from heme group (P < 0.05).

*The protective effect of spinach is due to its chlorophyll content*

In a second study we investigated whether the protective effect of spinach can be mimicked by an equimolar amount of chlorophyll. Therefore, rats were fed a control, a heme and a heme plus spinach diet identical to those in the first study and a heme plus chlorophyll diet. Food intake of the different groups in the second study was 18 g/day. Growth rate of the rats fed heme plus chlorophyll (5.4 ± 0.3 g/day) was higher than that of the other groups (4.2 ± 0.3 g/day).

Figure 2A shows that both chlorophyll and spinach completely prevented heme-induced epithelial hyperproliferation. The effect of the different diets on proliferation of colonic epithelial cells was reproducible between studies: the effects observed for the control, heme and heme plus spinach diets were the same in study 1 as in study 2.

Subsequently, to study alterations in the fate of colonocytes, we determined whether these diet-induced differences in cell turnover resulted in differences in exfoliation of colonocytes into the fecal stream. **Figure 2B** shows that dietary heme strongly reduced the amount of host DNA in the feces, compared with the control diet. Spinach and chlorophyll supplements in the heme diet abolished the heme-induced decrease in rat DNA excretion in feces. Similar results for the heme and heme plus spinach groups were observed in the first study (data not shown).

Analogous to the results observed in the first study, supplementation of heme increased cytotoxicity of the fecal water compared with the control diet. Supplementation of the heme diet with chlorophyll and spinach completely inhibited this heme-induced increase in cytotoxicity (**Fig. 2C**).

We showed earlier that a cytotoxic heme metabolite (heme factor) can be isolated by size exclusion chromatography, where its absorption at 400 nm coincides with cytotoxicity (23). We investigated the effect of chlorophyll on the formation of this heme factor. **Figure 3** shows the elution profiles for UV absorption (at 400 nm) and cytotoxicity of lipid extracts of pooled fecal water of the control, heme and heme plus chlorophyll groups. Only the fecal water extract of the heme group contained UV-absorbing cytotoxic components eluting around 6 min. The UV absorption spectra of these fractions showed that they contained the porphyrin structure of heme. Because native heme elutes later than 12 min (23), this implies that cytotoxicity is due to a heme metabolite of higher molecular weight.
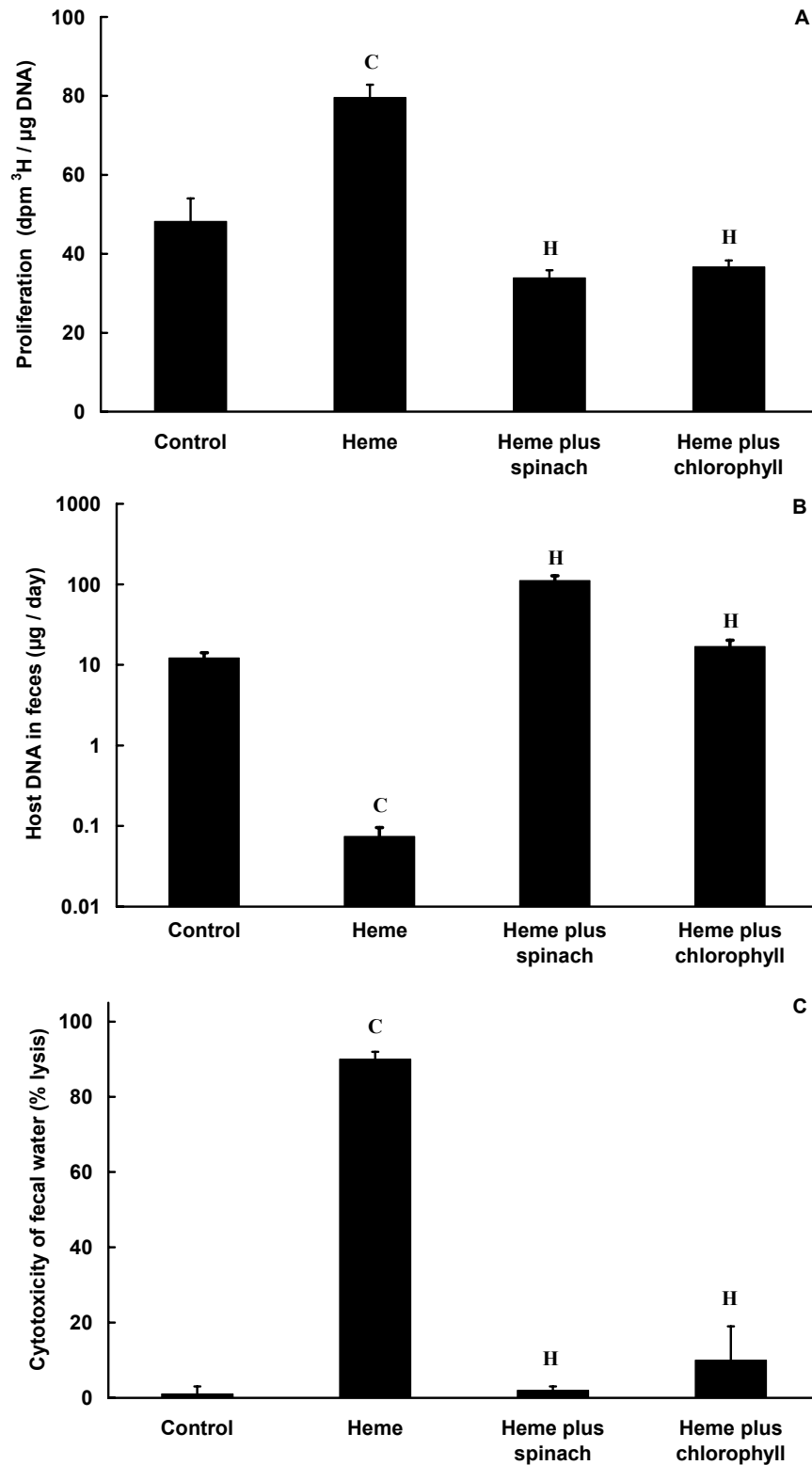
Figure 2. Effect of dietary heme, heme plus spinach and heme plus chlorophyll on: (**A**) colonic epithelial cell proliferation, determined by *in vivo* [methyl-^{3}H]thymidine incorporation into colonic mucosa; (**B**) the level of host DNA detected in feces, quantified by real-time PCR; (**C**) cytotoxicity of fecal water, determined with an erythrocyte bioassay. Results are mean ± SEM (n = 8). C Significantly different from control group; H significantly different from heme group (P < 0.05).
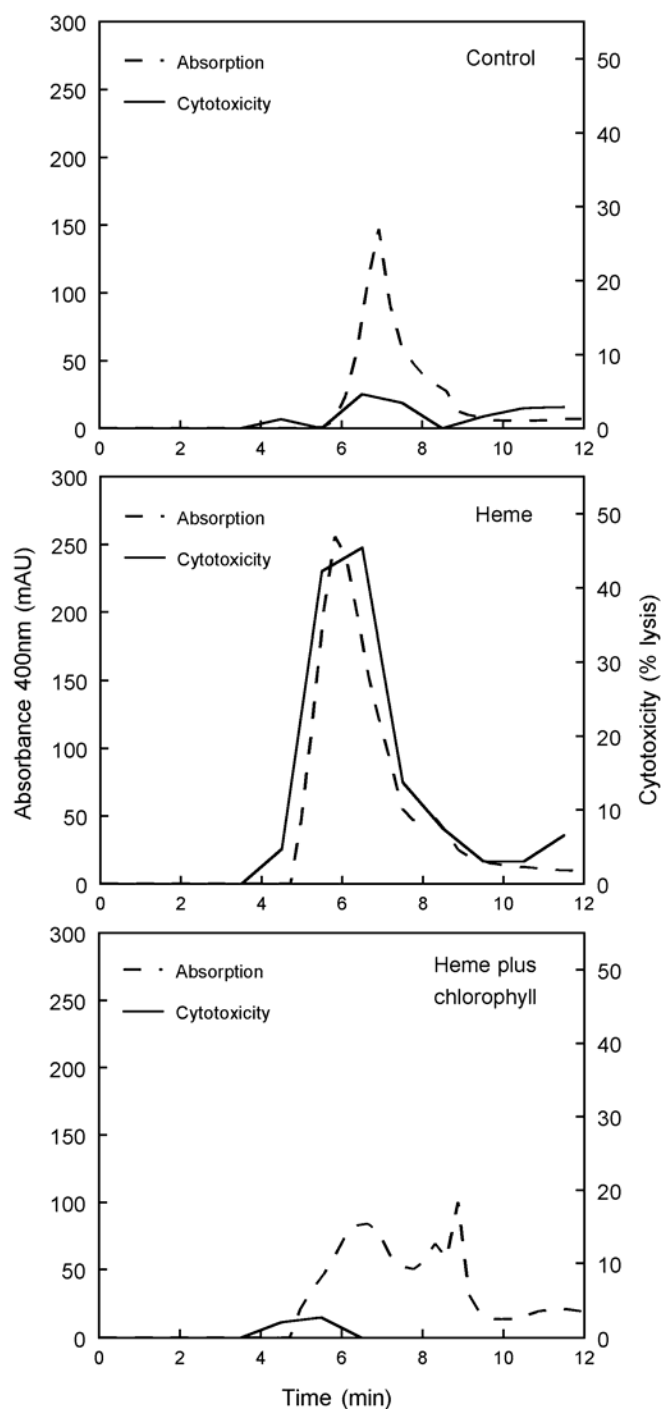
Figure 3. Representative size exclusion chromatograms of lipid extracts of pooled fecal waters from rats fed a control, heme or heme plus chlorophyll diet, measured by the absorption at 400 nm (dashed lines). One minute fractions were collected from 0 to 12 min and assayed for cytotoxicity using an erythrocyte bioassay (continuous lines).

It should be noted that cytotoxicity of the pooled fractions was always >90%, indicating that there was no loss of heme factor during extraction and chromatography. Unfortunately, we could not identify the molecular structure of the covalently bound heme metabolite because it resisted ionization in different types of mass spectrometers. Nevertheless, Figure 3 clearly shows that dietary chlorophyll blocks formation of the heme factor almost completely. A similar inhibition was found for the heme plus spinach group (data not shown).

Discussion

This study shows for the first time that spinach and chlorophyll inhibit heme-induced stimulation of colonic epithelial cell turnover. In line with our previous rat studies, dietary heme induced colonic cytotoxicity and compensatory hyperproliferation of colonic epithelial cells (9), which may increase endogenous mutations and thus colon cancer risk (12,24). This possible carcinogenic effect of heme has also been studied by Pierre et al. (25), who showed that dietary heme promotes luminal cytotoxicity and growth of aberrant crypt foci in rat colon to the same extent. These results imply that heme-induced cytotoxicity disturbs normal cell turnover in colonic epithelium. Colonocytes differentiate and mature during their migration along the crypt to the surface. At the end of their life-cycle these surface cells become senescent and are engulfed by stromal cells or exfoliated into the fecal stream (26,27). Our preliminary histological data indicate that heme damages surface colonocytes, which could explain the decreased exfoliation of senescent surface cells. The compensatory colonic hyperproliferation in the heme-fed rats implies that more surface cells are killed than are exfoliated in control rats. At present we do not know why heme also increases crypt column height and cell number (DNA content) in the mucosa. Whether this is due to inhibition of apoptosis in crypt cells requires more detailed immunohistochemical and cell kinetic studies. Nevertheless, the effects of heme on luminal cytotoxicity, colonocyte proliferation and mucosal cell number were prevented by supplementing the heme diet with spinach. Moreover, this protective effect of spinach could be mimicked completely by an equimolar amount of chlorophyll, indicating that other components in spinach, such as fiber, are not responsible.

Consistent with our previous work, we have shown that heme-induced cytotoxicity is due to the presence of an unknown lipid-soluble cytotoxic heme metabolite (heme factor) (23).

The heme factor is a covalently modified porphyrin, formed from heme in the gastrointestinal tract of the rat (9,23). We were unable to ionize this heme factor, so no mass spectrum could be obtained. Similar difficulties in identifying the structure of heme metabolites have been reported by others (28-30). Spinach increased heme recovery in the feces with equal intake of heme. This could indicate that spinach protects heme and prevents it from degradation. Purification of the heme factor in the second study showed that chlorophyll not only prevented heme degradation, but also inhibited formation of heme factor, concordant with the low cytotoxicity of the colonic contents.

How does chlorophyll inhibit heme-induced colonic cytotoxicity and subsequent adverse effects? The addition of chlorophyll to a heme diet prevented formation of heme factor. Because the molecular structure of the heme factor is still enigmatic, we can only speculate how spinach and chlorophyll inhibit its formation. Two possible inhibitory mechanisms can be proposed: competition in the solubilization process of heme and blocking of the modification sites of heme. Heme is poorly soluble in the stomach due to the low gastric pH (31). However, in order to form heme factor, heme must be solubilized. This solubilization is accomplished in the proximal small intestine by bile acids and other surfactants such as fatty acids and is probably the first important step in the formation of heme factor (32). Chlorophyll might prevent heme solubilization by competition for binding to bile acids and other surfactants in the proximal small intestine. Alternatively, chlorophyll could 'sandwich' heme to form hydrophobic heme–chlorophyll complexes and, as a result, block the covalent modification sites of heme and thus the formation of heme factor.

Most epidemiological studies show that red meat promotes and vegetable consumption decreases the risk for colon cancer (4,5,14). The mechanism underlying modulation of colon cancer risk by dietary intake of red meat and vegetables is still unknown. Butler et al. (33) suggest an association between colon cancer risk and heterocyclic aromatic amines formed during cooking of meat. However, this is not supported by other epidemiological evidence (34). In addition, Sinha et al. (35) showed that the level of some heterocyclic aromatic amines in cooked white meat exceeds the level in cooked red meat, which implies that heterocyclic amines cannot explain why red meat raises the risk while white meat does not. Besides, doses of heterocyclic aromatic amines required for carcinogenicity in animal studies far exceed the daily dietary intake by humans (10). Bingham et al. (36,37) proposed another mechanism, suggesting that heme-induced formation of N-nitroso compounds from red meat is responsible for the increased colon

cancer risk. However, green vegetables did not decrease the concentration of apparent N-nitroso compounds in a follow-up study from the same group (38). This could be due to the type of vegetables consumed and/or the small number of participants in the study and indicates that further studies on the modulation of colon cancer risk by N-nitroso compounds and components from green vegetables are needed.

Because our experimental diets mimicked the macro-nutrient composition of a low calcium, high meat and high fat diet our results may have implications for the human situation. We showed previously that dietary heme concentrations between 0.16 and 0.5 mmol/kg diet resulted in similar increases in cytotoxicity of the colonic contents and proliferation of the epithelial cells (23). Assuming that an average human diet consists of 450 g dry weight/day, 0.16 mmol/kg heme corresponds to 72 µmol heme/day. As beef contains up to 0.5 µmol heme/g wet weight (29), this implies a realistic beef intake of 150 g/day in humans.

In our rat studies a concentration of 1.2 mmol/kg chlorophyll inhibited the heme-induced cytotoxicity. Considering that spinach contains 0.12 mmol chlorophyll/100 g wet weight (39), people consuming 450 g of spinach/day could be protected from the adverse effects of dietary heme on the colonic mucosa. This protective effect of spinach is a maximum estimation and will be smaller if the effective inhibiting chlorophyll concentration is lower than the 1.2 mmol/kg used in the present study. The concentration dependence of this protective effect of chlorophyll is at present under investigation.

Figure 4 summarizes our studies on heme and chlorophyll, showing that modulation of colon cancer risk is a result of antagonistic interactions in the gut lumen. Our proposed mechanism implies that people who consume a diet high in red meat and low in green vegetables are especially at risk for colon cancer. We suggest that this heme–chlorophyll interaction should be taken into consideration in future epidemiological and experimental studies.

Diet-dependent effects

Response of colonic epithelium

Nutrient

Colonic lumen

Normal cells

↓

Heme → Heme factor → Cytotoxicity → Cell death → Hyperproliferation

↑ ⊖

Chlorophyll

↓

Aberrant crypts

↓

Tumor formation
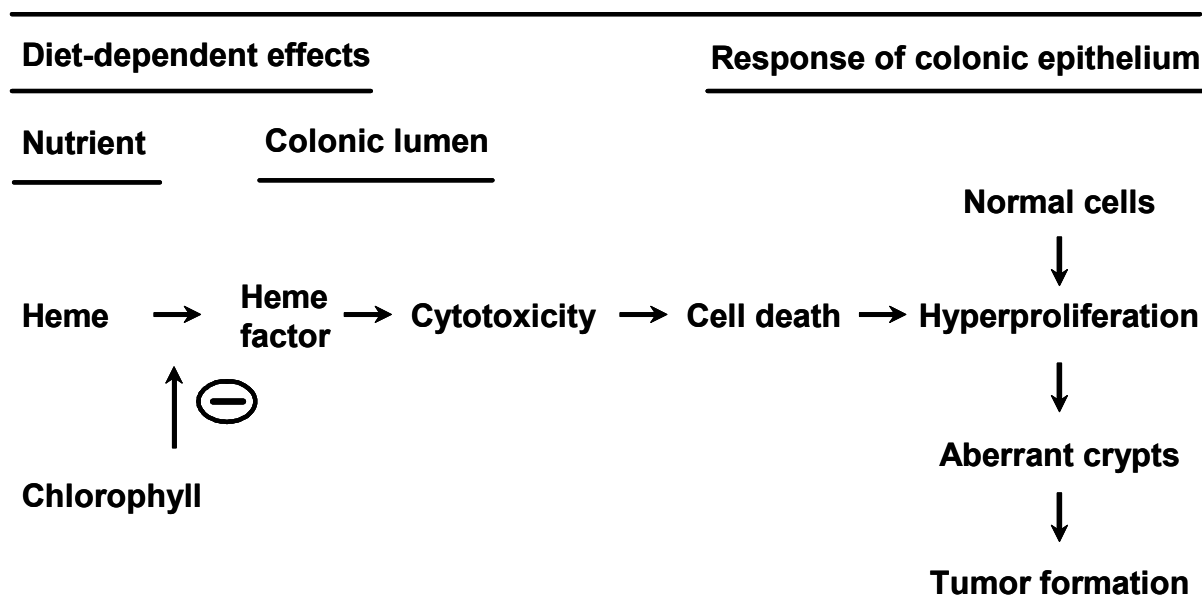
Figure 4. Proposed mechanism for the interaction between dietary heme and chlorophyll in the lumen of the colon and effects on the colonic epithelium.

Acknowledgments

References

1. Jemal, A., Murray, T., Samuels, A., Ghafoor, A., Ward, E. and Thun, M.J. (2003) Cancer statistics, 2003. *CA Cancer J Clin*, **53**, 5-26.
2. Potter, J.D. (1999) Colorectal cancer: molecules and populations. *J Natl Cancer Inst*, **91**, 916-932.
3. Armstrong, B. and Doll, R. (1975) Environmental factors and cancer incidence and mortality in different countries, with special reference to dietary practices. *Int J Cancer*, **15**, 617-631.
4. Norat, T., Lukanova, A., Ferrari, P. and Riboli, E. (2002) Meat consumption and colorectal cancer risk: dose-response meta- analysis of epidemiological studies. *Int J Cancer*, **98**, 241-256.
5. Giovannucci, E., Rimm, E.B., Stampfer, M.J., Colditz, G.A., Ascherio, A. and Willett, W.C. (1994) Intake of fat, meat, and fiber in relation to risk of colon cancer in men. *Cancer Res*, **54**, 2390-2397.
6. Kinzler, K.W. and Vogelstein, B. (1996) Lessons from hereditary colorectal cancer. *Cell*, **87**, 159-170.
7. Thilly, W.G. (2003) Have environmental mutagens caused oncomutations in people? *Nat Genet*, **34**, 255-259.

8. Terdiman, J.P. (2000) Genomic events in the adenoma to carcinoma sequence. *Semin Gastrointest Dis*, **11**, 194-206.
9. Sesink, A.L.A., Termont, D.S., Kleibeuker, J.H. and Van der Meer, R. (1999) Red meat and colon cancer: the cytotoxic and hyperproliferative effects of dietary heme. *Cancer Res*, **59**, 5704-5709.
10. Bingham, S.A. (1996) Epidemiology and mechanisms relating diet to risk of colorectal cancer. *Nutrition Research Reviews*, **9**, 197-239.
11. Baron, J.A. (2001) Intermediate effect markers for colorectal cancer. *IARC Sci Publ*, **154**, 113-129.
12. Lipkin, M. (1988) Biomarkers of increased susceptibility to gastrointestinal cancer: new application to studies of cancer prevention in human subjects. *Cancer Res*, **48**, 235-245.
13. Trock, B., Lanza, E. and Greenwald, P. (1990) Dietary fiber, vegetables, and colon cancer: critical review and meta- analyses of the epidemiologic evidence. *J Natl Cancer Inst*, **82**, 650-661.
14. Steinmetz, K.A. and Potter, J.D. (1996) Vegetables, fruit, and cancer prevention: a review. *J Am Diet Assoc*, **96**, 1027-1039.
15. Reeves, P.G., Nielsen, F.H. and Fahey, G.C., Jr. (1993) AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J Nutr*, **123**, 1939-1951.
16. Lapre, J.A. and Van der Meer, R. (1992) Diet-induced increase of colonic bile acids stimulates lytic activity of fecal water and proliferation of colonic cells. *Carcinogenesis*, **13**, 41-44.
17. Burton, K. (1956) A study of the conditions and mechanisms of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem J*, **62**, 315-323.
18. Van Lieshout, E.M., Van Doesburg, W. and Van der Meer, R. (2004) Real-time PCR of host DNA in feces to study differential exfoliation of colonocytes between rats and humans. *Scand J Gastroenterol*, **39**, 852-7.
19. van den Berg, J.W., Koole-Lesuis, R., Edixhoven-Bosdijk, A. and Brouwers, N. (1988) Automating the quantification of heme in feces. *Clin Chem*, **34**, 2125-2126.
20. Bligh, E.G. and Dyer, W.J. (1959) A rapid method of total lipid extraction and purification. *Canadian journal of biochemistry and physiology*, **37**, 911-917.
21. Lapre, J.A., Termont, D.S., Groen, A.K. and Van der Meer, R. (1992) Lytic effects of mixed micelles of fatty acids and bile acids. *Am J Physiol*, **263**, G333-G337.
22. Schwartz, S., Dahl, J., Ellefson, M. and Ahlquist, D. (1983) The "HemoQuant" test: a specific and quantitative determination of heme (hemoglobin) in feces and other materials. *Clin Chem*, **29**, 2061-7.
23. Sesink, A.L.A. (2001) *Red meat and colon cancer: a possible role for heme*. Rijksuniversiteit Groningen, Groningen.
24. Ames, B.N., Gold, L.S. and Willett, W.C. (1995) The causes and prevention of cancer. *Proc Natl Acad Sci U S A*, **92**, 5258-5265.
25. Pierre, F., Tache, S., Petit, C.R., Van Der Meer, R. and Corpet, D.E. (2003) Meat and cancer: haemoglobin and haemin in a low-calcium diet promote colorectal carcinogenesis at the aberrant crypt stage in rats. *Carcinogenesis*, **24**, 1683-1690.
26. Barkla, D.H. and Gibson, P.R. (1999) The fate of epithelial cells in the human large intestine. *Pathology*, **31**, 230-238.
27. Mayhew, T.M., Myklebust, R., Whybrow, A. and Jenkins, R. (1999) Epithelial integrity, cell death and cell loss in mammalian small intestine. *Histol Histopathol*, **14**, 257-267.
28. Atamna, H. and Ginsburg, H. (1995) Heme degradation in the presence of glutathione. A proposed mechanism to account for the high levels of non-heme iron found in the membranes of hemoglobinopathic red blood cells. *J Biol Chem*, **270**, 24876-83.
29. Schwartz, S. and Ellefson, M. (1985) Quantitative fecal recovery of ingested hemoglobin-heme in blood: comparisons by HemoQuant assay with ingested meat and fish. *Gastroenterology*, **89**, 19-26.
30. Rose, I.S., Young, G.P., St. John, D.J., Deacon, M.C., Blake, D. and Henderson, R.W. (1989) Effect of ingestion of hemoproteins on fecal excretion of hemes and porphyrins. *Clin Chem*, **35**, 2290-6.
31. Vaghefi, N., Nedjaoum, F., Guillochon, D., Bureau, F., Arhan, P. and Bougle, D. (2002) Influence of the extent of hemoglobin hydrolysis on the digestive absorption of heme iron. An in vitro study. *J Agric Food Chem*, **50**, 4969-4973.
32. Sesink, A.L.A., Termont, D.S., Kleibeuker, J.H. and Van Der Meer, R. (2000) Red meat and colon cancer: dietary haem, but not fat, has cytotoxic and hyperproliferative effects on rat colonic epithelium. *Carcinogenesis*, **21**, 1909-1915.
33. Butler, L.M., Sinha, R., Millikan, R.C., Martin, C.F., Newman, B., Gammon, M.D., Ammerman, A.S. and Sandler, R.S. (2003) Heterocyclic amines, meat intake, and association with colon cancer in a population-based study. *Am J Epidemiol*, **157**, 434-445.

34. Augustsson, K., Skog, K., Jagerstad, M., Dickman, P.W. and Steineck, G. (1999) Dietary heterocyclic amines and cancer of the colon, rectum, bladder, and kidney: a population-based study. *Lancet*, **353**, 703-707.

35. Sinha, R., Rothman, N., Brown, E.D., Salmon, C.P., Knize, M.G., Swanson, C.A., Rossi, S.C., Mark, S.D., Levander, O.A. and Felton, J.S. (1995) High concentrations of the carcinogen 2-amino-1-methyl-6-phenylimidazo- [4,5-b]pyridine (PhIP) occur in chicken but are dependent on the cooking method. *Cancer Res*, **55**, 4516-4519.

36. Bingham, S.A., Pignatelli, B., Pollock, J.R., Ellul, A., Malaveille, C., Gross, G., Runswick, S., Cummings, J.H. and O'Neill, I.K. (1996) Does increased endogenous formation of N-nitroso compounds in the human colon explain the association between red meat and colon cancer? *Carcinogenesis*, **17**, 515-523.

37. Cross, A.J., Pollock, J.R. and Bingham, S.A. (2003) Haem, not protein or inorganic iron, is responsible for endogenous intestinal N-nitrosation arising from red meat. *Cancer Res*, **63**, 2358-2360.

38. Hughes, R., Pollock, J.R. and Bingham, S. (2002) Effect of vegetables, tea, and soy on endogenous N-nitrosation, fecal ammonia, and fecal water genotoxicity during a high red meat diet in humans. *Nutr Cancer*, **42**, 70-77.

39. Khachik, F., Beecher, G.R. and Whittaker, N.F. (1986) Separation, Identification, and Quantification of the Major Carotenoid and Chlorophyll Constituents in Extracts of Several green Vegetables by Liquid Chromatography. *Jounal of Agricultural Food Chemistry*, **34**, 603-616.

Chapter 4

Natural Chlorophyll but Not Chlorophyllin Prevents Heme-Induced Cytotoxic and Hyperproliferative Effects in Rat Colon

Johan de Vogel, Denise S.M.L. Jonker-Termont, Martijn B. Katan, and Roelof van der Meer

Abstract

Diets high in red meat and low in green vegetables are associated with an increased risk of colon cancer. In rats, dietary heme, mimicking red meat, increases colonic cytotoxicity and proliferation of the colonocytes, whereas addition of chlorophyll from green vegetables inhibits these heme-induced effects. Chlorophyllin is a water-soluble hydrolysis product of chlorophyll that inhibits the toxicity of many planar aromatic compounds. The present study investigated whether chlorophyllins could inhibit the heme-induced luminal cytotoxicity and colonic hyperproliferation as natural chlorophyll does. Rats were fed a purified control diet, the control diet supplemented with heme, or a heme diet with 1.2 mmol/kg diet of chlorophyllin, copper chlorophyllin, or natural chlorophyll for 14 d (n=8/group). The cytotoxicity of fecal water was determined with an erythrocyte bioassay and colonic epithelial cell proliferation was quantified in $vivo$ by [methyl-^{3}H]thymidine incorporation into newly synthesized DNA. Exfoliation of colonocytes was measured as the amount of rat DNA in feces using quantitative PCR analysis. Heme caused a >50-fold increase in the cytotoxicity of the fecal water, a nearly 100% increase in proliferation, and almost total inhibition of exfoliation of the colonocytes. Furthermore, the addition of heme increased TBARS in fecal water. Chlorophyll, but not the chlorophyllins, completely prevented these heme-induced effects. In conclusion, inhibition of the heme-induced colonic cytotoxicity and epithelial cell turnover is specific for natural chlorophyll and cannot be mimicked by water-soluble chlorophyllins.

Introduction

Colon cancer was responsible for >0.5 million deaths worldwide and was the second leading cause of cancer death in Western countries in 2000 (1,2). Risk factors for colon cancer include a positive family history or environmental factors, with diet as a major modulator. In particular, diets high in red and processed meat, in contrast to white meat, are associated with increased colon cancer risk (3-5). However, people who consume a substantial amount of green vegetables have a reduced risk of colon cancer (6).

The mechanisms explaining the dietary modulation of the risk of colon cancer by intake of red meat and vegetables are still under debate. One mechanism was deduced from nutritional studies with rats. Sesink et al. (7) showed, in rats, that dietary heme (**Fig. 1A**), the iron-porphyrin pigment of red meat, increased cytotoxicity of the fecal stream. This resulted in increased exposure of the colonocytes to luminal irritants (7). Consequently, colonocyte proliferation increased, which is considered an important risk factor in the development of cancer (8-11). In line with these results, Pierre et al. (12,13) showed that dietary heme or meat supplemented to the diet promoted luminal cytotoxicity and increased the number and size of aberrant crypt foci in rat colon. Aberrant crypt foci are preneoplastic lesions that correlate with tumor incidence in most studies (14).

Remarkably, supplementation of this heme diet with freeze-dried spinach or an equimolar amount of natural chlorophyll inhibited the heme-induced cytotoxic and hyperproliferative effects (15). Chlorophyll (**Fig. 1B**) is the ubiquitous pigment in green leafy vegetables; like heme, it also has a planar porphyrin backbone. Chlorophyll is different from heme mainly by having the nonreactive magnesium instead of the highly reactive transition metal iron in the center of the porphyrin. In addition, chlorophyll has an esterified phytol tail instead of a propionic side chain. We speculated that chlorophyll "sandwiches" heme to form hydrophobic heme-chlorophyll complexes; as a result, it blocks radical-mediated heme metabolism and the heme-induced changes in epithelial cell turnover (15). In this study, we wanted to investigate whether this protective effect of natural chlorophyll is due to its porphyrin ring or requires the intact phytol-conjugated molecule. Therefore, we compared the possible protective effect of chlorophyllins with that of natural chlorophyll.

Chlorophyllins (**Fig. 1C**) are molecular analogs of chlorophyll studied for cancer prevention *in vitro* and *in vivo* because they might mimic the effects of chlorophyll (16-19). Chlorophyllins are food-grade molecules derived from chlorophyll.
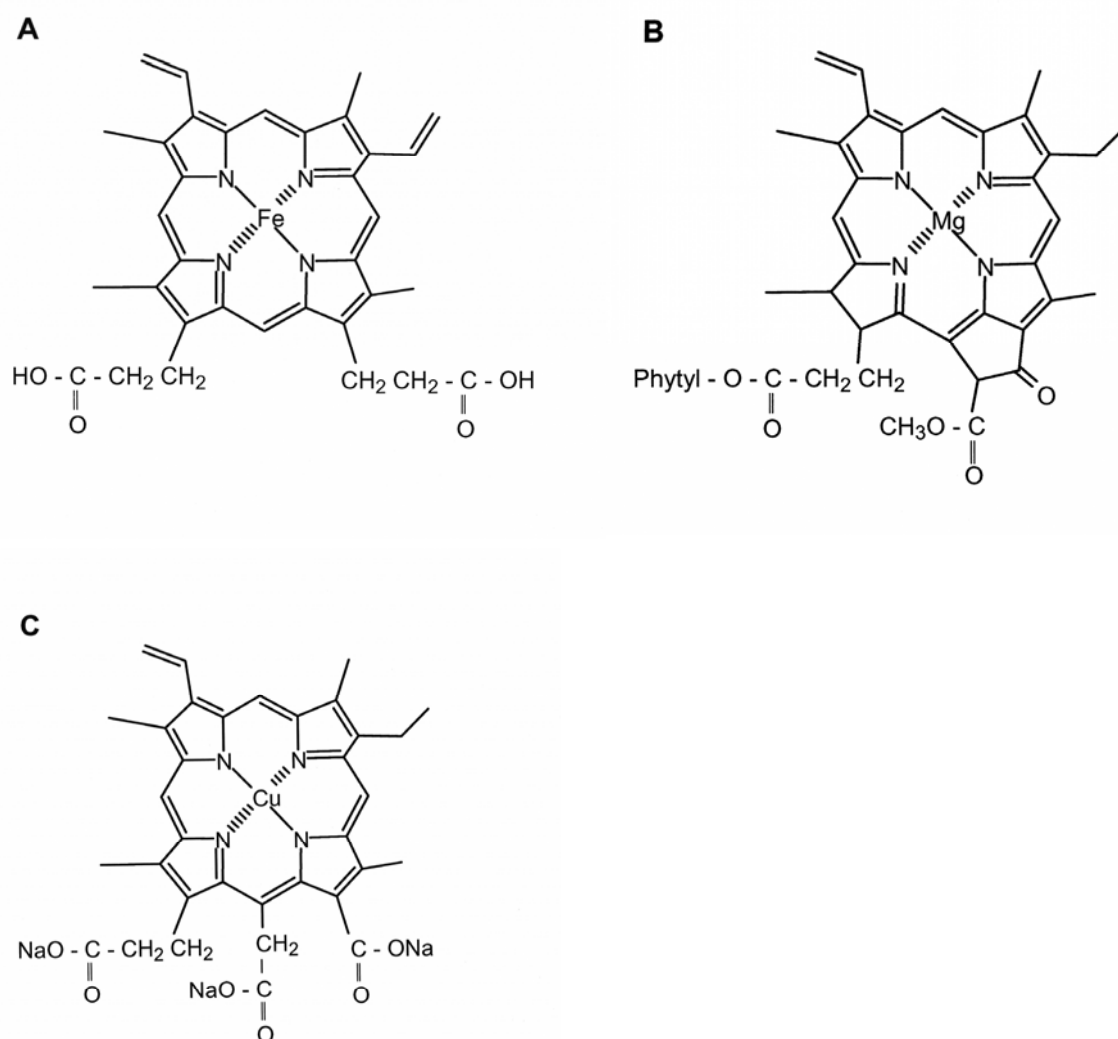
Figure 1. Chemical structures of the supplements used in the experimental diets: heme (**A**), chlorophyll a (**B**), and sodium copper chlorophyllin (**C**).

They are hydrophilic, due to hydrolysis of the phytol tail, and the magnesium in the center of the porphyrin ring is removed or replaced by another metal. In contrast to the limited *in vivo* studies with natural chlorophyll (20,21), chlorophyllins have received much more attention. Several studies indicate that chlorophyllins may have anticarcinogenic effects because their porphyrin macrocycle can either scavenge free radicals or form a complex with planar aromatic carcinogens and thus reduce their bioactivity (17,22,23).

Based on these data from the literature and because heme is also a planar aromatic compound, we suggest that the chlorophyllins block heme by complex formation. Blocking

of heme by chlorophyllins might inhibit its metabolism in the gut lumen as chlorophyll does (15). Consequently, chlorophyllin might also prevent the heme-induced luminal cytotoxicity and increased colonic epithelial cell turnover. We tested this in rats, supplementing their diets with heme, heme plus chlorophyllins, or heme plus chlorophyll.

Materials and Methods

Animals and diets

The experimental protocol was approved by the animal welfare committee of Wageningen University and Research Center. Outbred male SPF Wistar rats (8 wk old; WU, Harlan) were housed individually in metabolic cages in a room with controlled temperature (20°C-22°C), relative humidity (50–60%), and a 12-h light:dark cycle (lights on 0600–1800 h). Metabolic cages with wire-mesh bottoms made it possible to collect feces without urinary contamination. Rats were acclimated to the housing conditions for 5 d before the start of the experiment.

The body weight of the rats at the start of the experiment was 275 ± 1 g (mean ± SEM). For 2 wk, 5 groups of 8 rats were fed purified diets. The composition of the diets is given in **Table 1**. The heme-fed rats consumed a purified control diet supplemented with 0.5 mmol heme/kg diet (Sigma-Aldrich Chemie); 3 additional heme groups were supplemented with 1.2 mmol/kg Na-chlorophyllin, sodium copper chlorophyllin (Cu-chlorophyllin), (HaiNing FengMing Chlorophyll), or 1.2 mmol/kg chlorophyll (Japan Chlorophyll).

Food was administered to the rats just before dark to prevent possible degradation of the supplements. Food and demineralized drinking water were consumed ad libitum. Food intake and body weights were recorded every 2–4 d. Feces were collected quantitatively on d 11–14 of the experiment and frozen at –20°C.

In vivo colonic epithelial cell proliferation

After 14 d of experimental feeding, colon epithelial cell proliferation was quantified *in vivo* by measuring DNA replication, using [methyl-^{3}H]thymidine incorporation into DNA. Rats that had not been food deprived were injected i.p. with [methyl-^{3}H]thymidine (specific activity 925 GBq/mmol, dose 3.7 MBq/kg body weight, Amersham International) in 154 mmol/L NaCl. After 3 h, rats were killed by CO_2 inhalation. The colon was excised and

opened longitudinally. Colonic contents were removed, and the mucosa was scraped, homogenized in buffer, and analyzed as described previously (15).

Table 1. Composition of the experimental diets.

Ingredients	Control	Heme	Heme plus Na-chlorophyllin	Heme plus Cu-chlorophyllin	Heme plus chlorophyll
			g/kg		
Acid casein	200	200	200	200	200
Dextrose	532	532	531	531	532
Palm fat	160	160	160	160	148
Corn oil	40	40	40	40	40
Cellulose	20	20	20	20	20
Heme	--	0.326	0.326	0.326	0.326
Na-chlorophyllin	--	--	0.80	--	--
Cu-chlorophyllin	--	--	--	0.87	--
Chlorophyll	--	--	--	--	12
$CaHPO_4 \cdot 2H_2O^1$	3.44	3.44	3.44	3.44	3.44
$MgCl_2 \cdot 6H_2O^2$	--	--	0.25	--	--
Mineral mix[3]	35	35	35	35	35
Vitamin mix[3]	10	10	10	10	10

[1]Calcium was added as calcium phosphate ($CaHPO_4 \cdot 2H_2O$, Fluka Chemie Buchs, Switzerland).

[2]The heme plus Na-chlorophyllin diet was also supplemented with 1.2 mmol/kg magnesium chloride to adjust for the magnesium content of chlorophyll.

[3]The composition of the vitamin and mineral mixtures is according to the recommendation of the American Institute of Nutrition 1993 (24), except that calcium was omitted. In addition, tri-potassium citrate was added instead of KH_2PO_4, and choline was added as choline chloride.

Quantification of epithelial DNA in feces

We quantified host DNA in feces as a marker for epithelial exfoliation, as described earlier (15,25). Briefly, fecal host DNA was extracted from freeze-dried feces. The DNA in all isolates was of good purity (A_{260}/A_{280} 1.8). It was stored at 4°C, or –20°C for longer storage. The standard DNA used for quantification was isolated from rat spleen. Quantification was based on real-time PCR, performed with rat-specific probe and primers targeted to the β-globin gene sequence (25).

Cytotoxicity of fecal water

Fecal water was prepared by reconstituting a small amount of freeze-dried feces with double-distilled water to obtain a physiologic osmolarity of 300 mOsmol/L, as described

earlier (7). After preparation, the fecal waters were stored at –20°C until further analysis. Cytotoxicity of fecal water was quantified by potassium release of human erythrocytes after incubation with fecal water as described previously (7) and validated earlier with human colon carcinoma-derived Caco-2 cells (26). The potassium content of the erythrocytes was measured with an Inductive Coupled Plasma Absorption Emission Spectrophotometer (ICP-AES, Varian) and the cytotoxicity of fecal water was calculated and expressed as a percentage of maximal lysis.

Determination of heme in feces

The total amount of heme excreted in the feces was determined by a modified protocol of the HemoQuant assay (27). To quantify heme in feces, an acidified chloroform-methanol extract (28) was obtained from 30 mg of freeze-dried feces (final HCl concentration 1 mol/L). The chloroform phase was dried under nitrogen and dissolved in 0.45 mL of 250 mmol/L KOH. Subsequently, 0.45 mL of double-distilled water, 3.75 mL of 2-propanol, and 0.75 mL of 1.15 mol/L HCl were added to the samples, which were assayed for their heme content as described (27).

TBARS assay of fecal water

To determine lipid peroxidative processes in the lumen, TBARS in fecal water were quantified (7). The TBARS assay evaluates lipid peroxidation by quantifying the concentration of malondialdehyde (MDA) in fecal water (29). Briefly, fecal water was diluted 5-fold with double-distilled water. Subsequently, 100 µL of this solution was mixed with 100 µL of 8.1% SDS and 1 mL solution of 0.11 mol/L 2,6-di-tert-butyl-p-cresol, 0.5% TBA in 10% acetic acid (pH = 3.5). For background correction, TBA was omitted from the assay. TBARS were extracted, after heating for 60 min at 95°C, with 1.2 mL of n-butanol. The absorbance of this extract was measured at 532 nm (Spectramax plus, Bucher Biotec AG). The amount of TBARS was calculated as MDA equivalents using 1,1,3,3,-tetramethoxypropane as standard.

Determination of distribution coefficients

A method similar to that described by Kepczynski et al. (30) was used to determine the octanol/water distribution coefficient of heme, Na-chlorophyllin, Cu-chlorophyllin, and chlorophyll. The supplements were dissolved in 2 mL of octanol or PBS at pH 7.3 (final

concentrations < 4 μmol/L) and mixed with a vortex with an equal volume of PBS or octanol, respectively. After 20 min shaking and 10 min centrifugation at 3000 *g*, concentrations in both phases were determined using a spectrophotometer (Lambda 2, Perkin Elmer). The distribution coefficients of the supplements were defined by their concentration in octanol divided by their concentration in PBS.

Statistical analysis

All results are expressed as mean ± SEM (n = 8 per group). A commercially available package (Statistica 6.1, Statsoft) was used. Normality of the data was tested with the Shapiro Wilk test and homogeneity of variances was tested using Levene's test. In the case of normal distribution and equal homogeneity, 1-way ANOVA was performed to test for significant treatment effects followed by a Dunnett's post hoc test. In the case of non-Gaussian distribution of data, Kruskall-Wallis ANOVA was performed and, in addition, the nonparametric Mann-Whitney U test was used as a post hoc test. Bonferroni correction was made for the number of comparisons (n = 4). Differences were considered significant at P < 0.05.

Results

Addition of heme, chlorophyllins, or chlorophyll to the diets did not affect food intake (18.5 ± 0.5 g dry weight/d). Furthermore, the growth rate did not differ (4.1 ± 0.3 g/d) among the treatment groups.

We measured proliferation of the colonocytes *in vivo* to examine whether heme, chlorophyllins, or chlorophyll in the diet changed the response of the epithelial cells. The heme-supplemented group had an almost 100% increase in proliferation compared with the non-heme control group (**Fig. 2A**). Adding chlorophyllins to the heme diet did not inhibit the heme-induced hyperproliferation of colonic cells. In contrast, supplementing the heme diet with natural chlorophyll inhibited the heme-induced proliferation to a level similar to control values.

We quantified the level of host DNA in feces to study diet-induced differences in proliferation coincided with changes in exfoliation of the colonic epithelial cells (15). Compared with the control diet, dietary heme markedly reduced the amount of host DNA in the feces (**Fig. 2B**). Cu-chlorophyllin added to the heme diet did not inhibit this heme-

induced effect; addition of Na-chlorophyllin to the heme diet slightly increased the level of host DNA in feces compared with the effect of heme. However, adding chlorophyll to the heme diet completely prevented the heme-induced decrease in fecal host DNA excretion.
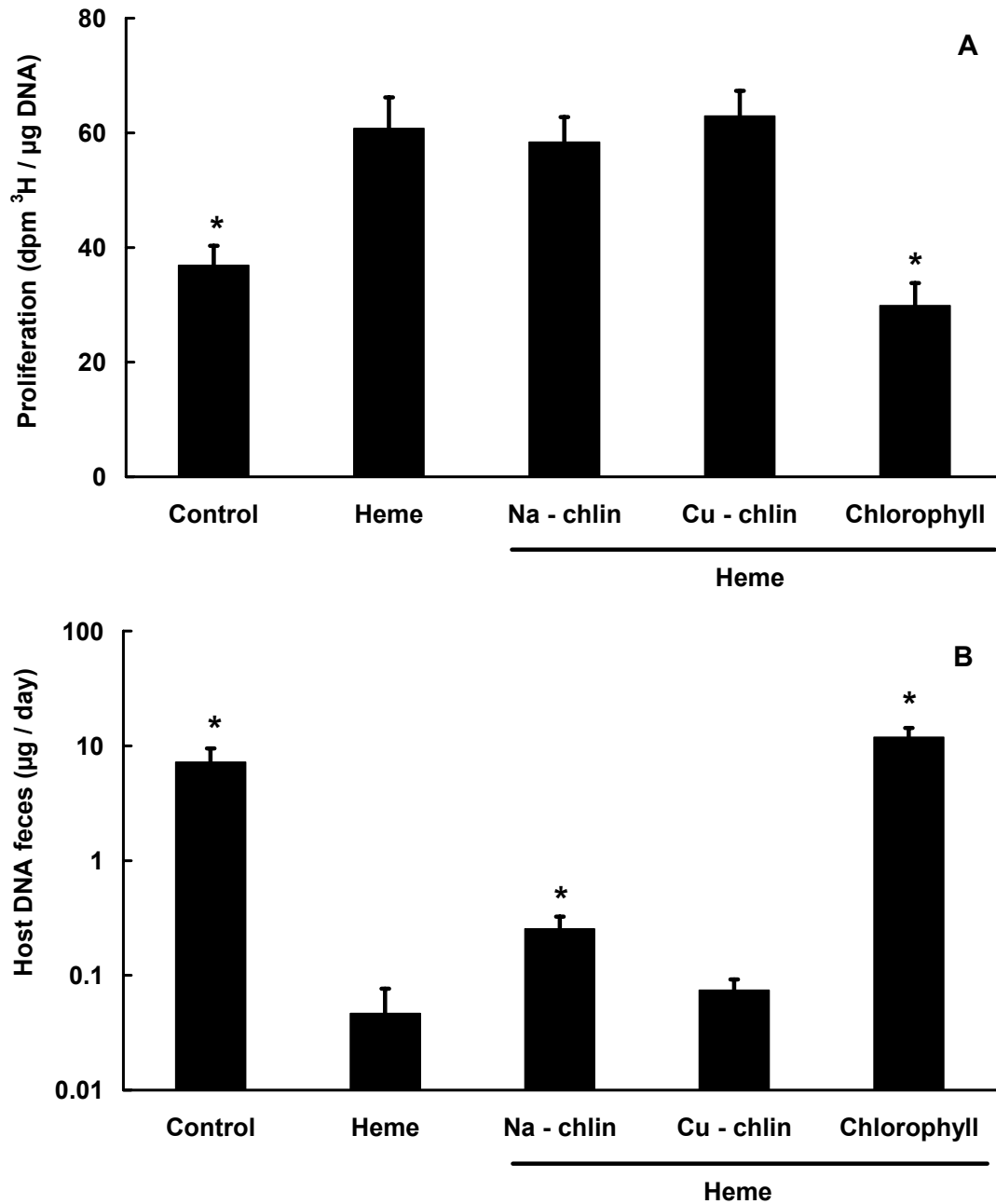
Figure 2. Effect of dietary heme, Na-chlin (Na-chlorophyllin), Cu-chlin (Cu-chlorophyllin), and chlorophyll on colonic epithelial cell proliferation, determined by *in vivo* [methyl-PP^{3}H]thymidine incorporation into colonic mucosa of rats (**A**) and the level of host DNA detected in feces of rats, quantified by real-time PCR (**B**). Values are mean ± SEM, n = 8. *Different from the heme group, $P < 0.05$.

We analyzed cytotoxicity of the fecal waters with an erythrocyte bioassay to determine whether the changes in colonic epithelial cell turnover resulted from differences in exposure to luminal irritants. Cytotoxicity of the fecal water from the heme group was 90%, which was >50-fold higher than in the control group (**Fig. 3**). The heme-induced cytotoxicity decreased significantly in the Na-chlorophyllin–supplemented heme group to 57%. In contrast, heme-induced cytotoxicity was blocked completely when chlorophyll was added to the heme diet.
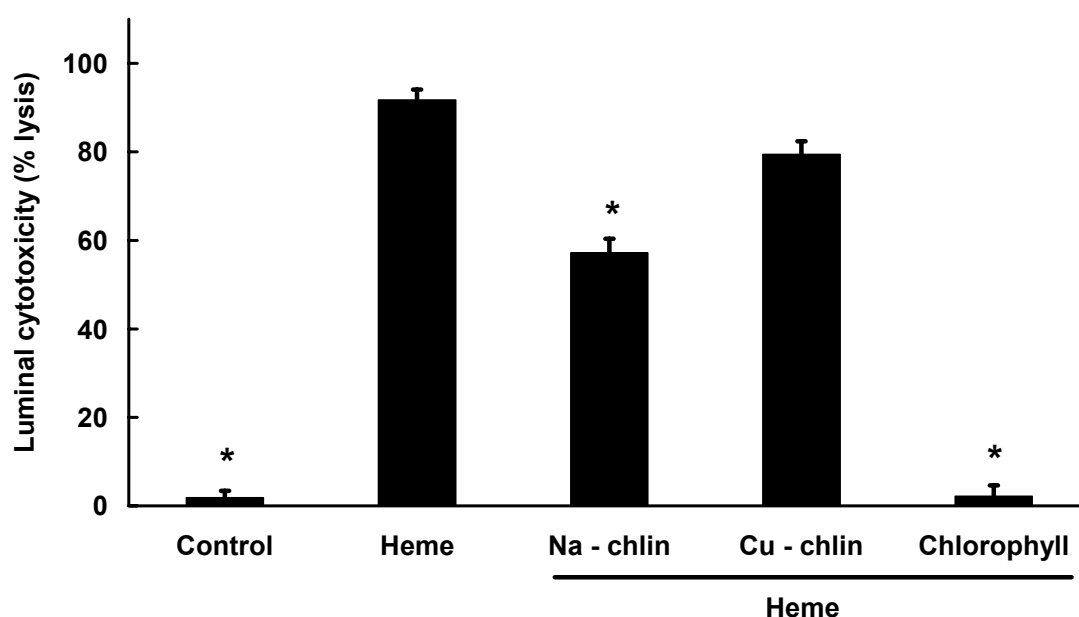
Figure 3. Effect of dietary heme, Na-chlin (Na-chlorophyllin), Cu-chlin (Cu-chlorophyllin), and chlorophyll on cytotoxicity of fecal water of rats, determined with an erythrocyte bioassay. Values are mean ± SEM, n = 8. *Different from heme group, $P < 0.05$.

We showed earlier that the detrimental heme-induced effects are mediated by luminal conversion of ingested heme (15,31). Therefore, we studied whether the dietary treatments affect intestinal heme metabolism. The amount of daily heme intake and excretion in the feces (**Table 2**) indicated that heme intake was nil in the control group and all of the heme-supplemented groups consumed 9 µmol/d. Obviously, fecal heme excretion was low in the control group. However, fecal heme excretion was also low in the heme group and in the heme plus chlorophyllin groups, indicating intensive catabolism of heme into other, non-porphyrin compounds even in the presence of chlorophyllin. In

contrast, when chlorophyll was added to the heme diet > 50% of the heme was detected as such in the feces, showing that natural chlorophyll inhibits conversion or catabolism of the heme. Control experiments with heme added to feces of chlorophyll-fed rats showed that chlorophyll did not interfere in the assay (data not shown).

Table 2. Effect of dietary heme, Na-chlorophyllin, Cu-chlorophyllin, and chlorophyll on intake, excretion, and catabolism of heme in rats[1].

	Control	Heme	Heme plus Na-chlorophyllin	Heme plus Cu-chlorophyllin	Heme plus chlorophyll
	μmol/day				
Heme intake	0	9.0 ± 0.2	9.1 ± 0.2	9.1 ± 0.2	9.4 ± 0.3
Fecal heme	0.4 ± 0.0	0.7 ± 0.0	1.1 ± 0.0 *	0.8 ± 0.0	4.9 ± 0.4 *
Catabolized heme	--	8.2 ± 0.2	8.0 ± 0.2	8.3 ± 0.2	4.5 ± 0.4 *

[1]Heme intake was calculated by multiplying individual daily food intake by the heme content of the diet. Heme in the fecal samples was determined by a modified HemoQuant assay. The amount of catabolized heme was calculated by subtracting fecal heme output from daily intake values of heme. Values are mean ± SEM, n = 8 *Different from heme, $p < 0.05$.

Subsequently, we measured TBARS in fecal water to examine whether heme-catalyzed lipid peroxidation in gut lumen could be inhibited by chlorophyllin and chlorophyll. TBARS increased 1.5-fold in fecal waters of the heme group compared with the control group (**Fig. 4**). TBARS in the heme plus chlorophyllin groups were similarly increased 1.5- to 2-fold, indicating no protective effects. In contrast, adding chlorophyll to the heme diet reduced formation of lipid radicals. To validate the assay, we also measured TBARS formation after adding heme in the assay or supplementing control fecal water with heme. No significant increases in TBARS were observed (data not shown).

Finally, we investigated whether the observed differential effects of chlorophyllin and chlorophyll were related to a difference in hydrophobicity of the molecules. Therefore, we determined the distribution coefficients of heme, chlorophyllin, and chlorophyll in octanol:water mixtures (**Table 3**). A high distribution coefficient indicates that a compound is very hydrophobic. The distribution coefficients of the supplements followed the order chlorophyll >> heme > Cu-chlorophyllin > Na-chlorophyllin. These results indicate that heme and chlorophyll will accumulate in a hydrophobic environment, whereas the chlorophyllins prefer a more hydrophilic environment. The low values of Na-chlorophyllin

and Cu-chlorophyllin are likely due to the 3 carboxyl groups on the porphyrin ring, and the high value for chlorophyll is due to the phytol tail attached to the porphyrin ring.
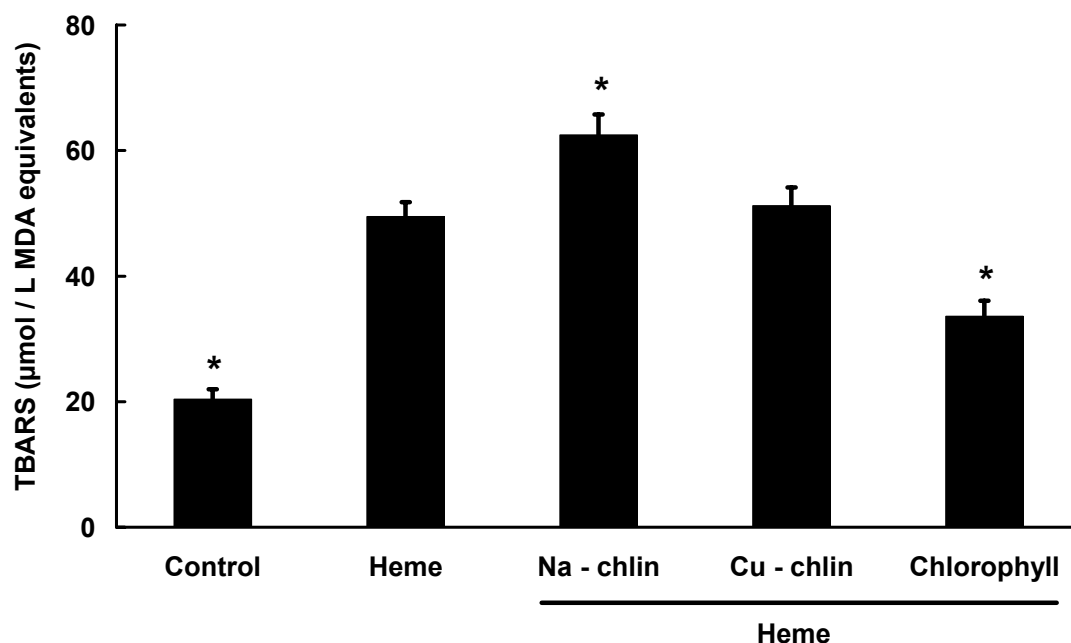
Figure 4. Effect of dietary heme, Na-chlin (Na-chlorophyllin), Cu-chlin (Cu-chlorophyllin), and chlorophyll on the presence of TBARS in fecal water, expressed as µmol/L MDA equivalents, used as marker for luminal lipid peroxidation products. Values are mean ± SEM, n = 8. *Different from heme group, $P < 0.05$.

Table 3. Distribution coefficients of heme, Na-chlorophyllin, Cu-chlorophyllin, and chlorophyll[1].

Dietary supplement	Distribution coefficient
Heme	9.0 ± 1.7
Na-chlorophyllin	0.5 ± 0.0
Cu-chlorophyllin	1.9 ± 0.2
Chlorophyll	162.2 ± 12.1

[1]Distribution coefficients were determined by measurement of the distribution of the components between octanol and water. Values are mean ± SEM, n ≥ 5.

Discussion

This study shows that chlorophyllins, in contrast to chlorophyll, do not inhibit the heme-induced carcinogenic effects in rats.

Heme, chlorophyll, and chlorophyllins are tetrapyrrole molecules containing a large number of conjugate double bonds within a planar ring system. Chlorophyllins are the water-soluble salts of the ubiquitous pigment chlorophyll found in green plants. Chlorophyllin is used in most studies as a model compound to mimic the effects of natural chlorophyll, probably because chlorophyll is unstable in solutions and insoluble in water (17,18). Several studies, and a human intervention study, described potent anticarcinogenic and antigenotoxic effects of chlorophyllins (17,19).

The chlorophyllins supplemented to our purified heme diets showed only a negligible inhibition of the heme-induced luminal cytotoxicity. This slight inhibition did not prevent the heme-induced changes in colonic cell turnover. In contrast, chlorophyll added to this heme diet inhibited all the heme-induced changes in luminal cytotoxicity and cell turnover.

Most of the heme ingested is delivered to the large bowel (32,33). There, a variable amount is converted to a range of iron-free porphyrins such as protoporphyrin, deuteroporphyrin, and pemptoporphyrin as a result of bacterial action (33). However, further degradation products such as di- and tripyrroles were not identified. The heme-induced cytotoxicity observed in our experiments results from the presence of a highly cytotoxic heme metabolite. This is a lipid-soluble, covalently modified porphyrin formed in the gut lumen of rats (7,31). Table 2 shows that chlorophyll, unlike the chlorophyllins, allowed most of the heme ingested to reach the feces without modification. This indicates that chlorophyll, but not chlorophyllin, prevents intestinal heme metabolism.

Our previous work showed that the presence of the heme metabolite in the colonic lumen damages the colon surface epithelial cells and consequently increases epithelial proliferation and inhibits apoptosis in the colonic crypts (15). Spinach or an equimolar amount of chlorophyll prevented this heme-induced effect (15). We hypothesized that this is due to a "sandwich" of heme with chlorophyll molecules. As a result, chlorophyll may block the chemical reactivity of heme and thus the formation of its cytotoxic metabolite. This "sandwich" could be due to pi-pi interactions between heme and chlorophyll in a hydrophobic complex, analogous to the mechanism described by Dashwood et al. (34) for

the interaction between chlorophyllins and planar aromatic compounds, such as heterocyclic amines.

The inhibition of heme-induced luminal cytotoxicity and increased colonic cell turnover by chlorophyll but not by chlorophyllins has to be explained in terms of structural differences between the molecules. One difference between the chlorophyllins and chlorophyll is the absence of a metal or the presence of copper in the center of the tetrapyrrole molecule, in contrast to magnesium in chlorophyll. Passage of chlorophyll through the stomach releases its magnesium due to the acidic pH (35,36). Magnesium is a divalent element like calcium, and intake of calcium precipitates bile acids (37) and stimulates precipitation of heme (7). Wang et al. (38) suggested that supplementation of magnesium in the diet might also precipitate bile acids. An increased magnesium concentration in the intestines might therefore precipitate heme or other components from the fecal matrix. Therefore, we adjusted the Na-chlorophyllin–supplemented diet with a concentration of magnesium equimolar to that in the chlorophyll diet. This was a minor (5.5%) increase in magnesium because of the high concentration of magnesium already present in the control (24) diet. Na-chlorophyllin supplementation to the heme diet caused only a minor inhibition of the heme-induced luminal cytotoxicity. Furthermore, no inhibitory effects on other colonic markers were observed, indicating that the magnesium of chlorophyll is not responsible for its inhibition of the heme-induced cell turnover.

We showed previously that the detrimental effects of heme coincide with heme-catalyzed lipid peroxidation in the gut lumen (7) and that both are inhibited by dietary antioxidants (12). This implies that heme has to be in close contact with fatty acids in the hydrophobic phase of the luminal contents. We now show that, in contrast to chlorophyllins, the addition of chlorophyll to a heme diet decreased this heme-induced lipid peroxidation (Fig. 4). This indicates that the chlorophyllins we used in our model cannot "sandwich" heme to inhibit a reaction between heme and fatty acids in the diet. Only chlorophyll might be able to "sandwich" heme and as a consequence inhibit the catalytic activity of heme in the generation of lipid hydroperoxides and the formation of a cytotoxic heme metabolite (39). The difference in hydrophobic behavior of chlorophyll and chlorophyllin is a consequence of a structural difference. Chlorophyll is extremely hydrophobic due to the presence of a phytol tail, which is retained during intestinal passage (36,40). On the other hand, chlorophyllins are hydrophilic as a result of the removal of the phytol tail from chlorophyll. A preference of heme and chlorophyll for a hydrophobic environment was confirmed by

their high octanol/water distribution coefficients (Table 3). The lower distribution coefficient of the chlorophyllins reflects their more hydrophilic character. Finally, heme and chlorophyllins are negatively charged under physiologic conditions such as prevail in the intestines, whereas chlorophyll remains a neutral molecule. These negative charges cause repulsive forces between heme and chlorophyllin, which may prevent formation of a complex between these molecules.

In summary, our data show that the heme-induced detrimental effects were inhibited only by natural chlorophyll and not by water-soluble chlorophyll derivatives. Extrapolation of these results to humans suggests that dietary protection against the increased risk of colon cancer due to high consumption of red meat can be offered only by consumption of green vegetables, and not by chlorophyllin supplements.

Acknowledgments

References

1. Stewart, B.W. and Kleihues, P. (eds.) (2003) *World Cancer Report*. IARC*Press*, Lyon.
2. Shibuya, K., Mathers, C.D., Boschi-Pinto, C., Lopez, A.D. and Murray, C.J. (2003) Correction: Global and regional estimates of cancer mortality and incidence by site: II. results for the global burden of disease 2000. *BMC Cancer*, **3**, 20.
3. Giovannucci, E., Rimm, E.B., Stampfer, M.J., Colditz, G.A., Ascherio, A. and Willett, W.C. (1994) Intake of fat, meat, and fiber in relation to risk of colon cancer in men. *Cancer Res*, **54**, 2390-2397.
4. Norat, T., Lukanova, A., Ferrari, P. and Riboli, E. (2002) Meat consumption and colorectal cancer risk: dose-response meta- analysis of epidemiological studies. *Int J Cancer*, **98**, 241-256.
5. Chao, A., Thun, M.J., Connell, C.J., McCullough, M.L., Jacobs, E.J., Flanders, W.D., Rodriguez, C., Sinha, R. and Calle, E.E. (2005) Meat consumption and risk of colorectal cancer. *Jama*, **293**, 172-82.
6. Potter, J.D. (1999) Colorectal cancer: molecules and populations. *J Natl Cancer Inst*, **91**, 916-932.
7. Sesink, A.L.A., Termont, D.S., Kleibeuker, J.H. and Van der Meer, R. (1999) Red meat and colon cancer: the cytotoxic and hyperproliferative effects of dietary heme. *Cancer Res*, **59**, 5704-5709.
8. Lipkin, M. (1988) Biomarkers of increased susceptibility to gastrointestinal cancer: new application to studies of cancer prevention in human subjects. *Cancer Res*, **48**, 235-245.
9. Kinzler, K.W. and Vogelstein, B. (1996) Lessons from hereditary colorectal cancer. *Cell*, **87**, 159-170.
10. Vogelstein, B., Fearon, E.R., Hamilton, S.R., Kern, S.E., Preisinger, A.C., Leppert, M., Nakamura, Y., White, R., Smits, A.M. and Bos, J.L. (1988) Genetic alterations during colorectal-tumor development. *N Engl J Med*, **319**, 525-32.
11. Ames, B.N., Gold, L.S. and Willett, W.C. (1995) The causes and prevention of cancer. *Proc Natl Acad Sci U S A*, **92**, 5258-5265.

12. Pierre, F., Tache, S., Petit, C.R., Van Der Meer, R. and Corpet, D.E. (2003) Meat and cancer: haemoglobin and haemin in a low-calcium diet promote colorectal carcinogenesis at the aberrant crypt stage in rats. *Carcinogenesis*, **24**, 1683-1690.

13. Pierre, F., Freeman, A., Tache, S., Van der Meer, R. and Corpet, D.E. (2004) Beef meat and blood sausage promote the formation of azoxymethane-induced mucin-depleted foci and aberrant crypt foci in rat colons. *J Nutr*, **134**, 2711-6.

14. Corpet, D.E. and Tache, S. (2002) Most effective colon cancer chemopreventive agents in rats: a systematic review of aberrant crypt foci and tumor data, ranked by potency. *Nutr Cancer*, **43**, 1-21.

15. de Vogel, J., Jonker-Termont, D.S., van Lieshout, E.M., Katan, M.B. and van der Meer, R. (2005) Green vegetables, red meat and colon cancer: chlorophyll prevents the cytotoxic and hyperproliferative effects of haem in rat colon. *Carcinogenesis*, **26**, 387-93.

16. Breinholt, V., Schimerlik, M., Dashwood, R. and Bailey, G. (1995) Mechanisms of chlorophyllin anticarcinogenesis against aflatoxin B1: complex formation with the carcinogen. *Chem Res Toxicol*, **8**, 506-14.

17. Dashwood, R.H. (1997) Chlorophylls as anticarcinogens. *International Journal of Oncology*, **10**, 721-727.

18. Sarkar, D., Sharma, A. and Talukder, G. (1994) Chlorophyll and chlorophyllin as modifiers of genotoxic effects. *Mutat Res*, **318**, 239-47.

19. Egner, P.A., Wang, J.B., Zhu, Y.R., Zhang, B.C., Wu, Y., Zhang, Q.N., Qian, G.S., Kuang, S.Y., Gange, S.J., Jacobson, L.P., Helzlsouer, K.J., Bailey, G.S., Groopman, J.D. and Kensler, T.W. (2001) Chlorophyllin intervention reduces aflatoxin-DNA adducts in individuals at high risk for liver cancer. *Proc Natl Acad Sci U S A*, **98**, 14601-6.

20. Harttig, U. and Bailey, G.S. (1998) Chemoprotection by natural chlorophylls in vivo: inhibition of dibenzo[a,l]pyrene-DNA adducts in rainbow trout liver. *Carcinogenesis*, **19**, 1323-6.

21. Blum, C.A., Xu, M., Orner, G.A., Dario Diaz, G., Li, Q., Dashwood, W.M., Bailey, G.S. and Dashwood, R.H. (2003) Promotion versus suppression of rat colon carcinogenesis by chlorophyllin and chlorophyll: modulation of apoptosis, cell proliferation, and beta-catenin/Tcf signaling. *Mutat Res*, **523-524**, 217-23.

22. Tachino, N., Guo, D., Dashwood, W.M., Yamane, S., Larsen, R. and Dashwood, R. (1994) Mechanisms of the in vitro antimutagenic action of chlorophyllin against benzo[a]pyrene: studies of enzyme inhibition, molecular complex formation and degradation of the ultimate carcinogen. *Mutat Res*, **308**, 191-203.

23. Pietrzak, M., Wieczorek, Z., Stachelska, A. and Darzynkiewicz, Z. (2003) Interactions of chlorophyllin with acridine orange, quinacrine mustard and doxorubicin analyzed by light absorption and fluorescence spectroscopy. *Biophys Chem*, **104**, 305-13.

24. Reeves, P.G., Nielsen, F.H. and Fahey, G.C., Jr. (1993) AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J Nutr*, **123**, 1939-1951.

25. Van Lieshout, E.M., Van Doesburg, W. and Van der Meer, R. (2004) Real-time PCR of host DNA in feces to study differential exfoliation of colonocytes between rats and humans. *Scand J Gastroenterol*, **39**, 852-7.

26. Lapre, J.A., Termont, D.S., Groen, A.K. and Van der Meer, R. (1992) Lytic effects of mixed micelles of fatty acids and bile acids. *Am J Physiol*, **263**, G333-G337.

27. van den Berg, J.W., Koole-Lesuis, R., Edixhoven-Bosdijk, A. and Brouwers, N. (1988) Automating the quantification of heme in feces. *Clin Chem*, **34**, 2125-2126.

28. Bligh, E.G. and Dyer, W.J. (1959) A rapid method of total lipid extraction and purification. *Canadian journal of biochemistry and physiology*, **37**, 911-917.

29. Ohkawa, H., Ohishi, N. and Yagi, K. (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem*, **95**, 351-8.

30. Kepczynski, M., Pandian, R.P., Smith, K.M. and Ehrenberg, B. (2002) Do liposome-binding constants of porphyrins correlate with their measured and predicted partitioning between octanol and water? *Photochem Photobiol*, **76**, 127-34.

31. Sesink, A.L.A. (2001) *Red meat and colon cancer: a possible role for heme*. Rijksuniversiteit Groningen, Groningen.

32. Young, G.P., Rose, I.S. and St John, D.J. (1989) Haem in the gut. I. Fate of haemoproteins and the absorption of haem. *J Gastroenterol Hepatol*, **4**, 537-45.

33. Young, G.P., St John, D.J., Rose, I.S. and Blake, D. (1990) Haem in the gut. Part II. Faecal excretion of haem and haem-derived porphyrins and their detection. *J Gastroenterol Hepatol*, **5**, 194-203.

34. Dashwood, R., Yamane, S. and Larsen, R. (1996) Study of the forces of stabilizing complexes between chlorophylls and heterocyclic amine mutagens. *Environ Mol Mutagen*, **27**, 211-8.

35. Ferruzzi, M.G., Failla, M.L. and Schwartz, S.J. (2001) Assessment of degradation and intestinal cell uptake of carotenoids and chlorophyll derivatives from spinach puree using an in vitro digestion and Caco-2 human cell model. *J Agric Food Chem*, **49**, 2082-9.

36. Baxter, J.H. (1968) Absorbtion of chlorophyll phytol in normal man and in patients with Refsum's disease. *Journal of lipid research*, **9**, 636-641.

37. Van der Meer, R., Termont, D.S. and De Vries, H.T. (1991) Differential effects of calcium ions and calcium phosphate on cytotoxicity of bile acids. *Am J Physiol*, **260**, G142-7.

38. Wang, A., Yoshimi, N., Tanaka, T. and Mori, H. (1994) The inhibitory effect of magnesium hydroxide on the bile acid-induced cell proliferation of colon epithelium in rats with comparison to the action of calcium lactate. *Carcinogenesis*, **15**, 2661-3.

39. Sawa, T., Akaike, T., Kida, K., Fukushima, Y., Takagi, K. and Maeda, H. (1998) Lipid peroxyl radicals from oxidized oils and heme-iron: implication of a high-fat diet in colon carcinogenesis. *Cancer Epidemiol Biomarkers Prev*, **7**, 1007-12.

40. Baxter, J.H. and Steinberg, D. (1967) Absorption of phytol from dietary chlorophyll in the rat. *J Lipid Res*, **8**, 615-20.

Chapter 5

Equimolar chlorophyll prevents dietary heme-induced detrimental effects on gene expression and cell turnover in rat colon mucosa

Johan de Vogel, Denise S.M.L. Jonker-Termont, Evelien Kramer, Jaap Keijer, Martijn B. Katan and Roelof van der Meer

Abstract

We have previously demonstrated that the association between high consumption of red meat and increased risk of colon cancer may be due to the heme content of meat. Dietary heme increases colonic luminal cytotoxicity and epithelial cell proliferation in rats. The addition of chlorophyll, mimicking green vegetables, to the heme diet prevents these heme-induced detrimental effects. However, these studies used a high dose of chlorophyll. In the present study, we determined the minimal dose of chlorophyll required to prevent all heme-induced detrimental effects. Groups of 8 rats were fed a purified humanized diet either alone (control) or supplemented with 0.5 mmol heme/kg in the presence of 0, 0.13, 0.25, 0.5, or 1.2 mmol/kg chlorophyll, respectively, for 14 days. Cytotoxicity of the luminal contents and colon epithelial cell proliferation were measured as carcinogenesis biomarkers. Cytotoxicity increased approximately forty-fold and proliferation doubled in rats fed heme. A dose of 0.25 mmol/kg or higher of chlorophyll prevented the heme-induced luminal cytotoxicity and colonic epithelial hyperproliferation. A dose of 0.5 mmol/kg or higher of chlorophyll prevented the heme-induced differential gene expression of colon mucosa, which was quantified to determine the response of the colon mucosa to diet-induced changes in the luminal contents. In addition, 0.5 and 1.2 mmol/kg of chlorophyll prevented most of the degradation of heme. Finally, *in vitro* precipitation experiments showed that heme binds chlorophyll with high affinity, which suggests that hydrophobic binding of chlorophyll to heme in the gut lumen blocks its reactivity. In conclusion, our results demonstrate that dietary chlorophyll in a molar ratio of 1:1 to heme is sufficient to prevent all heme-induced detrimental effects in rat colon.

Introduction

Colon cancer is a major cause of cancer death in affluent societies (1,2). The incidence of sporadic colon cancer varies widely between populations, due mainly to environmental risk factors, with special emphasis on dietary habits (3). Consumption of red meat and processed meat are important dietary components associated with an increased risk of colon cancer (4-7). In contrast, consumption of white meat (poultry or fish) is not (7,8). Furthermore, several studies indicate that the consumption of vegetables is associated with a decreased risk of colon cancer (2,9). This association is particularly linked to a protective effect of raw and green leafy vegetables (2,10-13).

The mechanism underlying dietary modulation of colon cancer risk is not fully understood. Mutational analysis of colon tumors indicate that dietary factors increasing colon cancer risk are probably not mutagens but luminal irritants that damage colon epithelial cells (14). This damage triggers compensatory tissue regeneration, which increases the risk of accumulation of endogenous mutations in tumor suppressor genes and oncogenes (15) and could ultimately increase the risk of colon cancer (16-19).

Previous studies from our group suggest that heme, the iron-porphyrin pigment of red meat, explains the association between red meat consumption and increased colon cancer risk (20,21). Sesink et al. (20) showed that dietary heme increases cytotoxicity of the fecal stream in rats, due to an increased amount of luminal irritants. This cytotoxicity resulted from the formation of a lipid-soluble cytotoxic heme metabolite, a covalently modified porphyrin, in the gastrointestinal tract of the rat (20,22). This increased cytotoxicity damaged colon surface epithelial cells, increased epithelial proliferation and probably inhibited apoptosis in the colonic crypts (22). Hyperproliferative cells are considered an important risk factor in the development of cancers (17,23). Supplementation of heme or meat in the diet also increases the number and size of aberrant crypt foci in rat colon (24). Aberrant crypt foci are pre-neoplastic lesions correlated with tumor incidence in most studies (25).

We have also shown previously that diet-induced changes in luminal irritants modulate gene expression of colon epithelial cells (26,27). The most striking result in these studies was a heme-induced ten-fold down-regulation of a novel mucosal pentraxin (Mptx) (27) and the differential expression of nine other genes from 365 pre-selected colon-expressed genes. Five of the nine were down-regulated more than two-fold and four were up-

regulated more then two-fold (26). These results imply that the expression of these genes is more susceptible to diet-induced changes in luminal irritants than the response observed on a physiological marker like epithelial cell proliferation. Therefore, these results provide us with an additional biomarker to study the response of colon mucosa to changes in composition of the luminal contents.

Recently we showed that consumption of green leafy vegetables might decrease the risk of colon cancer because supplementation of the 'high risk' heme diet with spinach or an equimolar amount of chlorophyll prevented heme-induced luminal cytotoxicity and changes in epithelial cell turnover (22). Chlorophyll is the green pigment ubiquitously present in green leafy vegetables. The molecular structure of chlorophyll consists of a phytol-esterified magnesium porphyrin and is therefore a structural analogue of heme. Concerning the mechanism, we speculated that chlorophyll complexes with heme in the hydrophobic phase of the luminal contents and blocks the radical-mediated heme degradation and heme-induced changes in epithelial cell turnover (22,28). This hypothesis predicts that an equimolar amount of chlorophyll is sufficient to block the reactivity of heme in the gut lumen. Thus determining the stoichiometry of the dietary chlorophyll and heme interaction has mechanistic implications. In addition, for the human diet it is important to determine how much green leafy vegetables are needed to prevent the detrimental effects of red meat consumption.

Therefore, we performed this study to determine the minimal dose of dietary chlorophyll required for complete prevention of all detrimental heme-induced effects in luminal cytotoxicity, epithelial proliferation, and gene expression in rat colon.

Materials and Methods

Animals and diets

The experimental protocol was approved by the animal welfare committee of Wageningen University and Research Centre. Approximately 8-week-old outbred male Wistar rats (WU, Harlan, Horst, The Netherlands) were housed individually in metabolic cages in a room with controlled temperature (20-22°C), relative humidity (50-60%) and light/dark cycle (lights on 6 a.m. to 6 p.m.). Animals were acclimatized to housing conditions for 5 days before the start of the experiment.

The rats were stratified by body weight and assigned to one of the six dietary groups (n=8/group). Body weight of the animals at the start of the experiment was 298 ± 3 g (mean ± SEM). During two weeks, six groups of eight rats were fed purified humanized diets (40 % energy as fat, 20 mmol/kg of calcium) as described in detail previously (22). The diets differed in heme and chlorophyll content. The six groups consisted of a control group and five heme groups with 0.5 mmol of heme/kg diet (Sigma-Aldrich, St Louis, MO) in the presence of: 0, 0.125, 0.25, 0.5, or 1.2 mmol of chlorophyll/kg diet (Japan Chlorophyll CO, Chiyoda-ku, Tokyo), respectively, dissolved in the fat portion of the diet. Food was administered to the rats just before dark to prevent possible degradation of the supplements. Food and demineralised drinking water were supplied *ad libitum*. Food intake and body weights were recorded every 2-4 days. Feces were quantitatively collected during days 11-14 of the experiment and frozen at -20°C.

In-vivo colonic epithelial proliferation

After fourteen days of experimental feeding, colon epithelial cell proliferation was quantified by measuring DNA replication *in vivo*, using ^{3}H-thymidine incorporation into DNA. Non-fasted rats were injected intraperitoneally with [methyl-^{3}H]thymidine (specific activity 925 GBq/mmol; dose 3.7 MBq/kg body weight; Amersham International, Amersham, UK) in 154 mM NaCl. After 2 h the rats were killed by CO_2 inhalation. The colon was excised and opened longitudinally. Colonic contents were removed by rinsing. The mucosa was scraped and divided in two equal parts. One part was homogenized in buffer and analyzed for ^{3}H-thymidine incorporation into DNA as described previously (22). The other half was frozen in liquid N_2 and used for RNA isolation.

Quantitative real-time PCR

Quantitative real-time PCR was performed to measure relative Mptx, keratin 20 (Krt20) and carbonic anhydrase I (Ca1) mRNA levels in the colon of control, heme-, and chlorophyll-fed rats. The liquid N_2-frozen colonic scrapings were homogenized (Ultraturrax Pro200: Pro Scientific Inc., Monroe, CT, USA) directly in ice-cold TRIzol reagent (Invitrogen, San Diego, CA, USA) and total RNA was isolated according to the manufacturers protocol. One microgram of Rneasy (Qiagen, Venlo, The Netherlands) purified total RNA was used for cDNA synthesis using iScript cDNA synthesis kit (Bio-Rad, Veenendaal, The Netherlands). Primers used for PCR: aldolase A (Aldoa) (BC064440;

forward, pos 142-164; reverse pos. 310-332), beta actin (Actb) (NM_031144; forward, pos. 347-369; reverse, pos. 636-662), Mptx (AY426671; forward, pos. 17-40; reverse, pos.210-237), Ca1 (XM_226922; forward, pos.577-599; reverse, pos.679-700), and Krt20 (NM_173128; forward, pos.1067-1086; reverse, pos.1236-1255).

For each reaction 2 µl of a 100X diluted cDNA sample was added to 23 µl pre-mix containing 0.4 µM of each primer and 12.5 µl 2X iQ SYBR green supermix (Bio-Rad, Veenendaal, The Netherlands). The sample was incubated at 95°C for 3 min, followed by 40 amplification cycles (15 s 95°C, 45 s 60°C) using the MyiQ Single-color real time PCR (Bio-Rad). Each sample was processed in duplicate and the PCR products were subjected to melting curve analysis. A negative control without cDNA template was run with every assay. Standard curves for Mptx, Krt20, Ca1, and the reference genes were generated, using serial dilutions of a reference sample (colon cDNA synthesized from a mixture of all RNA samples). Data were normalized against the housekeeping genes Aldoa and Actb (both assumed to be unaffected by dietary treatment).

Cytotoxicity of fecal water
Fecal water was prepared by reconstituting a small amount of freeze-dried feces with double-distilled water to obtain physiological osmolarity of 300 mOsmol/l, as described earlier (20). After centrifugation, the fecal waters were stored at –20°C until further analysis. Cytotoxicity of fecal water was quantified by potassium release of a human erythrocyte suspension after incubation with fecal water as described previously (20) and validated earlier with human colon carcinoma-derived Caco-2 cells (29). The potassium content of the erythrocytes was measured with an Inductive Coupled Plasma Absorption Emission Spectrophotometer (ICP-AES) (Varian, Mulgrave, Australia) and cytotoxicity of fecal water was calculated and expressed as percentage of maximal lyses.

Chemical analyses of feces
One of the important physiological functions of colonocytes is the reabsorption of minerals and water. Therefore, we measured the fecal cations sodium and potassium as markers for colon epithelial function. Feces were treated with 5% trichloroacetic acid for 1 h at room temperature and centrifuged for 2 min at 14 000 g. The supernatants were diluted with 0.05% CsCl, and sodium and potassium were analyzed with an ICP-AES.

The total amount of heme excreted in feces was determined by a previously described HemoQuant assay (30). To quantify heme in feces, an acidified chloroform-methanol extract (31) was obtained from 30 mg freeze-dried feces (final HCl concentration 1 M). The chloroform phase of the extracted samples was dried under nitrogen and dissolved in 0.45 ml 250 mM KOH. Subsequently, 0.45 ml double-distilled water, 3.75 ml 2-propanol, and 0.75 ml 1.15 M HCl was added to the samples and assayed for their heme content as described (30).

Quantification of epithelial DNA in feces

Host DNA in rat feces was quantified with real-time PCR as a marker for epithelial exfoliation (22,32). In short, DNA was isolated from rat feces using the QIAamp DNA stool mini kit (Westburg, Leusden, The Netherlands) and quantified with real-time PCR using rat specific primers and a probe with the β-globin gene as target sequence. Quantification of the DNA levels in feces was done against a standard curve of DNA isolated from rat spleen.

Chlorophyll – heme interaction in vitro

We studied whether heme interacts with chlorophyll and thus keeps the hydrophobic chlorophyll in aqueous solution after centrifugation. For comparison we used the known surfactants taurocholate (TC) (Sigma) and taurodeoxycholate (TDC) (Sigma). Heme stock solution (in 50 mM NaOH) was diluted in 50 mM phosphate buffer, pH 7.0, in 150 mM NaCl, to obtain a concentration range from 0 to 15 μM heme. Furthermore, TC and TDC stock solutions (in water) were diluted in PBS to obtain a concentration range from 0-5 mM. Chlorophyll A (Sigma) (in ethanol) was added to the diluted surfactants with a final concentration of 15 μM in the assay. Subsequently, the mixtures were mixed, incubated for 15 min at 37°C, and centrifuged for 5 min at 20 000 g. Solubility of the chlorophyll in the supernatant was quantified by measurement of the absorption at 667 nm using a spectrophotometer (Lambda 2; Perkin Elmer, Norwalk, CT). The results were verified by measurement of magnesium in the same supernatants on an ICP-AES.

Statistical analysis

All results are expressed as mean ± SEM (n=8 per group). We used Statistica 6.1 (Statsoft Inc., Tulsa, USA) for statistical analyses. Differences between the heme group and the

other dietary groups were tested for their significance using a Kruskall-Wallis ANOVA, followed by the non-parametric Mann-Whitney U test as a post-hoc test, as several parameters were not distributed normally. Bonferroni correction was made for the number of comparisons (n=5). Differences were considered statistically significant when $P < 0.05$.

Results

Animals fed the control or the experimental diets with heme and chlorophyll had similar food intakes (19.6 ± 0.7 g dry weight / day). No differences in growth rate (4.1 ± 0.4 g / day) or fecal output (0.8 ± 0.1 g dry weight / day) were observed (data not shown).

Effects in colonic lumen

The effects of the dietary treatment on the concentration of luminal irritants in the soluble fraction of the feces were measured with an *in vitro* cytotoxicity bioassay. **Figure 1** shows that the cytotoxicity of fecal water of heme-fed rats increased approximately seven-fold compared with the non-heme supplemented rats. Fecel water of rats fed a diet with a combination of 0.13 mmol/kg of chlorophyll and heme also had an approximately seven-fold increase in cytotoxicity. However, 0.25 mmol/kg of dietary chlorophyll or higher prevented the heme-induced cytotoxicity completely.
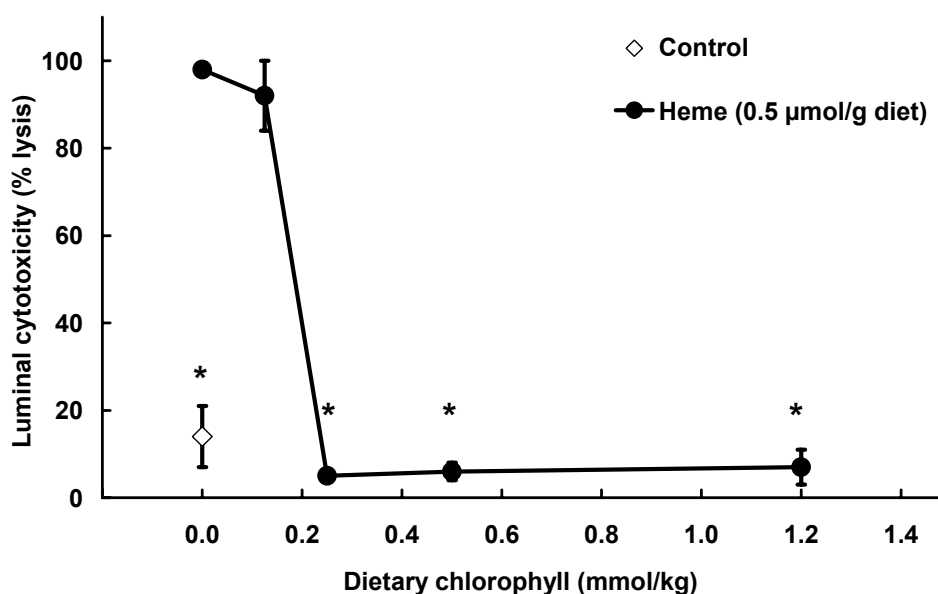
Figure 1. Effect of dietary heme and heme plus different concentrations of dietary chlorophyll on cytotoxicity of fecal water of rats, determined with an erythrocyte bioassay. The results are expressed as mean ± SEM, n = 8. *Different from heme group, P < 0.05.

Reabsorption of electrolyte-rich fluids, i.e. water rich in sodium and potassium ions, is one of the physiological functions of the colon. To examine whether dietary treatment alters this function, we measured the concentration of these cations in the feces. Dietary heme greatly increased the total fecal cations/day (**Table 1**). However, the combination of heme with a dose of 0.13 mmol/kg of chlorophyll prevented the heme-induced increase, and mixing heme with 0.25, 0.5, or 1.2 mmol/kg of chlorophyll completely normalized the values to levels observed in the rats fed the control diet.

We demonstrated previously that heme-induced cytotoxicity coincides with extensive luminal degradation of ingested heme (22,33) and that addition of spinach or chlorophyll to a heme diet inhibited this effect (22,28). Table 1 shows how different amounts of dietary chlorophyll supplemented to the heme diet modulated intestinal heme degradation. The rats not fed heme excreted a background level of ~0.3 µmol of heme/day in the feces, while rats on a diet supplemented with 0.5 µmol/kg of added heme excreted ~2 µmol of heme/day.

Table 1. Effect of heme and chlorophyll on the concentration of fecal cations and fecal heme excretion.

Group	1	2	3	4	5	6
Heme (mmol/kg diet)	0	0.5	0.5	0.5	0.5	0.5
Chlorophyll (mmol/kg diet)	0	0	0.13	0.25	0.5	1.2
Total fecal cations[1] (µmol/day)	102 ± 13 *	633 ± 37	260 ± 17 *	52 ± 7 *	71 ± 10 *	81 ± 9 *
Heme excretion (µmol/day)	0.24 ± 0.02 *	1.92 ± 0.21	2.79 ± 0.25	5.05 ± 0.45 *	7.33 ± 0.45 *	5.97 ± 0.32 *

[1]Total fecal cations represent the sum of the daily output of sodium and potassium. Results are expressed as mean ± SEM, n = 8. * Different from heme group, $P < 0.05$.

Addition of 0.5 mmol/kg of chlorophyll to the heme diet increased excretion of heme to an average of ~7.5 µmol/day. As intake of heme was almost equal in all heme-fed rats

(because all rats had similar food intakes), this result indicates that chlorophyll prevents intestinal catabolism of heme with saturation of the effect at equimolar concentrations of chlorophyll and heme in the diet.

Effects on colonic cell turnover

We measured proliferation of the colonic epithelial cells *in vivo* to determine whether diet-induced changes in luminal irritants influence the turnover of epithelial cells. Rats fed the heme diet showed a ~100% increased proliferation compared with the non-heme fed group (**Fig. 2A**). The lowest dose of 0.13 mmol/kg of chlorophyll added to the heme diet did not prevent the heme-induced hyperproliferation of colon epithelial cells. In contrast, doses of 0.25 mmol/kg of chlorophyll or higher mixed with the heme diet prevented heme-induced hyperproliferation completely.
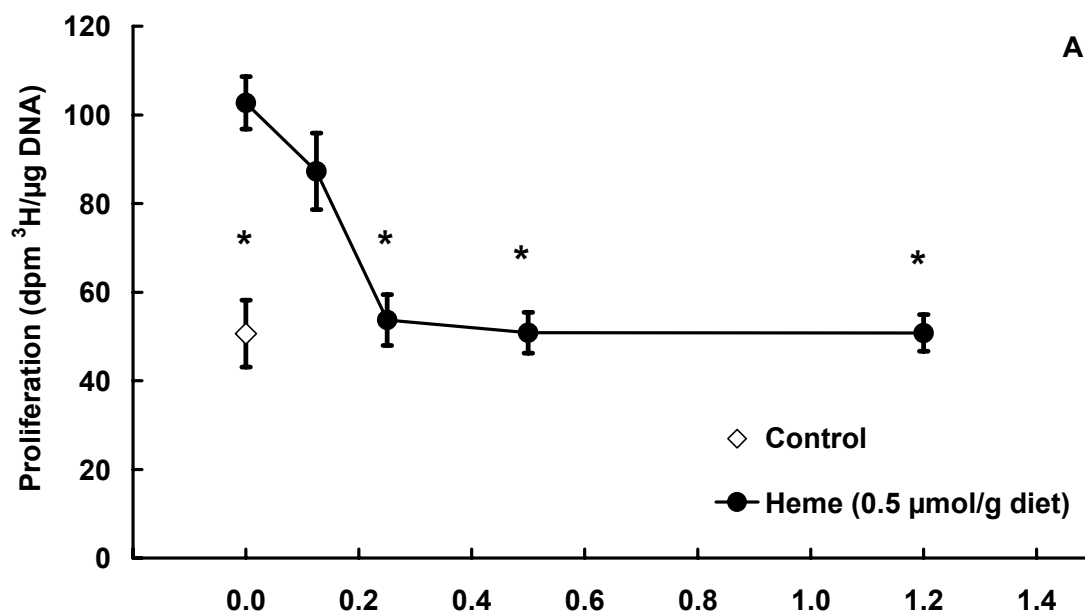
Figure 2A. Effect of dietary heme and heme plus different concentrations of dietary chlorophyll on colonic epithelial cell proliferation, determined by *in vivo* [methyl-^{3}H]thymidine incorporation into colonic mucosa of rats. The results are expressed as mean ± SEM, n = 8. *Different from the heme group, P < 0.05.

We also studied the amount of host DNA in the feces of the rats as a marker for the presence of exfoliated epithelial cells (32). **Figure 2B** shows that dietary heme reduced the amount of host DNA in the fecal matrix to almost zero compared with the non-heme

fed rats. Addition of 0.13 mmol/kg of chlorophyll to the heme diet did not prevent this heme-induced effect. However, concentrations of dietary chlorophyll of 0.25 mmol/kg or higher added to the heme diet prevented the heme-induced decrease in fecal host DNA to similar values as observed for the non-heme fed rats.
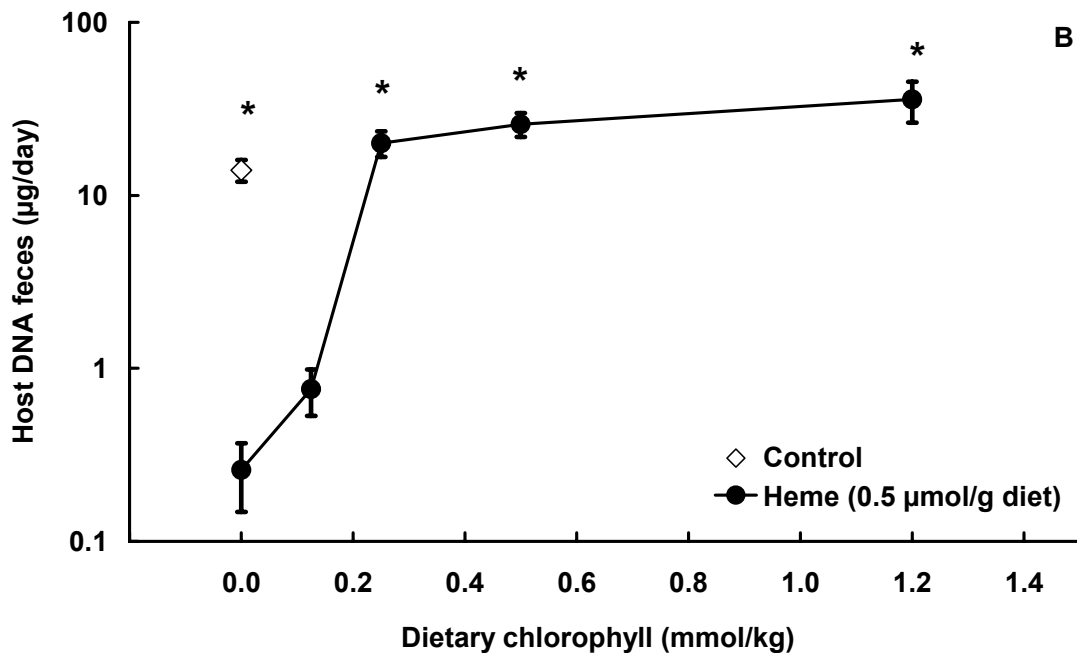
Figure 2B. Effect of dietary heme and heme plus different concentrations of dietary chlorophyll on the level of host DNA detected in feces of rats, quantified by real-time PCR. Note that a log scale is used for the y-axis. The results are expressed as mean ± SEM, n = 8. *Different from the heme group, $P < 0.05$.

These results show that the minimal dose of chlorophyll necessary to prevent heme-induced detrimental effects varies between 0.25 and 0.5 mmol/kg of chlorophyll. This variability may be due to different sensitivities of the markers used. Therefore, we also studied the protective effect of chlorophyll on the expression of some colon-expressed genes that were shown previously to be the most sensitive mucosal markers of diet-induced changes in the amount of luminal irritants (26,27).

Effects on colon epithelial gene expression

Van der Meer-van Kraaij et al. (26) showed that dietary heme induced a greater than two-fold differential gene expression in colon epithelial cells for ten genes from 365 pre-selected genes expressed in the colon mucosa (26). The three genes with the highest

heme-induced fold-changes were Mptx, which was more than ten-fold down-regulated, and Ca1 and Krt20 that were up-regulated more than two-fold (26,27). These changes in gene expression of epithelial cells seem to be a signature of the dietary treatment. The function of these changes in gene expression did not immediately provide a clear mechanistic explanation for the physiological effects demonstrated by the colon mucosa on the irritants present in the gut lumen (26). Furthermore, the function of Mptx, one of the 10 genes modulated by heme is unfortunately still unknown. We used quantitative real-time PCR to study the response of the colon mucosa on the presence of heme in combination with different concentrations of chlorophyll in the luminal contents. In line with our previous studies we reproduced the greater than ten-fold heme-induced down-regulation of Mptx and up-regulation of Ca1 and Krt20 (**Fig. 3A, B, and C**). Dietary chlorophyll prevented the heme-induced changes of Mptx, Ca1, and Krt20 when mixed with the heme diet in a concentration of 0.5 mmol/kg or higher.

Together, these results demonstrate that all heme-induced detrimental effects on the colon mucosa can be prevented by an equimolar dose of chlorophyll in the diet, which suggests that chlorophyll has a high affinity to block the metabolism of heme. To study this interaction we designed an *in vitro* solubilization assay for chlorophyll.

Figure 3. Effect of dietary heme and heme plus different concentrations of dietary chlorophyll on: the relative expression of (**A**) Mptx, (**B**) Ca1, and (**C**) Krt20 in colonic epithelial cells, determined by quantitative RT-PCR. Expression values represent mRNA levels relative to aldoa mRNA, similar results were obtained with mRNA levels relative to actb mRNA. Note that a log scale is used for the y-axis in A. The results are expressed as mean ± SEM, n = 8. *Different from heme group, $P < 0.05$.
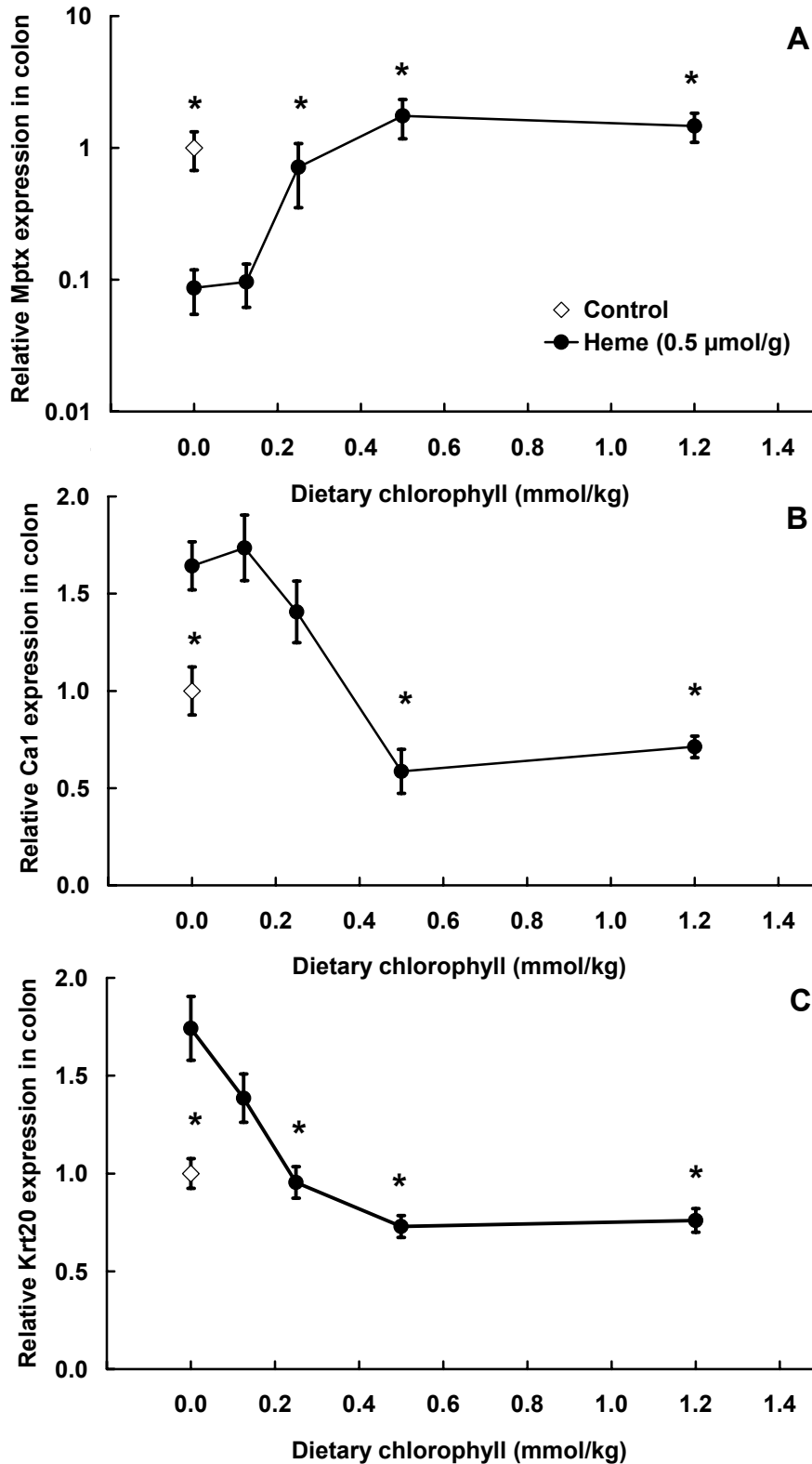
Interaction of chlorophyll and heme in vitro

Chlorophyll is insoluble in water and soluble in organic solvents. We hypothesized that if heme binds chlorophyll, it might dissolve the hydrophobic chlorophyll in an aqueous solution, because heme is an amphipathic surfactant. As shown in **Figure 4**, this is indeed the case; in fact, heme dissolved chlorophyll much more efficiently then the known hydrophobic bile salts taurodeoxycholate and taurocholate. Comparison of the effective concentration (EC_{50}) dissolving half of the chlorophyll showed that heme is able to dissolve chlorophyll in a much lower concentration than taurodeoxycholate and taurocholate (EC_{50}: 0.6, 15, and 30 µmol/l, respectively). Comparison of the concentration of magnesium in the supernatants with standard solutions resulted in similar EC_{50} values as described above (data not shown). Thus chlorophyll has a high hydrophobic binding affinity for heme which is much higher than that for bile acids.
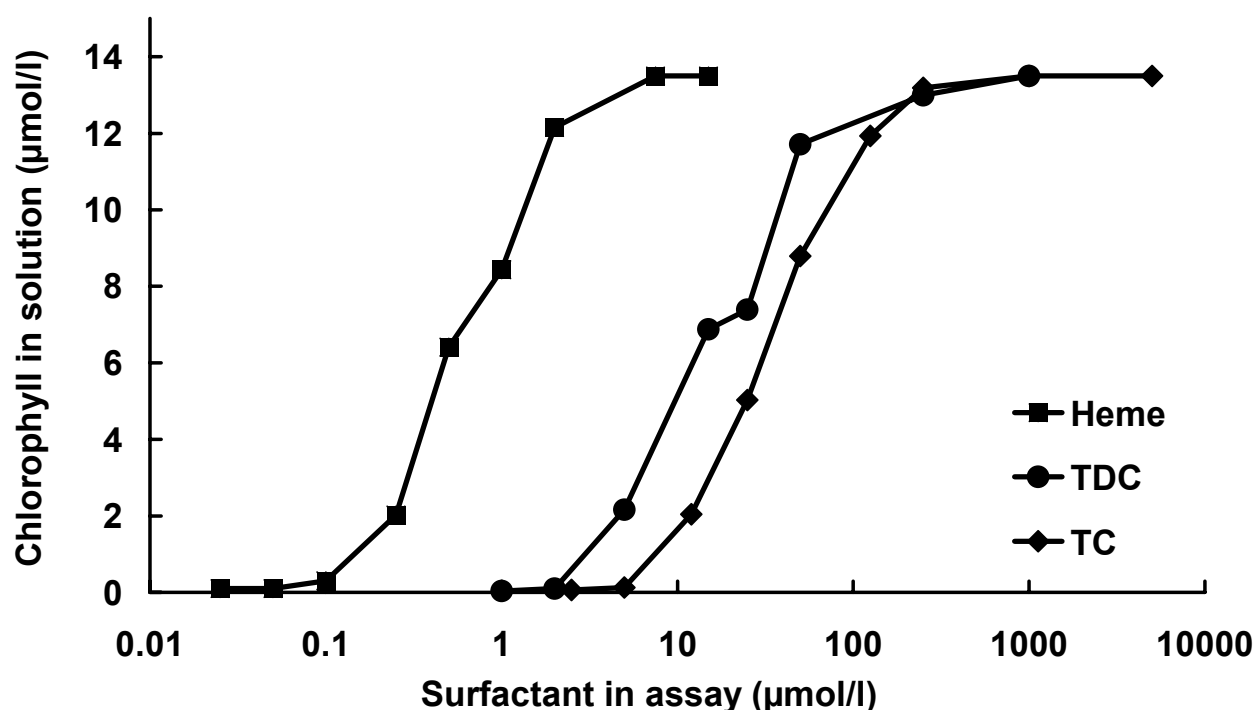
Figure 4. The effect of heme, taurocholate (TC), and taurodeoxycholate (TDC) on the solubility of chlorophyll in a phosphate buffered solution (pH 7.4). After centrifugation, chlorophyll was measured in the supernatant in a spectrophotometer at 666 nm. A representative graph of three independent experiments is shown.

Discussion

This study demonstrated that the minimum dose of chlorophyll required for prevention of all heme-induced changes in luminal cytotoxicity, epithelial proliferation, and gene expression in rat colon is 0.5 mmol/kg diet, which is the concentration equimolar to heme. Preliminary work demonstrated that dietary heme-induced cytotoxicity injured the colon surface epithelium (22). Furthermore, heme increased the output of fecal cations (20), which reflected an impaired reabsorption capacity of the colon due to the damaged colonocytes. Addition of chlorophyll to the heme diet prevented this heme-induced increase in fecal cation excretion. Thus chlorophyll prevents heme-induced damage to colonocytes and as a consequence normal reabsorptive functions of the colon are maintained.

The increased luminal cytotoxicity (20,22) in diets high in heme content is due to the presence of an unknown cytotoxic heme metabolite (20) and is completely prevented by dietary antioxidants (24). The formation of this cytotoxic heme metabolite coincides with lipid peroxidation in the gut lumen catalyzed by heme (20,28). Supplementation with dietary chlorophyll prevented this heme-induced lipid peroxidation (22).

In previous experiments, we used a dose of ~1.2 mmol/kg of chlorophyll to prevent fecal water cytotoxicity and colon epithelial hyperproliferation induced by 0.5 mmol/kg of dietary heme (22,28). In the present study, a chlorophyll dose of 0.25 mmol/kg prevented heme-induced luminal cytotoxicity and changes in epithelial cell turnover. However, heme degradation was maximally prevented at a dose of chlorophyll of 0.5 mmol/kg.

To substantiate the optimal effective dose, we analyzed the effects of dietary chlorophyll on heme-induced differential colonic gene expression as a more sensitive marker. Previous experiments (26,27) showed that diet-induced changes of the composition of the luminal contents resulted in a gene expression signature for each dietary treatment. As a marker for changes in colonic gene expression, we studied the differential gene expression of Mptx (that was down-regulated) and Ca1 and Krt20 (that were up-regulated) by dietary heme (26). Maximal prevention of the heme-induced differential gene expression was reached at a dose of 0.5 mmol/kg of chlorophyll and demonstrated that the changes in colonic gene expression were indeed more sensitive to dietary changes than other markers.

Our results show that all heme-induced detrimental effects on the colon mucosa can be prevented by an equimolar dose of chlorophyll in the diet. Previous *in vitro* studies demonstrated that the similar porphyrin structure of heme and chlorophyll forces them to accumulate in a hydrophobic environment (28). Chlorophyll has additional hydrophobicity because of its phytol tail, which is retained during intestinal passage (34,35). We hypothesized that a similar preference of chlorophyll and heme for the hydrophobic phase in the gut luminal contents creates an opportunity for chlorophyll to form a complex with heme (28).

For the formation of this complex, high affinity of chlorophyll for heme is a prerequisite. To test whether such affinity exists, we performed an *in vitro* solubilization experiment. Without surfactant, chlorophyll precipitates from the aqueous suspension but interaction with a surfactant solubilizes chlorophyll. We found that the affinity of chlorophyll to heme was 25 to 50-fold higher than for the hydrophobic bile acids taurodeoxycholate and taurocholate, respectively. This high affinity supports our hypothesis of the formation of a complex between heme and chlorophyll that prevents radical-mediated heme degradation and formation of the cytotoxic heme metabolite.

Based on these results, we propose the following mechanism (**Fig. 5A and B**). In the control diet heme accumulates at the water-lipid interphase of the gut contents. There they are present as monomers because of charge repulsion by their negative propionic side chains. Chlorophyll complexes these heme monomers in porphyrin stacks, blocking the reactivity of heme. The size of these stacks may vary from simple heterodimers to polymers of alternating heme and chlorophyll porphyrins. Depending on the size of these stacks about an equimolar amount of chlorophyll is required to trap all heme molecules. Additional research is necessary to determine the precise molecular interaction between chlorophyll and heme and how this blocks the pro-oxidant activity of heme.

Epidemiological studies often show no association between the consumption of total meat and the risk of colon cancer (36,37). In contrast, there is substantial evidence that consumption of high amounts of red meat and processed meat increase the risk of colon cancer (4-7). A protective effect on colon cancer risk was reported with high vegetable consumption (2,9). This evidence was particularly consistent for the consumption of raw vegetables and green leafy vegetables (11). However, several recent large cohort studies obtained inconsistent results for the association between vegetable consumption and the risk of colon cancer (12,38).
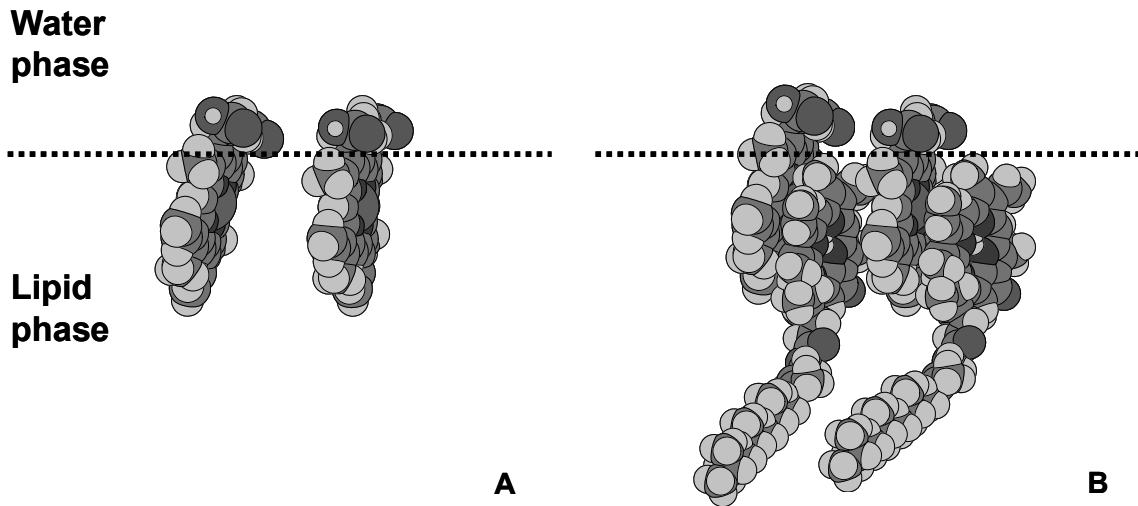
Figure 5. Cartoon of heme (**A**) and our proposed chlorophyll-heme stacking at the water-lipid interphase in the gut lumen (**B**).

An explanation for these inconsistent findings might be that total grams of different types of meat or vegetables are used as a measure of dietary intake. However, total grams of meats or vegetables mask a possible association with specific components such as heme or chlorophyll, because the concentration of heme in different types of meat and of chlorophyll in vegetables varies enormously (39,40). Beef, for example, has a high content of heme ~0.5 µmol/g wet weight compared with 0.03 µmol/g for chicken (39). Furthermore, green leafy vegetables such as spinach are high in chlorophyll with ~1 µmol/g wet weight, in contrast to, for example, broccoli that has a low content of chlorophyll with ~0.1 µmol/g (40,41). Therefore, we suggest that future epidemiological studies might use amounts of modulating ingredients, such as heme and chlorophyll, instead of total grams of meat and vegetables to determine risk exposure. Extrapolation of our results on the molar ratio of chlorophyll and heme in the animal diets to the human diet indicates that people consuming ~150 g of beef have to eat ~75 g of spinach or ~750 g of broccoli (40) to deliver enough chlorophyll to neutralize the detrimental effects of heme from beef.

Our proposed stacking of heme and chlorophyll in the gut lumen implies that chlorophyll has to be consumed together with heme for maximal protection. The need for this simultaneous consumption might explain why processed meats are even more associated with colon cancer risk then fresh meat (4,42). Processed meats are frequently eaten during lunch or as a snack between meals and not in combination with vegetables (43). In

contrast, consumption of red meat during dinner is most often combined with consumption of vegetables, at least in the Netherlands (43).

In conclusion, simultaneous consumption of a diet with 0.5 mmol/kg of heme and 0.5 mmol/kg of chlorophyll prevents all heme-induced detrimental effects in rat colon. Further studies are necessary to elucidate the exact mechanism of the protective effects of chlorophyll and the applicability of our results to humans.

Acknowledgments

References

1. Jemal, A., Tiwari, R.C., Murray, T., Ghafoor, A., Samuels, A., Ward, E., Feuer, E.J. and Thun, M.J. (2004) Cancer statistics, 2004. *CA Cancer J Clin*, **54**, 8-29.
2. *World Cancer Research Fund. Food, Nutrition and the prevention of cancer: a global perspective, 1997*, Washington, DC: American Institute for Cancer Research.
3. Armstrong, B. and Doll, R. (1975) Environmental factors and cancer incidence and mortality in different countries, with special reference to dietary practices. *Int J Cancer*, **15**, 617-631.
4. Norat, T., Lukanova, A., Ferrari, P. and Riboli, E. (2002) Meat consumption and colorectal cancer risk: dose-response meta- analysis of epidemiological studies. *Int J Cancer*, **98**, 241-256.
5. Chao, A., Thun, M.J., Connell, C.J., McCullough, M.L., Jacobs, E.J., Flanders, W.D., Rodriguez, C., Sinha, R. and Calle, E.E. (2005) Meat consumption and risk of colorectal cancer. *Jama*, **293**, 172-82.
6. Larsson, S.C., Rafter, J., Holmberg, L., Bergkvist, L. and Wolk, A. (2005) Red meat consumption and risk of cancers of the proximal colon, distal colon and rectum: the Swedish Mammography Cohort. *Int J Cancer*, **113**, 829-34.
7. Norat, T., Bingham, S., Ferrari, P., Slimani, N., Jenab, M., Mazuir, M., Overvad, K., Olsen, A., Tjonneland, A., Clavel, F., Boutron-Ruault, M.C., Kesse, E., Boeing, H., Bergmann, M.M., Nieters, A., Linseisen, J., Trichopoulou, A., Trichopoulos, D., Tountas, Y., Berrino, F., Palli, D., Panico, S., Tumino, R., Vineis, P., Bueno-de-Mesquita, H.B., Peeters, P.H., Engeset, D., Lund, E., Skeie, G., Ardanaz, E., Gonzalez, C., Navarro, C., Quiros, J.R., Sanchez, M.J., Berglund, G., Mattisson, I., Hallmans, G., Palmqvist, R., Day, N.E., Khaw, K.T., Key, T.J., San Joaquin, M., Hemon, B., Saracci, R., Kaaks, R. and Riboli, E. (2005) Meat, fish, and colorectal cancer risk: the European Prospective Investigation into cancer and nutrition. *J Natl Cancer Inst*, **97**, 906-16.
8. Giovannucci, E., Rimm, E.B., Stampfer, M.J., Colditz, G.A., Ascherio, A. and Willett, W.C. (1994) Intake of fat, meat, and fiber in relation to risk of colon cancer in men. *Cancer Res*, **54**, 2390-2397.
9. Riboli, E. and Norat, T. (2003) Epidemiologic evidence of the protective effect of fruit and vegetables on cancer risk. *Am J Clin Nutr*, **78**, 559S-569S.
10. Trock, B., Lanza, E. and Greenwald, P. (1990) Dietary fiber, vegetables, and colon cancer: critical review and meta- analyses of the epidemiologic evidence. *J Natl Cancer Inst*, **82**, 650-661.
11. Steinmetz, K.A. and Potter, J.D. (1996) Vegetables, fruit, and cancer prevention: a review. *J Am Diet Assoc*, **96**, 1027-1039.
12. Voorrips, L.E., Goldbohm, R.A., van Poppel, G., Sturmans, F., Hermus, R.J. and van den Brandt, P.A. (2000) Vegetable and fruit consumption and risks of colon and rectal cancer in a prospective cohort study: The Netherlands Cohort Study on Diet and Cancer. *Am J Epidemiol*, **152**, 1081-92.

13. Bueno-de-Mesquita, H.B., Ferrari, P. and Riboli, E. (2002) Plant foods and the risk of colorectal cancer in Europe: preliminary findings. *IARC Sci Publ*, **156**, 89-95.
14. Hollstein, M., Sidransky, D., Vogelstein, B. and Harris, C.C. (1991) p53 mutations in human cancers. *Science*, **253**, 49-53.
15. Thilly, W.G. (2003) Have environmental mutagens caused oncomutations in people? *Nat Genet*, **34**, 255-259.
16. Kinzler, K.W. and Vogelstein, B. (1996) Lessons from hereditary colorectal cancer. *Cell*, **87**, 159-170.
17. Lipkin, M. (1988) Biomarkers of increased susceptibility to gastrointestinal cancer: new application to studies of cancer prevention in human subjects. *Cancer Res*, **48**, 235-245.
18. Preston-Martin, S., Pike, M.C., Ross, R.K., Jones, P.A. and Henderson, B.E. (1990) Increased cell division as a cause of human cancer. *Cancer Res*, **50**, 7415-21.
19. Ames, B.N., Gold, L.S. and Willett, W.C. (1995) The causes and prevention of cancer. *Proc Natl Acad Sci U S A*, **92**, 5258-5265.
20. Sesink, A.L.A., Termont, D.S., Kleibeuker, J.H. and Van der Meer, R. (1999) Red meat and colon cancer: the cytotoxic and hyperproliferative effects of dietary heme. *Cancer Res*, **59**, 5704-5709.
21. Sesink, A.L.A., Termont, D.S., Kleibeuker, J.H. and Van Der Meer, R. (2000) Red meat and colon cancer: dietary haem, but not fat, has cytotoxic and hyperproliferative effects on rat colonic epithelium. *Carcinogenesis*, **21**, 1909-1915.
22. de Vogel, J., Jonker-Termont, D.S., van Lieshout, E.M., Katan, M.B. and van der Meer, R. (2005) Green vegetables, red meat and colon cancer: chlorophyll prevents the cytotoxic and hyperproliferative effects of haem in rat colon. *Carcinogenesis*, **26**, 387-93.
23. Terpstra, O.T., van Blankenstein, M., Dees, J. and Eilers, G.A. (1987) Abnormal pattern of cell proliferation in the entire colonic mucosa of patients with colon adenoma or cancer. *Gastroenterology*, **92**, 704-8.
24. Pierre, F., Tache, S., Petit, C.R., Van Der Meer, R. and Corpet, D.E. (2003) Meat and cancer: haemoglobin and haemin in a low-calcium diet promote colorectal carcinogenesis at the aberrant crypt stage in rats. *Carcinogenesis*, **24**, 1683-1690.
25. Corpet, D.E. and Tache, S. (2002) Most effective colon cancer chemopreventive agents in rats: a systematic review of aberrant crypt foci and tumor data, ranked by potency. *Nutr Cancer*, **43**, 1-21.
26. van der Meer-van Kraaij, C., Kramer, E., Jonker-Termont, D., Katan, M.B., van der Meer, R. and Keijer, J. (2005) Differential gene expression in rat colon by dietary heme and calcium. *Carcinogenesis*, **26**, 73-9.
27. Van Der Meer-Van Kraaij, C., Van Lieshout, E.M., Kramer, E., Van Der Meer, R. and Keijer, J. (2003) Mucosal pentraxin (Mptx), a novel rat gene 10-fold down-regulated in colon by dietary heme. *Faseb J*, **17**, 1277-85.
28. de Vogel, J., Jonker-Termont, D.S., Katan, M.B. and van der Meer, R. (2005) Natural chlorophyll but not chlorophyllin prevents heme-induced cytotoxic and hyperproliferative effects in rat colon. *J Nutr*, **135**, 1995-2000.
29. Lapre, J.A., Termont, D.S., Groen, A.K. and Van der Meer, R. (1992) Lytic effects of mixed micelles of fatty acids and bile acids. *Am J Physiol*, **263**, G333-G337.
30. van den Berg, J.W., Koole-Lesuis, R., Edixhoven-Bosdijk, A. and Brouwers, N. (1988) Automating the quantification of heme in feces. *Clin Chem*, **34**, 2125-2126.
31. Bligh, E.G. and Dyer, W.J. (1959) A rapid method of total lipid extraction and purification. *Canadian journal of biochemistry and physiology*, **37**, 911-917.
32. Van Lieshout, E.M., Van Doesburg, W. and Van der Meer, R. (2004) Real-time PCR of host DNA in feces to study differential exfoliation of colonocytes between rats and humans. *Scand J Gastroenterol*, **39**, 852-7.
33. Sesink, A.L., Termont, D.S., Kleibeuker, J.H. and Van der Meer, R. (2001) Red meat and colon cancer: dietary haem-induced colonic cytotoxicity and epithelial hyperproliferation are inhibited by calcium. *Carcinogenesis*, **22**, 1653-9.
34. Baxter, J.H. (1968) Absorbtion of chlorophyll phytol in normal man and in patients with Refsum's disease. *Journal of lipid research*, **9**, 636-641.
35. Baxter, J.H. and Steinberg, D. (1967) Absorption of phytol from dietary chlorophyll in the rat. *J Lipid Res*, **8**, 615-20.
36. Goldbohm, R.A., van den Brandt, P.A., van 't Veer, P., Brants, H.A., Dorant, E., Sturmans, F. and Hermus, R.J. (1994) A prospective cohort study on the relation between meat consumption and the risk of colon cancer. *Cancer Res*, **54**, 718-23.

37. Tiemersma, E.W., Kampman, E., Bueno de Mesquita, H.B., Bunschoten, A., van Schothorst, E.M., Kok, F.J. and Kromhout, D. (2002) Meat consumption, cigarette smoking, and genetic susceptibility in the etiology of colorectal cancer: results from a Dutch prospective study. *Cancer Causes Control*, **13**, 383-93.

38. Michels, K.B., Edward, G., Joshipura, K.J., Rosner, B.A., Stampfer, M.J., Fuchs, C.S., Colditz, G.A., Speizer, F.E. and Willett, W.C. (2000) Prospective study of fruit and vegetable consumption and incidence of colon and rectal cancers. *J Natl Cancer Inst*, **92**, 1740-52.

39. Schwartz, S. and Ellefson, M. (1985) Quantitative fecal recovery of ingested hemoglobin-heme in blood: comparisons by HemoQuant assay with ingested meat and fish. *Gastroenterology*, **89**, 19-26.

40. Khachik, F., Beecher, G.R. and Whittaker, N.F. (1986) Separation, Identification, and Quantification of the Major Carotenoid and Chlorophyll Constituents in Extracts of Several green Vegetables by Liquid Chromatography. *Jounal of Agricultural Food Chemistry*, **34**, 603-616.

41. Bohn, T. and Walczyk, T. (2004) Determination of chlorophyll in plant samples by liquid chromatography using zinc-phthalocyanine as an internal standard. *J Chromatogr A*, **1024**, 123-8.

42. Norat, T. and Riboli, E. (2001) Meat consumption and colorectal cancer: a review of epidemiologic evidence. *Nutr Rev*, **59**, 37-47.

43. Dutch-Nutrition-Foundation (1998) Zo eet Nederland 1998, resultaten van de voedselconsumptiepeiling 1998. Voedingscentrum, Den Haag.

Chapter 6

Heme and chlorophyll intake and the risk of colorectal cancer in the Netherlands Cohorts Study

Helena F. Balder, Johan de Vogel, Margje C.J.F. Jansen, Matty P. Weijenberg, Piet A. van den Brandt, Susanne Westenbrink, Roelof van der Meer, R. Alexandra Goldbohm

Abstract

Background: The evidence for red meat as a determinant of colorectal cancer remains equivocal, which might be explained by differences in heme content. Heme is the pro-oxidant, iron-containing porphyrin pigment of meat and its content depends on the type of meat. Chlorophyll from green vegetables might modify this association.

Methods: The Netherlands Cohort Study was initiated in 1986 when a self-administered questionnaire on risk factors for cancer was completed by 120 852 subjects aged 55-69 years. After 9.3 years of follow-up through the Cancer Registry, 1535 incident colorectal cancer cases (869 men and 666 women) were available. Nineteen of the 150 items in the validated dietary questionnaire related to consumption of specific types of fresh and processed meat. Heme iron content was calculated as a type-specific percentage of the total iron content and chlorophyll content of vegetables was derived from the literature.

Results: Multivariate rate ratios (RRs) for quintiles of heme iron intake and colon cancer were 1.00, 0.98, 1.04, 1.13, 1.29 (p-trend: 0.10) among men and 1.00, 1.31, 1.44, 1.18, 1.20 (p-trend: 0.56) among women, respectively. No consistent associations were observed for rectal cancer. RRs for colon cancer increased across successive quintiles of the ratio of heme to chlorophyll among men only (1.00, 1.08, 1.01, 1.32, 1.43; p-trend: 0.01). No associations were observed between fresh meat and colorectal cancer.

Conclusion: Our data suggest an elevated risk of colon cancer in men with increasing intake of heme iron and decreasing intake of chlorophyll. Further research is needed to confirm these results.

Introduction

Results from cohort studies suggest that consumption of (fresh) "red" meat is modestly associated with the risk of colon cancer (1-3). However, results differ between studies and over time. In some, no association was observed (4), while in others a strong association was seen (5,6). Recent results from seven cohort studies (7-13) only partly confirm a modest association with red meat (including processed meat in most cases) (9-12). Virtually all cohort studies found an increased risk for colon cancer with processed meat consumption (2,7-12). Results for rectal cancer were inconsistent.

An explanation for the inconsistent and relatively modest associations might be that not total red meat intake, defined by many authors as beef, pork and lamb, but heme increases risk. Heme is the iron porphyrin component of hemoproteins, such as hemoglobin and myoglobin and gives meat its red color. These proteins are digested in the upper gastrointestinal tract, releasing heme in the gut lumen (14). Heme is a pro-oxidant that has been previously demonstrated to increase colonic epithelial proliferation and toxicity of fecal water in rats (15). These effects were specific for heme because equimolar dietary ferric citrate and protoporphyrin did not increase proliferation and cytotoxicity (15). Dietary heme was metabolized in the gut lumen and resulted in the formation of a highly cytotoxic factor that damaged the colonic mucosa (16). This resulted in a compensatory hyperproliferation of the epithelium, which may increase the risk of colon cancer (17). In addition, Pierre et al. (18) showed that red meat and heme increased formation of aberrant crypt foci in rats. A role for heme in colorectal cancer etiology is further supported by Bingham et al. (19,20). They showed in randomized cross-over experiments in a metabolic suite that male volunteers, exposed to high amounts of red meat or heme, produced higher levels of fecal N-Nitroso compounds than when exposed to the same amounts of white meat or ferrous iron.

Previous results from the Netherlands Cohort Study indicated that fresh meat consumption as such was not a risk factor for colon cancer (4). However, intake of iron was found to be a risk factor in men (21). Lee et al. (22) previously reported that the relative risks for (proximal) colon cancer increased more than twofold across categories of heme iron intake in the Iowa Women's Health Study, especially among women who drink alcohol. Larsson et al. (23) also observed an increased risk for colon cancer with a high heme iron intake in a female Swedish cohort, but the association was equally likely as that for meat as such.

To date, heme intake is usually assessed by applying a fixed factor to the total iron content of all meat items, regardless of the origin of the meat. However, it is apparent from the literature that not only the absolute total iron content differs substantially between meat from different origins, but also the percentage iron from heme, which is high in beef and low in poultry and fish (24). In order to assess heme intake more accurately, we took the origin of the meat, i.e. beef, pork, chicken, etc., into account, by estimating the heme content of specific types of meat based on published literature.

In addition to a hypothesized positive association between heme intake and colorectal cancer risk, we hypothesized that chlorophyll, the ubiquitous pigment in green vegetables, modifies this association. Chlorophyll is structurally similar to heme, as it contains a porphyrin ring similar to that of heme but with a central, non-reactive, magnesium instead of iron atom. Chlorophyll may block the reactivity of heme in the gastrointestinal tract and thus prevent the formation of cytotoxic heme metabolites. We previously demonstrated in rats that addition of spinach or purified chlorophyll to a heme diet inhibited heme metabolism in the gut and prevented the heme-induced formation of a cytotoxic factor that increased colonic cytotoxicity and epithelial proliferation (16).

The aim of this paper was to determine the association of heme in combination with chlorophyll intake with risk of colorectal cancer in the Netherlands Cohort Study on Diet and Cancer, a population-based prospective cohort study with 9.3 years of follow-up. To our knowledge, this has not previously been studied in an observational setting.

SUBJECTS AND METHODS

Study population

The study design of the Netherlands Cohort Study on diet and cancer (NLCS) has been reported in detail elsewhere (25). The NLCS was initiated in 1986 when a self-administered questionnaire on dietary habits, lifestyle characteristics, medical history and other potential risk factors for cancer was completed by 58 279 men and 62 573 women aged 55-69 years. After baseline exposure measurement, a subcohort of 5000 subjects was randomly sampled from the large cohort. Following the case-cohort approach, this subcohort was followed-up for vital status and migration to estimate person time at risk accumulated in the cohort. The cohort-at-large has been followed-up for incident cancer by

record linkage to the Netherlands Cancer Registry and the Netherlands Pathology Registry (PALGA) for 9.3 years (26).

After excluding subjects who reported prevalent cancer other than skin cancer at baseline and subjects with incomplete or inconsistent dietary information, 869 male and 666 female cases with primary colon (ICD-O 153.0-153.9) or rectal cancer (ICD-O 154.0 and 154.1) and 2156 male and 2215 female subcohort members were available for analysis.

The Netherlands Cohort Study (NLCS) has been approved by the institutional review boards of the TNO Nutrition and Food Research Institute (Zeist) and Maastricht University (Maastricht).

Questionnaire

The dietary part of this questionnaire consisted of a 150-item semi-quantitative food frequency questionnaire (FFQ) on the usual intake of food and beverages in the year preceding the start of the study, which was validated against a 9-day diet record (27). The Spearman correlation coefficients for fresh meat, processed meat (meat products), fish, and vegetables were 0.46, 0.54, 0.53, and 0.38 respectively. As the classification of foods into food groups did not entirely overlap, due to differences in the coding of recipes between the FFQ and record, the observed correlation between the food groups was likely to be lower than the true. On average, the FFQ captured between 85 and 98% of the absolute intake of energy, animal protein and total (fresh) meat assessed by the record, but only 57% of processed meat.

Questionnaire data of all cases and subcohort members have been key-entered twice and blinded with respect to case/subcohort status to avoid random and systematic coding errors.

Assessment of heme iron intake

The FFQ contained 14 items on consumption of meat with the hot meal (mainly fresh meat, including chicken), 5 items on consumption of processed meat (meat products) used as sandwich filling and 3 items on fish consumption. To derive an individual serving size of fresh meat, a question was included on the quantity of meat usually purchased (per person, per meal). For chicken and fish, standard serving sizes were used. Fresh meat was defined as meat that has not undergone some form of preservation and includes beef, pork, minced meat, chicken, liver and other meat (i.e., horse and lamb). Processed meat

was defined as meat items that have undergone some form of preservation. i.e., smoking, fermentation, and/or treatment with nitrate and/or nitrite salt ("curing"). Meat items in the questionnaire were converted into mean daily consumption in grams.

We decided to use the total iron content of each heme-containing food item from the Dutch Food Composition Database (28), which was used to calculate nutrient intake in the NLCS, as a starting point, as this takes into account the specific types, cuts and fat composition of the Dutch food items. To investigate the levels of heme for different types of meat, poultry and fish, a literature search was performed. The results were used to derive the mean percentage heme iron relative to total iron for each specific origin of the meat (i.e., beef, pork, chicken, fish, etc). We selected only those studies that measured total iron directly and, after lipid extraction, heme iron in the same meat sample (24,29-32). The average percentages were 65, 39, and 26 for cooked beef, pork, and chicken or fish, respectively. Multiplying the type-specific percentages of heme iron with the total iron content (mg/g) yielded heme-iron contents for all heme-containing food items in the NLCS database. Individual mean daily intake of heme iron was assessed by multiplying the estimated heme-iron content with the mean daily intake of the relevant food items.

Assessment of chlorophyll intake

With regard to vegetable consumption, participants were asked to report their frequency of consumption of a number of vegetables, both in summer and in winter. Usual serving sizes were asked for string beans and cooked endive only, the mean of which served as an indicator for serving sizes of all cooked vegetables (33). Standard serving sizes were used for lettuce and other raw vegetables. For tomatoes and sweet peppers, consumption was asked in pieces per week and month respectively, during summer and winter. We used the derived individual portion sizes to convert these frequencies and amounts to consumption in grams per day. Items included in the questionnaire covered almost all vegetables and fruits eaten regularly at baseline measurement.

To assess chlorophyll intake, publications reporting chlorophyll content in vegetables were searched. However, this information appeared to be scarce in published literature. Khachik et al (34) reported total chlorophyll contents for: broccoli, cabbage, spinach, brussels sprouts, and kale. We classified all vegetables from the NLCS food frequency questionnaire into five categories most resembling these five vegetables analyzed by Khachik et al. Throughout the classification of the vegetables we also took the level of

green coloring, assumed to correlate with chlorophyll content, and the shape of the vegetables into account, as vegetables growing on a head usually have a core that is pale compared to the outer leaves. The five vegetable categories were: not green colored (white, yellow, red etc) vegetables (0 mg/100 g), pale green vegetables with a light core (leek, broad beans, 2 mg/100 g), green vegetables (brussels sprouts, green cabbage, green beans, green peppers, 6 mg/100 g), green leafy vegetables (endive, spinach, lettuce, 130 mg/100 g), dark green leafy vegetables (kale, 185 mg/100 g).

To check our classification of the vegetables included in our questionnaire but not analyzed by Khachik et al, we also measured the chlorophyll content of some of these vegetables relative to that of spinach, which was used as reference. We extracted chlorophyll from freeze-dried endive, lettuce, tomato, and spinach (bought in a local supermarket) by washing them with 80% acetone until colorless (35). The concentration chlorophyll in the extracts of these vegetables was determined by comparing their absorption spectra with those of standard chlorophyll a and b solutions in 80% acetone (Sigma) on a spectrophotometer (Lambda 2; Perkin Elmer, Norwalk, CT). Our classification of the vegetables concerned in the five chlorophyll categories was in agreement with their classification based on the chemical analysis. Mean daily intake of chlorophyll was calculated by multiplying the estimated chlorophyll content of the vegetables with their respective intake.

Statistical analyses

All analyses were conducted separately for men and women. Cox proportional hazards models were constructed to estimate hazard ratios and 95% confidence intervals relating intake of heme iron and chlorophyll to the incidence of colorectal, colon and rectal cancer (Stata version 9; Stata Corporation, College Station, Texas). The proportional hazards assumption was tested using Schoenfeld residuals. Because of the case-cohort design, the 95% confidence intervals were corrected for the additional variance introduced by using a random sampled subcohort instead of the complete cohort, by using the robust option. Rate ratios for heme iron were estimated for quintiles based on the sex-specific distribution in the subcohort and as a continuous variable with an increment of 1 mg/day. Two-sided tests for trend in the rate ratios were assessed by fitting ordinal exposure categories as continuous variables. To evaluate whether early symptoms of disease

before diagnosis could have influenced the results, early cases (diagnosed within 2 years after baseline) were also excluded from the analyses.

Age at baseline, education, cigarette smoking, non-occupational physical activity, body mass index, intake of energy, alcohol, folate, and fiber and total vegetable consumption were considered as potential confounders based on their association with risk of colorectal cancer. Of these, age at baseline (years), cigarette smoking status (never, former, current), non-occupational physical activity (<30, 30-60, 60-90, >90 min/day), body mass index (continuous, kg/m^2), alcohol intake (gender-specific categories), and total vegetable consumption (continuous, g/day) were included in the confounder-adjusted models as they were associated with heme intake and appeared to affect the RR estimates. Family history of colorectal cancer in first or second degree relatives, a strong determinant of colorectal cancer risk, was added to the multivariate models to reduce residual variation.

To evaluate the combined effect of heme and chlorophyll on the risk of colorectal, colon and rectum cancer, indicator variables were included in the regression model, representing tertiles of heme iron by tertiles of the intake of chlorophyll, using the lowest tertile of heme and the highest tertile of chlorophyll as the reference category. Total vegetable consumption was analyzed in the same manner. Presence of effect modification (on the multiplicative scale) was tested using the interaction term of the continuous heme and chlorophyll variables. Biological interaction was assessed by comparing the sum of the main effects with the combined effect (36). In addition, risk of colorectal cancer was estimated for quintiles of the molair ratio of heme to chlorophyll. Subjects with a high score on an error index for measurement of vegetable consumption, which was available in the dataset (33), were excluded from this analysis to avoid unrealistic high and low ratios.

Colon cancer was also divided in proximal (cecum through transverse colon; ICD-O codes 153.0,153.1, 153.4, 153.5, 153.6) and distal cancer (splenic flexure through sigmoid colon; ICD-O codes 153.2, 153.3, 153.7) for the analyses of main effects of heme, chlorophyll, and the heme/chlorophyll ratio.

Modification of the heme-colorectal cancer association by alcohol consumption was assessed by dividing subjects in three (gender-specific) categories of alcohol consumption.

Results

A description of the 869 male and 666 female colorectal cancer cases and 2156 male and 2215 female subcohort members is presented in **Table 1**. On average, cases were older, more overweight, and more likely to report a family history of colorectal cancer than members of the subcohort. Heme intake was higher among men compared to women and heme intake was positively associated with BMI, total energy and alcohol intake, fresh meat, processed meat, and total vegetable consumption in both genders.

Table 2A and B presents rate ratios for heme intake and colorectal cancer estimated in a Cox proportional hazard model. After adjustment for confounders, there was an indication of a consistent positive, but not statistically significant association between heme iron intake and risk of colorectal and colon cancer in men, which became more evident after exclusion of cases diagnosed in the first 2 years of follow-up (Table 2). The RR for colon cancer and the continuous heme variable was 1.16 (95% CI: 0.94-1.43) per increment of heme intake of 1 mg/day. After additional adjustment for fresh and processed meat, the RR increased to 1.38 (95% CI: 0.99-1.92) per increment of 1 mg heme per day. No association was observed for male rectal cancer, or for both cancer sites among women. Results for proximal and distal colon cancer showed that the associations were not confined to a particular site (data not shown).

Table 3 presents rate ratios for heme-related variables that may alternatively explain the relation between heme intake and colon cancer. Whereas among men no association was observed between total iron and fresh meat and colon cancer, a small increased risk was seen for men eating more than 20 g processed meat per day (RR: 1.33; 95%CI: 0.89-1.99).

Table 1. Mean (SD) daily intake of heme iron (mg/day) according to categories of non-dietary and dietary characteristics of participants of the Netherlands Cohort Study.

| | Men | | | | | | Women | | | | | |
| | Subcohort | | | Colorectal cancer cases | | | Subcohort | | | Colorectal cancer cases | | |
	n	%	Heme iron (mg/day)	n	%	Heme iron (mg/day)	n	%	Heme iron (mg/day)	n	%	Heme iron (mg/day)
All	2156	100	1.16 (0.51)	869	100	1.17 (0.53)	2215	100	0.98 (0.48)	666	100	0.97 (0.43)
Age (y)												
55-59	834	39	1.19 (0.51)	244	28	1.25 (0.62)	869	39	0.98 (0.46)	165	25	0.99 (0.44)
60-64	746	35	1.16 (0.51)	325	37	1.14 (0.46)	738	33	0.95 (0.45)	254	38	0.94 (0.40)
65-69	576	27	1.13 (0.52)	300	35	1.14 (0.53)	608	27	0.99 (0.55)	247	37	0.97 (0.44)
BMI (kg/m^2)												
< 23	425	20	1.06 (0.50)	149	17	1.07 (0.54)	597	27	0.90 (0.48)	154	23	0.88 (0.44)
23-24.9	689	32	1.16 (0.51)	262	30	1.20 (0.55)	600	27	0.95 (0.48)	174	26	0.96 (0.37)
25-26.9	565	26	1.18 (0.49)	231	27	1.13 (0.51)	418	19	0.99 (0.45)	138	21	0.98 (0.45)
≥ 27	404	19	1.23 (0.56)	195	22	1.28 (0.52)	525	24	1.06 (0.49)	173	26	1.04 (0.44)
Family history of colon cancer												
no	2044	95	1.16 (0.52)	783	90	1.18 (0.54)	2092	94	0.98 (0.49)	609	91	0.96 (0.42)
yes	112	5	1.11 (0.41)	86	10	1.16 (0.51)	123	6	0.97 (0.45)	57	9	1.00 (0.47)
Cigarette smoking status												
never	272	13	1.12 (0.53)	90	10	1.15 (0.51)	1287	58	0.98 (0.49)	406	61	0.96 (0.41)
former	1105	51	1.14 (0.49)	521	60	1.15 (0.52)	459	21	0.96 (0.46)	139	21	0.97 (0.42)
current	779	36	1.21 (0.53)	258	30	1.23 (0.58)	469	21	0.98 (0.49)	121	18	0.99 (0.49)
Non-occupational physical activity (min/day)												
< 30	392	18	1.15 (0.55)	136	16	1.20 (0.56)	547	25	0.97 (0.51)	199	30	0.98 (0.46)
30-60	661	31	1.15 (0.50)	268	31	1.14 (0.53)	679	31	0.96 (0.45)	193	29	0.97 (0.40)
60-90	401	19	1.15 (0.48)	177	20	1.19 (0.53)	491	22	0.97 (0.46)	149	22	0.93 (0.41)
> 90	678	31	1.18 (0.52)	277	32	1.20 (0.53)	468	21	1.03 (0.52)	117	18	1.00 (0.44)
Total energy intake (median MJ/day)												
Q1 (m:6.5; w:5.1)*	430	20	0.94 (0.45)	183	21	0.98 (0.46)	442	20	0.82 (0.42)	141	21	0.85 (0.45)
Q2 (m:7.9; w:6.1)	431	20	1.07 (0.48)	175	20	1.10 (0.50)	444	20	0.92 (0.48)	141	21	0.90 (0.42)
Q3 (m:8.9; w:6.9)	431	20	1.16 (0.48)	200	23	1.15 (0.47)	443	20	0.96 (0.42)	138	21	0.96 (0.37)
Q4 (m:10; w:7.8)	432	20	1.21 (0.47)	159	18	1.24 (0.53)	445	20	1.00 (0.51)	131	20	1.06 (0.39)
Q5 (m:12; w:9.2)	432	20	1.42 (0.55)	152	17	1.45 (0.63)	441	20	1.17 (0.51)	115	17	1.09 (0.46)

Alcohol intake (g/day)

303	14	1.06 (0.50)	111	13	1.04 (0.62)	681	31	0.95 (0.54)	229	34	0.94 (0.44)
447	21	1.13 (0.52)	199	23	1.15 (0.54)	765	34	0.96 (0.48)	206	31	0.93 (0.43)
585	27	1.13 (0.50)	190	22	1.17 (0.50)	394	18	1.00 (0.40)	120	18	1.00 (0.41)
480	22	1.22 (0.49)	202	23	1.19 (0.46)	201	9	1.04 (0.45)	57	9	1.11 (0.40)
313	15	1.27 (0.53)	162	19	1.30 (0.58)	73	3	1.07 (0.48)	29	4	1.09 (0.31)

Row labels: 0 / 0.1- 4.9 / 5-14.9 / 15-29.9 / ≥30

Total vegetables consumption (median g/day)

432	20	1.05(0.47)	183	21	1.08 (0.49)	446	20	0.86 (0.38)	147	22	0.93 (0.40)
431	20	1.11 (0.44)	160	18	1.12 (0.50)	443	20	0.96 (0.44)	129	19	0.92 (0.44)
435	20	1.17 (0.51)	181	21	1.15 (0.48)	449	20	0.96 (0.44)	129	19	0.95 (0.41)
432	20	1.21 (0.53)	174	20	1.20 (0.51)	439	20	1.03 (0.50)	135	20	1.04 (0.42)
426	20	1.26 (0.58)	171	20	1.34 (0.64)	438	20	1.07 (0.61)	126	19	0.99 (0.46)

Row labels: Q1 (m:102; w:106)* / Q2 (m:144; w:149) / Q3 (m:177; w:183) / Q4 (m:218; w:224) / Q5 (m:295; w:300)

Total fresh meat intake (median g/day†)

425	20	0.75 (0.38)	188	22	0.76 (0.38)	443	20	0.55 (0.30)	114	17	0.50 (0.28)
429	20	0.98 (0.31)	173	20	1.04 (0.38)	441	20	0.83 (0.30)	129	19	0.86 (0.28)
441	20	1.12 (0.38)	181	21	1.21 (0.48)	438	20	1.00 (0.37)	157	24	1.01 (0.35)
428	20	1.29 (0.41)	172	20	1.27 (0.43)	448	20	1.10 (0.37)	137	21	1.10 (0.36)
433	20	1.66 (0.54)	155	18	1.68 (0.56)	445	20	1.39 (0.58)	129	19	1.28 (0.43)

Row labels: Q1 (m:56; w:45)* / Q2 (m:86; w:74) / Q3 (m:103; w:91) / Q4 (m:124; w:108) / Q5 (m:158; w:146)

Total processed meat intake, g/day (median)

201	9	0.81 (0.46)	78	9	0.85 (0.51)	296	13	0.67 (0.43)	87	13	0.67 (0.37)
684	32	0.98 (0.41)	277	32	1.00 (0.43)	987	45	0.86 (0.40)	295	44	0.88 (0.40)
610	28	1.14 (0.43)	239	28	1.15 (0.44)	539	24	1.06 (0.43)	169	25	1.03 (0.35)
661	31	1.47 (0.53)	275	32	1.46 (0.58)	393	18	1.38 (0.51)	115	17	1.31 (0.38)

Row labels: 0 (0) / 0.1-9.9 (5) / 10 -19.9 (14) / ≥20 (32)

Total iron intake (median mg/day)

430	20	0.85 (0.33)	178	20	0.88 (0.34)	443	20	0.70 (0.31)	144	22	0.71 (0.31)
433	20	1.01 (0.38)	158	18	1.00 (0.33)	442	20	0.85 (0.34)	145	22	0.89 (0.37)
429	20	1.15 (0.43)	191	22	1.12 (0.40)	441	20	0.94 (0.37)	127	19	0.95 (0.32)
435	20	1.28 (0.51)	159	18	1.28 (0.52)	446	20	1.01 (0.41)	137	21	1.10 (0.43)
429	20	1.52 (0.59)	183	21	1.58 (0.68)	443	20	1.38 (0.64)	113	17	1.24 (0.50)

Row labels: Q1 (m:9.5; w:8.5)* / Q2 (m:11; w:10) / Q3 (m:13; w:11) / Q4 (m:15; w:13) / Q5 (m:17; w:15)

* Q: quintiles, median values for men (m) and women (w).
† based on raw weight.

Table 2A. Rate ratios (RRs) and 95 percent confidence intervals (95% CI) of colorectal, colon, and rectal cancer according to quintiles of heme iron intake of **men** in the Netherlands Cohort Study (9.3 years of follow-up).

	Men					P_Trend	Continuous*
	Q1	Q2	Q3	Q4	Q5		
Median heme intake (mg/day)	0.60	0.87	1.08	1.34	1.85		18 688
Person years	3716	3767	3709	3779	3718		
Colorectal cancer							
Cancer cases (n)	167	164	190	179	169		869†
RR (age-adjusted)	1	0.99	1.16	1.08	1.06		1.07
95% CI	ref	0.76-1.28	0.90-1.49	0.84-1.39	0.82-1.37	0.46	0.91-1.24
RR (multivariate) ‡	1	0.99	1.13	1.06	1.16		1.11
95% CI	ref	0.75-1.29	0.86-1.48	0.80-1.39	0.87-1.55	0.27	0.93-1.33
RR (> 2 yrs)	1	1.11	1.20	1.20	1.32		1.15
95% CI	ref	0.83-1.49	0.90-1.61	0.89-1.62	0.96-1.80	0.08	0.95-1.39
Colon cancer							
Cancer cases (n)	108	100	109	114	108		539
RR (age-adjusted)	1	0.94	1.03	1.07	1.06		1.04
95% CI	ref	0.69-1.27	0.76-1.39	0.80-1.45	0.78-1.43	0.47	0.86-1.25
RR (multivariate) ‡	1	0.98	1.04	1.13	1.29		1.16
95% CI	ref	0.71-1.35	0.75-1.44	0.82-1.56	0.92-1.81	0.10	0.94-1.43
RR (> 2 yrs)	1	1.08	1.07	1.29	1.50		1.22
95% CI	ref	0.76-1.53	0.75-1.53	0.90-1.83	1.03-2.17	0.02	0.98-1.52
Rectal cancer							
Cancer cases (n)	59	64	82	66	62		333
RR (age-adjusted)	1	1.08	1.41	1.10	1.08		1.11
95% CI	ref	0.74-1.58	0.98-2.01	0.76-1.60	0.74-1.58	0.69	0.89-1.39
RR (multivariate) ‡	1	1.00	1.30	0.97	0.98		1.04
95% CI	ref	0.68-1.49	0.89-1.90	0.65-1.45	0.64-1.50	0.84	0.80-1.35
RR (> 2 yrs)	1	1.17	1.44	1.10	1.06		1.04
95% CI	ref	0.75-1.80	0.95-2.19	0.71-1.72	0.66-1.71	0.94	0.79-1.38

* per increment of 1 mg heme/day.

† number of colorectal cancer cases is lower than number of colon plus rectal cancer cases due to cases with tumors in both sites.

‡ adjusted for age at baseline (y), body mass index (kg/m^2), family history of colorectal cancer(yes/no), cigarette smoker (never/former/current), non-occupational physical activity (<30, 30-60, 60-90, >90 min/day), total energy intake (kJ), consumption of alcohol: 0-4.9, 5-14.9, 15-29.9, ≥30 g/day, and total vegetable consumption (g/day).

Table 2B. Rate ratios (RRs) and 95 percent confidence intervals (95% CI) of colorectal, colon, and rectal cancer according to quintiles of heme iron intake of **women** in the Netherlands Cohort Study (9.3 years of follow-up).

	Women						
	Q1	Q2	Q3	Q4	Q5	P_{Trend}	Continuous*
Median heme intake (mg/day)	0.47	0.71	0.92	1.13	1.54		
Person years	4038	4028	3965	3991	4013		20 035
Colorectal cancer							
Cancer cases (n)	119	133	149	137	128		666
RR (age-adjusted)	1	1.12	1.29	1.16	1.11		0.96
95% CI	ref	0.84-1.48	0.98-1.70	0.88-1.54	0.83-1.47	0.46	0.81-1.13
RR (multivariate) ‡	1	1.14	1.39	1.19	1.22		1.00
95% CI	ref	0.84-1.54	1.03-1.87	0.87-1.62	0.89-1.68	0.22	0.83-1.21
RR (> 2 yrs)	1	1.09	1.47	1.18	1.20		0.99
95% CI	ref	0.78-1.51	1.08-2.02	0.85-1.65	0.86-1.69	0.24	0.81-1.20
Colon cancer							
Cancer cases (n)	84	108	108	96	88		484
RR (age-adjusted)	1	1.29	1.32	1.15	1.08		0.90
95% CI	ref	0.94-1.77	0.97-1.81	0.84-1.59	0.78-1.50	0.94	0.74-1.09
RR (multivariate) ‡	1	1.31	1.44	1.18	1.20		0.96
95% CI	ref	0.94-1.85	1.02-2.01	0.83-1.68	0.83-1.74	0.56	0.77-1.20
RR (> 2 yrs)	1	1.26	1.56	1.26	1.19		0.97
95% CI	ref	0.87-1.83	1.09-2.23	0.86-1.83	0.80-1.77	0.43	0.78-1.22
Rectal cancer							
Cancer cases (n)	35	26	41	43	40		185
RR (age-adjusted)	1	0.73	1.15	1.23	1.17		1.12
95% CI	ref	0.43-1.23	0.72-1.83	0.77-1.95	0.73-1.88	0.14	0.88-1.43
RR (multivariate) ‡	1	0.72	1.19	1.27	1.23		1.12
95% CI	ref	0.40-1.27	0.72-1.98	0.76-2.13	0.73-2.07	0.11	0.86-1.46
RR (> 2 yrs)	1	0.69	1.18	1.07	1.20		1.05
95% CI	ref	0.37-1.29	0.68-2.05	0.60-1.91	0.68-2.12	0.25	0.76-1.43

* per increment of 1 mg heme/day.

† number of colorectal cancer cases is lower than number of colon plus rectal cancer cases due to cases with tumors in both sites.

‡ adjusted for age at baseline (y), body mass index (kg/m^2), family history of colorectal cancer(yes/no), cigarette smoker (never/former/current), non-occupational physical activity (<30, 30-60, 60-90, >90 min/day), total energy intake (kJ), consumption of alcohol: 0, 0.1-4.9, 5-14.9, ≥15 g/day, and total vegetable consumption (g/day).

Table 3. Multivariate-adjusted* rate ratios (RRs) and 95 percent confidence intervals (95% CI) of colorectal, colon, and rectal cancer according to total iron intake, total fresh meat, and processed meat consumption of men and women in the Netherlands Cohort Study (9.3 years of follow-up).

	Men						Women					
	Q1	Q2	Q3	Q4	Q5	P_{Trend}	Q1	Q2	Q3	Q4	Q5	P_{Trend}
Colorectal cancer												
Total iron RR	1	0.92	1.16	1.02	1.34		1	1.22	1.01	1.11	1.08	
95% CI	ref	0.69-1.23	0.86-1.55	0.75-1.39	0.93-1.93	0.12	ref	0.91-1.63	0.74-1.39	0.80-1.55	0.72-1.62	0.90
Fresh meat RR	1	1.00	0.91	0.93	0.82		1	1.12	1.30	1.13	1.10	
95% CI	ref	0.77-1.30	0.70-1.19	0.72-1.22	0.62-1.08	0.15	ref	0.82-1.52	0.97-1.75	0.83-1.53	0.80-1.51	0.57
Processed meat (categories) RR	1	1.02	0.98	1.18			1	1.04	1.13	1.05		
95% CI	ref	0.74-1.41	0.71-1.36	0.84-1.64		0.25	ref	0.78-1.39	0.82-1.55	0.74-1.48		0.62
Colon cancer												
Total iron RR	1	0.90	0.98	0.85	1.30		1	1.30	0.89	1.12	1.14	
95% CI	ref	0.64-1.26	0.69-1.40	0.58-1.24	0.84-2.01	0.43	ref	0.94-1.81	0.62-1.29	0.77-1.64	0.73-1.80	0.91
Total fresh meat RR	1	1.01	1.07	1.04	0.88		1	1.04	1.30	1.07	1.00	
95% CI	ref	0.74-1.38	0.79-1.47	0.76-1.43	0.63-1.23	0.59	ref	0.73-1.47	0.94-1.80	0.76-1.51	0.70-1.45	0.89
Processed meat (categories) RR	1	1.11	1.09	1.33			1	1.00	1.06	1.07		
95% CI	ref	0.75-1.63	0.73-1.63	0.89-1.99		0.13	ref	0.72-1.39	0.74-1.51	0.73-1.57		0.61
Rectal cancer												
Total iron RR	1	1.02	1.54	1.39	1.44		1	0.97	1.41	1.13	1.11	
95% CI	ref	0.67-1.56	1.02-2.32	0.89-2.16	0.85-2.45	0.08	ref	0.57-1.66	0.83-2.38	0.63-2.04	0.53-2.30	0.63
Total fresh meat RR	1	1.03	0.73	0.81	0.78		1	1.42	1.30	1.37	1.40	
95% CI	ref	0.72-1.48	0.49-1.07	0.56-1.18	0.53-1.14	0.09	ref	0.83-2.43	0.76-2.22	0.80-2.35	0.81-2.42	0.33
Processed meat (categories) RR	1	0.90	0.83	0.96			1	1.19	1.42	1.01		
95% CI	ref	0.58-1.40	0.52-1.31	0.60-1.53		0.99	ref	0.71-2.02	0.81-2.50	0.54-1.90		0.83

* adjusted for age at baseline (y), body mass index (kg/m^2), family history of colorectal cancer (yes/no), cigarette smoker (never/former/current), non-occupational physical activity (<30, 30-60, 60-90, >90 min/day), total energy intake (kJ), consumption of alcohol (for men: 0-4.9, 5-14.9, 15-29.9, ≥30 g/day; for women: 0, 0.1-4.9, 5-14.9, ≥15 g/day) and total vegetable consumption (g/day).

The combined effect of heme and chlorophyll intake was evaluated by calculating the risk of colorectal cancer for heme in tertiles of chlorophyll intake (**Tables 4 and 5**). The category that we hypothesized to be associated with the lowest risk (low heme in combination with high chlorophyll) was used as the reference category. High intake of heme in combination with low intake of chlorophyll appeared to be associated with an elevated risk of colon cancer in men (RR: 1.58; 95%CI: 0.99-2.54). There was no evidence of effect modification on the multiplicative scale (p-value for interaction: 0.40) and weak evidence for biological interaction assessed on the additive scale. Similar results were observed for the combined effect of heme and vegetables, although the dose-response association was less consistent. The hypothesized highest risk category corresponded with a RR of 2.02 (95% CI: 1.22-3.34) compared to the lowest risk category. Neither for male rectal cancer nor for female colon or rectal cancer an association emerged in the hypothesized direction. We also estimated the heme colon cancer risk for the molar ratio of heme to chlorophyll. Multivariate RRs (95% CIs) for successive quintiles of the heme/chlorophyll ratio compared to the lowest quintile were 1.08 (0.77-1.51), 1.01 (0.72-1.41), 1.32 (0.95-1.84), and 1.43 (1.03-1.97) in men (p-value for trend: 0.01) (**Fig. 1**) and 1.33 (0.94-1.87), 1.15 (0.81-1.64), 1.34 (0.95-1.89), and 1.12 (0.78-1.59) in women (p-value for trend: 0.61). These results were very similar for proximal and distal colon cancer (data not shown).

Tables 4 and 5 also address the association of heme and colorectal cancer across three levels of alcohol consumption. In men, no evidence of a differential association was observed. In women, a small elevated risk of colon and rectal cancer was observed for those in the highest tertile of heme intake and drinking more than 5 g alcohol per day as compared to non-drinkers with a low heme intake (RR for colorectal cancer: 1.48, 95% CI: 1.00-2.19).

Table 4. Multivariate-adjusted* rate ratios (RR) and 95% confidence interval (95%CI) of colorectal, colon, and rectal cancer by tertiles of heme iron intake, according to tertiles of dietary intake of chlorophyll, total vegetables consumption, and alcohol consumption among **men**.

	Colorectal cancer			Colon cancer			Rectal cancer		
	Heme iron (median mg/day)			Heme iron (median mg/day)			Heme iron (median mg/day)		
	Tertile 1 (0.67) RR (95%CI)	Tertile 2 (1.08) RR (95%CI)	Tertile 3 (1.74) RR (95%CI)	Tertile 1 (0.67) RR (95%CI)	Tertile 2 (1.08) RR (95%CI)	Tertile 3 (1.74) RR (95%CI)	Tertile 1 (0.67) RR (95%CI)	Tertile 2 (1.08) RR (95%CI)	Tertile 3 (1.74) RR (95%CI)
Chlorophyll (median intake mg/day)									
tertile 3 (86)	1 (ref)	1.17 (0.79-1.73)	1.14 (0.78-1.68)	1 (ref)	1.06 (0.65-1.71)	1.16 (0.73-1.85)	1 (ref)	1.45 (0.85-2.47)	1.12 (0.65-1.93)
tertile 2 (48)	1.21 (0.82-1.77)	1.13 (0.77-1.67)	1.19 (0.80-1.78)	1.14 (0.72-1.82)	1.09 (0.68-1.75)	1.30 (0.80-2.09)	1.32 (0.76-2.26)	1.20 (0.69-2.07)	1.02 (0.57-1.82)
tertile 1 (26)	1.13 (0.77-1.65)	1.31 (0.90-1.92)	1.34 (0.90-1.99)	1.27 (0.81-1.99)	1.30 (0.82-2.06)	1.58 (0.99-2.54)	0.87 (0.49-1.55)	1.37 (0.80-2.34)	0.98 (0.54-1.76)
Vegetables (median intake g/day)									
tertile 3 (283)	1 (ref)	1.41 (0.96-2.07)	1.28 (0.88-1.87)	1 (ref)	1.50 (0.92-2.44)	1.47 (0.91-2.36)	1 (ref)	1.30 (0.78-2.16)	1.01 (0.60-1.69)
tertile 2 (178)	1.35 (0.92-1.99)	1.16 (0.78-1.72)	1.24 (0.83-1.85)	1.70 (1.05-2.73)	1.39 (0.85-2.26)	1.46 (0.89-2.4))	0.88 (0.51-1.50)	0.89 (0.52-1.54)	0.95 (0.55-1.64)
tertile 1 (113)	1.16 (0.79-1.71)	1.25 (0.85-1.84)	1.37 (0.90-2.07)	1.39 (0.86-2.24)	1.26 (0.77-2.06)	2.02 (1.22-3.34)	0.83 (0.48-1.43)	1.20 (0.71-2.01)	0.63 (0.34-1.18)
Alcohol consumption (g/day)‡									
0-4.9 (1.4)	1 (ref)	1.06 (0.76-1.49)	1.06 (0.74-1.50)	1 (ref)	0.90 (0.60-1.34)	1.03 (0.68-1.55)	1 (ref)	1.48 (0.90-2.43)	1.17 (0.69-2.00)
5-14.9 (10)	0.75 (0.51-1.09)	0.80 (0.55-1.16)	1.07 (0.72-1.59)	0.68 (0.43-1.06)	0.77 (0.50-1.18)	1.13 (0.72-1.79)	0.96 (0.55-1.68)	1.01 (0.58-1.75)	0.95 (0.50-1.77)
≥15 (31)	1.15 (0.81-1.63)	1.24 (0.90-1.70)	1.13 (0.82-1.56)	0.99 (0.65-1.50)	1.01 (0.69-1.48)	1.06 (0.73-1.54)	1.50 (0.91-2.48)	1.77 (1.11-2.82)	1.30 (0.79-2.13)

* adjusted for age at baseline (y), body mass index (kg/m^2), family history of colorectal cancer (yes/no), cigarette smoker (never/former/current), non-occupational physical activity (<30, 30-60, 60-90, >90 min/day), total energy intake (kJ), and consumption of alcohol (except for analysis of alcohol).

‡ additional adjustment for total vegetable consumption (g/day).

Table 5. Multivariate-adjusted* rate ratios (RR) and 95% confidence interval (95%CI) of colorectal, colon, and rectal cancer by tertiles of heme iron intake, according to tertiles of dietary intake of chlorophyll, total vegetables consumption, and alcohol consumption among **women**.

	Colorectal cancer			Colon cancer			Rectal cancer		
	Heme iron (median mg/day)			Heme iron (median mg/day)			Heme iron (median mg/day)		
	Tertile 1 (0.53) RR (95%CI)	Tertile 2 (0.91) RR (95%CI)	Tertile 3 (1.47) RR (95%CI)	Tertile 1 (0.53) RR (95%CI)	Tertile 2 (0.91) RR (95%CI)	Tertile 3 (1.47) RR (95%CI)	Tertile 1 (0.53) RR (95%CI)	Tertile 2 (0.91) RR (95%CI)	Tertile 3 (1.47) RR (95%CI)
Chlorophyll (median intake mg/day)									
tertile 3 (84)	1 (ref)	1.13 (0.73-1.75)	1.21 (0.80-1.84)	1 (ref)	0.99 (0.61-1.60)	0.96 (0.60-1.54)	1 (ref)	1.65 (0.73-3.76)	2.23 (1.04-4.79)
tertile 2 (48)	1.08 (0.71-1.66)	1.32 (0.88-2.00)	1.43 (0.94-2.19)	0.98 (0.61-1.57)	1.24 (0.79-1.96)	1.27 (0.79-2.04)	1.51 (0.68-3.36)	1.55 (0.70-3.44)	2.11 (0.97-4.60)
tertile 1 (25)	1.12 (0.74-1.70)	1.23 (0.81-1.86)	1.10 (0.71-1.69)	1.07 (0.67-1.69)	1.21 (0.77-1.92)	1.04 (0.64-1.68)	1.40 (0.63-3.11)	1.17 (0.52-2.66)	1.40 (0.61-3.21)
Vegetables (median intake g/day)									
tertile 3 (284)	1 (ref)	0.94 (0.61-1.43)	1.11 (0.74-1.66)	1 (ref)	0.92 (0.58-1.48)	0.96 (0.61-1.53)	1 (ref)	0.86 (0.40-1.85)	1.43 (0.73-2.77)
tertile 2 (184)	0.78 (0.51-1.19)	1.17 (0.78-1.75)	0.97 (0.64-1.48)	0.75 (0.46-1.21)	1.15 (0.73-1.81)	0.92 (0.58-1.48)	0.80 (0.38-1.70)	1.08 (0.54-2.16)	1.07 (0.53-2.18)
tertile 1 (117)	0.97 (0.65-1.45)	1.02 (0.68-1.53)	1.08 (0.71-1.65)	1.01 (0.65-1.58)	1.04 (0.66-1.64)	1.04 (0.65-1.69)	0.75 (0.36-1.57)	0.81 (0.39-1.68)	1.13 (0.55-2.32)
Alcohol consumption (median g/day)‡									
0	1 (ref)	1.65 (1.13-2.42)	1.24 (0.83-1.86)	1 (ref)	1.91 (1.24-2.95)	1.26 (0.79-2.01)	1 (ref)	0.93 (0.47-1.83)	1.20 (0.63-2.30)
0.1-4.9 (1.9)	1.11 (0.75-1.64)	1.02 (0.69-1.52)	1.08 (0.72-1.64)	1.36 (0.88-2.10)	1.02 (0.64-1.61)	1.10 (0.68-1.77)	0.52 (0.24-1.12)	0.99 (0.51-1.90)	1.02 (0.52-2.03)
≥5 (17)	1.18 (0.76-1.81)	1.15 (0.76-1.73)	1.48 (1.00-2.19)	1.24 (0.76-2.04)	1.26 (0.79-2.00)	1.50 (0.95-2.36)	1.05 (0.51-2.15)	0.82 (0.40-1.68)	1.41 (0.76-2.61)

* adjusted for age at baseline (y), body mass index (kg/m^2), family history of colorectal cancer (yes/no), cigarette smoker (never/former/current), non-occupational physical activity (<30, 30-60, 60-90, >90 min/day), total energy intake (kJ) and consumption of alcohol (except for analysis of alcohol).

‡ additional adjustment for total vegetable consumption (g/day).
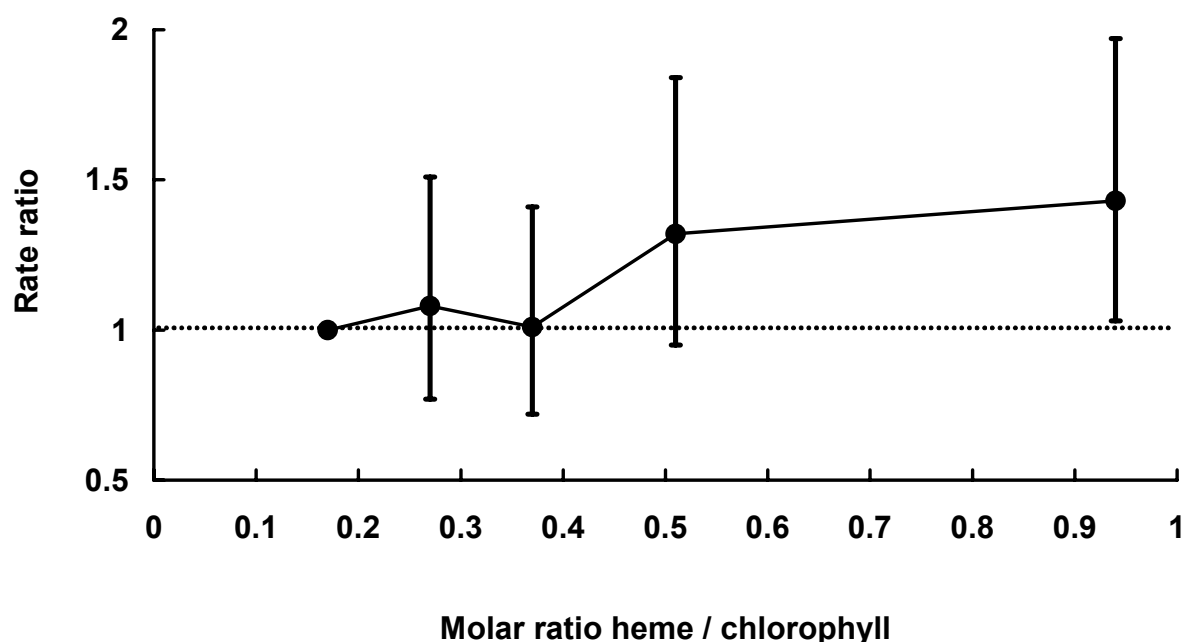
Figure 1. Rate ratios (dots) and 95% confidence intervals (vertical bars) of colon cancer in men according to quintiles of the molar ratio of heme to chlorophyll intake (p-trend: 0.01).

Discussion

In this cohort study with 539 male and 448 female incident colon and 333 male and 185 female incident rectal cancer patients, a small positive association was observed between heme intake and colon cancer among men, but not among women. After stratification by chlorophyll intake, the male subjects with the lowest chlorophyll and the highest heme intake had the highest risk of colon cancer and this was also shown by the statistically significant positive association between the heme/chlorophyll ratio and colon cancer in men. No consistent associations were observed for rectal cancer, both in men and women. The prospective design and the completeness of follow-up of our study (>95% (37)) ensured that information bias due to disease status and selection bias due to loss of follow-up are unlikely. In addition, the associations did not appear to be spuriously produced by symptoms of early, but not yet diagnosed disease, as the exclusion of cases diagnosed within the first two years of follow-up made the associations more evident, indicating that early cases had a somewhat lower heme intake than later cases.

Our study also has some limitations. Measurement error can be substantial in dietary assessment. The validation study of our food frequency questionnaire has shown that it performs relatively well (27), but measurement error will still have attenuated associations. Furthermore, as neither heme nor chlorophyll was included in the Dutch food composition table, the content of these compounds was estimated on the basis of reported levels in the literature. This may have resulted in additional measurement error, although the estimation of heme intake was based on more available data than that of chlorophyll intake. However, although there is a considerable chance of misclassification, it is unlikely that this misclassification would be differential with regard to the endpoint. Also, heme-iron content values as calculated in this study (i.e., based on type-specific percentage of total iron content) appeared to be in reasonable agreement with absolute heme-iron values in meat and fish available from the same literature sources. Finally, even though our results for heme support prior biological hypotheses and agree with studies in rats (15) and in humans (20), we can not rule out the possibility of chance findings within small subgroups.

Previously published results on meat and colorectal cancer risk in the NLCS, based on 3.3 years (4) and 7.3 years (21) of follow-up, showed no association between consumption of total fresh meat and fish and colon cancer risk in men and women. A positive association for both men and women was initially observed for processed meat and colon cancer (4), but diminished, particularly in women, after longer follow-up. The current results for fresh meat and colon cancer, based on extended follow-up, are still in line with the previous.

The weak, but consistent association between heme intake and colon cancer observed in men, was driven by consumption of (cooked) beef, which contains 3-4 times as much heme as (cooked) pork. The contribution of each type of meat to the total fresh meat consumption was approximately 40, 42, 14, and 4% for beef, pork, chicken, and other meat (including horse and lamb) in this population. The relative high proportion of pork may explain the absence of an association with fresh meat in our study. Other cohorts have frequently used "red meat", which includes beef, pork and lamb, as key variable in meat and cancer studies, but have only rarely reported on the association between colorectal cancer and specific types of meat, also because some frequently used food frequency questionnaires did not distinguish beef from pork consumption. In North-American cohorts, consumption of beef is roughly three times higher than that of pork. However, recent results from the European Prospective Investigation into Cancer and

Nutrition (EPIC) showed a stronger association of colorectal cancer risk with pork than with beef plus veal (12).

The rate ratios for heme and colon cancer became stronger when additional adjustment for fresh and processed meat was performed, indicating that a meat effect, if any, is more likely to be due to heme and not to other constituents or preparation methods of meat. Most epidemiologic studies that determined the risk of specific constituents of (red) meat considered only total dietary iron (38). However, as approximately 90% of the total dietary iron consists of non-heme iron, associations of total dietary iron with colorectal cancer in most epidemiologic studies may be attenuated by other compounds contained in the non-meat sources of this non-heme iron. Two studies examined associations among colon cancer incidence and dietary intake of heme iron among women (22,23). Both observed an increased risk of colon cancer with increasing heme-iron intake; however this association was confined to proximal colon tumors in the Iowa Women's Health Study (22), whereas the study in Sweden likely observed the association for distal tumors only (11). In both studies, the positive association between heme iron and colon cancer was stronger among women who consumed alcohol than among those who consumed little or no alcohol. Neither study took the type of meat into account in the assessment of heme iron as they applied a fixed percentage (40%) to the total iron content of all meat items. Although we did not observe an association between heme intake and colon cancer among women, we observed a suggestion of an association with colon and rectal cancer among women who drank more than 5 g alcohol (0-1 glass) per day, which is comparable to the level above which an effect was seen in Iowa and Sweden (22,23).

We did not beforehand anticipate a different association for men and women. However, there are several plausible explanations for the different findings. An explanation could be the total intake of heme and chlorophyll. Men are known to consume more food in general and more meat and relatively less vegetable than women, as was also evident from our data. Alternatively, it could also be hypothesized that, as women need more iron due to menstrual losses, and as heme iron is more easily absorbed compared to non-heme iron, relatively more iron from heme is absorbed in women, so that less heme is available during lifetime up to menopause to form the cytotoxic factor in the bowel.

Our results are also consistent with those of experimental studies among human volunteers (19,20). As these studies were conducted in men only, we do not know whether women would have shown a different effect of red meat and heme.

Evidence of a protective effect of (green) vegetables for colorectal cancer risk (39,40) stimulated us to investigate the interaction of heme and spinach in an animal model study. We demonstrated that spinach or an equimolar amount of chlorophyll inhibited dietary heme-induced luminal cytotoxicity and damage to colonic mucosa in rats (16). The addition of chlorophyll to a heme diet prevented the formation of a cytotoxic heme metabolite. We speculated that chlorophyll traps heme in hydrophobic heme-chlorophyll complexes in the gut lumen and as a result blocks the pro-oxidant activity of heme (16,41). This mechanism implies that heme and chlorophyll must be consumed simultaneously. The present study indicates that an increase in risk is indeed associated with an increase in the heme iron to chlorophyll ratio, although the evidence for biological interaction between heme and chlorophyll was not strong. However, our FFQ was not designed to capture combinations of specific types of meat with specific types of vegetables in the same meal, even though vegetables are mostly eaten in combination with meat, in particular in this population with traditional dietary habits. We can therefore expect that biological interaction, if present, is underestimated, due to dilution effects of the heme and chlorophyll variables calculated as mean daily intake. A human experimental study has not observed inhibition of heme-induced formation of N-nitroso compounds by vegetables, but the vegetables used in that study (broccoli, peas, and Brussels sprouts) were low in chlorophyll compared to high levels of heme in the type and quantities of meat used (42). Our rat studies showed only detrimental dietary heme-induced effects in the absence or at low concentrations of chlorophyll (manuscript in preparation). Furthermore, processed meats, in contrast to fresh meats, are often not consumed together with vegetables. This might also explain why the association with processed meats and colorectal cancer is clearer than for fresh meats.

In conclusion, we hypothesized that heme might be positively associated with colon cancer risk despite absence of an association with meat and that chlorophyll intake would modify this association. Our results confirm these hypotheses in part, but further research is needed, particularly on the role of chlorophyll and the association in women.

Acknowledgments

References

1. Norat, T. and Riboli, E. (2001) Meat consumption and colorectal cancer: a review of epidemiologic evidence. *Nutr Rev*, **59**, 37-47.
2. Sandhu, M.S., White, I.R. and McPherson, K. (2001) Systematic review of the prospective cohort studies on meat consumption and colorectal cancer risk: a meta-analytical approach. *Cancer Epidemiol Biomarkers Prev*, **10**, 439-46.
3. Norat, T., Lukanova, A., Ferrari, P. and Riboli, E. (2002) Meat consumption and colorectal cancer risk: dose-response meta- analysis of epidemiological studies. *Int J Cancer*, **98**, 241-256.
4. Goldbohm, R.A., van den Brandt, P.A., van 't Veer, P., Brants, H.A., Dorant, E., Sturmans, F. and Hermus, R.J. (1994) A prospective cohort study on the relation between meat consumption and the risk of colon cancer. *Cancer Res*, **54**, 718-23.
5. Willett, W.C., Stampfer, M.J., Colditz, G.A., Rosner, B.A. and Speizer, F.E. (1990) Relation of meat, fat, and fiber intake to the risk of colon cancer in a prospective study among women. *N Engl J Med*, **323**, 1664-72.
6. Giovannucci, E., Rimm, E.B., Stampfer, M.J., Colditz, G.A., Ascherio, A. and Willett, W.C. (1994) Intake of fat, meat, and fiber in relation to risk of colon cancer in men. *Cancer Res*, **54**, 2390-7.
7. Chao, A., Thun, M.J., Connell, C.J., McCullough, M.L., Jacobs, E.J., Flanders, W.D., Rodriguez, C., Sinha, R. and Calle, E.E. (2005) Meat consumption and risk of colorectal cancer. *Jama*, **293**, 172-82.
8. Kojima, M., Wakai, K., Tamakoshi, K., Tokudome, S., Toyoshima, H., Watanabe, Y., Hayakawa, N., Suzuki, K., Hashimoto, S., Ito, Y. and Tamakoshi, A. (2004) Diet and colorectal cancer mortality: results from the Japan Collaborative Cohort Study. *Nutr Cancer*, **50**, 23-32.
9. English, D.R., MacInnis, R.J., Hodge, A.M., Hopper, J.L., Haydon, A.M. and Giles, G.G. (2004) Red meat, chicken, and fish consumption and risk of colorectal cancer. *Cancer Epidemiol Biomarkers Prev*, **13**, 1509-14.
10. Wei, E.K., Giovannucci, E., Wu, K., Rosner, B., Fuchs, C.S., Willett, W.C. and Colditz, G.A. (2004) Comparison of risk factors for colon and rectal cancer. *Int J Cancer*, **108**, 433-42.
11. Larsson, S.C., Rafter, J., Holmberg, L., Bergkvist, L. and Wolk, A. (2005) Red meat consumption and risk of cancers of the proximal colon, distal colon and rectum: the Swedish Mammography Cohort. *Int J Cancer*, **113**, 829-34.
12. Norat, T., Bingham, S., Ferrari, P., Slimani, N., Jenab, M., Mazuir, M., Overvad, K., Olsen, A., Tjonneland, A., Clavel, F., Boutron-Ruault, M.C., Kesse, E., Boeing, H., Bergmann, M.M., Nieters, A., Linseisen, J., Trichopoulou, A., Trichopoulos, D., Tountas, Y., Berrino, F., Palli, D., Panico, S., Tumino, R., Vineis, P., Bueno-de-Mesquita, H.B., Peeters, P.H., Engeset, D., Lund, E., Skeie, G., Ardanaz, E., Gonzalez, C., Navarro, C., Quiros, J.R., Sanchez, M.J., Berglund, G., Mattisson, I., Hallmans, G., Palmqvist, R., Day, N.E., Khaw, K.T., Key, T.J., San Joaquin, M., Hemon, B., Saracci, R., Kaaks, R. and Riboli, E. (2005) Meat, fish, and colorectal cancer risk: the European Prospective Investigation into cancer and nutrition. *J Natl Cancer Inst*, **97**, 906-16.
13. Lin, J., Zhang, S.M., Cook, N.R., Lee, I.M. and Buring, J.E. (2004) Dietary fat and fatty acids and risk of colorectal cancer in women. *Am J Epidemiol*, **160**, 1011-22.
14. Young, G.P., Rose, I.S. and St John, D.J. (1989) Haem in the gut. I. Fate of haemoproteins and the absorption of haem. *J Gastroenterol Hepatol*, **4**, 537-45.
15. Sesink, A.L.A., Termont, D.S., Kleibeuker, J.H. and Van der Meer, R. (1999) Red meat and colon cancer: the cytotoxic and hyperproliferative effects of dietary heme. *Cancer Res*, **59**, 5704-5709.

16. de Vogel, J., Jonker-Termont, D.S., van Lieshout, E.M., Katan, M.B. and van der Meer, R. (2005) Green vegetables, red meat and colon cancer: chlorophyll prevents the cytotoxic and hyperproliferative effects of haem in rat colon. *Carcinogenesis*, **26**, 387-93.

17. Lipkin, M. (1988) Biomarkers of increased susceptibility to gastrointestinal cancer: new application to studies of cancer prevention in human subjects. *Cancer Res*, **48**, 235-245.

18. Pierre, F., Tache, S., Petit, C.R., Van Der Meer, R. and Corpet, D.E. (2003) Meat and cancer: haemoglobin and haemin in a low-calcium diet promote colorectal carcinogenesis at the aberrant crypt stage in rats. *Carcinogenesis*, **24**, 1683-1690.

19. Bingham, S.A., Hughes, R. and Cross, A.J. (2002) Effect of white versus red meat on endogenous N-nitrosation in the human colon and further evidence of a dose response. *J Nutr*, **132**, 3522S-3525S.

20. Cross, A.J., Pollock, J.R. and Bingham, S.A. (2003) Haem, not protein or inorganic iron, is responsible for endogenous intestinal N-nitrosation arising from red meat. *Cancer Res*, **63**, 2358-2360.

21. Bausch-Goldbohm, R.A., Voorrips, L.E., Bos, G. and van den Brandt, P.A. (2001) Wel of geen relatie tussen vlees en dikkedarmkanker? Nieuw licht op tegenstrijdige resultaten. *Voeding Nu*, **3**, 9-12.

22. Lee, D.H., Anderson, K.E., Harnack, L.J., Folsom, A.R. and Jacobs, D.R., Jr. (2004) Heme iron, zinc, alcohol consumption, and colon cancer: Iowa Women's Health Study. *J Natl Cancer Inst*, **96**, 403-7.

23. Larsson, S.C., Adami, H.O., Giovannucci, E. and Wolk, A. (2005) Re: Heme iron, zinc, alcohol consumption, and risk of colon cancer. *J Natl Cancer Inst*, **97**, 232-3; author reply 233-4.

24. Carpenter, C.E. and Mahoney, A.W. (1995) Evaluation of methods used in meat iron analysis and iron content of raw and cooked meats. *Journal of Agricultural Food Chemistry*, **43**, 1824-7.

25. van den Brandt, P.A., Goldbohm, R.A., van 't Veer, P., Volovics, A., Hermus, R.J. and Sturmans, F. (1990) A large-scale prospective cohort study on diet and cancer in The Netherlands. *J Clin Epidemiol*, **43**, 285-95.

26. Van den Brandt, P.A., Schouten, L.J., Goldbohm, R.A., Dorant, E. and Hunen, P.M. (1990) Development of a record linkage protocol for use in the Dutch Cancer Registry for Epidemiological Research. *Int J Epidemiol*, **19**, 553-8.

27. Goldbohm, R.A., van den Brandt, P.A., Brants, H.A., van't Veer, P., Al, M., Sturmans, F. and Hermus, R.J. (1994) Validation of a dietary questionnaire used in a large-scale prospective cohort study on diet and cancer. *Eur J Clin Nutr*, **48**, 253-65.

28. Voorlichtingsbureau voor de Voeding. NEVO table: Dutch Food Composition Table 1986 - 1987. *1986, The Hague*.

29. Clark, E.M., Mahoney, A.W. and Carpenter, C.E. (1997) Heme and total iron in ready-to-eat chicken. *Journal of Agricultural Food Chemistry*, **45**, 124-6.

30. Kalpalathika, P.V.M., Clark, E.M. and Mahoney, A.W. (1991) Heme iron content in ready-to-serve beef products. *Journal of Agricultural Food Chemistry*, **39**, 1091-3.

31. Kongkachuichai, R., Napatthalung, P. and Charoensiri, R. (2002) Heme and nonheme iron content of animal product commonly consumed in Thailand. *Journal of Food Composition*, **15**, 398-98.

32. Lombardi-Boccia, G., Martinez-Dominguez, B., Aguzzi, A. and Rincon-Leon, F. (2002) Optimazation of heme iron analyses in raw and cooked red meat. *Food Chemistry*, **78**, 505-10.

33. Voorrips, L.E., Goldbohm, R.A., Verhoeven, D.T., van Poppel, G.A., Sturmans, F., Hermus, R.J. and van den Brandt, P.A. (2000) Vegetable and fruit consumption and lung cancer risk in the Netherlands Cohort Study on diet and cancer. *Cancer Causes Control*, **11**, 101-15.

34. Khachik, F., Beecher, G.R. and Whittaker, N.F. (1986) Separation, Identification, and Quantification of the Major Carotenoid and Chlorophyll Constituents in Extracts of Several green Vegetables by Liquid Chromatography. *Jounal of Agricultural Food Chemistry*, **34**, 603-616.

35. Coombs, J., Hall, D.O. and Long, S.P. (1985) *Techniques in bioproductivity and photosynthesis*. Oxford: Pergamon.

36. Rothman, K.J. (2002) *Epidemiology. An introduction*. New York: Oxford University Press.

37. Goldbohm, R.A., van den Brandt, P.A. and Dorant, E. (1994) Estimation of the coverage of Dutch municipalities by cancer registries and PALGA based on hospital discharge data. *Tijdschrift Sociale Gezondheidszorg*, **72**, 80-4.

38. Nelson, R.L. (2001) Iron and colorectal cancer risk: human studies. *Nutr Rev*, **59**, 140-8.

39. *World Cancer Research Fund. Food, Nutrition and the prevention of cancer: a global perspective, 1997*, Washington, DC: American Institute for Cancer Research.

40. Steinmetz, K.A. and Potter, J.D. (1996) Vegetables, fruit, and cancer prevention: a review. *J Am Diet Assoc*, **96**, 1027-1039.

41.   de Vogel, J., Jonker-Termont, D.S., Katan, M.B. and van der Meer, R. (2005) Natural chlorophyll but not chlorophyllin prevents heme-induced cytotoxic and hyperproliferative effects in rat colon. *J Nutr*, **135**, 1995-2000.
42.   Hughes, R., Pollock, J.R. and Bingham, S. (2002) Effect of vegetables, tea, and soy on endogenous N-nitrosation, fecal ammonia, and fecal water genotoxicity during a high red meat diet in humans. *Nutr Cancer*, **42**, 70-77.

Chapter 7

General discussion

Epidemiological studies show that consumption of diets with a high percentage of red meat and processed meat, in contrast to white meat, is associated with an increased risk of colon cancer (1). Consumption of vegetables and especially green vegetables is associated with a decreased risk of colon cancer (2,3). The mechanism by which consumption of red meat and green leafy vegetables modulates colon cancer risk is still unknown. **Therefore we studied what molecular mechanism explains the effects of consumption of red meat and green leafy vegetables on the risk of colon cancer.**

Heme

Red meat could be associated with increased colon cancer risk because of its high content of heme (4,5). Previous studies showed that metabolites of the poorly absorbed dietary heme increased the amount of irritants in the gut lumen and caused hyperproliferation in the colon mucosa of rats (6). We recently demonstrated that this epithelial hyperproliferation was accompanied with injury to the colon surface epithelium (chapter 2). Furthermore, heme-induced cytotoxicity decreased apoptosis in the colon mucosa. This implies that heme disturbs cell homeostasis of colonic epithelium. Homeostasis is the balance between cell proliferation at the base of the crypt and cell loss and death at the luminal surface (**Fig. 1**).
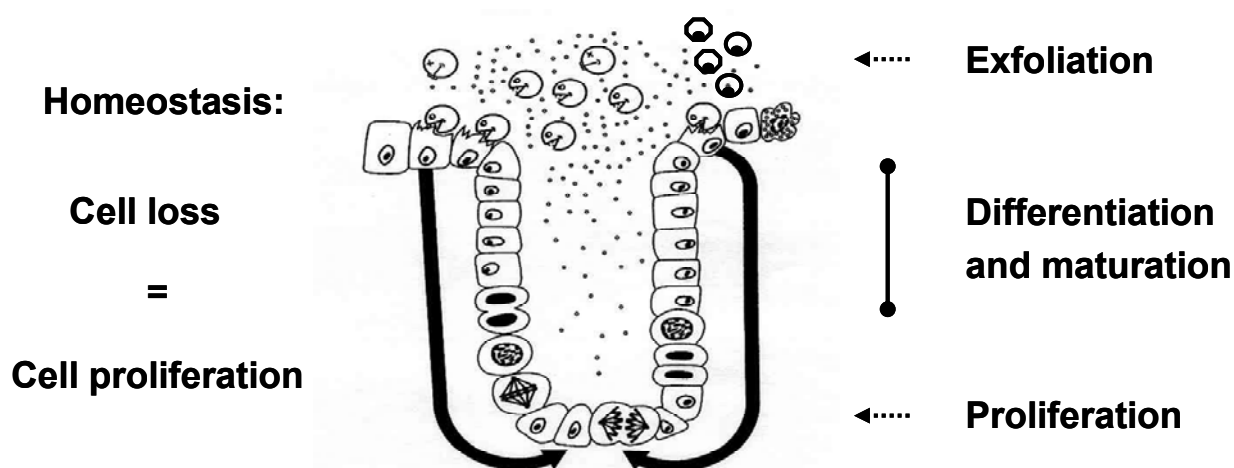
Figure 1. Homeostasis in the colonic crypt. The pacmans represent dietary heme-induced injury to the surface epithelium.

Disruption of homeostasis, i.e. increased proliferation and decreased apoptosis, could increase the risk of acquiring endogenous mutations in tumor suppressor genes and oncogenes initiating colon epithelial cell carcinogenesis.

Colon tissues of rats fed heme did not show signs of inflammation, indicating that the integrity of the basal lamina is maintained. We speculate that heme-induced injury to the surface epithelial cells is repaired by a process of epithelial restitution. During this process epithelial cells bordering the wound are stimulated to migrate and replace the injured cells, maintaining integrity of the surface epithelium. This repair process is regulated by numerous peptide and non-peptide factors that come from the luminal environment or are produced by a variety of mucosal and submucosal cells (7-9). In the mean time, signals sent to the stem cell compartment at the base of the crypt (arrows in Fig. 1) stimulate epithelial proliferation to restore cells lost from the crypt surface.

Besides heme-induced modulation of physiological markers, we also showed that changes in the amount of luminal irritants modulated gene expression of some colon-expressed genes (10). Most striking was a heme-induced 10-fold down-regulation of a novel mucosal pentraxin (Mptx) (10,11). The high fold-changes in expression of colon-expressed genes imply that the expression of these genes is very susceptible to diet-induced changes in luminal irritants compared with the response observed on a physiological marker like epithelial cell proliferation. The functional implications of the changes in gene expression are unfortunately not yet clear and should be the subject of future research. Nevertheless, these results provided us with an extra biomarker to study the response of colon mucosa to changes in composition of the luminal contents. We studied dietary heme-induced effects in our rat-model only for two weeks, but it was also shown that long-term feeding of heme, after carcinogen induction, not only increased cytotoxicity but also the number of aberrant crypt foci in rat colon (12). Aberrant crypt foci are considered early markers for the development of colon tumors.

Based on current epidemiological evidence combined with our animal experimental results we suggest that intake of heme modulates colon cancer risk and explains the different associations between red meat and white meat and the risk of colon cancer.

Heterocyclic amines and apparent *N*-nitroso compounds

The association between red meat and colon cancer risk might also be explained by the intake of carcinogens formed during preparation of the meat. When meat is heated to high

temperatures for a substantial amount of time, mutagenic heterocyclic amines (HCA) are formed as a result of the reaction between creatinine (from meat or fish), amino acids and sugars (13). However, an association between HCA exposure and the risk of colon cancer is questionable because levels of exposure for humans are very low and probably insufficient to produce cancers by themselves (14-16). In addition, the concentration of some HCAs in cooked white meat exceeds the level in cooked red meat (17). Furthermore, mutation fingerprints of human colon tumors are mainly associated with C-to-T transitions (18) and not with typical G-to-T transversions or G-to-A transitions characteristic for HCAs (19). We feel, therefore, that it is not likely that HCAs cause the increased colon cancer risk observed after prolonged high consumption of red meat.

Alternatively, consumption of red meat could increase the presence of *N*-nitroso compounds (NOCs), which might be important in colon carcinogenesis. These NOCs are formed endogenously within the colon from amines and amides produced primarily by bacterial decarboxylation of amino acids that are N-nitrosated in the presence of a nitrosating agent, such as NO (20). Transitions of G-to-A in the K-*ras* gene (oncogene) observed in colorectal cancers could be characteristic for alkylating agents such as NOCs (21). However, in our opinion G-to-A transitions are not specific for NOCs. First, NOCs do not give a specific mutation fingerprint but rather a random profile of transitions and transversions (22). Second, the G-to-A transition in the sense strand could be equivalent to a C-to-T transition in the antisense strand, which is characteristic of an endogenous mutation not repaired after cell division (23). Nevertheless, Bingham and co-workers (24,25) showed a dose-response effect between red meat consumption in humans and formation of apparent NOCs in feces, whereas consumption of white meat did not have an effect on fecal NOC levels (24). Furthermore, recent experiments from Cross et al. (26) demonstrated that a supplement of heme (as liver pâté and blood sausage) almost mimicked the effect of red meat consumption on the formation of fecal apparent NOCs. This suggests that heme has a crucial role in the formation of NOCs.

Consumption of green vegetables is possibly associated with a decreased risk of colon cancer. Therefore, NOC formation was also studied after consumption of a diet with a combination of red meat and green vegetables but this did not prevent the formation of apparent NOCs (27). However, this could be due to the use of broccoli, brussels sprouts, and green peas, which are vegetables with low chlorophyll contents (Fig. 5B, Introduction). Taken together, apparent NOCs might be important in risk modulation of colon cancer,

even though the structures of these NOCs and their effects on the colon mucosa are still unknown. At least, the above results indicate that further studies on the role of heme, protein, and green vegetable consumption in the formation of apparent NOCs are needed.

Heme, chlorophyll, and chlorophyllin

We studied whether spinach, as a model for green leafy vegetables, modifies the detrimental heme-induced effects on colonic cell turnover in rats. We were able to show that all detrimental heme-induced effects were prevented after mixing spinach through the heme diet (28). Furthermore, the preventive effects of spinach could be totally mimicked by addition of equimolar amounts of chlorophyll to the heme diet (28). This could imply that the association between the consumption of green vegetables and a decreased risk of colon cancer is due to prevention of the detrimental heme-induced effects by chlorophyll. Chlorophyll is a phytol-esterified magnesium porphyrin and thus a structural analogue of heme. In an additional rat intervention study we demonstrated that chlorophyllin, a hydrophilic analogue of chlorophyll, did not prevent detrimental heme-induced effects on the colon mucosa (29). Thus optimal prevention of the detrimental heme-induced effects requires intact chlorophyll.

These initial studies used a high dose of chlorophyll (1.2 mmol/kg), which extrapolates to consumption of 450 g of spinach daily to neutralize the effects of heme in humans. However, we demonstrated with a dose-response study that even a low dose of chlorophyll (0.5 mmol/kg) daily prevents all heme-induced changes in luminal cytotoxicity, epithelial proliferation, and gene expression in rat colon.

Chlorophyll mimicked the effect of spinach entirely when mixed with the heme diet. This does not imply that chlorophyll is the only protective component in green leafy vegetables that might decrease the risk of colon cancer. Other components in green leafy vegetables such as folic acid might also be associated with a decreased risk of colon cancer. However, we focused on a protective effect of spinach and chlorophyll in the context of an interaction with heme.

A molecular mechanism

All heme-induced effects are mediated by a cytotoxic heme factor (heme factor), which is a lipid-soluble covalently modified porphyrin formed in the gut lumen of the rat (30). The molecular structure of this heme factor is still unknown, which hampers the formulation of a

molecular mechanism of its formation in the gut lumen. However, based on the increased presence of lipid peroxidation products in feces of heme-fed rats compared with controls, we speculate that radical reactions catalyzed by heme are involved in the formation of the heme factor. This is in line with results showing that antioxidants prevented the heme-induced cytotoxicity in rat colon (31). Chlorophyll, but not chlorophyllin, inhibited this heme-induced lipid peroxidation. To simulate heme reactivity *in vivo*, we studied pro-oxidant reactivity of heme towards polyunsaturated fatty acids *in vitro.* Addition of chlorophyll in this model decreased this reactivity (data not shown). However, the heme-induced reactivity was also inhibited after addition of chlorophyllin. The discrepancy between chlorophyll and chlorophyllin preventing the formation of the heme factor *in vivo* and inhibiting heme reactivity *in vitro* might be explained by the molecular characteristics of these components. Heme and chlorophyllin are both negatively charged under physiological conditions such as prevail in the intestines. In contrast, chlorophyll remains a neutral molecule. The negative charges of heme and chlorophyllin might cause repulsive forces and prevent formation of a complex. However, this does not explain why chlorophyllin inhibits pro-oxidant heme reactivity *in vitro*. An alternative explanation is that heme and chlorophyll dissolve in a hydrophobic environment*,* while chlorophyllin prefers a hydrophilic environment (chapter 4). We speculate that dietary heme dissolves in the hydrophobic phase of the gut contents and catalyzes lipid peroxidation. Chlorophyllin prefers the hydrophilic phase of the gut contents and is not near heme to scavenge radicals or obstruct covalent modifications to the heme molecule. In contrast, chlorophyll is very hydrophobic and, like heme, poorly absorbed in the gut lumen (32,33). Furthermore, chlorophyll and heme have a high affinity towards each other (chapter 5). Therefore, chlorophyll might interact with heme and scavenge free radicals or prevent covalent modifications to the heme molecule *in vivo*.

We demonstrated that dietary chlorophyll equimolar to heme prevented all heme-induced detrimental effects. In **figure 2A and B** we speculate how heme and chlorophyll are present in the gut lumen. Figure 2A shows a part of a micelle with heme. For simplicity, we left out interaction of heme with other surfactants such as bile acids, fatty acids and phospholipids. In this micelle, heme is susceptible to covalent modifications. Figure 2B shows the interaction between heme and chlorophyll. This interaction prevents the formation of the heme factor. More insight into the stoichiometry of the heme-chlorophyll

complex might be obtained *in vitro* with for example a chlorophyll titration study of the pro-oxidant reactivity of heme.

Water phase

Lipid phase

Water phase

Lipid phase

A

B
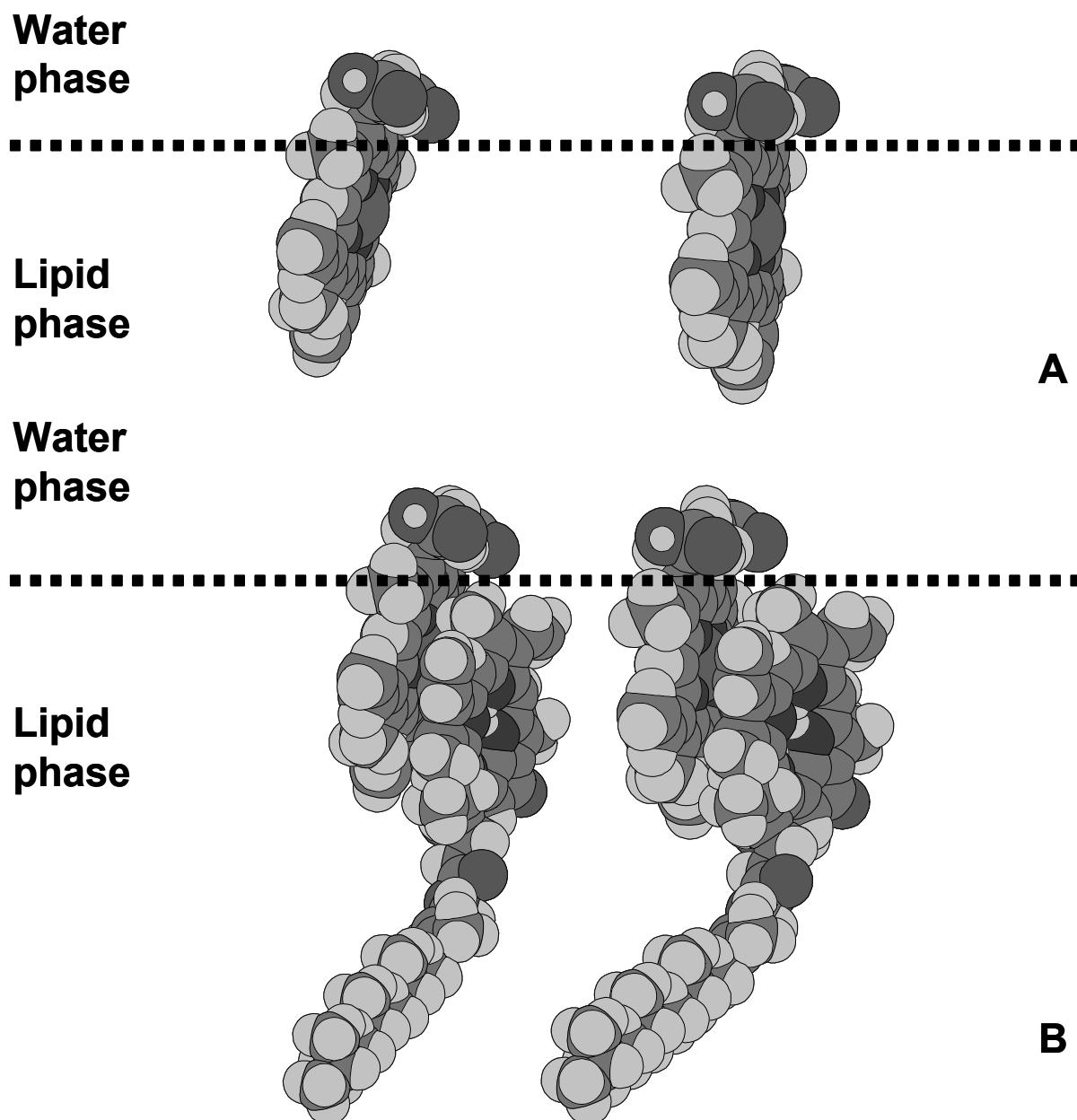
Figure 2. Presence of heme and chlorophyll in the intestinal lumen. Porphyrin ring part of heme molecules in the micellar phase of the gut contents (**A**). Interaction of heme and chlorophyll in the micellar phase (**B**).

If chlorophyll functions as antioxidant *in vivo*, studying the effect of known fat-soluble dietary antioxidants such as quercetin or vitamin E on the formation of the heme factor might provide more insight into the protective mechanism of chlorophyll. Pierre et al. (31)

have already demonstrated that a mixture of a fat-soluble antioxidant, butylated hydroxyanisole, and a more water-soluble antioxidant such as rutein inhibited the heme-induced cytotoxicity and formation of aberrant crypt foci. However, the possible differential effect of these components is not known.
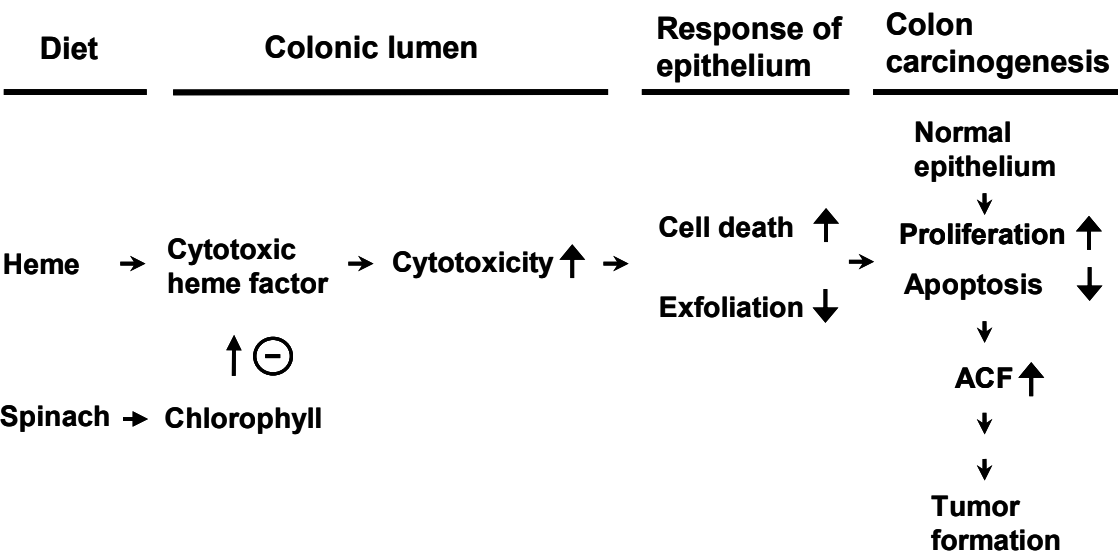
Effects of heme and chlorophyll in humans

To validate the results we obtained in our rat studies we would like to study the effects of heme and chlorophyll on the colon mucosa of humans too. However, it should first be studied whether the heme factor is indeed formed in the intestinal lumen of humans after red meat or heme consumption. Secondly, sampling of colon mucosal tissue during a diet intervention study to examine epithelial cell turnover is not easily achieved. An alternative approach was to study data from the Netherlands Cohort Study on diet and cancer to examine an association between heme intake and the risk of colon cancer and whether this association could be modulated by chlorophyll (chapter 6). We were the first to study a role of chlorophyll in the risk of colon cancer and we could demonstrate that increasing the molar heme to chlorophyll ratio was associated with an increased colon cancer risk in men. No consistent associations between heme intake and colon cancer risk were observed in women. Intake of heme to chlorophyll in ratios > 0.5 increased colon cancer risk in humans, which was close to the ratio heme to chlorophyll of > 1 that induced detrimental effects on the colon mucosa in rats (chapter 5). The differences might be accounted for by measurement errors in calculation of heme and chlorophyll intake in humans, as Spearman correlation coefficients for fresh meat, processed meat (meat products), fish, and vegetables were only 0.46, 0.54, 0.53 and 0.38 respectively. Possibly more important is the different timing of heme and chlorophyll intake. Our animal studies show that simultaneous intake is crucial for the prevention of the detrimental heme-induced effects. It is likely that people do not consume all green vegetables simultaneously with red meat. Thus, in humans part of the chlorophyll from green vegetables can not interact with heme from red meat and this may explain why the critical heme to chlorophyll ratio is lower in humans than in rats. Since we were the first to study the association between heme and chlorophyll consumption and the risk of colon cancer, other cohort studies, such as the European Prospective Investigation into Cancer might be used to examine this association further. The more studies supporting our hypothesis the more

likely the effect of chlorophyll is indeed real, which will eventually lead to better dietary advice to the general public.

Conclusion

We demonstrated that dietary heme and chlorophyll modulated the cytotoxicity of the colonic contents and consequently colonic epithelial cell homeostasis in rats (**Fig. 3**), which is an important step in early colon carcinogenesis. Furthermore, we verified our animal experimental data in humans and demonstrated that increasing the heme to chlorophyll ratio was associated with an increased risk of colon cancer in men. Our results suggest that chlorophyll interacts with heme (in a molar ratio of heme to chlorophyll of 1 to 1) in the hydrophobic phase of the gut lumen of the rat and prevents the formation of the cytotoxic heme factor.

Figure 3. Proposed mechanism of the effects of dietary heme and chlorophyll on carcinogenesis in rat colon. ACF: aberrant crypt foci.

References

1. Norat, T., Lukanova, A., Ferrari, P. and Riboli, E. (2002) Meat consumption and colorectal cancer risk: dose-response meta- analysis of epidemiological studies. *Int J Cancer*, **98**, 241-256.
2. *World Cancer Research Fund. Food, Nutrition and the prevention of cancer: a global perspective, 1997*, Washington, DC: American Institute for Cancer Research.
3. Riboli, E. and Norat, T. (2003) Epidemiologic evidence of the protective effect of fruit and vegetables on cancer risk. *Am J Clin Nutr*, **78**, 559S-569S.

4. Lee, D.H., Anderson, K.E., Harnack, L.J., Folsom, A.R. and Jacobs, D.R., Jr. (2004) Heme iron, zinc, alcohol consumption, and colon cancer: Iowa Women's Health Study. *J Natl Cancer Inst*, **96**, 403-7.
5. Larsson, S.C., Adami, H.O., Giovannucci, E. and Wolk, A. (2005) Re: Heme iron, zinc, alcohol consumption, and risk of colon cancer. *J Natl Cancer Inst*, **97**, 232-3; author reply 233-4.
6. Sesink, A.L.A., Termont, D.S., Kleibeuker, J.H. and Van der Meer, R. (1999) Red meat and colon cancer: the cytotoxic and hyperproliferative effects of dietary heme. *Cancer Res*, **59**, 5704-5709.
7. Mammen, J.M. and Matthews, J.B. (2003) Mucosal repair in the gastrointestinal tract. *Crit Care Med*, **31**, S532-7.
8. Dignass, A.U. (2001) Mechanisms and modulation of intestinal epithelial repair. *Inflamm Bowel Dis*, **7**, 68-77.
9. Podolsky, D.K. (1999) Mucosal immunity and inflammation. V. Innate mechanisms of mucosal defense and repair: the best offense is a good defense. *Am J Physiol*, **277**, G495-9.
10. van der Meer-van Kraaij, C., Kramer, E., Jonker-Termont, D., Katan, M.B., van der Meer, R. and Keijer, J. (2005) Differential gene expression in rat colon by dietary heme and calcium. *Carcinogenesis*, **26**, 73-9.
11. Van Der Meer-Van Kraaij, C., Van Lieshout, E.M., Kramer, E., Van Der Meer, R. and Keijer, J. (2003) Mucosal pentraxin (Mptx), a novel rat gene 10-fold down-regulated in colon by dietary heme. *Faseb J*, **17**, 1277-85.
12. Pierre, F., Freeman, A., Tache, S., Van der Meer, R. and Corpet, D.E. (2004) Beef meat and blood sausage promote the formation of azoxymethane-induced mucin-depleted foci and aberrant crypt foci in rat colons. *J Nutr*, **134**, 2711-6.
13. Skog, K. (1993) Cooking procedures and food mutagens: a literature review. *Food Chem Toxicol*, **31**, 655-75.
14. Wakabayashi, K., Nagao, M., Esumi, H. and Sugimura, T. (1992) Food-derived mutagens and carcinogens. *Cancer Res*, **52**, 2092s-2098s.
15. Bingham, S.A. (1996) Epidemiology and mechanisms relating diet to risk of colorectal cancer. *Nutrition Research Reviews*, **9**, 197-239.
16. Augustsson, K., Skog, K., Jagerstad, M., Dickman, P.W. and Steineck, G. (1999) Dietary heterocyclic amines and cancer of the colon, rectum, bladder, and kidney: a population-based study. *Lancet*, **353**, 703-707.
17. Sinha, R., Rothman, N., Brown, E.D., Salmon, C.P., Knize, M.G., Swanson, C.A., Rossi, S.C., Mark, S.D., Levander, O.A. and Felton, J.S. (1995) High concentrations of the carcinogen 2-amino-1-methyl-6-phenylimidazo- [4,5-b]pyridine (PhIP) occur in chicken but are dependent on the cooking method. *Cancer Res*, **55**, 4516-4519.
18. Harris, C.C. and Hollstein, M. (1993) Clinical implications of the p53 tumor-suppressor gene. *N Engl J Med*, **329**, 1318-27.
19. Schut, H.A. and Snyderwine, E.G. (1999) DNA adducts of heterocyclic amine food mutagens: implications for mutagenesis and carcinogenesis. *Carcinogenesis*, **20**, 353-68.
20. Bingham, S.A. (2000) Diet and colorectal cancer prevention. *Biochem Soc Trans*, **28**, 12-6.
21. Bos, J.L. (1989) ras oncogenes in human cancer: a review. *Cancer Res*, **49**, 4682-9.
22. Singer, B. and Kusmierek, J.T. (1982) Chemical mutagenesis. *Annu Rev Biochem*, **51**, 655-93.
23. Kinzler, K.W. and Vogelstein, B. (1996) Lessons from hereditary colorectal cancer. *Cell*, **87**, 159-170.
24. Bingham, S.A., Hughes, R. and Cross, A.J. (2002) Effect of white versus red meat on endogenous N-nitrosation in the human colon and further evidence of a dose response. *J Nutr*, **132**, 3522S-3525S.
25. Hughes, R., Cross, A.J., Pollock, J.R. and Bingham, S. (2001) Dose-dependent effect of dietary meat on endogenous colonic N-nitrosation. *Carcinogenesis*, **22**, 199-202.
26. Cross, A.J., Pollock, J.R. and Bingham, S.A. (2003) Haem, not protein or inorganic iron, is responsible for endogenous intestinal N-nitrosation arising from red meat. *Cancer Res*, **63**, 2358-2360.
27. Hughes, R., Pollock, J.R. and Bingham, S. (2002) Effect of vegetables, tea, and soy on endogenous N-nitrosation, fecal ammonia, and fecal water genotoxicity during a high red meat diet in humans. *Nutr Cancer*, **42**, 70-77.
28. de Vogel, J., Jonker-Termont, D.S., van Lieshout, E.M., Katan, M.B. and van der Meer, R. (2005) Green vegetables, red meat and colon cancer: chlorophyll prevents the cytotoxic and hyperproliferative effects of haem in rat colon. *Carcinogenesis*, **26**, 387-93.

29. de Vogel, J., Jonker-Termont, D.S., Katan, M.B. and van der Meer, R. (2005) Natural chlorophyll but not chlorophyllin prevents heme-induced cytotoxic and hyperproliferative effects in rat colon. *J Nutr*, **135**, 1995-2000.
30. Sesink, A.L.A. (2001) *Red meat and colon cancer: a possible role for heme*. Rijksuniversiteit Groningen, Groningen.
31. Pierre, F., Tache, S., Petit, C.R., Van Der Meer, R. and Corpet, D.E. (2003) Meat and cancer: haemoglobin and haemin in a low-calcium diet promote colorectal carcinogenesis at the aberrant crypt stage in rats. *Carcinogenesis*, **24**, 1683-1690.
32. Young, G.P., St John, D.J., Rose, I.S. and Blake, D. (1990) Haem in the gut. Part II. Faecal excretion of haem and haem-derived porphyrins and their detection. *J Gastroenterol Hepatol*, **5**, 194-203.
33. Young, G.P., Rose, I.S. and St John, D.J. (1989) Haem in the gut. I. Fate of haemoproteins and the absorption of haem. *J Gastroenterol Hepatol*, **4**, 537-45.

Samenvatting

- Celhoeveelheid in balans

- Heem

- Spinazie en chlorofyl

- Mechanisme

- Mensen

- Conclusie

Johan de Vogel, Aloys Sesink en Roelof van der Meer

Dikke darmkanker is één van de belangrijkste doodsoorzaken van kanker in de westerse wereld. In Nederland wordt jaarlijks bij 9000 mensen de diagnose dikke darmkanker gesteld en 4400 mensen overlijden jaarlijks door dikke darmkanker. De kans dat iemand darmkanker krijgt, hangt niet alleen af van erfelijke factoren, maar ook van omgevingsfactoren zoals de samenstelling van de voeding. Uit bevolkingsstudies blijkt, dat het eten van veel rood vlees de kans op darmkanker met 70% verhoogt, dit in tegenstelling tot het eten van wit vlees (zoals kip of kalkoen). Regelmatig eten van rauwe groenten en groene bladgroenten kan de kans op het ontstaan van darmkanker juist verkleinen. Het onderzoek beschreven in dit proefschrift geeft een mechanistische verklaring hoe het eten van rood vlees en groene bladgroenten de kans op darmkanker beïnvloedt.

Celhoeveelheid in balans

Voedingsstoffen die niet door de dunne darm worden opgenomen, komen in de dikke darm terecht. Ze kunnen daar de kans op darmkanker beïnvloeden, omdat sommige van deze stoffen irriterend zijn voor de cellen in de darmwand. Deze irritatie kan leiden tot beschadiging van de darmwand. Om de wand te herstellen en "gezond" te houden, moeten de beschadigde cellen vervangen worden en nieuwe cellen versneld worden aangemaakt. De hoeveelheid cellen in de darmwand is daardoor altijd in balans. Het verstoren van deze balans verhoogt de kans op darmkanker, want er is minder tijd beschikbaar om mutaties in het DNA gevormd tijdens de celdeling te repareren. Opeenstapeling van deze mutaties kan leiden tot ongebreidelde groei van epitheelcellen en uiteindelijk resulteren in de vorming van darmtumoren. We hebben in eerste instantie in studies met ratten onderzocht hoe voedingsstoffen de wand van de dikke darm beïnvloeden. Aangezien het darmepitheel van de rat en de mens sterk op elkaar lijkt en omdat uit eerder onderzoek bleek, dat voedingseffecten op de dikke darm vergelijkbaar zijn tussen rat en mens, kunnen we de resultaten van onze rattenstudies gebruiken om het risico voor de mens in te schatten.

Heem

De aanwezigheid van heem (**fig. 1A**) is een belangrijk verschil tussen rood en wit vlees. Heem is de ijzerdrager van rood vlees, die van belang is voor het zuurstoftransport en verantwoordelijk is voor de rode kleur van het vlees. Heem is één van de

voedingscomponenten die slecht wordt opgenomen door de dunne darm en het eten van veel rood vlees zorgt er dus voor, dat er veel heem in de dikke darm terechtkomt. Toevoeging van heem aan de voeding van ratten bleek te resulteren in verhoogde oxidatieve stress waardoor destructieve moleculen, bekend als radicalen gevormd werden. Deze toevoeging ging ook gepaard met een sterke verhoging van de concentratie irriterende stoffen in de darminhoud. Dit had tot gevolg, dat de cellen van de darmwand zich sneller gingen delen.
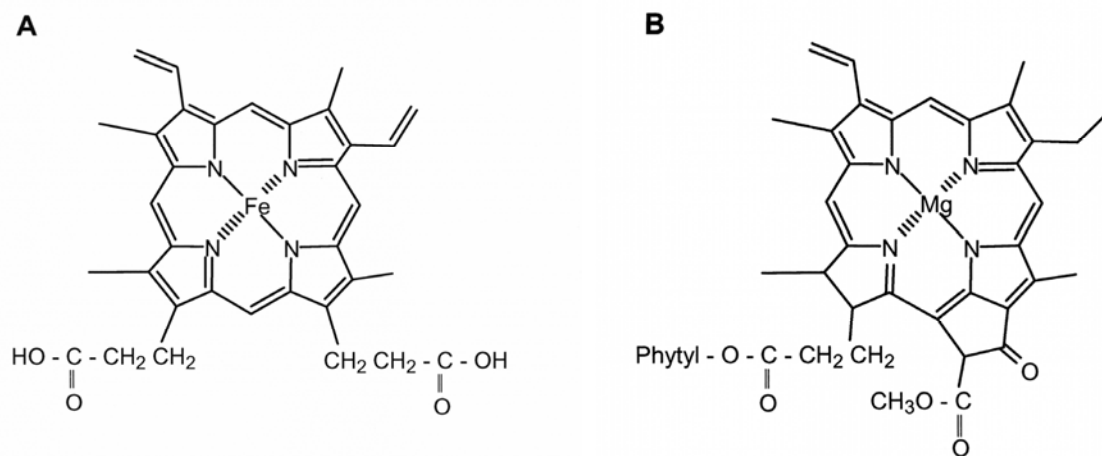
Figuur 1. (**A**) Moleculaire structuur van heem, (**B**) moleculaire structuur van chlorofyl, phytyl: $CH_2CHC_2H_3(CH_2CH_2CH_2C_2H_4)_3CH_3$.

We hebben nu uitgezocht dat deze verhoogde hoeveelheid irriterende stoffen in de darminhoud inderdaad schade gaf aan de cellen van de darmwand. Verder werd duidelijk dat niet alleen de celdeling, maar ook het proces van doodgaan van de darmcellen verstoord was. Heem blijkt de balans in de regulatie van de hoeveelheid cellen in de darmwand te veranderen. Deze ontregeling kan de kans op afwijkende cellen in de darmwand sterk verhogen. Hoe heem de verhoogde irritatie veroorzaakt is nog niet duidelijk. We weten wel dat niet heem zelf, maar een metaboliet van heem verantwoordelijk is voor de schadelijke effecten van heem. We hebben aanwijzingen dat heem zich aan andere componenten bindt tijdens het verblijf in de darminhoud en dat de aanwezigheid van oxidatieve stress belangrijk is voor de vorming van deze verbinding.

Spinazie en chlorofyl

Zoals eerder gezegd, blijkt het eten van groene groenten de kans op darmkanker te verminderen. Eén van de belangrijkste kenmerken van groene bladgroenten is de hoge concentratie aan chlorofyl (**Fig. 1B**). Chlorofyl is een molecuul dat in de plant betrokken is bij de fotosynthese. Chlorofyl lijkt qua structuur heel sterk op heem, waardoor we dachten dat chlorofyl mogelijk de schadelijke effecten van heem kon remmen. Allereerst hebben we onderzocht, of de effecten van heem in ons rattenmodel geremd konden worden door spinazie. Dit als voorbeeld van een typische groene bladgroente met een hoge concentratie aan chlorofyl. Hiervoor hebben we spinazie toegevoegd aan een voeding met heem en de effecten vergeleken met een controlevoeding en een voeding met alleen heem. Evenals in voorgaande proeven verhoogde heem de hoeveelheid irriterende stoffen in de darminhoud en de delingssnelheid van de cellen van de darmwand. Echter, de ratten die de gecombineerde voeding van heem plus spinazie kregen, bleken geen verhoging te hebben van de hoeveelheid irriterende stoffen in de darminhoud; met als gevolg dat de delingssnelheid van de cellen van de darmwand vergelijkbaar bleef met die van de controlegroep. Om onze hypothese verder te onderzoeken hebben we ook de effecten van pure chlorofyl getest. Dit onderzoek was deels een herhaling van het spinazie-experiment, maar nu met een extra groep ratten die een voeding kregen met heem (0.5 mmol/kg) plus pure chlorofyl (1.2 mmol/kg). Wat bleek... de toevoeging van chlorofyl aan een heemvoeding gaf dezelfde bescherming als de spinazie! Dit wijst erop, dat chlorofyl de belangrijkste component in spinazie is die verantwoordelijk is voor de beschermende werking.

Mechanisme

De belangrijkste moleculaire kenmerken van chlorofyl zijn de aanwezigheid van een porfyrine ring en een lange "staart" van 20 koolstof atomen. Nadat we het beschermende effect van chlorofyl gevonden hadden, waren we erg geïnteresseerd in het mechanisme hoe chlorofyl de schadelijke effecten van heem kon voorkomen. Daarom hebben we een proef gedaan waarbij we chlorofyl vervangen hebben door chlorofyline om te bestuderen welk deel van het chlorofyl verantwoordelijk is voor het beschermende effect. Chlorofyline is een water oplosbare vorm van chlorofyl waarbij de lange staart van chlorofyl weg is. Echter het mixen van chlorofyline met heem resulteerde niet in dezelfde beschermende

effecten als chlorofyl en geeft dus aan, dat voor de preventie van de schadelijke heem effecten, het gehele chlorofyl molecuul vereist is.

Verder is bekend dat chlorofyl nauwelijks opgenomen of afgebroken wordt gedurende de passage door de dunne darm. Hierdoor ontstaat een hoge concentratie chlorofyl in de dikke darm. Hoe chlorofyl zijn beschermende werking precies uitoefent is nog niet bekend. Wel is duidelijk dat chlorofyl voorkomt dat heem een irriterende werking uitoefent op de cellen van de darmwand. Chlorofyl zou kunnen fungeren als antioxidant, waarbij de heem geïnduceerde oxidatieve stress wordt geremd. Een andere optie is, dat heem gevangen wordt in een complex waarbij chlorofyl verhindert dat er binding van andere componenten aan heem plaatsvindt. Het exacte mechanisme is echter onderwerp van toekomstige studies.

In bovengenoemde experimenten werden chlorofyl en spinazie gebruikt in een dosis, die bij mensen vergelijkbaar zou zijn met een consumptie van ongeveer een halve kilo spinazie per dag om de effecten van heem te neutraliseren. Om te onderzoeken of deze hoge dosis vereist was, hebben we een dosis proef gedaan, waarbij we verschillende concentraties chlorofyl gemengd hebben met het heem voer. Uit deze studie bleek dat de schadelijke werking van 0.5 mmol/kg heem volledig voorkomen werd door 0.5 mmol/kg chlorofyl. Concentraties heem en chlorofyl in de verschillende voedingsmiddelen verschillen sterk (zie fig. 4B en 5B uit de introductie), wat zou kunnen beteken dat de schadelijke effecten van de consumptie van 150 gram rood vlees worden geremd bij een gelijktijdige consumptie van 75 gram spinazie of meer dan 750 gram broccoli.

Mensen

Bij voorkeur hadden we deze studies natuurlijk allemaal bij mensen uitgevoerd. Om praktische redenen zijn we echter begonnen met het bestuderen van voedingseffecten in ratten. Om toch dichter bij de mens te komen, hebben we de voeding van de deelnemers aan de Nederlandse Cohortstudie naar voeding en kanker (NLCS) bestudeerd. In deze studie bekeken we, of het eten van vlees en meer specifiek heem geassocieerd was met de kans op dikke darmkanker en of een eventuele associatie beïnvloed werd door het eten van chlorofyl. Uit de resultaten bleek dat inderdaad het eten van heem een associatie had met een verhoogd risico op darmkanker bij mannen (alhoewel dit net niet significant was), in tegenstelling tot het eten van de totale hoeveelheid vlees per dag.

Wij waren de eerste onderzoekers die in mensen de associatie van chlorofyl met het risico op darmkanker hebben onderzocht. Chlorofyl zelf verminderd niet het risico op darmkanker maar we konden wel aantonen dat het verhogen van de verhouding tussen inname van heem en chlorofyl resulteerde in een verhoogd risico (**Fig. 2**), wat erop kan duiden dat de verhouding van heem en chlorofyl in de maaltijd belangrijk is voor het risico op darmkanker. Bij vrouwen zijn geen consistente associaties gevonden tussen heem en chlorofyl en het risico op darmkanker. De verhouding heem-chlorofyl bij mannen voor een verhoogd risico was 0,5 en dit was redelijk in de buurt van een verhouding hoger dan 1 die in de rattenstudies de balans tussen celdeling en celdood verstoorde. De associatie tussen chlorofyl en heem en het risico op darmkanker zal nu ook in andere studies onderzocht moeten worden om te bestuderen of het niet een toevallige vinding is geweest die we hebben gedaan.
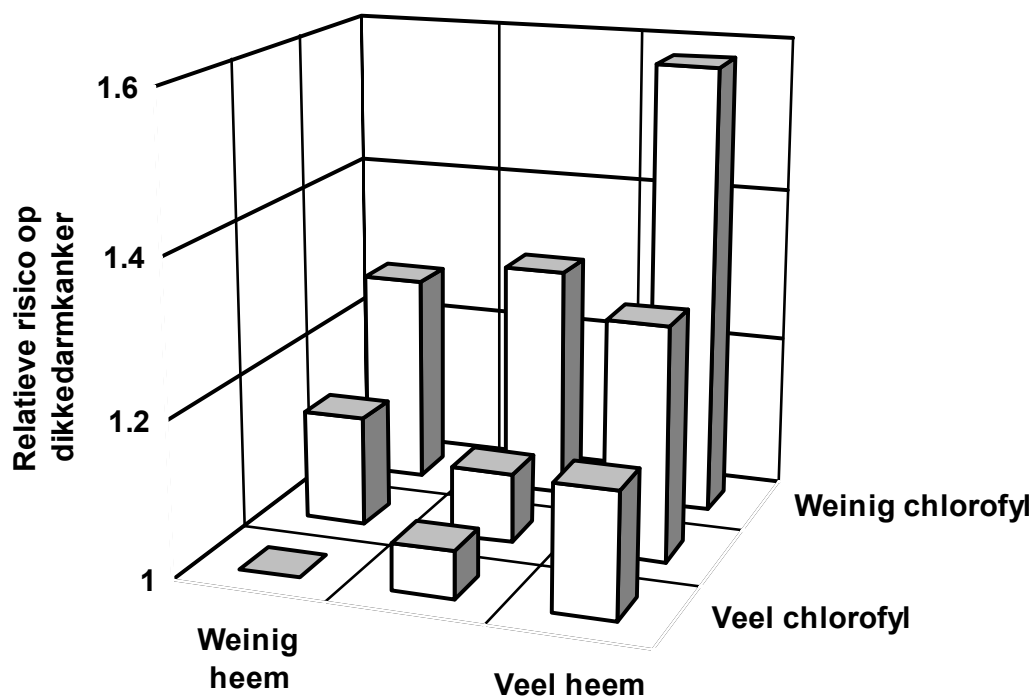
Figuur 2. Relatieve risico op dikke darmkanker bij mannen in de NLCS. Langs de x-as de hoeveelheid gegeten heem, de y-as de hoeveelheid gegeten chlorofyl en op de verticale z-as het relatieve risico van dikke darmkanker. Het eten van weinig heem en veel chlorofyl is gesteld op een relatief risico van 1 (1 is geen invloed van de voeding op het risico).

Conclusie

De resultaten uit de NLCS laten zien dat het verhogen van de verhouding heem-chlorofyl geassocieerd is met een verhoogd risico op darmkanker in mannen. Uit onze rattenstudies blijkt dat de schadelijke werking van irriterende stoffen in de dikkedarminhoud, veroorzaakt door heem uit rood vlees, geneutraliseerd kan worden door chlorofyl uit groene groenten. Voorlopige resultaten suggereren dat chlorofyl een interactie aangaat met heem (met een moleculaire ratio van 1:1) in de darminhoud van de rat. Met als gevolg dat de heem-geïnduceerde verstoring in de balans tussen celdeling en celdood in de darmwand wordt voorkomen. Dit mechanisme zou kunnen verklaren, waarom consumptie van groene groenten geassocieerd is met een verminderde kans op dikke darmkanker.

Dankwoord

Vier jaar onderzoek doen, wat een geweldige tijd is het geweest. Op sommige momenten denk je alleen dat het heel wat meer jaren van je leven gaat vergen. Echter met hulp van de juiste mensen is het toch gelukt. In dit hoofdstuk heb ik mooi de gelegenheid deze mensen te bedanken.

Een promotie is te vergelijken met de voorbereiding van een sporter voor een groot evenement zoals bijvoorbeeld een olympische spelen. Nu wil ik mij in het geheel niet vergelijken met een atleet maar wel met de faciliteiten waar topsporters gebruik van maken. Wat zou het namelijk geworden zijn zonder goede mensen om mij heen en toereikende financiën.

Gelukkig bestond de technische staf ((co)promomotor) uit een uitstekend duo met ten eerste Roelof. Je beschikt over een voor mij ongeëvenaarde kennis over metabolisme en de biochemie, hebt altijd ideeën te over, verder was en ben je altijd kritisch en konden we samen onze passie voor onderzoek delen (meestal na 17.00h en soms Marieke aan de lijn). Daarnaast wil ik Martijn bedanken. Er was af en toe wat discussie en soms liep het moeizaam maar uiteindelijk is er een goede organisatie ontstaan met als resultaat natuurlijk dit proefschrift. Gelukkig had je ook altijd ergens een bite vrij in de organiser om een afspraak in te roosteren voor het eindeloos reviewen van mijn manuscripten.

Maar waar heb je nu tegenwoordig nog coaches zonder assistentcoach. Denise, jij was altijd aanspreekbaar voor directe hulp over lab en aanverwante zaken (misschien bel ik je nog wel een keer midden in de nacht om...). Zonder jouw inzet en ongeremd enthousiasme was het me nooit gelukt en daarom ben ik je ook zeer dankbaar, dat je me zelfs tot het allerlaatste moment bij wilt staan als paranimf.

Ook kan een sporter niet functioneren zonder verzorgers, in de wetenschap werkt dat net zo en het waren er veel:

Nutsen (Arjan, Ingeborg, Aloys, Corinne, Carolien, Hans, Mischa, Wim, Leontien, Chantal, Karina, Esther, Sandra, Marloes, Marleen, Gabriële, Wendy en Fanny) en andere mede NIZO-ers, jullie stonden altijd klaar om te helpen bij proeven bij welk apparaat dan ook of bij andere problemen maar ook om een lolletje te maken, veel dank daarvoor.
Evelien, Cindy, Jaap en Marjolein uit de moleculaire A003 hoek kan ik natuurlijk niet vergeten, al die maandagmiddagsessies die eigenlijk nooit op tijd afwaren maar toch wel weer in leuke ideeën

resulteerden. Na een paar jaar werd A003 uitgebreid met collega's van TNO voeding en de Universiteit Maastricht. Elleny, Suzanne, Margje, Sandra, Matty en Piet bedankt voor de leuke samenwerking en pittige bijeenkomsten, die mij verder hebben geholpen in de epidemiologie en uiteindelijk resulteerden in een mooi manuscript.

Het team uit Groningen ook hartelijk dank voor de samenwerking. Wytske, al die crypten en cellen die je geteld hebt, het moet een enorm karwei zijn geweest en Jan ook bedankt voor je toch wel erg nuchtere kijk op ons heemonderzoek en voor het reviewen van ons manuscript.

Uiteraard ben ik alle mensen erg erkentelijk voor hun bijdrage tijdens mijn promotietijd. Maar alle ratten die niet vrijwillig hun leven hebben gelaten voor onze proeven mag ik zeker niet vergeten. Gelukkig zijn we een stuk verder gekomen en is het niet voor niets geweest. Hiermee samenhangend wil ik ook alle mensen van het CKP bedanken voor het verzorgen van de dieren, de hulp tijdens de sectie's en de prettige samenwerking.

Alle collega's van de afdeling Humane Voeding heel erg bedankt voor de belangstelling en goede samenwerking.

Dan is er een speciaal plekje voor de collega's uit de 'NIZO kantoortuin'. Sandra, Esther, Gabriële, Jan, Arjen, Marloes en Marleen, dank voor de samenwerking en de leuke tijd. Als er wat afleiding nodig was kon er in de tuin altijd wel iets op korte termijn geregeld worden. Sandra, jij als mede-NIZO-bureau-eigenaar en paranimf, fijn dat we altijd over van alles hebben kunnen kletsen, zowel tijdens als buiten werktijd. Het was natuurlijk wel een beetje droevig, dat je na je promotie van plekje ging wisselen maar gelukkig was onze vriendschap daar tegen bestand en ik hoop dat we die nog lang kunnen voortzetten.

Ook wil ik het WCFS en alle collega's van WCFS programma 1 tot en met 3 bedanken voor respectievelijk het verzorgen van de financiën en de prettige samenwerking. Deelnemen met mede-WCFS AIO's in het WCFS topforum was ook geweldig. De vele mensa-happen, brainstormen, uitjes en lunches had ik niet willen missen. Hopelijk worden er voor de huidige AIO's nog een paar van de overgebleven ideeën uitgevoerd. Collega's vanuit het WCFS bestuurscentrum ook hartelijk dank voor het altijd klaarstaan om mij de WCFS regels nogmaals rustig uit te leggen en voor alle andere hand en span diensten die de afgelopen 4 jaar verrricht zijn. Ik werk dan ook met plezier 4 jaar verder voor het WCFS.

Nog 4 jaar? Ja inderdaad, maar nu in Maastricht. Het is even rijden maar meer dan de moeite waard. Ik wil graag Ronald, alle mensen in het SHOCK team, van de middenlob en de diverse

kamergenootjes bedanken voor de mooie tijd en de prettige samenwerking die we tot nu toe al hebben gehad.

Luitjes uit het westen noem ik ze altijd, (Gemmeke, Hidde, Janine en Rohin) super bedankt voor de altijd aanwezige steun. We hebben het altijd druk, maar toch weet ik dat ik op jullie kan bouwen en dat de vriendschap altijd hecht zal blijven, waar we dan ook wonen of werken. Alle fietsmaatjes en de Aussies ook heel erg bedankt voor de heerlijke ontspannen tijd die we samen hebben kunnen doorbrengen. Tijdens de gehele promotie heb ik nog op kamers mogen wonen in Wageningen. Ik wil dan ook alle oud huisgenootjes bedanken voor de leuke tijd en de talloze Kolonisten sessies en film/voetbal avonden die we op 217 hebben beleefd.

Uiteraard wil ik ook Dirk, Reini, Michiel, Mirjam en Quibus heel hartelijk danken voor de fijne tijd samen. Ik voelde me meteen welkom in de familie en het was fantastisch zoals jullie altijd meeleefden en geïnteresseerd waren in het onderzoek. Mam en Pap fantastisch mooi dat jullie me in de gelegenheid hebben gesteld om zover te komen. Fijn dat ik altijd op jullie kan rekenen. Terwijl ik met de laatste loodjes bezig was, heb je weer iets unieks in elkaar gezet. Pa, ontzettend veel dank voor de omslag.

Dan als laatste de allerbelangrijkste persoon. Helemaal super dat ik jou ontmoet heb Heleen en na Australië zette de vonken de zaak flink in brand. Fijn dat we altijd alles kunnen delen en veel samen kunnen lachen. Nu moet je straks nummer 1 warmhouden en ik ben benieuwd hoe dat voelt. Met je ervaring kan ik me dan vast gaan voorbereiden. Bedankt voor alles en ik hoop dat we nog lang samen kunnen dreamen (of was het nu dweamen?).

Bedankt allen,
Johan
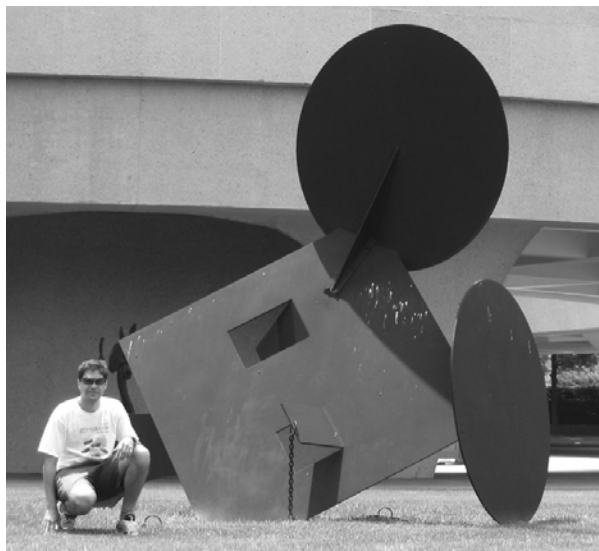
Curriculum vitae

Johan de Vogel was born on the 9th of June 1978 in Rotterdam, The Netherlands. After completing secondary school (HAVO at the Laurens College, Rotterdam) in 1996, he started a bachelor at Hogeschool Rotterdam & Omstreken (Hoger Laboratorium Onderwijs) to study medical biology. As part of the study he worked for 9 months at the division of Experimental Hematology at the Leiden University Medical Centre on the influence of radiation on the IL-8 induced stem cell mobilisation in mice. After his graduation in 1999, the grown interest in biological science inspired him to study biology at Leiden University. During this study he conducted two research projects focussed on molecular biology and biochemistry. The subject of the first project was to study the mutational analysis of the TMV-elicitor of the hypersensitive response at the Institute of Molecular Plant Science, division plant viruses at Leiden University. The second project was at the division Nutritional Supplements of Numico Research in Wageningen with subject: the role of ribose in muscle metabolism. In 2001 he received his MSc degree and started working for the Wageningen Centre for Food Sciences (WCFS) at NIZO food research in Ede on the project Dietary Modulation of Colon Cancer Risk under supervision of Dr. R. van der Meer and Prof. Dr. M.B. Katan. He is currently working as a post-doc on the project Diet, Insulin Resistance and Chronic Inflammation for the WCFS at the division Human Biology of Maastricht University.

Publications

- Abbink TE, De Vogel J, Bol JF, Linthorst HJ. Induction of a hypersensitive response by chimeric helicase sequences of tobamoviruses U1 and Ob in N-carrying tobacco. Mol Plant Microbe Interact. 2001 Sep;14(9):1086-95.

- De Vogel J, Jonker-Termont DS, van Lieshout EM, Katan MB en van der Meer R. Green vegetables, red meat and colon cancer: chlorophyll prevents the cytotoxic and hyperproliferative effects of haem in rat colon. Carcinogenesis. 2005 Feb;26(2):387-93.

- De Vogel J, Jonker-Termont DS, Katan MB en van der Meer R. Green vegetables and colon cancer: Natural chlorophyll but not chlorophyllin prevents heme-induced cytotoxic and hyperproliferative effects in rat colon. Journal of Nutrition. 2005; 135: 1995-2000

- Balder HF, de Vogel J, Jansen M.C.J.F, Weijenberg M.P., van den Brandt P.A., Westenbrink S, van der Meer R en Goldbohm R.A.. Heme and chlorophyll intake and colorectal cancer risk in the Netherlands Cohort Study. Cancer Epidemiology Biomarkers and Prevention. Accepted with minor revisions

- De Vogel J, Kramer E, Jonker-Termont DS, Keijer J, Katan MB en van der Meer R. Equimolar chlorophyll prevents dietary heme-induced detrimental effects on gene expression and cell turnover in rat colon mucosa. Submitted

- De Vogel J, Boersma-van Eck W, Sesink A, Jonker-Termont DS, Kleibeuker J en van der Meer R. Dietary heme injures surface epithelium and consequently disturbs epithelial cell turnover in rat colon mucosa. To be submitted

- De Vogel J, Sesink A, Van der Meer R. Darmkanker, rood vlees en groene groenten. Chlorofyl heeft mogelijk een beschermende werking. Voeding Nu 2005; 7(4): 25-27

Patent:

- De Vogel, J., Jonker-Termont, D.S., Van der Meer, R. Use of chlorophyll or analogues thereof for the prevention of cytotoxic effects of dietary heme on the intestinal mucosa. Reference Number: 03078182.7

Training and supervision plan

Discipline specific activities

Bioinformation Technology - 1, VLAG

Ecophysiology of the GI-tract, VLAG

ESPEN Tracer Methodology in Metabolism, Maastricht University

General Courses

Scientific writing in English, CENTA

Radiation expert 5B, Larenstein, Velp

Proefdierkunde, University of Utrecht

Optional courses and activities

Nutrition and Sports, VLAG, 2004

Journal club NIZO food research 2001-2005

NWO-voeding, Papendal, The Netherlands 2001-2005

PhD study tour Human Nutrition, Australia, 2003

Digestive Disease Week, New Orleans, USA, 2004

International research conference on food, nutrition, and cancer, Washington, USA, 2004