The effect of different diets on faecal markers related to colorectal cancer risk: studies with vegetarians and urolithiasis patients

Citation for published version (APA):

van Faassen, A. (1996). *The effect of different diets on faecal markers related to colorectal cancer risk: studies with vegetarians and urolithiasis patients*. [Doctoral Thesis, Maastricht University]. Maastricht University Press. <https://doi.org/10.26481/dis.19960510af>

Document status and date:

Published: 01/01/1996

DOI:

[10.26481/dis.19960510af](https://doi.org/10.26481/dis.19960510af)

Document Version:

Publisher's PDF, also known as Version of record

Please check the document version of this publication:

- A submitted manuscript is the version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher's website.
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- The final published version features the final layout of the paper including the volume, issue and page numbers.

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**The effect of different diets on faecal
markers related to colorectal cancer risk**
Studies with vegetarians and urolithiasis patients

CIP-GEGEVENS KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Faassen, Arendina van

The effect of different diets on faecal markers related
to colorectal cancer risk : studies with vegetarians and
urolithiasis patients / Arendina van Faassen. -

Maastricht : Universitaire Pers Maastricht. - Ill.

Thesis Rijksuniversiteit Limburg, Maastricht. - With ref.

ISBN 90-5278-213-X

Subject headings: diets / colorectal cancer / urolithiasis.

Illustraties en omslag:

G. van Zonneveld, Audiovisuele Dienst, Academisch Ziekenhuis Maastricht

Productie en druk:

Datawayse | Universitaire Pers Maastricht

The effect of different diets on faecal markers related to colorectal cancer risk

Studies with vegetarians and urolithiasis patients

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Rijksuniversiteit Limburg te Maastricht,
op gezag van de Rector Magnificus, Prof. Mr. M.J. Cohen,
volgens het besluit van het College van Dekanen,
op vrijdag 10 mei 1996
om 16.00 uur

door

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Het onderzoek was mogelijk door financiële ondersteuning van de Nederlandse Kankerbestrijding - Koningin Wilhelmina Fonds en de Stichting Zuivel, Voeding en Gezondheid. Beide organisaties droegen ook bij in de drukkosten van het proefschrift.

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Summary

Colorectal cancer is the second most common cancer among women and the third most common cancer among men in most Westernized countries. The treatment of colorectal cancer by surgery, farmaco- and radiotherapy has a 5 years survival of ca. 50% . Therefore special attention has been focussed on prevention. Colorectal cancer occurrence appears strongly influenced by environmental factors, among others diet. Some risk is inherited, but groups with dietary habits and lifestyle which are largely different from the general population like Seventh-day Adventists, Mormons and vegetarians, have lower mortality rates.

Studies on the pathogenesis of colorectal cancer show that it results from the accumulation of multiple mutations in oncogenes and tumor suppressor genes. The latency between the first mutation and the occurrence of the disease is at least 20 years. Therefore biological markers of risk have been sought for in this long continuum of the pathogenetic chain. Because the colon and rectum are exposed both to blood and intestinal contents, markers of exposure may be found in both compartments. Remnants of the intestinal contents are found in faeces.

From literature before starting our studies (**chapter 1**) we hypothesized that a high dietary intake of meat, and a low intake of dietary fibre and calcium are related to a high risk of colorectal cancer. The concentrations of the secondary bile acids deoxycholic acid and lithocholic acid and of the long chain fatty acids in total faeces and in faecal water on one hand, but also faecal pH and faecal bulk are the faecal markers that are most probably related to colorectal cancer risk and that might be sensitive to dietary modulation.

The aim of this thesis was to study whether habitual dietary habits which are different in intake of meat, fibre and calcium (omnivorous, lacto-ovo-vegetarian, vegan and calcium-restricted diet of urolithiasis patients) will affect faecal markers for colorectal cancer risk. If the relations found are consistent with the data on diet and colorectal cancer risk, the biomarkers can be used as intermediate endpoints in epidemiological studies. If the data on biomarkers are explaining biologically the relation between diet and colorectal cancer risk, the hypotheses about diet and colorectal cancer are strengthened.

An accurate and valid analytical method for the determination of the concentration of faecal deoxycholic and lithocholic acid was developed (**chapter 2**). The method used in our next studies, which was based on this analytical method, gave figures for the main bile acids in human faeces that are similar to those acquired by methods from other laboratories. Moreover minor bile acids like the 3β -isomers of deoxycholic and lithocholic acid and the 7- and 12-keto bile acids could be quantitated (**chapter 8**). Deoxycholic acid, lithocholic acid and their 3α -isomers are called secondary bile acids, because they are made by the intestinal bacteria from the primary bile acids chenodeoxycholic and cholic acid, which are formed in the liver.

In a strictly controlled study with male, young volunteers, the lacto-ovo-vegetarian and the vegan diet lowered the concentration of secondary bile acids in total faeces and enhanced faecal bulk and defaecation frequency compared to the mixed diet. The effects are most probably caused by the high amount of dietary fibre. The concentration of faecal secondary bile acids was higher on the lacto-ovo-vegetarian diet than on the vegan diet, most probably due to the higher amount of calcium in this diet. The vegan diet caused a lower faecal pH than the mixed and lacto-ovo-vegetarian diet (**chapter 3**).

The intake of dietary fibre is consistently negatively associated with the risk of colorectal cancer in retrospective epidemiological studies. Also a faecal bulk higher than 200 g/d was associated with a 3 times lower colorectal cancer risk in a correlation study. In a small study, patients with adenomatous polyps in the large bowel, which are considered as precursors of colorectal cancer, had higher concentrations of secondary bile acids in the faeces than healthy controls. Also, ulcerative colitis patients with high concentrations of secondary bile acids in faeces, had a higher risk on colorectal cancer than patients with low concentrations. Therefore the concentration of secondary bile acids in faeces may be used as an intermediate point in epidemiological studies. The role of faecal bulk, faecal pH and defaecation frequency have to be studied more intensively in prospective epidemiological studies before using them as a marker related to colorectal cancer. Moreover, the relation between intake of dietary fibre and colorectal cancer risk is strengthened by our results (**chapter 8**).

Habitual lacto-ovo-vegetarian men and women of 59 - 67 yrs did not have lower concentrations of bile acids in total faeces but the concentration of secondary bile acids in the aqueous phase of the faeces was lower than in omnivores. The aqueous phase of faeces is obtained by high speed centrifugation and is supposed to be in contact with the colonic epithelial cells. Faecal bulk and defaecation frequency were higher in the vegetarians than in omnivores and also higher in men than in women (**chapter 4**). Thirty percent of

the variance in the concentration of deoxycholic acid in faecal water was explained by the intake of saturated fat while 24 % could be explained by the intake of cholesterol. Another twenty percent was explained by sex and faecal bulk. Also, twenty percent of the variance in faecal bulk and defaecation frequency was explained by the intake of dietary fibre, especially from fruit and vegetables. Faecal pH did not differ between omnivores and vegetarians, but forty-seven percent of the variance in faecal pH was explained by the calcium concentration in faeces and the faecal concentration of one of the main short chain fatty acids, butyrate.

The intake of saturated fat is not associated with an increased risk of colorectal cancer in all epidemiological studies. However a high consumption of food like red meat and eggs, are found to be associated with an increased risk. The relation between the concentration of secondary bile acids in faecal water and colorectal cancer risk has not yet been studied in large epidemiological studies (chapter 8).

Research by others showed that calcium-supplementation lowered the concentration of bile acids in faecal water. Moreover, epidemiological and experimental studies reported that a high intake of calcium may protect against colorectal carcinogenesis. The effects of a calcium-restriction on markers of colorectal cancer risk were not yet studied. We studied the effects in rats of a binder of calcium and magnesium (ethylene diamine tetra acetic acid, EDTA) (chapter 5). Moreover, in male volunteers the effects were studied of a medication that is used by a group of urolithiasis patients to prevent hyperabsorption of calcium: Calcisorb® (chapter 6). In both studies an increase was expected in the concentrations of bile acids and long chain fatty acids in the aqueous part of the colonic content and faeces respectively, however no significant changes could be detected. On the other hand, the concentrations of bile acids and long chain fatty acids in total faeces decreased. The results may be explained by the lowering of the bioavailability of calcium below 1000 mg/day: there are indications from literature that calcium-supplementation above 1000 mg/day increases faecal bulk, which will decrease the concentration of deoxycholic acid in faecal water as can be derived from our study with the habitual omnivores and vegetarians. The concentrations of calcium and magnesium in faecal water were lower after consuming these binders. The possible role of these concentrations in influencing colorectal cancer risk has to be studied further in animal and epidemiological studies, because both minerals also inhibit directly cell proliferation.

The adverse effects of the calcium-restricted diet of urolithiasis patients with hypercalciuria type II on faecal markers related to colorectal cancer risk, were studied in a randomized clinical trial. These patients have an increased

absorption of calcium, which results in a high urinary excretion of calcium. The calcium-restricted diet might enhance the concentration of bile acids and long chain fatty acids in faecal water. However, this diet lowered the concentration of bile acids and long chain fatty acids in faecal water and its in vitro toxicity to red blood cells, most probably as a result of the lower intake of (saturated) fat and cholesterol that accompanies the calcium-restriction due to the omission of dairy products. The in vitro toxicity of faecal water was most strongly correlated with the concentration of long chain fatty acids in faecal water. Others have shown that the in vitro toxicity of faecal water to red blood cells correlates strongly with the in vitro toxicity to colonic epithelial cells and with hyperproliferation of epithelial cells of the large bowel in rats. Long chain fatty acids are suspected of increasing colorectal cancer risk because like bile acids, they activate protein kinase C. Protein kinase C is a key enzyme in cell proliferation. The relation between long chain fatty acids in faecal water and colorectal cancer risk has to be studied more deeply in epidemiological studies. Faecal pH did not change, but baseline values were positively associated with the concentration of calcium in faeces and negatively with the in vitro cytotoxicity of faecal water. Baseline values of faecal bulk correlated positively with the intake of calcium as measured by the urinary plus faecal excretion of calcium, especially above 1000 mg/day. Moreover, sixty-five percent of the variation in the change in faecal bulk was explained by the change in calcium-intake measured as describe above.

In this study, also the effects of the calcium-restricted diet on risk factors for recurrent stone formation were checked. The urinary calcium-excretion decreased slightly and only in men; the urinary oxalate excretion decreased, although not significantly. The citrate excretion did not change (**chapter 7**).

The results of the clinical trial and recent literature prompted us to propose a new scheme for the diagnosis and dietary treatment of urolithiasis patients with calcium-oxalate stones, which has to be studied for efficiency and feasibility (**chapter 8**). Patients with a primary stone, should be screened by medical, clinical laboratory, dietary and antropometric methods. If there are no metabolic disturbances, a prudent diet as described by the Dutch guidelines for a healthy diet (moderate in meat, eggs, sugar and sodium, sufficient in calcium and fluid and rich in vegetables and fruits) should be advised. Recurrent patients should be studied on a calcium-rich, prudent diet and a calcium-low, prudent diet, in which the extra calcium is derived from tablets. Patients with decreasing urinary risk factors like the excretion of calcium, oxalate, urate, citrate and/or magnesium and whose urinary indicators of diet like ureum and sodium also decrease on the calcium-rich prudent diet, should follow the prudent diet described above. Patients who show no decrease of urinary calcium excretion on the calcium-rich diet, but

who respond to the calcium-low diet, are diagnosed as hypercalciuria type II and treated with a calcium- and oxalate-restricted and fluid enhanced diet. Further research is needed to select all patients that develop an increased risk for osteopenia on this diet.

Based on the results of our studies and on the literature, the model for the role of faecal constituents in the pathogenesis of colorectal cancer and for the influences of several dietary factors on the faecal constituents has to be adapted (chapter 8). Faecal constituents like long chain fatty acids and hydrophobic bile acids in faecal water will increase the proliferation of colonic epithelial cells, which is associated with a higher expression of mutations. It is concluded that there are strong indications that vegan diets will be protective for colorectal cancer due to their low saturated fat and cholesterol content and their high amount of dietary fibre and resistant starch. Both will lead to low concentrations of hydrophobic bile acids and long chain fatty acids in faecal water. Moreover, the high amount of dietary fibre and resistant starch will enhance the production of butyrate in the colon, which will lower the hyperproliferation of epithelial cells. Lacto-ovo-vegetarian diets which are not that low in saturated fat as vegan diets, may be protective because of their high amount of dietary fibre and their high calcium-content. Dietary fibre and a high intake of calcium will enhance faecal bulk and therefore lower the amount of hydrophobic bile acids and long chain fatty acids in faecal water. A high intake of calcium will also enhance faecal pH, thereby resulting in a decrease of the amount of undissociated secondary bile acids. These undissociated acids are hydrophobic and therefore cytotoxic. Omnivorous diets which normally are low in dietary fibre, sometimes short in calcium and mostly rich in (saturated) fat and cholesterol from meat and eggs, may enhance the colorectal cancer risk.

The risk of recurrent urolithiasis in men which are genetically predisposed to calcium-oxalate stones, may be diminished slightly by a diet restricted in calcium. The risk of colorectal cancer may not be increased when the omitted milkproducts are not substituted by products rich in animal protein like meat and eggs or by other food products. However in women, the same diet may be ineffective and may even increase their risk of osteoporosis. A prudent diet as described by the Dutch guidelines for a healthy diet, should fulfill all requirements at the same time with regard to the risk of recurrent urinary calcium-oxalate stone forming, osteoporosis and colorectal cancer.

Introduction and objectives

1.1. Diet, biological markers and colorectal cancer risk

Cancer is the second cause of death in the Netherlands after heart and vascular diseases (1). Of each 100 subjects, 26 die of cancer. Colorectal cancer (CRC) is diagnosed each year in about 7000 Dutch men and women. It is the second most common cancer among women, behind breast cancer and the third most common one among men, after cancer of the lung and prostate (2). Also in other Western industrialised countries, the age-stratified incidence varied between 0.3 and 0.5/1000 with the Netherlands on a third place after the USA and Canada. The treatment of CRC by surgery and radiotherapy has a five year survival rate of 50% (3). Therefore special attention has been focused on prevention.

CRC occurrence appears to be strongly influenced by environmental factors, among others diet. The mortality rates generally increase among migrants from low to high-incidence areas and groups with dietary and lifestyle habits different from the general population like Seventh-day Adventists, Mormons and vegetarians have lower mortality rates (4).

Some risk on CRC is inherited. Familial adenomatous polyposis and Gardner's syndrome are relatively uncommon syndromes in which extensive colonic polyp formation leads to the development of colon cancer. Some families have a high incidence of CRC, without extensive polyp formation. These groups are named hereditary non-polyposis CRC types.

Studies on the genetics of CRC resulted in a view that CRC results from the accumulation of multiple mutations in oncogenes and tumor suppressor genes (5). Some mutations are inherited, while others are the result of environmental factors. They may differ between cancer of the colon and rectum and between subsites within the colon (6). However uncertainties in the classification at the border of colon and rectum prompted researchers often to combine them to CRC.

FIGURE 1
Combination of metabolic and epidemiological studies using biomarkers

The incidence of CRC is highest at ages above 75 yrs (3). The latency between the exposure that causes the first mutation and the occurrence of CRC is supposed to be at least 20 yrs. Biological markers (biomarkers) of CRC risk in this long continuum of the pathogenetic chain have been sought. These markers of risk are variables which values different from normal predict subsequent risk. They can be divided in three broad classes: markers of exposure, of effect and of susceptibility. At least 7 components are distinguished in the pathogenetic chain: markers of exposure, internal dose, effective dose, early biological effect, altered structure/function, clinical disease and of progression (7). In the research on diet and cancer biomarkers of exposure have been used in metabolic and epidemiological investigations, for example studies on the effect of diet on faecal bulk and the relationship between this variable and CRC (see Figure 1). If the relations found are consistent with the data on diet and CRC risk, the biomarkers can be used as intermediate points in epidemiological studies. This kind of research has been called metabolic epidemiology (8). It is the variant of molecular epidemiology (9), in which the biomarkers are metabolites reflecting the effective dose (7). Moreover, if the data on biomarkers are explaining biologically the relation between diet and CRC risk, the hypotheses about diet and CRC risk are strengthened.

CRC arises from the epithelial cells of the large intestine. The colon has one layer of cylindrical cells with a large amount of crypts, the rectum several layers of squamous cells. These cells are exposed both to the intestinal contents and to blood. The colon is exposed to blood from the arteria mesenterica inferior, the rectum to the same and to blood from the arteria hypogastrica (10). Therefore markers of exposure may be found in blood or in the intestinal contents. An example of exposure by blood may be the concentration of folic acid in serum (11), which is a marker of the dietary intake and distribution of folic acid. A high intake of folic acid is associated with a low risk of CRC (12). A marker of exposure by the intestinal contents may be the concentration of bile acids (BA) in faeces, which is influenced by diet (13).

1.2. Urolithiasis, diet and colorectal cancer risk

Urinary stones are a common reason of patients to visit their physician. The incidence in Germany (14) and the Netherlands (15) is about 0.5 % per year. Of these stones 0.12 % is a first stone (16), comparable to the incidence of diabetes in the Netherlands (17). At least 50 % of the stone patients have a second event within 5 yrs when they are not treated against recurrence (18). The stones are formed in the renal calyces. At some time they are detached and start their drainage. When the stones reach the ureters, severe pain occurs. Moreover the stones can damage the tissues. Therapies are surgery, extracorporeal shock wave lithotripsy, or passing the stone with the urine. However the risk of recurrence can be lowered only by metaphylaxis. This should be based on risk factors or if possible on the etiological causes. There are several kinds of stones. About seventy five percent of the stones in adults of Western, industrialised countries are calcium (Ca) oxalate stones, sometimes combined with phosphate (19,20).

Risk factors for renal stone disease (urolithiasis) in general are genes, occupation, climate and diet. A small number of gene loci (3 a 4) are probably the principal contributors to the observed heritability of Ca-oxalate stone disease. Moreover adult men have a twofold higher risk for stones than women. The related genes may determine the levels of Ca, oxalate and citrate excreted in urine (21).

Professions with a sedentary style have a higher incidence of stones. Moreover when military subjects were placed in (sub)tropical areas the incidence of urolithiasis increased (22).

Data from World War II show that when a country undergoes food and protein deprivation, the frequency of urinary stones decreases (23). Vegetarians in England have a 8 fold lower prevalence of stones than the general population of the same social class (24).

Urolithiasis patients with hypercalciuria type II are treated during the rest of their life with a Ca-restricted diet (25), because they have an increased absorption of Ca. Patients with hypercalciuria type I do not respond strongly enough to a Ca-restricted diet and therefore are treated with Calcisorb[®], an oral binder of Ca composed of sodium cellulose phosphate (26). A low intake of Ca however, has been related to a high risk of CRC (27). Therefore urolithiasis patients with hypercalciuria type I and II may ultimately experience a higher risk of CRC. Because the diagnosis of hypercalciuria type I and II is not made in most hospitals, classical epidemiological studies cannot be used to study the risk of these patients. The number of urolithiasis patients with hypercalciuria type I is too small for epidemiological studies, therefore the effect of their therapy on risk for CRC has to be studied in healthy volunteers.

1.3 Review of the literature before 1990

1.3.1 Dietary factors and CRC risk

A higher intake of **meat**, a lower intake of **fibre** and a lower intake of **milkproducts** were associated with a higher incidence of colon cancer in two Scandinavian countries (28). In a Canadian hospital based case-control study, the intake of **saturated fat** was positively associated with the incidence of CRC (29). In a hospital based case-control study in Greece, a higher intake of meat and a lower intake of vegetables appeared to have the highest risk of CRC (30). The intake of Ca was lower in CRC patients than in controls in the USA (27). Also a cohort study in the USA showed a protective effect of Ca and vitamin D (31). In the USA, **animal fat** appeared to be a risk factor in a prospective study with women. (32). **Total energy** and **protein** intake was associated in a population based case-control study in Australia with an increased risk for colon and rectal cancer in both men and women consuming a low **fibre** diet (33).

1.3.2 Dietary factors and faecal markers of CRC risk

Cummings et al showed that the intake of dietary fibre from cereals and from fruit and vegetables increased **daily faecal wet weight** (34,35). Also a high

intake of starch increased faecal wet weight (36). Consumption of fibre rich products increased defaecation frequency (37). Daily ingestion of large amounts of pectin-rich forms of dietary fibre such as 7 oranges (38), an experimental vegan diet (39) or 35 g commercial pectin (40) by young healthy males increased **faecal pH**. Large amounts of other forms of dietary fibre such as corn bran (41) or non-starch polysaccharides from wheat bran (42) did not lower faecal pH. Thornton et al.(43) suggested that faecal pH also is influenced by the intake of polymeric carbohydrates. Consuming 150 g fat mainly of animal origin increased the faecal concentration of the main **bile acids** (BA) deoxycholic acid (DC) and lithocholic acid (LC) in total faeces (44). The ratio primary to secondary BA (cholic acid to DC and chenodeoxycholic acid to LC) increased after a diet rich in dietary fibre (45). The concentration of BA in faecal water increased after an intake of 90 g corn oil, while the concentration decreased after consuming 50 g less dietary fat (46). Allinger et al (47) described that the concentration of DC in faecal water decreased after shifting from a mixed to a lactovegetarian diet. A diet with approximately 100 g saturated fat increased the concentration of **long chain fatty acids** (LCFA)in total faeces, while the concentration of BA did not increase (48). Newmark et al (49) hypothesised that Ca could bind the BA and LCFA in the intestine, thereby making them insoluble and thus less harmful for the colonic epithelium.

Also non-dietary factors influence the faecal biomarkers. Males have larger faecal bulk and higher defaecation frequencies than females (41,42). Hormonal factors like those in pregnancy and the luteal phase of the ovulatory cycle decrease defaecation frequency (50). Bingham et al showed that physical activity does not seem to influence transit time or defaecation frequency in healthy subjects (51). Obese subjects have higher faecal concentrations of BA (52). Patients who had a cholecystectomy, have higher concentrations of faecal DC (53).

1.3.3 Faecal markers and CRC risk

Higher **faecal wet weight** was found in populations with a lower risk (28, 54-56). No data are available from case-control and prospective studies, most probably because of difficulties in getting reliable data.

Defaecation frequency did not differ significantly between population groups or between cases and controls (56,57). Although the hypothesis of a shorter time of contact between noxious agents in faeces and the colonic mucosa is biologically attractive, transit time and colorectal cancer incidence were not associated in a case-control study in Greece (58). With regard to the correla-

tion studies, this may be due to the methods used to determine transit time, especially when they are long (59); in case-control studies the absence of an association may be a consequence of the disease rather than a cause. It may also result from non-representative case recruitment e.g. irregular defaecation of some colon cancer cases has been reported (60).

A high **faecal pH** has been associated with an increased risk of colorectal cancer in correlation studies between countries (55), within countries (61), as well as in a case-control study in the U.S.A. (62). Moreover, Samelson et al (63) reported that acidification of stools and shortened transit time due to dietary supplementation of rats with lactulose or sulphate diminished the number of colon tumours after treatment with dimethylhydrazine. On the other hand, rats treated with dimethylhydrazine and a low colonic pH due to consumption of high amounts of guar or pectin showed a higher tumour incidence than controls (64). So, a short transit time may have been the protective factor in the study of Samelson et al.

Breuer and Goebell (65) showed in their review, that in epidemiological correlation studies and in some case-control studies an increased concentration of **DC in total faeces** was associated with a higher risk of CRC. Moreover, Hill et al (66) found in a prospective study with ulcerative colitis patients a high CRC risk for the concentration of DC in faeces. The ratio of primary to secondary bile acids was lower in high-risk patient groups (67). The ratio of LC to DC was higher in patients than controls (68). In spontaneous colon cancer-bearing rats, a higher concentration of DC resulted in a higher incidence of tumours in the proximal colon (69). Application of DC and LC to the colonic mucosa or feeding diets that enhance the concentration of DC and LC in the colon, promoted chemically induced colorectal tumours in rats (70,71).

More recently, Stadler et al (72) detected higher concentrations of **DC in faecal water** in patients than in controls. It has been postulated by Newmark et al (49) that this concentration might be a better marker for CRC risk than the concentration in total faeces, because this concentration represents the amount of DC which is in contact with the colonic mucosa. Rafter et al (73) reported that a high concentration of DC combined with a high pH showed the most severely damaging effect on a perfused colon. DC, but not LC have been shown to be mutagenic (74), although the results could not be reproduced (75). Both were co-mutagenic (76,77), co-carcinogenic (78,79) and mitogenic (80). Increased cell proliferation would favour the occurrence and expression of genetic damage.

LCFA are shown also to be mitogenic (81). Populations at high risk for CRC had high concentrations of oleic acid, one of the main LCFA in faeces (82).

Short chain fatty acids (SCFA), the principal organic anions in the colon, have a trophic effect on the colonic epithelium (83). Colonic cells receive most of their nutrition from luminal SCFA, especially butyric acid (84). In addition, butyric acid affects a number of cellular enzymes associated with cell differentiation and reduces the growth rate of human colorectal adenocarcinoma cell lines in vitro (85). Patients with adenomatous polyps or colon cancer had lower butyrate ratios in colonic contents than normal subjects (86). Adenomatous polyps in the colon are considered as precancerous lesions (87).

1.3.4 Diet, deoxycholic acid and colorectal cancer risk.

Studies on diet, faecal markers and CRC risk have been reported for humans and experimental animals, especially for DC. A higher meat, lower fibre and lower milk consumption coincided with a higher concentration of BA in faeces and a higher incidence of colon cancer in two Scandinavian countries (28). A high intake of fibre and a low concentration of BA in faeces occurred in the Scandinavian area with a low incidence of CRC (55). In a Japanese study, the concentration of DC in faeces was positively associated with the intake of fat and the incidence of colonic cancer (88). Nigro et al (89) showed that in rats a higher concentration of beef fat in the diet caused a higher concentration of DC in faeces and a higher incidence of colonic tumours.

From these data, we hypothesised that the intake of meat, dietary fibre and Ca may be related to the risk of CRC, that the concentration of DC in total faeces and in faecal water, the concentration of LCFA in total faeces and in faecal water, faecal pH and faecal bulk may constitute faecal markers that are related to CRC risk and sensitive to dietary modulation. We applied the first part of the approach shown in Figure 1 on the role of diet in the aetiology of CRC by studying the effects of habitual diets differing in meat, fibre and Ca (omnivorous, vegetarian, vegan and Ca-restricted diets) on the faecal markers of biological effective dose related to CRC risk.

2. Objectives

The objectives of the investigations described in this thesis were:

1. to develop an accurate and valid analytical method for the determination of the concentrations of secondary faecal BA as markers of CRC risk

2. to study the effects of an omnivorous, a lacto-vegetarian and a vegan diet in a controlled metabolic study with volunteers on faecal bulk, pH, defaecation frequency and BA in faeces as markers of CRC risk
3. to analyse the same faecal markers in habitual omnivores and vegetarians
4. to determine the effects of Calcisorb[®], which is given to urolithiasis patients with hypercalciuria type I, on the same faecal markers in a randomized, double-blind, placebo-controlled trial with volunteers preceded by a study with another Ca-binder (ethylene diamine tetra acetic acid, EDTA) in rats
5. to study the effects of the Ca-restricted diet of urolithiasis patients with hypercalciuria type II on the same faecal markers in a randomized clinical trial

The results of our studies with humans (metabolic, correlation and experimental) were compared with those from other reported studies and discussed from a pathogenetic point of view. Also the significance of the results for the CRC risk of omnivorous and vegetarian diets, the Calcisorb[®]-treatment of urolithiasis patients with hypercalciuria type I and the Ca-restricted diet of urolithiasis patients with hypercalciuria type II were discussed. Some conclusions were drawn.

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Determination of individual human faecal bile acids by gas-liquid chromatography after enzymatic deconjugation and simultaneous solvolysis and methylation using dimethoxypropane

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Based on: Clin. Chim. Acta 152 (1985) 231-239

Introduction

In recent years there has been a renewed interest in the measurement of faecal bile acids, because of their possible involvement in colorectal carcinogenesis (1,2).

Several methods have been employed for the determination of individual bile acids in human faeces (3-8). Major problems in the measurement are the extraction of bile acids from the particulate matter in the stool and interference of fatty acids and neutral steroids in the gas-liquid chromatographic (GLC) analysis. Furthermore, conjugated and/or sulfated bile acids can be present in human faeces; therefore, a deconjugation and solvolysis step should be included in the analytical procedure (9-15).

Recently, a very detailed method has been described in which all faecal bile acid metabolites are measured, including conjugated and sulfated (16). The procedure, however, is time-consuming and needs expensive and not widely available equipment like capillary GLC-mass spectrometry.

We developed a rapid GLC procedure for the determination of individual human faecal bile acids, using enzymatic deconjugation and subsequently solvolysis and methylation in one step with 2,2-dimethoxypropane.

Materials and methods

Chemicals

All reagents for extraction and analysis were of analytical grade and obtained from Merck Darmstadt (FRG). β -Cholyglycine hydrolase (EC 35.12.24; spec. act. 7194 U/mg protein; 1.39 mg protein/ml) was obtained from Schwarz-Mann (Orangeburg, NY, USA).

Bile acid standards

The following reference bile acids were obtained from Steraloids Inc. (Wilton, NH, USA): lithocholic acid (LC), deoxycholic acid (DC), chenodeoxycholic acid (CDC), cholic acid (C) and 7-ketodeoxycholic acid (7-K-DC). Taurocholic acid (TC) and glycocholic acid (GC) were obtained from Calbiochemical (San Diego, CA, USA). The sulfated bile acids 3 α -sulfolithocholic acid (SLC), 3 α -sulfodeoxycholic acid (SDC) and sulfochenodeoxycholic acid (SCDC; consisting of 70% 3 α -sulfo CDC and 30% 7 α -sulfo CDC) were obtained from P.L.-Biochemicals (Milwaukee, WI, USA). All reference bile acids were > 95% pure.

Radiolabelled [24-¹⁴C]lithocholic acid (50 mCi/mmol) and [24-¹⁴C]deoxycholic acid (60 mCi/mmol) were obtained from the Radiochemical Centre (Amersham, UK). [24-¹⁴C]Chenodeoxycholic acid (50 mCi/mmol) was obtained from New England Nuclear (Boston, MA, USA).

Instruments

Faeces were homogenized using a Philips mixer and freeze-dried subsequently in a Breda Scientific Freeze dryer LY-S-FM. Gas-liquid chromatography was performed on a F & M 402 high efficiency chromatograph (Hewlett Packard, Avondale, USA) with a U-shaped all glass column, 1.20 m X 3 mm i.d., packed with 1% OV-210 on Gaschrom Q, 100-120 mesh (Chrompack, Middelburg, The Netherlands). Operating conditions for GLC were: oven temperature 235°C, temperature injection point 250°C, temperature flame ionisation detector 245°C. Nitrogen was used as a carrier gas, flow rate 20

ml/min; hydrogen 40 ml/min, air 310 ml/min. Retention times and peak areas were measured with a computing integrator (Minigrator, Spectra-Physics, Eindhoven, The Netherlands) and chromatograms recorded on a recorder of Honeywell Controls LTD (UK). Radioactivity was determined in a Philips liquid scintillation counter PW4700 (Philips, The Netherlands). Combustion of faecal samples for determination of total ^{14}C -radioactivity was performed in a sample oxidator (17).

Collection and preparation of faeces

Fresh voided faeces were collected and frozen in solid CO_2 . After weighing, this was diluted with a known amount of distilled water and homogenized with a mixer at a temperature of 80°C (to speed up thawing and denature bile acid converting enzymes). A sample (10 g) was immediately frozen (-20°C), lyophilized for 48 h and stored at -20°C until analysis.

Analytical procedure

The method is a modification of the Grundy technique (3). The procedure is illustrated in the flow sheet (Fig. 1). The procedure is described here stepwise.

Enzymatic deconjugation 50 mg lyophilized faeces are weighed in a glass stoppered centrifuge tube. Two millilitres of 0.1 mol/l sodium acetate buffer (pH 5.7 with 2.0 g EDTA/l and 0.2 ml β -mercaptoethanol/l) and 50 μl cholyglycine hydrolase are added. Incubation is carried out overnight in a waterbath at 37°C .

Saponification To each sample 0.1 ml 5 mmol/l 7-K-DC are added as an internal standard to correct for losses during the procedure. One millilitre of 10 mol/l sodium hydroxide, 6 ml methanol and some boiling chips are added and the mixture is refluxed for 2 h at boiling point.

Extraction of neutral steroids Neutral steroids are extracted 3 times in petroleum ether after shaking and centrifugation.

Solvolysis and methylation After the extraction of the neutral steroids 1.0 ml HCl and 7.0 ml DMP/ml aqueous solution is added. After mixing, incubation is carried out for 18 h in the dark (to prevent formation of pigment polymers) at 37°C . After this overnight incubation the mixture is evaporated under a stream of nitrogen at 50°C . Beyond this temperature, a dark brown residue will appear due to the formation of pigment polymers.

FIGURE 1

Flow sheet of the method for the analysis of human faecal bile acids.

Extraction of bile acids 7 ml aqua dest is added to the residue and the methylated bile acids are extracted 3 times with 10 ml diethylether. After completion the diethylether is evaporated under a stream of nitrogen.

Derivatisation The methylated bile acids are converted into their trifluoroacetates by addition of 0.2 ml trifluoroacetic anhydride to the residue and incubation in closed tubes for 20 min at 37° C.

Gas-liquid chromatography The trifluoroacetic anhydride is evaporated and the residue dissolved in 0.2 ml acetone. After injecting a 1 µl sample in the gaschromatograph, the bile acids are identified by comparing the retention times to those of reference bile acids and for one keto bile acid with a reference in the literature on a QF-1 column. The amount of bile acid in each sample is calculated by dividing the peak area of the bile acid by that of the internal standard, multiplied by the amount of internal standard added to the sample and corrected for the response factor of the flame ionizing detector.

Liquid scintillation counting In case recovery experiments with radiolabelled bile acids are performed, 0.1 ml of the final acetone mixture is transferred into a counting vial and evaporated. The residue is dissolved in a PPO/POPOP mixture (5:0.2/litre) in toluene and counted with external standardization for quench correction.

Determination of accuracy and precision The accuracy of the method is determined by measuring the recoveries of added reference bile acids to lyophilized faeces. Furthermore, the recovery of endogenously present DC and CDC in the stool of two patients (injected with ¹⁴C-labelled DC and CDC for kinetic studies) is determined by comparing radioactivity in the final acetone extracts with radioactivity measured after direct combustion of the faecal samples. The intra-assay precision is evaluated by calculating the CV values from the duplicate measurements of LC and DC in a control sample on different days. The inter-assay precision is calculated from the means of the duplicate measurements each day.

Results

In Fig. 2A, a GL chromatogram of a faecal sample with secondary bile acids is shown. Figure 2B shows a GL chromatogram of a faecal sample in which a considerable quantity of primary bile acids are present. A reasonable separation of bile acids is achieved within 10 min. Unfortunately on an OV-210 column isolithocholic acid (3β-hydroxy-5β-cholanic acid) cannot be

FIGURE 2
 GLC of faecal bile acids in two different stools. (A) with secondary bile acids and a minor fraction of primary bile acids; (B) with secondary and primary bile acids.

TABLE 1
 Recovery of radiolabelled reference bile acids added to faeces

No. of samples	Added bile acid	Recovery (% $\pm$ SD)
10	[24- 14 C]LC	89.7($\pm$ 1.6)
12	[24- 14 C]DC	86.3($\pm$ 1.7)
10	[24- 14 C]CDC	85.8($\pm$ 2.6)

separated from lithocholic acid. In contrast, isodeoxycholic acid (3β,12α-dihydroxy-5β-cholanic acid) can be separated from deoxycholic acid. At the end of the run keto bile acids are eluted, of which 3β,12-keto-5β-cholanic acid and 3α,12-keto-5β-cholanic acid can be identified. However, these keto bile acids are not always completely separated from cholic acid.

Recoveries of added radiolabelled reference bile acids are shown in Table 1 and proved to be sufficient and reproducible. Recoveries of two conjugated, four unconjugated and three sulfated bile acids, measured by GLC, are

TABLE 2

Recovery of reference bile acids added to faeces, determined by GLC

No. of samples	Added bile acid	Recovery (% $\pm$ SD)
8	LC	95.8($\pm$ 5.0)
8	DC	95.5($\pm$ 6.5)
8	CDC	96.2($\pm$ 8.2)
4	C	92.8($\pm$ 4.0)
4	GC	85.5($\pm$ 0.7)
4	TC	84.5($\pm$ 7.7)
8	SLC	100.0($\pm$ 10.2)
8	SDC	101.3($\pm$ 3.0)
8	SCDC	98.0($\pm$ 5.4)

TABLE 3

Recovery of ^{14}C from faeces on 4 consecutive days ^a

Patient 1 ^b [24- ^{14}C]DC	Radioactivity after combustion (disintegrations/g faeces)	Radioactivity after extraction (disintegrations/g faeces)	Recovery %
Day 1	1220	1040	85.2
Day 2	8455	7336	86.7
Day 3	6477	5601	86.5
Day 4	10525	8257	78.4
Patient 2 ^c [24- ^{14}C]CDC			
Day 1	8419	7441	88.4
Day 2	16193	12891	79.6
Day 3	7077	6890	97.4
Day 4	5076	4383	86.3

^a After intravenous injection of [24- ^{14}C]DC or [24- ^{14}C]CDC in two patients.^b Patient with malignant adenoma of the colon and secondary bile acids in faeces.^c Patient with bile acid induced diarrhoea and only primary bile acids in faeces.

sufficient and reproducible as is shown in Table 2. Recoveries of two endogenous bile acids (DC and CDC) were determined in two patients. In one patient (injected with [24- ^{14}C]DC) only secondary bile acids were present in the stool; in the other patient (injected with [24- ^{14}C]CDC) only primary bile acids were detected in the stool. Faeces was collected during four days. Combustion of faeces in a sample oxidator (± 1 g wet faeces) was used as a reference. The efficacy of the combustion had been determined by adding a

known amount of $[24\text{-}^{14}\text{C}]\text{DC}$ to wet fresh voided faeces. The recovery of the $^{14}\text{C}_{\text{O}_2}$ was $96.7 \pm 6.9\%$ ($n=5$) of the calculated radioactivity. After extraction of the faeces of the two patients, the recovery of DC was $84.2 \pm 3.9\%$ (mean $\pm$ SD) and of CDC $87.9 \pm 7.3\%$ (mean $\pm$ SD), compared to combustion in the sample oxidator, as is shown in Table 3. The intra-assay precision for the determination of the most prominent faecal bile acids LC and DC was 2.8 and 4%, respectively. The inter-assay precision was 9.0% and 7.2% for LC and DC, respectively.

Discussion

Most extraction procedures of faecal bile acids are complex, use several purification steps and are time-consuming. The Grundy method (3), which is regarded as our reference procedure does not allow the measurement of sulfated bile acids and some keto bile acids. Furthermore, the strong alkaline hydrolysis which is used, can give rise to artefacts (18), like the formation of unsaturated derivatives of sulfated chenodeoxycholic acid (23). In our method alkaline hydrolysis is replaced by enzymatic deconjugation. The conjugated bile acids are hydrolysed sufficiently.

Bile acids are extracted from the particulate matter by refluxing in an alkaline methanolic solution. We use methanol instead of ethanol to prevent the formation of bile acid ethylesters. The extraction of endogenous DC and CDC is acceptable by refluxing for two hours. The extraction efficiency of endogenous LC could not be detected unfortunately. However, Podesta et al extracted 99% of the radioactivity in faeces of a healthy volunteer, who ingested $[24\text{-}^{14}\text{C}]\text{CDC}$, by refluxing 2 h in an alkaline ethanolic solution (9). Since CDC is normally almost completely dehydroxylated to LC (+ isoLC) in the large bowel, the radioactivity would be present in this last fraction.

After the saponification step neutral steroids must be extracted with petroleum ether, because coprostanon can interfere with the gas liquid chromatographic analysis on OV-210. This compound is eluted at the same time as (iso)lithocholic acid.

The extent of sulfation of bile acids in human faeces is still debated. Some investigators found up to 40% sulfated bile acids in human faeces (9,10), whereas, others found only a minor fraction ($< 5\%$) sulfated bile acids in human stool (11,12,14,15). Because sulfated bile acids (mainly sulfated lithocholic acid) can be present in human faeces we decided to carry out a

solvolysis procedure. The solvolysis procedure, originally described by Javitt (19) and applied to serum by Cantafora et al (20), seemed appropriate to use for faecal bile acids. An advantage of the method is the simultaneous methylation of the free bile acids that occurs in the acidified anhydrous mixture (21). To split CDC-7-sulfate incubation overnight at 37°C is warranted (22). We have tried to measure the amount of unsulfated bile acids by extraction of the bile acids after saponification with diethylether at an acidic pH. However, in accordance with the observations of Pageaux et al (23) added sulfolithocholic acid was partly solvolysed during this procedure. Even when the extraction was performed at a lower temperature or higher pH partial solvolysis took place. So, quantitative measurement of unsulfated bile acids was not possible.

Recently, a rapid extraction procedure for the determination of faecal bile acids was published (8). Recoveries, however, were measured qualitatively using thin-layer chromatography or quantitatively by enzymatic determination.

In conclusion, the proposed method for the determination of the major bile acid metabolites in human stool by GLC is rapid and does allow the determination of a considerable number (about 48) of faecal samples in 3 days. The simultaneous solvolysis and methylation of the bile acids after saponification is of particular advantage. Conjugated and sulfated bile acids, which may be present in human faeces, are included in the determination of the individual bile acids.

Acknowledgements

This study was supported by The Netherlands Cancer Foundation (NUKC 78-4 and 83-10), The Netherlands Organisation for Advancement of Pure Research (ZWO 94-38) and the University of Nijmegen.

The authors are grateful for the technical assistance of Mrs. A. van Schaik at the beginning of the study.

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Bile acids, neutral steroids, and bacteria in faeces as affected by a mixed, a lacto-ovovegetarian, and a vegan diet

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Based on: Am. J. Clin. Nutr. 46 (1987) 962-967

Abstract

In a metabolic ward 12 healthy male subjects consumed mixed Western (M), lacto-ovovegetarian (L), and vegan (V) diets in a randomized order for 20 d each. The concentrations of deoxycholic acid, isolithocholic acid, and total bile acids in 4-d composites of faeces on the L and V diets were significantly lower than on the M diet. The chenodeoxycholic-to-isolithocholic plus lithocholic acid ratio was significantly higher on the V diet. The concentrations of coprostanol and of coprostanol plus cholesterol were highest on M diet and lowest on V diet. The number of faecal lactobacilli and enterococci on the V diet was significantly lower than on the M or the L diets. This study showed a decrease in the concentration of faecal (secondary) bile acids by the L and the V diets and an alteration of the faecal flora composition by the V diet.

Introduction

In epidemiological studies the mortality of colorectal cancer has been associated with the diet. For omnivores a higher mortality is reported for colorectal cancer than for vegetarians (1, 2) and vegans (3). A high-fat low-fiber diet is associated with a high risk of developing colorectal cancer (4).

However, lifestyle factors other than the diet also may be responsible for the difference in colorectal cancer mortality between groups with different dietary habits. To determine the role of the diet in developing colorectal cancer, many studies have been performed on the influence of fat, fiber, and meat on faecal characteristics, which also are associated with this form of cancer (5, 6). We studied the combined effect of three types of habitual diets, ie, mixed Western (M), lacto-ovo-vegetarian (L), and vegan (V) diets, on faecal concentrations of bile acids, neutral steroids, and bacteria.

Materials and methods

Subjects and diets

The study was a diet-controlled, Latin-square design involving 12 male Caucasians, aged 20-27 y, selected from a pool of volunteers because of general health condition as assessed by hematological and clinical examination, dietary habits (nonvegetarian), and trustworthiness. Two identical experiments were carried out with six volunteers each. They were housed in the institute's controlled metabolic ward and continued their normal daily routine. A working group responsible for human nutrition studies performed at the institute approved this study.

After an adaptation period of 8 d on a mixed diet, the three experimental diets were provided in randomized order for 20 d each. The energy and nutrient contents of the diets were determined as described earlier (7). The mean values of four daily samples of each diet are shown in Table 1. The nutrient composition of the diet was based on a descriptive study of the nutritional status of Dutch lacto-ovo-vegetarians and omnivores (8) aged 18-30 y. The diets differed in the amount and composition of fat and dietary fiber and in composition of the protein. The percentage of meat protein was zero in the L and V diets and 46 in the M diet. The percentage of milk protein was zero in the V diet and 28 and 54 in the M and L diets, respectively. The percentage of vegetable protein in the V, L, and M diets were 100, 46, and 26, respectively. The food was prepared in the diet kitchen; portions were weighed to the nearest gram and deep frozen if necessary. Sugar, soft drinks, and jelly were supplied when the body weight, as measured in the adaptation period, had decreased > 2%.

Faecal steroids

Faeces were collected at -20° C using deep-freeze toilets (9). Composites of 4-d collections were thawed overnight at room temperature, homogenized,

TABLE 1
Nutrient composition of the diets

Nutrient	Mixed diet (M)	Lacto-ovovegetarian diet (L)	Vegan diet (V)
Metabolizable energy (MJ)	11.8	12.9	13.0
Metabolizable energy (kcal)	2820	3080	3110
Protein (energy %)	17	16	17
Fat (energy %)	38	32	33
Available carbohydrates (energy %)	45	52	50
Linoleic acid (energy %)	5	1.1	17
Saturated fatty acids (energy %)	1.3	14	8
Neutral detergent dietary fiber (g)	22	28	40
Hemicellulose (g)	11	13	22
Cellulose (g)	7	10	12
Lignin (g)	4	5	6

and frozen in portions. From a thawed portion 0.5 g was weighed into a glass-stoppered centrifuge tube. The concentration of faecal steroids was measured by a modification of the method for faecal bile acids as described earlier (10). Enzymatic deconjugation was omitted because in healthy subjects most faecal bile acids are deconjugated (11). Hyodeoxycholic acid and 5 α -cholestane (Steraloids Inc, Wilton) were used as internal standards for the determination of acidic steroids (bile acids) and neutral steroids (cholesterol and vegetable steroids such as β -sitosterol), respectively. Sulfated steroids (12) were split by addition of 7 ml 2,2-dimethoxypropane per ml faecal water, acidification to 0.1 N HCl, and incubation overnight at 37° C. After neutralization with NaOH in methanol, the anhydrous mixture was evaporated under a nitrogen stream. Esters were hydrolysed and bile acids were extracted from the particulate matter with a 76% (vol/vol) methanolic solution containing 1 N NaOH. The neutral steroids and fatty acids were extracted with petroleum ether after acidifying the mixture with acetic acid to pH 4. The neutral steroids were trifluoracetylated after evaporation. Bile acids were extracted with diethyl ether after acidifying to pH < 1 with concentrated HCl and then methylated in methanol-2,2 dimethoxypropane-concentrated HCl, 10:10:1 (vol/vol) by incubating overnight at room temperature in the dark. After evaporation of the reagents, the methylated bile acids were trifluoracetylated. Neutral steroid trifluoracetates and bile acid methyl ester trifluoracetylates were analyzed on a 25 m x 0.45 mm id capillary glass column coated with 0.4 μ m CP Sil 5 (Chrompack, Middelburg, the Netherlands) using a Packard Becker 420 gas chromatograph (Packard Instrument Co, Inc, Downers Grove, IL) with a solid injector. Operation and conditions were as

follows: column oven temperature 224° C; injector port temperature 235° C; hydrogen flame detector 260° C; carrier flow of helium, 8 ml/min; and split-flow 15 ml/min. Peaks were identified by comparing their relative retention times with those of pure standards. The amount of each steroid was calculated from the relative peak height and amount of internal standard used. Peak height of each steroid was corrected for detector response. Samples from the three different dietary periods of one person were processed simultaneously.

Faecal bacteria

The microbial composition of two faecal samples taken on different days during the last week of a dietary period was determined. Immediately after defaecation, subsamples from different regions of the stool were pooled in a prereduced transport medium (13). Prereduced media and an anaerobic glovebox were used for the analyses of anaerobic microorganism. Total anaerobes and clostridia were determined on blood agar and SuaP-agar (14), respectively. Bifidobacteria were enumerated on bifidobacteria agar and Bacteroides species on KVL agar (15). Isolates were confirmed by gas chromatographic analysis of the volatile fatty acids and lactic and succinic acids in culture broths.

Ambient conditions were used for analysis of aerobic microorganisms. The total number of aerobes, enterobacteriaceae, lactobacilli, and enterococci were estimated using tryptone soya glucose agar (TSGA) (CM 131, Oxoid Ltd, Hants, UK, supplemented with 1 % glucose), violet red bile glucose (VRBG) (Oxoid CM 485), Rogosa (5413, Merck, Amsterdam, the Netherlands), and kanamycin milk agar (13), respectively.

Statistical analysis

Analysis of variance was applied to detect differences in faecal characteristics during the dietary periods and the two experiments. The effects of the diets were corrected for possible residual influences of one preceding experimental diet period (16). Variables that were not normally distributed, even after transformation, were analyzed using the signed-rank test.

Results

All 12 subjects completed the study and adhered faithfully to the dietary regimen. There were no reports of any health problems. No statistically

TABLE 2

Concentrations of faecal bile acids during mixed (M), lacto-ovovegetarian (L), and vegan (V) periods

Bile acid	Concentration in $\mu\text{mol/g}$ wet weight ^a		
	M	L	V
Deoxycholic acid (DC)	1.95 (0.8-3.2)	1.52 (0.6-2.7)*	1.29 (0.2-2.1)*#
Isolithocholic acid (ILC)	0.90 (0.5-1.3)	0.69 (0.1-1.1)*	0.52 (0.1-0.8)*
Lithocholic acid (LC)	1.36 (0.1-2.0)	1.14 (0.1-1.7)	1.08 (0.1-1.9)
Chenodeoxycholic acid (CDC)	0.15 (0.0-0.4)	0.14 (0.0-0.4)	0.17 (0.0-0.3)#
Ursocholic acid (UDC)	0.13 (0.1-0.3)	0.15 (0.0-0.3)	0.14 (0.1-0.2)
7-ketodeoxycholic acid (7-K-DC)	0.00 (0.0-0.1)	0.00 (0.0-0.1)	0.00 (0.0-0.1)
7-ketolithocholic acid (7-K-LC)	0.21 (0.0-0.5)	0.17 (0.1-0.5)	0.13 (0.0-0.3)
DC+ILC+LC+CDC+UDC+7-K-DC+7-K-LC	4.61 (3.0-7.3)	3.77 (2.1-5.7)*	3.34 (1.0-5.2)*
CDC/ILC + LC	0.06 (0.0-0.2)	0.10 (0.0-0.5)	0.15 (0.0-0.5)*#

^a Mean values with minimum and maximum values in parentheses.

* Significantly different from their respective values in the M period ($p < 0.05$).

Significantly different from their respective values in the L period ($p < 0.05$).

significant residual effects of the preceding diet period and no differences in results between the two experiments were observed. Therefore, results are given for the 12 subjects.

Faecal bile acid concentrations were influenced by the diets (Table 2). The faecal concentration of deoxycholic acid (DC), isolithocholic acid (ILC), and the sum of DC, ILC, lithocholic acid (LC), chenodeoxycholic acid (CDC), ursodeoxycholic acid (UDC), 7-ketodeoxycholic acid (7-K-DC), and 7-ketolithocholic acid (7-K-LC) decreased significantly ($p < 0.05$) during the L and V diets. During the V diet, the concentration of DC was lower and that of CDC higher than during the L diet. For ILC there was a trend ($0.05 < p < 0.1$) to a lower concentration on the V diet compared with the L diet and for LC a trend to a lower concentration on the V diet compared with the M diet. The CDC:(ILC + LC) ratio, which is a measure for the primary-to-secondary bile acids ratio, was significantly higher ($p < 0.05$) during the V diet than during the M and L diets.

The concentrations of faecal cholesterol and coprostanol and their sum were significantly lower ($p < 0.05$) during the V diet than the L diet (Table 3). The concentrations of coprostanol and of cholesterol plus coprostanol on the L diet was lower than the M diet; for cholesterol there was a trend ($0.05 < p < 0.1$) to a lower concentration on the L diet than on the M diet. The cholesterol-to-coprostanol ratio, which is a measure of the cholesterol

TABLE 3
Concentrations of faecal cholesterol and coprostanol during mixed (M), lacto-ovovegetarian (L) and vegan (V) periods

Neutral steroid	Concentration in $\mu\text{mol/g}$ wet weight ^a		
	M	L	V
Coprostanol	9.50 (1.6-20.7)	6.67 (2.8-11.4)*	3.62 (1.1-9.6)*#
Cholesterol	2.17 (0.2-4.8)	1.60 (0.2-4.0)	0.91 (0.1-2.5)*#
Cholesterol + coprostanol	11.68 (3.8-24.5)	8.27 (3.2-15.0)*	4.53 (1.8-12.1)*#
Cholesterol/Coprostanol	0.39 (0.0-2.2)	0.23 (0.0-0.4)	0.28 (0.1-0.7)

^a Mean values with minimum and maximum values in parentheses.
 * Significantly different from their respective values in the M period ($p < 0.05$).
 # Significantly different from their respective values in the L period ($p < 0.05$).

TABLE 4
Faecal cholesterol-to-coprostanol and 24-ethyl cholesterol (β -sitosterol)-to-24-ethyl coprostanol ratios of one volunteer during successive mixed (M), vegan (V), and lacto-ovovegetarian (L) periods

Ratio	M ₁	M ₂	M ₃	V ₁	V ₂	V ₃	L ₁	L ₂	L ₃
Cholesterol:Coprostanol	2.3	2.2	2.2	2.0	0.3	0.3	0.2	0.3	0.2
24-ethylcholesterol: 24-ethyl coprostanol	2.8	3.4	2.9	1.8	0.10	0.1	0.1	0.1	0.1

hydrogenating activity of the faecal flora, did not change significantly. For one volunteer, however, lower cholesterol-to-coprostanol and ethyl cholesterol (β -sitosterol, a vegetable steroid)-to-ethyl coprostanol ratios were found after changing from the M to the V and then to the L diets (Table 4). In the first 4-d composite of faeces of this volunteer on the V diet, the ratios still were similar to the ones on the M diet.

The mean counts of anaerobic microorganisms in the faeces during the different dietary periods differed only slightly (Table 5). The V and L diets reduced clostridia counts to some extent in eight volunteers. The numbers of lactobacilli and enterococci were significantly ($p < 0.05$) lower on the V diet compared with the L diet. The number of lactobacilli was significantly ($p < 0.05$) higher on the M diet than on the V diet. In some individuals the differences were > 2 log units.

TABLE 5
 Number of faecal bacteria during mixed (M), lacto-ovovegetarian (L), and vegan (V) periods

Faecal bacteria	Number (log viable count/g wet weight)					
	M		L		V	
	Mean	Range	Mean	Range	Mean	Range
Total anaerobes	9.9	9.0-10.8	9.9	9.4-10.3	9.9	8.9-10.6
Clostridia	7.9	6.8-8.8	7.5	6.7-8.3	7.4	6.5-8.4
Bifidobacteria	8.8	< 7-10.3	8.7	7.7-9.5	8.9	< 7-10.1
Bacteroides	9.5	9.0-10.5	9.5	9.2-10.1	9.6	8.6-9.9
Total aerobes	6.9	6.2-7.7	7.2	5.6-8.5	6.7	5.7-8.4
Enterobacteriaceae	6.4	4.9-7.7	6.6	5.0-8.4	6.0	< 4-8.4
Enterococci	5.3	3.5-6.4	5.8	4.4-7.3	5.1 [*]	< 3-6.7
Lactobacilli	4.9	3.2-6.4	5.4	4.9-6.7	4.1 ^{*#}	< 3-6.4

^{*} Significantly different from their respective values in the L period (p < 0.05).

[#] Significantly different from their respective values in the M period (p < 0.05).

Discussion

The aim of this study was to measure the influence of habitual diets on faecal characteristics that have been associated with the risk of developing colorectal cancer, ie, the faecal concentrations of bile acids, neutral steroids, and bacteria. The concentrations of the secondary bile acids DC and ILC and also of the sum of the measured bile acids, which are positively associated with the risk of colorectal cancer (17,18), was highest on the M diet. This diet provided the highest amount of fat and meat and the lowest amount of dietary fiber. A higher excretion of bile acids and a lower faecal wet weight (19) was found on this diet than on the L and V diets, causing the higher bile acid concentration.

The enhancing effect of fat on the DC concentration was described for beef fat (20). The lowering effect of dietary fiber on the concentrations of total bile acids was described for fiber from bran (7,21), for a fiber-rich diet (22) and for vegetarian diets (3,23,24). The DC concentration was lower on the V diet than on the L diet. The fat content of both diets was equal and so were the resulting faecal weights. However, the V diet contained no milk products and more polyunsaturated fatty acids than the L diet. The hypothesis has been put forward that milk products increase the excretion of DC because of

gastrointestinal binding to insoluble calcium phosphate (25). Polyunsaturated fatty acids were found to increase the excretion of faecal bile acids only, at high cholesterol levels of the diet (26). The V diet contained almost no cholesterol.

The CDC:(ILC + LC) (primary-to-secondary bile acid) ratio, which is negatively associated with the risk of colorectal cancer (3,24), was highest on the V diet. The ratio of the other primary bile acid (C) to its secondary products (IDC + DC) could not be determined because of the poor separation of C and DC in 30% of the chromatograms. A higher ratio has been described after consumption of a diet rich in fiber (27). The V diet contained the highest amount of dietary fiber, especially of fermentable hemicellulose. The higher ratio of CDC:(ILC + LC), mainly because of the higher concentration of CDC, may be caused by a lower 7 α -dehydroxylating activity of faecal bacteria because faecal pH was lower on the V diet (28, 29). It should be realized however that the secondary-to-primary bile acid ratio is not the only variable determining the concentration of secondary bile acids. The concentration of primary bile acids is even more important.

The concentration of faecal cholesterol plus coprostanol, which has been positively associated with the risk of colorectal cancer in epidemiological studies (4), but which promoting effect on chemically induced colon carcinogenesis is uncertain (30), was highest on M diet and lowest on V diet. The concentration of coprostanone could not be measured because of its degradation during the analytical procedure. However, this compound is a minor bacterially degraded product of cholesterol (31). The lower amount of fat and the higher amount of dietary fiber in the L diet compared with the M diet will be responsible for the lower concentration of cholesterol plus coprostanol in the faeces during the L diet, and the almost zero amount of cholesterol in the V diet for the lowest faecal concentration of cholesterol plus coprostanol on this diet (18,26,32,33).

The cholesterol-to-coprostanol ratio, which has been reported to be higher in persons belonging to groups at high risk of colon cancer (34), did not change in most of the subjects. Only one volunteer, who was a low converter of cholesterol and ethyl cholesterol (β -sitosterol)(35) on the M diet, became a high converter when consuming the V and L diets. The reason for this may be his rapid gastrointestinal transit on the M diet, which slowed down on the L and V diets (36).

The anaerobes-to-aerobes ratio, which has been shown to be higher in individuals with an increased risk of colorectal cancer (37), did not differ on the three diets. It should be realized, however, that in this ratio organisms with

potential totally different metabolism are combined and therefore this ratio probably is not relevant in bowel flora studies. Total numbers of faecal enterococci and lactobacilli were clearly reduced on the V diet compared with the other diets. For some volunteers a difference of > 2 log units was observed. A possible explanation may be the increased inhibitory action of bile acids at the lower pH value on this diet (38). Until now nonextreme experimental diets have been reported to cause only minor changes in faecal flora composition (6,39).

In a low-risk population (Finland) a greater number of faecal lactobacilli was reported (40). The Finnish diet contains a large amount of milk products (41) and, therefore, is comparable to our L diet, on which we found a higher number (as compared with the M diet) of faecal lactobacilli in 8 of 12 volunteers.

This study shows a decrease of the concentration of faecal (secondary) bile acids by the V and L diets. It also was observed that the composition of the faecal flora was changed after consuming the V diet for 2 wk.

Acknowledgements

We wish to thank Jacques T.N.M. Thissen, MSc, and Michel Wedel, MSc, for statistical support; Miss A. Wesstra for composing the diets; our subjects for eating the diets; Mr L. van Ginkel for the assays of faecal bile acids and neutral steroids; and Mr E.J. van Overeem for the determination of faecal bacteria.

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Bile acids and pH values in total faeces and in faecal water from habitually omnivorous and vegetarian subjects

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Based on: Am. J. Clin. Nutr. 58 (1993) 917-922

Abstract

Twenty habitually omnivorous subjects and 19 habitual lacto-ovo-vegetarians aged 59-65 yrs collected faeces during 4 consecutive days. The concentrations of bile acids in total faeces did not differ between the omnivores and vegetarians, but the bile acid concentrations in faecal water were significantly lower in the vegetarians. The concentration of the colorectal cancer- predicting bile acid deoxycholic acid in faecal water was explained by the intake of saturated fat and the daily faecal wet weight ($r^2=0.50$). Faecal pH did not differ between the omnivores and vegetarians. This parameter was significantly ($p<0.05$) explained by the intake of calcium ($r^2=0.30$); 24-h faecal wet weight and defaecation frequency were significantly higher in the vegetarians. In conclusion, our vegetarian subjects had a lower concentration of deoxycholic acid in faecal water, higher faecal wet weight and a higher defaecation frequency than the omnivorous subjects.

Introduction

Omnivores have a higher colorectal cancer mortality than vegetarians (1,2). In a previous study, we found a lower concentration of deoxycholic acid (DC), one of the main faecal bile acids, in total faeces and a lower pH of total faeces

in subjects on a vegan diet compared to those with an omnivorous diet (3). DC may be a promoting factor for colorectal cancer. In epidemiological correlation and some case-control studies (reviewed in 4) and in a prospective study with ulcerative colitis patients an increased concentration of DC in total faeces was associated with a high risk on colorectal cancer (5). The ratio of primary to secondary bile acids was lower in high-risk patient groups (6). The ratio of the other main faecal bile acid, lithocholic acid (LC), to DC was higher in patients than control subjects (7). In experimental studies on rats (8), a higher concentration of DC resulted in a higher incidence of colorectal tumours.

A high faecal pH has been associated with an increased risk of colorectal cancer in correlation studies between countries (9-11), within countries (12,13), as well as in case-control studies (14,15). However, in a more recent case-control study no difference in gastrointestinal pH measured by a pH sensitive radiotelemetry capsule, was found (16). Acidification of stools of rats and enhanced transit time due to dietary supplementation with lactulose or sulphate diminished the number of colon tumours after treatment with dimethylhydrazine (17). On the other hand rats treated with dimethylhydrazine and a low colonic pH due to consumption of high amounts of guar or pectin showed a higher tumour incidence than controls (18).

More recently higher concentrations of DC in faecal water (19) and plasma (20) have been detected in patients than in control subjects. It has been postulated (21) that the concentration of DC in faecal water might be a better marker for colorectal cancer risk than the concentration in total faeces, because this concentration represents the amount of DC which will be in contact with the colonic mucosa. A high concentration of DC combined with a high pH showed the most severely damaging effect on a perfused colon (22).

Our previous study was performed under metabolically controlled conditions and only bile acids and pH in total faeces were measured. In the present study we measured bile acids and pH in total and in the aqueous phase of faeces of habitual omnivores and vegetarians.

Methods

Recruitment of subjects

Subjects were recruited in two ways. One hundred forty-nine omnivores were recruited at random from a larger age- (55-69 yrs) and sex-stratified sample

(n=594), drawn from 23 municipalities in the Netherlands which participate in a pilot investigation for a cohort study on diet and cancer (23). They were asked to assess faecal pH using a strip of pH paper (Combur-3-test; Boehringer, Mannheim, Germany) and send the self assessed samples to the laboratory (24). Additionally, 11 vegetarians were recruited by the Dutch Vegetarian Society to perform the same activities, and then 10 more to send only faecal samples.

The study protocol was approved by the Medical Ethics Committees of the State University Limburg and the TNO-Nutrition and Toxicology Institute.

A structured dietary history interview was taken from the respondents at their homes (25). The frequency and amount (number of standard portions) of food items were asked. Portion sizes of important food items were checked by weighing. To minimize the effects of seasonal variation, the reference period covered the whole year preceding the interview. Nutrient intake was calculated using the standardized Dutch Uniform Encoding System (26) and the 1984 release of the National computerized Dutch Nutrient Databank (27). This databank is prepared by a national committee.

The individuals who volunteered for self-assessment of faecal pH received at home a styrofoam box with 25 kg dry ice (-78° C), a plastic bucket that fit into the toilet (Emergo, Landsmeer, The Netherlands) and coded plastic bags that fit into the bucket. On 4 consecutive days, the plastic bags containing the stools were transferred to the dry ice to freeze quickly. Defaecation date and time, and contamination with urine were recorded on a form. After 12.00 h on the fourth day, the box containing the remaining dry ice, the stool samples and the form were mailed to the laboratory.

Laboratory methods

The stools were weighed to the nearest gram and stored in a freezer at -20° C. The defaecation frequency was calculated from the number of stools between 15.00 h on the first day and 12.00 h on the fourth day. At the end of the study period of three mo the stools were thawed at 4° C to avoid pH changes and mixed inside the bag by kneading vigorously. The homogeneous-looking mixture was transferred to plastic bowls and mixed again by stirring with a wooden spatula. The pH was measured with a pH electrode for small samples at two positions and the average pH was recorded. The pH meter was calibrated with buffers of pH 4 and 7.

Measurement of the pH of homogenized faeces with an electrode for small samples appeared to be feasible and reproducible, although a few samples took about 1 minute before the value stabilized. Nevertheless, most samples could be measured immediately. The CV as calculated from 20 duplicate measurements was 2 %. Only the first two stools were analyzed because only a fraction of the subjects had three bowel movements during the sampling period.

Samples of 20 omnivores who were randomly selected and samples from all 19 vegetarians were used for the analysis of bile acids. Eight-gram samples of subsequently produced stool samples were combined per subject. This way of pooling results in an averaged concentration of exposure to the colon. Faecal water was isolated by centrifuging the wet faeces for 2 h at $25,000 \times g$ in the 250 ml bucket of a hook-rotated centrifuge rotor (XL-80; Beckman, Palo Alto, CA). A good separation between faecal water and pellet was achieved in all faecal samples.

Bile acids in total faeces were isolated as earlier (28). The methyl esters of bile acids were silylated with a pyridin/Hydrox-sil (Chrompack, Middelburg, The Netherlands) mixture (3:1 vol/vol) for 30 min at 80°C . After evaporation of the reagent, the residue was dissolved in hexane and centrifuged for 5 min at $1000 \times g$. The supernate was analyzed on a capillary fused silica column 25 m length and with 0.32 mm internal diameter coated with $0.2 \mu\text{m}$ CP Sil 19 CB (Chrompack) combined with a capillary fused silica column 5 m length and with 0.25 mm internal diameter coated with $0.25 \mu\text{m}$ CP Sil 8 CB by using a Dani 8521-a gas chromatograph (DANI SpA, Monza, Milan, Italy) and a splitless, hot-needle injection technique. Operation conditions were as follows: the column oven temperature 6 sec at 200°C , 2.5 min increasing at $20^{\circ}\text{C}/\text{min}$, 10 min at 250°C , 8 min increasing at $3^{\circ}\text{C}/\text{min}$ and then 15 min 275°C . Temperature of the injector port was 285°C , the hydrogen flame detector was 300°C . The head pressure was $0.8 \times 10^5 \text{ Pa}$ helium. Peaks were identified by comparing their relative retention times with those of pure standards from Sigma (Instruchemie, Hilversum, The Netherlands). The amount of each steroid was calculated from the peak height and detector response relative to the internal standard (hyodeoxycholic acid).

Bile acids from faecal water were isolated by extraction with diethylether after acidifying with diluted HCl. Gas chromatography was performed as described above. The pH of faecal water was measured at 37°C with a glass pH electrode.

Differences in dietary intake and faecal values between omnivores and vegetarians and between males and females were assessed by Student's *t*-test. Variables with skewed distributions were logarithmically transformed. Linear regression was used to examine determinants of the faecal variables (29).

Results

Of the omnivores, 17 males and 30 females volunteered to collect stools on four consecutive days about 9 weeks after the dietary survey. The stools of 6 subjects (11%) were not used for determination of faecal pH, because they were already thawed on arrival at the laboratory. The stools from 4 subjects (7%) were not analyzed for faecal pH, because it was indicated on the accompanying form that the faeces were contaminated with urine. Seven vegetarian males and 14 females collected stools; 11 of them filled in the dietary questionnaire.

Table 1 shows that the habitual vegetarians had significantly ($p < 0.05$) lower intake of saturated fat, monounsaturated fat and animal protein, whereas the intake of polyunsaturated fat, dietary fibre and vegetable protein was significantly higher. The intake of calcium tended to be higher in the vegetarians than in the omnivores ($p < 0.1$).

The concentrations of bile acids in total faeces did not differ between the omnivorous and vegetarian groups (Table 2). Moreover, the ratio of primary bile acids - chenodeoxycholic acid (CDC) and cholic acid (C) - to their secondary bile acids - LC plus isolithocholic acid (ILC) and DC plus isodeoxycholic acid (IDC) - did not differ between the two groups. The LC:DC ratio was marginally lower for vegetarians (Table 2).

Table 3 shows that faecal bulk was significantly larger for vegetarians than omnivores ($p < 0.05$), and defaecation frequency was higher. Moreover, males had a higher defaecation frequency than females in both vegetarians and omnivores ($p < 0.05$). Fourty-two of the volunteers collected at least two stools. The mean pH values of these two stools did not differ significantly between omnivorous and vegetarian groups ($p > 0.05$), nor did the pH of pooled faecal water.

The concentrations of the following bile acids in faecal water were significantly lower for vegetarians than omnivores: IDC, DC, CDC, C and the sum of

TABLE 1
Nutrient intake of omnivores and vegetarians^a

Nutrient	omnivores (n=20)	vegetarians (n=11)
Energy (kJ/d)*	9229 ± 2606	9232 ± 1509
Fat (g/d)*	92.9 ± 33.9	81.7 ± 22.7
Saturated	42.8 ± 16.9	30.5 ± 14.9 [#]
Monounsaturated	33.8 ± 12.6	28.5 ± 8.2 [#]
Polyunsaturated	14.0 ± 6.3	19.7 ± 6.7 [#]
P:S ^b	0.32 ± 0.13	0.68 ± 0.90 [#]
Cholesterol (g/d)	352 ± 114	177 ± 124 [#]
Fiber (g/d)*	27.3 ± 6.5	40.8 ± 11.0 [#]
From cereal*	11.4 ± 4.7	17.8 ± 5.6 [#]
From fruit and vegetables*	15.8 ± 4.9	23.1 ± 9.3 [#]
Protein (g/d)	76.3 ± 19.6	64.2 ± 18.1
animal	51.0 ± 16.4	28.4 ± 17.0 [#]
vegetable	25.5 ± 5.7	35.8 ± 6.5 [#]
Carbohydrates (g/d)	242 ± 56	261 ± 68
Mono- and disaccharides	119 ± 45	110 ± 33
Polysaccharides	122 ± 28	139 ± 41
Calcium (mg/d)	887 ± 309	1155 ± 480 ^{\$}
vitamin C (mg/d)*	118 ± 56	134 ± 76

^a Mean ± SD

^b Ratio of polyunsaturated to saturated fat

* Logarithmically transformed in t-test

[#] Significantly different from omnivores, $p < 0.05$

^{\$} Marginally different from omnivores, $p < 0.1$

the analyzed bile acids (Table 4). In the vegetarians the percentage of ILC + LC was higher, whereas the percentage of IDC + DC was lower in the aqueous phase of faeces leading to a higher ratio of ILC + LC to IDC + DC for faecal water. The ratio of DC in the aqueous phase to DC in total faeces was significantly lower for vegetarians than omnivores (mean ± SD) 0.044 ± 0.024 versus 0.018 ± 0.016 .

To explain these results in terms of dietary intake, regression analyses were performed by using different models. The models that explained most of the variance in the faecal parameters are shown in Table 5. Fifty percent of the variance in the concentration of DC in faecal water could be explained by the intake of saturated fat, faecal bulk and sex. In omnivores the same amount of variance was explained by sex and intake of saturated fat. Thirty-nine percent

TABLE 2
Concentration of bile acids in total faeces from habitual omnivores (n=20) and vegetarians^a

Bile acid	Omnivores (n=20)	Vegetarians (n=19)
$\mu\text{mol/g}$ wet weight		
Isolithocholic acid (ILC)	0.8 (0.4-1.2)	0.8 (0.2-1.5)
Lithocholic acid (LC) [*]	1.5 (0.6-2.2)	1.4 (0.5-3.1)
Isodeoxycholic acid (IDC) [*]	0.7 (0.3-1.3)	0.6 (0.1-2.0)
Deoxycholic acid (DC) [*]	2.1 (0.9-3.2)	2.2 (0.8-4.8)
Chenodeoxycholic acid(CDC) [*]	0.1 (0.0-0.4)	0.1 (0.0-0.2)
Cholic acid (C) [*]	0.2 (0.0-1.0)	0.1 (0.0-0.5)
Ursodeoxycholic acid (UDC) [*]	1.1 (0.3-3.4)	1.2 (0.6-2.0)
Sum (ILC + LC + IDC + DC + CDC + C + UDC) [*]	6.5 (3.3-10.0)	6.4 (2.9-12.3)
CDC/ILC + LC	0.06 (0.01-0.21)	0.06 (0.02-0.15)
C/IDC + DC	0.07 (0.01-0.30)	0.06 (0.01-0.28)
LC/DC	0.73 (0.47-0.96)	0.65 (0.45-0.87) [#]
ILC + LC/IDC + DC	0.84 (0.53-1.09)	0.79 (0.61-1.06)

^a mean, range in parentheses.
^{*} Logarithmically transformed in t-test
[#] Marginally different from omnivores, p < 0.1

TABLE 3
Faecal variables of habitual omnivores and vegetarians^a

Faecal variable	Omnivores	Vegetarians
Bulk (g/24 h) [*]	119 ± 61 [19]	189 ± 70 [15] [#]
Dry weight (% by wt)	27.1 ± 4.5 [20]	27.9 ± 6.6 [19]
Faecal water (ml/g faeces)	0.33 ± 0.09 [20]	0.34 ± 0.15 [19]
Defaecation frequency (number of stools/24 h) [*]	0.9 ±0.3 [20]	1.5 ± 0.6 [17] [#]
Bile acid excretion $\mu\text{mol/day}$) [*]	505 ± 150 [18]	489 ± 178 [15]
Mean pH of two stools [*]	6.8 ± 0.3 [23]	6.8 ± 0.5 [17]
Faecal water pH	6.9 ± 0.6 [20]	7.1 ± 0.6 [21]

^a mean ± SD; n in brackets
^{*} Logarithmically transformed in t-test
[#] Significantly different from omnivores, p < 0.05.

of the variance in faecal bulk was explained by the daily amount of cereals, fruits and vegetables and sex. Defaecation frequency was explained significantly by the intake of fibre from fruits and vegetables and by sex. Unexpectedly, 30% of the variance in faecal pH was explained by the intake of calcium. Figure 1 shows the regression line between these two variables.

TABLE 4

Concentration of bile acids in faecal water from habitual omnivores and vegetarians^a

Bile acid	Omnivores (n=20)	Vegetarians (n=19)
	$\mu\text{mol/l}$	
Isolithocholic acid (ILC)*	10 (2-28)	8 (0-23)
Lithocholic acid (LC)*	23 (7-97)	21 (6-94)
Isodeoxycholic acid (IDC)*	27 (10-77)	15 (3-38) [#]
Deoxycholic acid (DC)*	87 (23-247)	34 (4-77) [#]
Chenodeoxycholic acid (CDC)*	6 (0-19)	2 (0-10) [#]
Cholic acid (C)*	19 (0-83)	5 (0-22) [#]
Ursodeoxycholic acid (UDC)*	11 (2-23)	13 (2-48)
Sum (ILC + LC + IDC + DC + CDC + C + UDC)*	187 (59-442)	98 (34-213) [#]
DC + IDC/Sum	0.6 (0.4-0.9)	0.5 (0.2-0.7) [#]
LC + ILC/Sum	0.2 (0.1-0.4)	0.3 (0.1-0.5) [#]
(ILC + LC/IDC+ DC)*	0.4 (0.1-1.1)	0.8 (0.2-2.1) [#]

^a mean; range in parentheses.

* Logarithmically transformed in t-test

[#] Significantly different from omnivores, $p < 0.05$.

TABLE 5

Linear regression of faecal parameters^a

Dependent variable	Explanatory variable	β	SE β	r^2
¹⁰ Log DC in faecal water ($\mu\text{mol/l}$)	Sex (1=male, 2=female)	0.16	0.11	0.50
	¹⁰ Log saturated fat (g/d)	0.87*	0.24	
	¹⁰ Log faecal bulk (g/24h)	- 0.41 [#]	0.22	
¹⁰ Log faecal bulk (g/24 h)	Sex (1=male, 2=female)	- 0.16 [#]	0.08	0.39
	¹⁰ Log cereal fiber (g/d)	0.46*	0.22	
	¹⁰ Log fruit and vegetable fiber (g/d)	0.59*	0.23	
¹⁰ Log defaecation frequency (stools/24 h)	Sex (1=male, 2=female)	- 0.16*	0.07	0.35
	¹⁰ Log cereal fiber (g/d)	0.25	0.18	
	¹⁰ Log fruit and vegetable fiber (g/d)	0.47*	0.19	
¹⁰ Log mean pH of two stools	Calcium (mg/day)	5×10^{-5} *	2×10^{-5}	0.30

^a n= 28.* $p < 0.05$.[#] $p < 0.1$.

FIGURE 1
 mean pH of two stools = 6.1 ± 0.0006 Ca-intake. SE β = 0.0003; r^2 = 0.24

Discussion

The aim of this study was to determine differences between omnivorous and vegetarian groups in faecal variables that have been associated with the risk of developing colorectal cancer, i.e. bile acids (especially deoxycholic acid) and faecal pH.

The volunteers were visited at their homes. Only those who were able to answer the dietary questions and to collect stools in the proper way were entered into the study.

Intake of nutrients from supplements were not included in the dietary data. Only 3% of Dutch omnivores of age 50 - 64 yrs use vitamin C pills and only 1% use calcium supplements (30). Of the vegetarians, 30% use supplements, calcium among others, and 25% use vitamin C supplements (31). Therefore, the differences between omnivores and vegetarians in intake of calcium and vitamin C are probably higher than calculated.

The concentration of DC in total faeces did not differ between omnivores and vegetarians, but the concentration of DC in faecal water was significantly

lower in the vegetarians. Turjman et al (32) found lower concentrations of DC in the total faeces of vegetarians, but the diets of the males studied contained significantly less fat than those of the omnivores (33). In the present study the vegetarians did not consume significantly less fat than the omnivores (Table 1).

Our results agree with those of others: a lower concentration of DC in faecal water when volunteers changed from a normal diet to a low-fat diet (34) and from a mixed to a lacto-ovo-vegetarian diet (35). The lower concentration of DC in faecal water was explained significantly by the intake of saturated fat and faecal bulk. Saturated fat may enhance the concentration of DC in faecal water by delivering saturated fatty acids to the gastrointestinal tract, which displace DC from its insoluble calcium salt (36). Most probably, the higher faecal bulk in vegetarians caused a lower concentration of DC in the aqueous phase due to binding of DC solid material such as fibre, bacteria, calcium or magnesium ions (37). It also confirms why animal or saturated fat and low fiber have been associated with the risk of colorectal cancer and its precursors, colorectal adenomas (38).

The higher ratios of LC + ILC to DC + IDC in total faeces from the omnivores agree with the higher ratios in patients with colorectal cancer than in control subjects (7). We also found a negative correlation between the ratio LC + ILC to IDC + DC and faecal bulk (39). On the basis of the analytical method used by Owen et al (7), the concentrations of IDC + DC and ILC + LC in our study are comparable with the concentrations of DC and of LC in the study of Owen et al.

Neither the mean pH values of the two stool samples nor the pH values of faecal water differed between the omnivorous and vegetarian groups, although diets differed in contents of dietary fibre. In metabolic studies faecal pH is lowered in young healthy males by the daily ingestion of large amounts of pectin-rich forms of dietary fibre such as 7 oranges (40), an experimental vegan diet (41) or 35 g commercial pectin (42). About 18 g of fibre from oat-bran reduced faecal pH in older, male and female volunteers (43). Large amounts of other forms of dietary fibre such as corn bran (44) or non-starch polysaccharides from wheat bran (43,45) did not lower faecal pH.

Thornton et al (46) suggested that faecal pH was influenced by the intake of polymeric carbohydrates. In the regression analyses in the present study faecal pH was not associated with this dietary variable. Pectin intake would have been higher in the vegetarians, because vitamin C intake was higher.

However, a lowering effect of pectin on faecal pH in the vegetarians would have been counteracted by an enhancing effect of their higher intake of calcium. Our results show a significant positive correlation between the mean pH of the two stools and the intake of calcium. This correlation was much lower between calcium intake and the pH of pooled faecal water. Pooling samples for the measurement of pH is probably not the best method, because samples may influence each other. In experimental studies supplemental calcium (about 1200 mg as calcium carbonate) increased the faecal pH by 0.3 units (47,48). Also, supplementation of rats with calcium phosphate increased faecal pH (49). This effect may be explained by the neutralizing effect of calcium in the colon on the poorly absorbed short chain fatty acids like lactate and succinate (50). The effect of reducing the calcium intake from 1000 to 500 mg/day on faecal pH (and bile acids) is currently being studied in a clinical trial in the Department of Urology of the State University of Limburg.

Other significant differences in faecal parameters related to colorectal cancer risk included the higher faecal bulk in the vegetarians. A higher faecal bulk has been associated with a lower risk of colorectal cancer (51). The variance in this faecal variable could be partly explained by the intake of dietary fiber from cereals. It could not be explained by the intake of polymeric carbohydrates as reported before (52). After adjustment for fiber intake, males still had a larger faecal bulk than females, although the difference was not statistically significant as have been reported previously (45).

The higher defaecation frequency in vegetarian than in omnivorous subjects corresponds with findings based on a questionnaire in the United States (53). Defaecation frequency could be explained partly by sex and the intake of dietary fiber from fruits, vegetables and cereals. In metabolic studies, defaecation frequency increased by the consumption of bran, pectin, cereals, fruits and vegetables (54). The higher defaecation frequency in vegetarians may be explained to a great extent by the higher degree of physical activity of most vegetarians (55). The relation between defaecation frequency and colorectal cancer risk is currently being studied in the Dutch prospective study on diet and cancer (23). The accuracy of the question about defaecation frequency in this study is being studied in our clinical trial mentioned above.

The lower defaecation frequency in females than in males in omnivores as well as in vegetarians (2.3 versus 3.0/day and 3.8 versus 5.4 respectively) agrees with the longer gastrointestinal transit time of females (42). It also supports the hypothesis (56) that a difference in transit time causes a higher risk of colorectal cancer in females.

From our results it appears that the lower risk of colorectal cancer in vegetarians cannot be explained by lower concentrations of bile acids in total faeces. On the other hand, the lower concentration of DC in the faecal water may contribute to the explanation of this difference. This amount of the cancer-promoting bile acid will be in contact with the colonic mucosa. The lower concentration of DC in faecal water in vegetarians may be the consequence of the stronger binding of fatty acids than DC to calcium and binding of DC to the increased amounts of solid faecal material. Moreover, the shorter time of contact between this promotor and the colonic mucosa due to the higher defaecation frequency may also contribute to the protection against the development of colorectal cancer in vegetarians.

Lacto-ovo-vegetarians had a lower concentration of DC in faecal water, probably because of the lower intake of saturated fat and the larger amount of faeces. The faecal pH was not lower in the vegetarians, probably because these subjects had a higher intake of calcium.

Acknowledgements

We thank A. Huldij, H. Brants of the TNO-Toxicology and Nutrition Institute for collection of the dietary interviews and the stool samples; and Dr. R. van der Meer of the Department of Nutrition, Netherlands Institute for Dairy Research (NIZO) for stimulating discussions and critically reviewing the manuscript.

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Effects of a calcium binder on solubility of bile acids and fatty acids in the large intestine of the rat

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Based on: Cancer Lett. 81 (1994) 77-80

Abstract

Kidney stone patients with hypercalciuria type I are treated with an oral calcium binder. Lower intakes of calcium (Ca) in the range of 0 - 1500 mg/day have been associated with an increased incidence of colorectal cancer. The aim of this study is to analyze the effects of feeding ethylene diamine tetracetic acid sodium salt (EDTA), a strong, non-absorbable binder of Ca, on the solubility of bile acids (BA) and long chain fatty acids (LCFA) in the large intestine of the rat. We have shown that the concentrations of soluble BA and LCFA in the large intestine contents remained constant while the concentration of total BA and LCFA decreased. Therefore, lowering the amount of Ca available for binding BA or LCFA is unlikely to increase the risk of colorectal cancer by that method.

Introduction

We are treating kidney stone patients with hypercalciuria type I with an oral calcium binder (1). Lower intakes of calcium (Ca) in the range of 0 - 1500 mg/day have been associated with a higher risk of colonic cancer in epidemiological studies (2- 5). Higher intakes of Ca (1000 - 3000 mg/day) have been associated with a reduced incidence of colorectal cancer in experimental studies in humans (6,7) and animals (8). The protective effect of Ca has been explained by the precipitation of bile acids (BA) and long chain

fatty acids (LCFA) in the large intestine, by that decreasing the contact of these toxic compounds with epithelial colon cells (9). Another mechanism may be the direct stimulation of epithelial cell differentiation (10).

We studied the effect of calcium binding by feeding rats ethylene diamine tetracetic acid sodium salt (Na_2EDTA), a strong, non-absorbable binder of Ca. The aim of the study was to analyze the effects of lower availability of colonic Ca on the solubility of BA and LCFA in the large intestine of the rat.

Materials and methods

Animals and their treatment

Twenty male SPF BN/M rats, 12 weeks old and weighing approximately 250 g, were obtained from the Centralized Experimental Animal Facilities of the University of Limburg and divided at random into 4 groups of 5 animals. The animals were housed under standardized conditions; each group in a separated macrolon cage with sterilized woodshavings as bedding material. They were fed ad libitum with a commercially available rat chow (SRM-A, Hope farms, Woerden, The Netherlands) containing approximately 1.4% Ca and 0.2% magnesium and had free access to demineralized drinking water, acidified with HCl to approximately pH 3.

At day 0 all animals received subcutaneously 0.5 ml of a ferri-dextrane solution (Prevan-200, AUV, Cuyck, the Netherlands) containing 200 mg Fe^{3+} per ml to prevent iron deficiency. For group 2 this was repeated after 7 days and for group 3 the injection was repeated after 7 and after 14 days.

Three groups were given twice daily at approximately 8.00 and 16.00 h 2 ml of water containing 10% (w/v) EDTA by gavage. The fourth group served as a control group and was given twice daily 2 ml of water by gavage. After 7 days the control group (group 0) and group 1 was euthanized with a CO_2/O_2 mixture (11), a blood sample taken by aorta-puncture in tubes containing EDTA for hemoglobin measurement. In the morning the contents of caecum and colon were collected for measurement of soluble and total BA, LCFA and Ca. After two weeks group 2 and after three weeks group 3 was treated in the same way.

Faecal water is difficult to separate from faeces of rats. We therefore analyzed soluble BA and LCFA from caecal contents. Due to limited amounts of caecal water per rat, the caecal contents of each group were pooled. Total BA and LCFA were analyzed in the colonic contents of each rat.

The concentrations of soluble BA and LCFA were analyzed in the supernatants of the pooled caeca of 5 rats of a group after centrifuging during 2 h at 25,000 x g. The concentrations of total BA and total LCFA were analyzed in the contents of the colon of individual rats after freeze-drying.

The analysis of bile acids was performed as described before (12) using 7-ketodeoxycholic acid instead of hyodeoxycholic acid as internal standard. Briefly, total bile acids were isolated after hydrolysis in 76% (v/v) methanolic solution containing 1 N NaOH. After extraction of neutral steroids and fatty acids with petroleum ether and acidifying the mixture to pH < 1 with concentrated HCl, bile acids were extracted with diethyl ether. They were then methylated in methanol/2,2 dimethoxypropane/concentrated HCl and silylated with pyridin/Hydrox-sil mixture (Chrompack, Middelburg, The Netherlands). The derivatives were analyzed on a combination of a capillary fused silica column coated with CP Sil 19 CB and one coated with CP Sil 5 CB. Soluble bile acids were isolated with the same method starting hydrolysis of faecal water in 100% methanol containing 1.25 M NaOH.

The concentrations of unesterified LCFA were measured as following: freeze dried contents of the colon were spiked with an internal standard (heptadecanoic acid) and incubated during 1 h at roomtemperature with methanol containing 1 mM HCl. After stopping the reaction with water, the LCFA were extracted with hexane. The derivatives were analysed on a 50 m x 0.25 mm i.d. capillary glass column coated with 0.2 um CP Sil88 (Chrompack, Middelburg, The Netherlands) using a Dani 8521-a gaschromatograph (DANI S.p.A., Monza,Milan,Italy). Operation conditions were as follows: column oven temperature rising at 4° C/min from 160° C to 230° C; injector port temperature 230° C; hydrogen flame detector 250° C; head pressure 1.6 Bar He; split-flow 50 ml/min. Faecal waters were dried in a vacuum-concentrator (Heto,Instrumentenhandel,The Hague,The Netherlands). After methylation and extraction with hexane, the derivatives were analyzed by a splitless, hot-needle injection technique. Operation conditions were: column oven temperature 2 min at 80° C, rising at 30° C/min from 80° C to 160° C and at 4° C/min from 160° C to 230° C; injector port temperature 230° C; hydrogen flame detector 250° C; head pressure 2.5 Bar He. With this method the variability of LCFA in total faeces was lower than 5 %. The variability of

the analysis of soluble LCFA was lower than 10% for the main LCFA (C16:0, C18:0, C18:1 and C18:2) making about 90% of the total LCFA in rats, while the variability of the analysis of the other LCFA was approximately 20%. Only main LCFA are analyzed, therefore.

The concentrations of calcium in colonic contents were analyzed by atomic absorption spectrophotometry after destruction of freeze dried material by 3 h boiling in 20% H₂O₂ and 1 N HCl at the Laboratory of Clinical Chemistry of the University Hospital Maastricht.

Differences between groups are analyzed using Student's t-test.

Results

The animals stayed healthy during the study as observed by the technicians giving them daily the EDTA-solution by gavage. The concentration of hemoglobin in blood remained constant.

The concentrations of BA in the supernatant of centrifuged caecal contents remained constant during the experiment, although the concentration of total BA in the colon content decreased considerably (Table 1).

The concentrations of LCFA in the supernatant of centrifuged caecal contents of groups 1 to 3 were much lower than those of the controls (group 0). The concentration of total LCFA in the colonic contents was significantly lower for groups 2 and 3 when compared with group 0 (Table 2).

TABLE 1.

Concentrations of soluble bile acids in pooled caecal contents and of total bile acids in colonic contents of rats fed EDTA

Group no.	Soluble bile acids in pooled caecal contents (μmol/l)	Total bile acids in colonic contents (μmol/g wet weight ± SD)
0	76	1.3 ± 0.5
1	70	0.9 ± 0.3
2	99	0.6 ± 0.1*
3	75	0.4 ± 0.1*

* Significantly different from group 0 ($p < 0.05$)

TABLE 2

Concentrations of soluble long chain fatty acids in pooled caecal contents and of total long chain fatty acids in colonic contents of rats fed EDTA

Group no.	Soluble long chain fatty acids ($\mu\text{mol/l}$)	Total long chain fatty acids ($\mu\text{mol/g}$ wet weight $\pm$ SD)
0	244	16.8 ± 3.3
1	29*	14.0 ± 3.7
2	56*	$11.5 \pm 4.1^*$
3	21*	$10.8 \pm 2.7^*$

* Significantly different from group 0 ($p < 0.05$)

TABLE 3

Concentrations of calcium in and weights of colonic contents of rats fed EDTA

Group no.	Calcium ($\text{mmol/g} \pm \text{SD}$)	Weight ($\text{g} \pm \text{SD}$)	'Excretion' ($\text{mmol/animal} \pm \text{SD}$)
0	0.185 ± 0.025	1.10 ± 0.40	0.21 ± 0.09
3	0.120 ± 0.031	2.28 ± 0.44	0.26 ± 0.06

The concentration of Ca in the colonic contents was lower for group 3 than for group 0, but multiplying the concentration with the weight of the colonic contents showed a higher 'excretion' of calcium by group 3 than by group 0 (Table 3).

Discussion

EDTA is a very efficient binder of cations with two free protons like calcium, magnesium, etc. We therefore fed rats with EDTA to study the effect of free calcium-binding on solubility of BA and LCFA in the gastrointestinal tract. A high solubility of these compounds is associated with an increased risk of developing colorectal cancer.

The pattern of faecal bile acids in our rats is slightly different from humans; that of fecal LCFA is similar (results not shown).

Assuming a consumption of 15 g chow per day, the molar proportion of EDTA to Ca intake would be 1:5. However only 30% of the Ca in the chow

is available as free calcium (results not shown and personal communication of Hope Farms). Therefore the ratio between the intake of EDTA and Ca was about 1:2.

Because rats normally eat during twilight hours and EDTA-gavage was at 8.00 and 16.00 h, the intake of EDTA and Ca was about the same time.

The total concentration of BA in the colon contents decreased after EDTA-feeding. Most likely this is caused by a lower excretion of BA in the colon, because multiplying the total concentration in wet weight with the weights of the colonic contents showed a significantly lower 'excretion' by group 4 than group 0. This lower excretion will be due to less precipitated BA by calcium and magnesium, which were bound by EDTA. The amount of soluble BA remained constant despite the decreasing amount of BA.

The total concentration of LCFA decreased after EDTA-feeding, although the products of concentration and weights of colonic contents showed no significantly lower 'excretion' of LCFA due to EDTA-treatment. This will be due to the stronger binding of Ca to LCFA than to EDTA (9). The amount of soluble LCFA decreased strongly after feeding EDTA. This cannot be explained by a lower pH due to lower calcium availability (13), because the pH of caecal water increased from 5.7 in group 0 to 6.5 in group 3 (results not shown). It may be explained by pulling Ca which is bound to EDTA in the stomach, into the intestine. After the production of free LCFA in the small intestine by splitting dietary triglycerides, the higher amount of Ca is bound to the LCFA, resulting in lower concentrations of soluble LCFA in groups 1-3.

This study shows that lowering the amount of Ca available for binding BA or LCFA is unlikely to increase the risk of colorectal cancer by that method, because it does not increase the concentrations of soluble BA or LCFA in the large intestine and it lowers the concentration of total faecal BA and LCFA. Whether this effect also occurs in humans consuming between 0 and 1500 mg Ca per day, has to be shown in our clinical trial with kidney stones patients, who are prescribed a calcium-poor diet (500 mg/d) due to their hypercalciuria type II.

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Effects of Calcisorb® on faecal bile acids and fatty acids in human volunteers

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Submitted

Abstract

The intake of calcium (Ca) is negatively associated with colorectal cancer (CRC) risk. The aim of this study was to investigate in a double blind, placebo controlled trial, the effects of the Ca-binder Calcisorb®, which is given to kidney stone patients with hypercalciuria type I, on risk factors for CRC risk, bile acids (BA) and long chain fatty acids (LCFA) in faecal water. Results show that the concentration of BA and LCFA in faecal water did not change, although the urinary excretion of Ca and magnesium and the concentration of Ca and magnesium in faecal water decreased. The daily excretion of BA and LCFA acids decreased significantly ($p < 0.05$) during the Calcisorb®-period. In conclusion binding dietary Ca and magnesium with Calcisorb® from a diet with a relatively low amount of fat does not enhance the solubility of BA and LCFA in faecal water.

Introduction

Lower intakes of calcium (Ca) are associated with an increased risk of colorectal cancer (CRC) in some epidemiological studies (1-4), but recently no significant association (5) and no significant association after adjustment for vitamin D intake (6) were found. In experimental studies in humans (7,8) and animals (9) Ca-supplementation decreases rectal epithelial cell proliferation.

The protective effect of Ca has been explained by the precipitation of bile acids (BA) and long chain fatty acids (LCFA), decreasing the contact of these toxic compounds with epithelial colorectal cells [10]. Another mechanism may be the direct stimulation of epithelial cell differentiation by Ca (11).

Until now, the potential modulating effect of Ca has been studied by supplementing the diet with Ca from pharmaceutical preparations (12,13) or by omitting the calcium from milk (14).

The aim of this study is to investigate the effects of Calcisorb® on the solubility of BA and LCFA in the faeces as markers for colorectal cancer risk. Calcisorb® is used in the therapy of kidney stones with hypercalciuria type I (15). This product contains sodium cellulosephosphate and binds cations like calcium (Ca) and magnesium (Mg). It is not absorbed from the intestinal tract and hydrolysis in the large intestine is minimal (information from the supplier). While the incidence of hypercalciuria type I is low, the placebo-controlled trial was performed with healthy volunteers.

Materials and methods

Subjects

Two adult male subjects (AB,MH) participated in a pilot study. For the placebo-controlled trial six healthy male volunteers were recruited from the staff of the University of Limburg. They were 23 (2x), 45 (2x) and 46 yrs old. They were all without medication which might influence Ca, bile acid and/or fatty acid metabolism. All subjects gave their informed consent, and all of them finished the study.

Experimental protocol

The study protocol of the trial was approved by the Medical Ethics Committee of the University Hospital Maastricht.

In the pilot study the subjects consumed 15 g Calcisorb® (Riker/3M, Zoeterwoude, The Netherlands) per day for three weeks. Faeces were sampled for one day before the trial, weekly during the Calcisorb® intake and one week after stopping the supplementation.

Before starting the trial, the six volunteers recorded their diet during three days (Thursday, Friday and Saturday) in specially designed diaries. They were asked to maintain their usual dietary habits during those days and during the trial. The major moments of Ca intake were analyzed by a dietician. At those moments the subjects were asked to take their prescribed dose of Calcisorb® or placebo (see below) during one week. At the end of the second period the volunteers again recorded their diet during three days (Sunday, Monday and Tuesday).

A double-blind cross-over design was used to eliminate bias due to subjects' or investigators' attitudes. To control for carry-over effects there was one week interval between both treatment periods. Subjects were divided at random between the group which started with the Calcisorb® and the one which started with the placebo.

Before starting the experiment and at the end of both experimental periods, the volunteers sampled urine for 2 x 24 hours during weekdays and all stools for 2 x 24 hours during the weekend. During 7 days before sampling stools the subjects took 10 radiopaque cubic pellets (Resprecare Medical B.V, Den Haag, The Netherlands; specific gravity 1.36 mg/mm³) three times a day for correcting excretion values for faecal flow.

All faecal and urinary samples were coded so that the origin of the samples was unknown during processing and chemical analysis.

Experimental products

The Calcisorb® was supplied following the prescription of the supplier: 15 g per day. This amount showed to have rigorous effects on faecal bile acids and fatty acids in the pilot study. It contains 3 mmol phosphate per gram (information from Riker and results of our analysis). The placebo (also 15 g per day) was made by the Dept. of Clinical Pharmacy, Academic Hospital Maastricht and consisted of microcrystalline, depolymerized cellulose (Avicel PH 101) coloured with a small amount of ferric oxide. This placebo had the same texture, colour and taste as Calcisorb® and did not swell in water. Both products were easy to consume after mixing e.g. in coffee or apple mash.

Dietary assessment

The dietary records were coded by a dietitian using the Dutch Uniform Encoding System (16). Nutrient intake was calculated using the 1990 release of the National Computerized Dutch Nutrient Databank (17). Stability of the diet during the two experimental periods was checked by measurement of sodium, urea and creatinine in the 24 h urine (18,19).

Urinary collection and analyses

Urine was sampled during 2 x 24 h using a small sampler (8 cm diameter x 15 cm height)(U-mate P, Sumitomo Bakelite Co, Tokyo, Japan) with the lower part filled with 1.7 g boric acid as preservative. It is based on the principle of the sampler described by Tochikubo (20). The apparatus is double-bottomed and has a pipe-shaped scale with a stopcock leading to the lower compartment. The diameter of the pipe is 1/50 of that of the upper compartment. The urine is voided into the main compartment. A constant fraction, can be sampled from the pipe and stored in the subcompartment. The remainder of the urine in the main compartment can be discarded. By repeating this manipulation 1/50 of the total urine excreted during 24 hours can be collected in the small cup. With this small apparatus, 24 h urine can be sampled very easily and without having to carry a big jerrycan of 2,5 l. The sampler can be cleaned and disinfected by soap and hypochlorite.

Urinary analyses were performed by the Dept. of Clinical Chemistry of the Academic Hospital Maastricht. Creatinin was measured by a colorimetric assay of the Dimension® clinical chemistry system (Dupont de Nemours, Wilmington, U.S.A.) and urea by the method of the Dimension system. Sodium was analyzed by flame spectroscopy on a photometer (model 943, Instrumentation Laboratory Incorporation, Milan, Italy). Ca and Mg were determined by atomic absorption spectrophotometry (model AA/AE 551, Instrumentation Laboratory Incorporation, Milan, Italy.) with long path air acetylene burner (IL 43005-02). Phosphate was determined as described by the Dimension®-system.

Stool collection and analyses

Stools were collected in coded plastic bags which were folded in a plastic bucket which fits into the toilet (Emergo, Landsmeer, The Netherlands). The stools were placed immediately after defaecation between four frozen ele-

ments of a freezing pack that were surrounded by styrofoam plates in a cardboard box. In this manner sampling materials and faecal samples can be easily mailed and the samples stay below 5° C during four days. Faecal pH was stable at this temperature.

In the laboratory the stools were weighed, flattened for x-raying and stored in a freezer at -20° C. X-rays were made using AGFA-Gevaert Curix RPI films of 24 x 30 cm in Kodak X-omatic cassettes in a Philips camera type Practix. The samples (4 per plate) were illuminated at 60 cm distance by 50 kV for 0.2 sec. The pellets were counted on a lighted bank.

After thawing the samples overnight at 4° C, they were mixed inside the bag by kneading vigorously. Faecal water was obtained by centrifugation of 24 g of stools for 2 h at 25,000 x g at 20° C in the 250 ml bucket of a hook-rotated centrifuge rotor (XL-80; Beckman, Palo Alto, CA). In this way a good separation between faecal water and pellet could be achieved in all samples. Faecal water was carefully aspirated and the pH was measured at room temperature. Samples were stored at -20° C for further analyses. A part of the homogenized faeces was freeze-dried, and the percentage dry weight calculated.

The analysis of BA was performed as described before (21). The concentrations of unesterified LCFA were measured by a the method described before (22). Briefly, freeze dried faeces (100 mg) and faecal waters (100 µl) were spiked with 100 µl of an internal standard (heptadecanoic acid), dissolved in 0.5 ml methanol and 50 µl concentrated HCl and incubated during 1 h at room temperature. After stopping the methylation reaction with 0.5 ml water, the LCFA were extracted with 2 x 1 ml hexane. The derivatives were analyzed by gas-liquid chromatography as described before. The variability of the analysis of the main LCFA (C16:0, C18:0, C18:1 and C18:2) in total faeces and in faecal water was lower than 20 %, while the variability of the analysis of the other LCFA was higher. Only main LCFA were analyzed, therefore. No saponification of myristate cholesterylester occurred during the procedure and only little saponification of α -lecithin (7-16).

After ashing of the freeze-dried faeces and digestion in 50 perchloric acid and 7.5 hydrogen peroxide, Ca, Mg and phosphate were measured as described above. The concentrations in faecal water were analyzed as described by Van der Meer (10).

Results are given as mean $\pm$ SD. Each value is the mean of measurements of two consecutive days. Differences between means at the end of both experimental periods were tested by the Student's t-test for paired samples.

Results

In the pilot study the concentrations of BA and LCFA in faecal water did not change after taking Calcisorb[®]. The concentration in total faeces however, decreased by 50 after one week and returned to the initial values one week after stopping the supplementation. Based on these strong effects in both subjects after one week, we decided to perform the trial during one week with 6 subjects and to apply a one week washout period.

The diet of the volunteers in the trial reflected a typical omnivorous Dutch diet (23) (Table 1). One subject had diarrhea a few days before sampling of the faeces during his Calcisorb[®]-period. Moreover, this was the only person with a higher excretion of Ca in urine during his Calcisorb[®]-period than during the placebo-period. During the Calcisorb[®]-period, he probably consumed much more dietary protein than during the placebo-period, because also his excretion of urea, phosphorous and creatinine was much higher during this period. Therefore the data of this subject are treated separately from the data of the other five subjects.

Urinary markers of dietary intake (Table 2) showed that the intake of sodium and protein did not differ significantly between the periods.

Table 3 shows the total urinary plus faecal excretion of Ca, Mg and phosphorous during the placebo and the Calcisorb[®]-period. The Ca-excretion did not differ between both periods, but the Mg-excretion was lower in the Calcisorb[®]-period. It occurred in each subject. As expected, the phosphorous-excretion was much higher in the Calcisorb[®]-period.

The excretion of Ca and Mg with urine decreased significantly (Table 4). The mean decrease of Ca was 1.5 mmol/d, that of Mg 2.5 mmol/d. The excretion of phosphorous increased with 7 mmol/d.

The effects of Calcisorb[®] on general faecal variables are shown in Table 5. Faecal bulk was significantly ($p < 0.05$) lower in the Calcisorb[®]-period. The effects on characteristics of faecal water are collected in Table 6. The pH of faecal water did not differ significantly. The amount of faecal water was

TABLE 1

Dietary intake of the volunteers before the experiment

	mean $\pm$ SD (n=6)
Energy (kcal/d)	2390 $\pm$ 695
Total protein (g/d)	77.7 $\pm$ 16.4
Animal protein (g/d)	44.8 $\pm$ 14.3
Total fat (g/d)	85.7 $\pm$ 24.4
Saturated fat (g/d)	33.8 $\pm$ 12.7
Mono-unsaturated fat (g/d)	33.2 $\pm$ 13.1
Poly-unsaturated fat (g/d)	14.6 $\pm$ 2.9
Fibre (g/d)	26.9 $\pm$ 7.9
Calcium (mg/d)	1005 $\pm$ 449
Phosphorous (mg/d)	1591 $\pm$ 517

TABLE 2

Urinary markers of dietary intake during the placebo and the Calcisorb[®]-period (mean $\pm$ SD) (n=5; values of omitted subject between brackets)

	placebo	Calcisorb [®]	difference
sodium (mmol/d)	134 $\pm$ 25 [117]	158 $\pm$ 49 [113]	24.3 $\pm$ 32
urea (mmol/d)	340 $\pm$ 113 [342]	307 $\pm$ 57 [546]	33 $\pm$ 117
creatinine (mmol/d)	19 $\pm$ 4 [23]	16 $\pm$ 5 [30]	3 $\pm$ 4

TABLE 3

Total urinary plus fecal excretion of calcium, magnesium and phosphorous during the placebo- and the Calcisorb[®]-period (mean $\pm$ SD) (n=5; values of omitted subject between brackets)

	placebo	Calcisorb [®]	difference
calcium (mmol/d)	30.5 $\pm$ 5.5 [31.1]	26.7 $\pm$ 5.4 [62.6]	3.8 $\pm$ 5.8
magnesium (mmol/d)	21.1 $\pm$ 3.5 [22.4]	16.9 $\pm$ 2.8 [49.1]	4.2 $\pm$ 2.1*
phosphate (mmol/d)	61.6 $\pm$ 5.7 [67.3]	97.1 $\pm$ 8.6 [130.9]	35.5 $\pm$ 5.6 [#]

* p<0.05

[#] p<0.01

TABLE 4

Effects of Calcisorb[®] on urinary excretion of calcium, magnesium and phosphorous (mean $\pm$ SD) (n=5; values of omitted subject between brackets)

	placebo	Calcisorb [®]	difference
calcium (mmol/d)	3.6 $\pm$ 1.4 [6.4]	2.1 $\pm$ 1.3 [9.5]	1.5 $\pm$ 0.8*
magnesium (mmol/d)	5.0 $\pm$ 1.3 [7.2]	2.5 $\pm$ 1.2 [5.2]	2.5 $\pm$ 0.7#
phosphorous (mmol/d)	31.4 $\pm$ 8.5 [42.0]	38.0 $\pm$ 7.7 [69.0]	6.6 $\pm$ 8.9

* p<0.05

p<0.01

TABLE 5

Effects of Calcisorb[®] on general faecal variables (mean $\pm$ SD)

(n=5; values of omitted subject between brackets)

	placebo	Calcisorb [®]	difference
Defaecation frequency (stools/d)	1.2 $\pm$ 0.5 [2]	1.1 $\pm$ 0.2 [2]	0.1 $\pm$ 0.2
Faecal bulk (g wet weight/d)	259 $\pm$ 60 [188]	204 $\pm$ 62 [302]	55 $\pm$ 37*
Dry weight (%)	30.0 $\pm$ 5.1 [33.0]	27.7 $\pm$ 3.6 [24.2]	2.3 $\pm$ 5.6
Faecal water (ml/24 g)	7.4 $\pm$ 1.6 [8.6]	5.4 $\pm$ 1.9 [5.3]	2.0 $\pm$ 1.3*

* p<0.05

significantly lower ($p<0.01$) after taking Calcisorb[®]. The concentrations of Ca and Mg were lower after consuming Calcisorb[®], those of phosphorous higher. The amount of solubilized Ca (calculated as the amount of Ca in the aqueous part of the faeces divided by the amount in total faeces) was significantly lower in the Calcisorb[®] period. Concentrations of BA and/or LCFA in faecal water did not change significantly due to the intake of Calcisorb[®] (see also Figure 1 and 2). Faecal excretion of BA and LCFA decreased significantly (Table 7). Faecal excretion of phosphorous increased strongly in the Calcisorb[®]-period.

TABLE 6
Effects of Calcisorb® on constituents of faecal water (mean ± SD)
(n=5; values of omitted subject between brackets)

	placebo	Calcisorb®	difference
pH	6.6 ± 0.2 [6.2]	6.4 ± 0.2 [5.9]	0.2 ± 0.3
Bile acids (µmol/l)	130 ± 44 [200]	306 ± 309 [150]	176 ± 317
Long chain fatty acids (mmol/l)	1.4 ± 1.8 [3.2]	1.0 ± 0.5 [1.3]	0.4 ± 1.3
Calcium (mM)	10.2 ± 6.1 [14.8]	5.5 ± 2.9 [26.8]	4.7 ± 5.6
Magnesium (mM)	20.2 ± 5.9 [40.2]	7.6 ± 2.1 [28.7]	12.6. ± 5.5 [#]
Inorganic phosphate (mM)	19.7 ± 10.7 [39.5]	39.8 ± 9.3 [15.3]	20.1 ± 12.9 [*]
Ratio Ca in water to Ca in total faeces (µmol/µmol)	0.03 ± 0.01 [0.04]	0.01 ± 0.01 [0.03]	0.02 ± 0.01 [*]

^{*} p<0.05

[#] p<0.01

TABLE 7
Effects of Calcisorb® on faecal excretion values (mean ± SD)
(n=5; values of omitted subject between brackets)

	placebo	Calcisorb®	difference
Bile acids (mmol/d)	1.1 ± 0.3 [0.6]	0.7 ± 0.1 [1.5]	0.4 ± 0.3 [*]
Long chain fatty acids (mmol/d)	9.4 ± 2.5 [10.0]	4.1 ± 1.5 [15.7]	5.3 ± 2.4 [#]
Calcium (mmol/d)	27.0 ± 5.0 [24.7]	24.6 ± 4.6 [53.1]	2.4 ± 6.1
Magnesium (mmol/d)	16.2 ± 3.1 [15.3]	14.4 ± 2.2 [44.0]	1.7 ± 1.9
Phosphorous (mol/d)	30.2 ± 6.4 [25.3]	59.1 ± 3.9 [61.6]	28.9 ± 6.2 [#]

^{*} p<0.05

[#] p<0.01

FIGURE 1
Individual values of bile acids in faecal water (µmol/l)

FIGURE 2
Individual values of long chain fatty acids in faecal water (µmol/l)

Discussion

Intake of 15 g Calcisorb[®] or placebo per day did not cause discomfort, although gastrointestinal discomfort has been described for Calcisorb[®] in some persons (15). Compared to values before the trial, faecal excretion and faecal water increased a little during the placebo-period (see below). The difference between daily excretion of phosphorous in the Calcisorb[®]- and placebo-period (35 mmol/d) can be explained by the daily intake of 15 g Calcisorb[®] which contains 3 mmol phosphorous per gram.

The lower excretion of Ca and Mg with urine in the Calcisorb[®]-period shows that the absorption of Ca and Mg is diminished by the Calcisorb[®]. The higher excretion of phosphorous will have been caused by the hydrolysis of some Calcisorb[®] in the gastrointestinal tract (information from the supplier).

The faecal wet weight and faecal water in the Calcisorb[®]-period were equal to the ones before the trial. Therefore the placebo will have increased the faecal weight and the amount of faecal water a little. Although the placebo consisted of microcrystalline, depolymerized cellulose that does not swell (results not shown), most probably the cellulose will have bound a certain amount of water.

The lower amount of Ca and Mg in faecal water after the Calcisorb[®] intake shows that the binding of Ca and Mg to the Calcisorb[®] is still present in the faeces. The higher phosphate concentration in faecal water will be caused by the small hydrolysis of Calcisorb[®]. A lower concentration of Ca in faecal water may cause a higher risk of colorectal cancer (11).

We did not detect a diminishing effect of Ca-binding on faecal pH as expected from our previous cross-sectional study with different intakes of Ca (21) and the dietary study with milk low in Ca by Govers et al (14). This may be due to the range of Ca-intake in this study. A difference in urinary excretion of 1.5 mmol/d multiplied by an absorption of about 30 gives 4.5 mmol = 180 mg/d. Therefore the range of intake was between 1000 and 800 mg/d. In the study of Govers the intake of Ca varied between 1800 and 800 mg/d. The scatter diagram of faecal pH and Ca-intake from our cross-sectional study (21) shows no relation between both variables below a Ca-intake of about 1000 mg/d.

The excretion of Ca and Mg with the faeces is not enhanced during the Calcisorb[®]-period. It is difficult to detect the small difference in Ca and Mg absorption (1.5 and 2.5 mmol/d respectively) among the large amount of faecal Ca and Mg (27 and 16 mmol/d respectively).

The “Ca-and Mg-binding” by Calcisorb[®] did not enhance the concentrations of BA or LCFA in faecal water as expected from the hypothesis of Newmark et al (24). In one subject (see Figure 1) the concentration of BA was enhanced extremely in the Calcisorb[®]-period. These high concentrations have been found before (12). We could not explain this high concentration by faecal or dietary factors.

The daily excretion of BA and LCFA decreased significantly after taking Calcisorb^R. This corresponds well with an increased excretion of BA and LCFA after supplementation with Ca (10).

The diets in this study contained less fat than the amount for which Newmark proposed his hypothesis (85 compared to 150 g/d). A high fat diet will cause a high concentration of BA and LCFA in faeces. A low Ca-diet may then have a more pronounced effect on BA and LCFA in faecal water than a low fat diet. Such an effect has been reported for rats (25).

In conclusion, binding dietary Ca and Mg in the intestinal tract with Calcisorb[®] from a diet with a relatively low amount of fat apparently does not enhance the solubility of BA and LCFA in faecal water. Kidney stone patients who use this diet and drug, most likely will have no enhanced risk of developing colorectal cancer by this way. The importance of a low concentration of Ca in faecal water as a risk factor for colorectal cancer has to be studied in animal and epidemiological studies.

Acknowledgements

We wish to thank Mrs. M. van der Westerlaken, M.Sc. of the Dept. of Clinical Pharmacy, Academic Hospital Maastricht for preparing and testing the placebo and randomizing the volunteers. Mr. Walenkamp of Riker/3M, Zoeterwoude provided us with the Calcisorb[®] and its specifications. Mr. L. Donselaar of the Dept. of Clinical Chemistry, Academic Hospital Maastricht coordinated the clinical chemistry of urine and faecal samples and Mr. M. Bost of the Centralized Animal Services made the X-rays of the stools. Dr. R. van der Meer of the Dept. of Nutrition, Netherlands Institute for Dairy Research (NIZO), Ede has critically reviewed the manuscript. The volunteers are gratefully acknowledged for their dedication.

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The calcium-restricted diet of urolithiasis patients with hypercalciuria type II: a benefit for kidney stones but a risk for colorectal cancer?

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Submitted

Abstract

The calcium (Ca)-restricted diet of urolithiasis patients with hypercalciuria type II may decrease the urinary Ca-excretion but increase biochemical markers of risk for colorectal cancer (CRC). We allocated twenty-nine patients from 6 hospitals at random into an experimental (Ca-restriction to 500 mg /day) and a control (no restrictions) group for one month. During the trial the experimental group consumed 800 mg less Ca than the control group, but also less animal protein, mono- en disaccharides, polysaccharides, cholesterol, saturated fatty acids, energy, phosphorous and sodium. The urinary Ca-excretion did not decrease significantly, but the oxalate excretion decreased, although not significantly either. The concentration of bile acids and long chain fatty acids in faecal water and its cytotoxicity decreased. We conclude that our Ca-restricted diet, which turned out to be lower in Ca, animal protein and fat due to the omission of dairy products, may be a benefit for male hypercalciuria type II patients without enhancing their risk for CRC. For women, a diet restricted in animal protein and table salt may be more effective against urolithiasis, safer for their bones and of equal protection against CRC.

Introduction

Urolithiasis patients diagnosed as hypercalciuria type II are those patients that respond to a calcium- (Ca) and sodium-deprived diet during the metabolic screening of recurrent stone formers (1). They have an increased intestinal absorption of Ca (35 compared to a normal value of 25) . This is most probably caused by higher levels of 1,25-dihydroxy vitamin D₃ (2). Our therapeutic diet of these patients consists of a diet moderately restricted in Ca (500 mg/d), oxalate (< 400 mg/d: discourage of spinach, rhubarb, red beets; light-brewed black tea) and sodium (100 - 150 mmol/d). Dietary excesses of animal protein (> 50 g/d) were eliminated (3).

Lower intakes of Ca have been associated with an increased risk of colorectal cancer (CRC) in some epidemiological studies (4,5), in experimental studies in humans (6) and animals (7,8). Therefore the Ca-restricted diet prescribed to hypercalciuria type II patients may enhance the risk for CRC.

This paper describes the effect of the Ca-restricted diet of our hypercalciuria type II patients on metabolic risk factors for CRC : pH, bile acids (BA), long chain fatty acids (LCFA) and cytotoxicity of faecal water (8).

Methods

The study protocol was approved by the Medical Ethics Committee of the University Hospital Maastricht (PF8).

Patients with recurrent urolithiasis were metabolically screened as described by Pak et al (1) using a computerized program. Before the metabolic screening, the patients filled in a structured dietary questionnaire (9), improved by measures for portion size and by more detailed questions about calcium, salt and oxalate-intake. Urologists of six Dutch hospitals participated in the study, using the same version (2.0.1) of the computerprogram.

Patients with an urinary Ca-excretion on their regular diet > 5 mmol/d and an excretion below this value after one week on a Ca- (400 mg/d) and sodium- (100 mmol/d) restricted diet, were diagnosed as hypercalciuria type II. These patients were invited to participation by their urologist, after which informed consent was obtained. Exclusion criteria were using laxatives, cholesterol-lowering medicines, gallbladder disease and bone disorders. After the decision to participate, the patients received detailed information and materials to collect 24 h urine and 48 h faeces. The projectmanager explained

the trial extensively to the patients by telephone and a data-plan was made together. Randomization between the experimental (Ca-restricted) and the control (regular diet) group were performed within strata for Ca-intake (less and more than 1000 mg/d) and sex.

Before and after the study period of 4 weeks, fasting urine and blood were sampled in the hospital of the patient. At both sampling times a form with questions about defaecation frequencies and problems during sampling was filled in by the patients. Moreover at the end of the four weeks, the patients filled in the dietary questionnaire mentioned before, to measure dietary practices during the trial.

The dietary questionnaire of the respondents was controlled by the dietitian of the patient's hospital and calculated for Ca and other nutrients by a dietitian of the Dept. of Dietetics of the University hospital in Maastricht using the standardized Dutch Uniform Encoding System (10) and the 1992 release of the National Computerized Dutch Nutrient Databank, NEVO (11). This databank is prepared and regularly updated by a national committee. The experimental subjects visited their dietitian to discuss the Ca-restricted diet. The amount of fluid was prescribed to 2.5 l per day. The Ca-intake was limited to 500 mg/d. In the Netherlands this is mainly achieved by omitting dairy products like cheese and other milk (products). No lower intake than 500 mg/d was allowed, because at low intakes of Ca, oxalate absorption might be increased. Oxalate-rich vegetables like spinach or rhubarb were discouraged. Light-brewed black tea was allowed to a maximum of 1 liter, because of its high content of oxalic acid, which may result in a high excretion of oxalate in urine. The diet was normalized in relation to animal protein (50 g/d) and salt (4500mg = 200 mmol Na/d) because these dietary factors increase the Ca-excretion with urine (1). Alcoholic drinks were restricted to 4 drinks/d because the alcohol. may lead to a high excretion of Ca in urine (12).

Fasting urine was sampled after discarding the first urine of the morning at the patient's hospital. It was poured directly into a glass tube, covered with a drop of toluene and frozen at -20° C. Fasting blood was sampled in lithium heparin, immediately centrifuged during 10 min at 1500 x g, the plasma portioned in plastic tubes and the tubes frozen at -20° C.

Urine during 2 x 24 h was sampled by the patients on weekdays using the U-mate P of Sumitomo Bakelite Co, Tokyo, Japan with the lower part filled with 1.7 g boric acid as preservative. It is based on the principle of the one described by Tochikubo (13), taking 1/50 of every urine voidance. With this

small apparatus, 24 h urine can be sampled very easy without carrying a big jerrycan of 2.5 l. The sampler can be cleaned and disinfected by soap and hypochlorite. Combined with a clear description of usage, about 30 man and women between 20 and 65 yrs old used this apparatus without reporting to have difficulties. We controlled the validity of urine sampling by analyzing the creatinine excretion (1).

Faeces during 48 h were collected in the weekend using a plastic bucket that fits into the toilet (Emergo, Landsmeer, The Netherlands) and coded plastic bags that fit into the bucket. Immediately after defaecation, the bags containing the stools were placed between four frozen elements of a freezing pack that is surrounded by styrofoam plates in a cardboard box. In this manner sampling materials and faecal samples can be easy mailed and the samples stayed below 5° C during four days. Seven days before collection the patients swallowed 10 radio-opaque pellets (Resprecare Medical B.V., The Hague, The Netherlands), three times a day to correct excretion values for faecal flow (14). Each collected stool was weighed, flattened for x-raying and stored in a freezer at -20° C. X-rays were made using AGFA-Gevaert Curix RPI films of 24 x 30 cm in Kodak X-omatic cassettes in a Philips camera type Practix. The samples (4 per plate) were illuminated at 60 cm distance by 50 kV for 0.2 sec. The pellets were counted on a lighted bank.

pH, BA and LCFA in total faeces and in faecal water were measured as described by van Faassen (15). The cytotoxicity of faecal water was determined by a modification of the method described by Govers (16): 40 µl of faecal water was mixed with 40 µl 154 mM NaCl. After preincubation for 5 min at 37° C, 20 µl of a washed human erythrocyte suspension was added (final hematocrit 5%) and incubated for 3 h at 37° C. Each hour the tubes were vortexed. The intact erythrocytes were precipitated by centrifuging for 1 min at 1500 x g, washed three times with 154 mM NaCl, and after acidification with trichloroacetic acid (final 5% w/v) and subsequent centrifugation (1 min at 10,000 x g), the supernatant was diluted 25 times with 0.05 CsCl. The potassium content of the solution was measured by atomic emission spectrophotometry (Perkin Elmer 1100). Simultaneously, erythrocytes were incubated in 154 mM NaCl (0% hemolysis) and in double-distilled water (100% hemolysis). Percent hemolysis was calculated from these 0 and 100 controls.

In urine creatinine was measured by a colorimetric assay of the Dimension® clinical chemistry system (Dupont de Nemours, Wilmington, U.S.A.). Sodium was analyzed by flame spectroscopy on a photometer (model 943, Instrumentation Laboratory Incorporation, Milan, Italy), Ca and Mg on an

atomic absorption spectrophotometer (model AA/AE 551, Instrumentation Laboratory Incorporation, Milan, Italy.) with a long path air acetylene burner (IL 43005-02). Phosphate, urea and uric acid were determined as described by the Dimension®-system. Sulphate was analyzed by a turbidimetric method using polyethylene glycol 6000 (17). Oxalate and citrate were measured by enzymatic kits of Sigma and Boehringer, respectively.

Ca, Mg and phosphorous in faeces were analyzed as described above after incubating wet faeces in 4 N hydrogen chloride and 7.5 hydrogen peroxide at 100°C for 1 hour, centrifuging 10 min 3000 x g and filtering through Filter Sampler, a standard model of blood serum filters of Porex Medical, Fairburn, Georgia, U.S.A. Their concentrations in faecal water were analyzed after incubating faecal water in 5 trichloroacetic acid during 1 h and centrifuging 2 min 10,000 x g (18).

Absolute values and differences in changes of dietary constituents and variables of blood, urine and faeces between experimental and control subjects were analyzed by Student's t-test or Wilcoxon test if the variables were not normally distributed. Correlations between (shifts in) parameters were calculated by the Pearson correlation coefficient or Spearman correlation coefficient if the variables were not normally distributed (19).

Results

Twenty-nine subjects from six hospitals volunteered into the study. Six male and two female patients were invited by their urologist, but did not want to participate. Of three patients (one male and two females) we did not receive the dietary questionnaire, sent by mail. The fasting urine and blood of two patients were not collected properly by their hospital. Twenty-four hour urine of four patients were not collected correctly as shown by creatinine excretion values. Two female patients refused to collect faeces and the faecal analyses of two patients were unreliable; one because she had a gall-bladder operation and the other because the photograph of the pellets in his faeces was not valid. Therefore dietary data of 26 subjects, the data of bone metabolism of 27 subjects and urinary and faecal excretion values of 25 subjects were statistically analyzed

The values at the beginning of the trial and the changes in dietary intakes of the patients from the control and from the Ca-restricted group are shown in Table 1. The changes (value after 4 weeks minus value at the beginning) in intake of Ca, animal protein, mono- en disaccharides, polysaccharides,

TABLE 1
Mean (SD) of baseline values and changes in nutrients

Dietary constituent	Control diet (n=13)		Ca-restricted diet (n=13)	
	baseline	change	baseline	change
Energy (kJ/d)	9690 (2830)	- 400 (1270)	12130 (3650)	- 3420 (3070) [#]
Total protein (g/d)	75 (20)	- 4 (14)	98 (31)	- 37 (24) [*]
Animal protein (g/d)	48 (17)	- 5 (11)	67 (27) [*]	- 33 (29) [#]
Total fat (g/d)	100 (31)	- 9 (19)	133 (45) [*]	- 46 (39)
Saturated fat (g/d)	40 (13)	- 5 (8)	54 (17) [*]	- 26 (13)
Cholesterol (mg/d)	237 (86)	- 24 (43)	320 (115) [*]	- 144 (108) [*]
Carbohydrates (g/d)	251 (98)	- 3 (48)	317 (108)	- 76 (75) [#]
Mono- + disaccharides (g/d)	111 (64)	0 (48)	155 (68)	- 47 (64) [*]
Polysaccharides (g/d)	140 (50)	- 3 (23)	162 (56)	- 29 (35) [*]
Fibre (g/d)	28.5 (9.4)	1.1 (7.9)	27.9 (8.7)	- 2.7 (6.9)
Calcium (mmol/d)	23.2 (9.2)	- 1.5 (6.9)	31.2 (16.8)	- 17.8 (18.5) [*]
Phosphorous (mmol/d)	47.5 (13.6)	- 1.1 (9.5)	57.9 (19.3)	- 23.5 (17.1) [*]
Sodium (mmol/d)	182 (47)	- 11 (23)	203 (39)	- 43 (23) [#]
Fluid (l/d)	2.9 (0.8)	- 0.1 (0.7)	2.9 (1.0)	- 0.3 (1.1)

^{*} p < 0.05

[#] p<0.01

TABLE 2
Mean (SD) baseline values and changes in urinary plus faecal excretion of minerals

Balance variable (mmol/d)	Control diet (n=11)		Ca-restricted diet (n=9)	
	baseline	change	baseline	change
calcium	29.1 (14.9)	2.8 (28.5)	32.3 (22.6)	- 17.0 (25.4) [*]
magnesium	21.1 (14.0)	- 3.1 (17.8)	18.8 (10.8)	- 6.7 (12.1)
phosphorous	53.4 (19.3)	- 6.3 (24.7)	50.1(22.1)	- 11.8 (22.1)

^{*} P<0.05

cholesterol, saturated fatty acids, energy, phosphorous and sodium were significantly higher for the experimental group than the control group. The initial intake of animal protein, total fat and saturated fat were significantly higher in the restricted group. The Ca-intake appeared higher initially in the experimental group although not significantly.

TABLE 3

Mean (SD) of baseline values and changes in urinary variables

Urinary variable	Control diet (n=13)		Ca-restricted diet (n=12)	
	baseline	change	baseline	change
urine (l/d)	1.6 (0.4)	0.06 (0.6)	1.7 (0.8)	0.05 (1.1)
urea (mmol/d)	334 (102)	- 9.2 (75.5)	342 (154)	- 31 (132)
sodium (mmol/d)	153 (53)	- 0.5 (46.5)	169 (66)	- 25 (88)
creatinine (mmol/d)	13 (4)	0 (3)	13 (4)	- 1 (6)
calcium (mmol/d)	6.1 (1.4)	- 0.2 (2.5)	6.3 (2.3)	- 0.7 (2.9)
oxalate (mmol/d)	0.38 (0.17)	0.05 (0.15)	0.43 (0.25)	- 0.12 (0.27)*
citrate (mmol/d)	2.4 (1.3)	- 0.3 (1.0)	3.1 (1.4)	- 0.3 (1.6)
sulphate (mmol/d)	17.7 (8.2)	- 0.3 (8.1)	19.1 (6.5)	- 3.8 (9.0)
phosphate (mmol/d)	31.3 (9.6)	- 1.9 (6.5)	33.0 (13.9)	- 5.3 (14.8)
magnesium (mmol/d)	4.7 (1.4)	- 0.4 (1.4)	5.0 (2.7)	0.1 (2.4)

* p < 0.1

TABLE 4

Mean (SD) of baseline values and changes in faecal variables

Faecal variable	Control diet (n=13)		Ca-restricted diet (n=13)	
	baseline	change	baseline	change
Defaecation frequency (stools/d)	2.1 (2.6)	- 0.9 (2.6)	1.8 (2.6)	0.0 (0.1)
Faecal bulk (g wet weight/d)	226 (192)	- 2 (24)	229 (179)	- 10 (20)
Faecal dry weight (%)	26.1 (4.6)	- 0.8 (4.0)	24.0 (5.0)	0.5 (6.3)
Bile acids in total faeces (μ mol/g wet weight)	4.3 (1.3)	0.2 (1.8)	4.4 (1.2)	2.3 (6.8)
Long chain fatty acids in total faeces (μ mol/g wet weight)	41 (18)	2 (27)	40 (29)	- 16 (29)
Bile acid excretion (μ mol/d)	907 (657)	- 56 (891)	999 (719)	- 102 (1431)
Long chain fatty acid excretion (mmol/d)	7.7 (5.8)	2.0 (12.4)	8.7 (7.3)	- 5.9 (7.2)*

* p < 0.1

The combined urinary and faecal excretion values excretion values in urine and faeces (Table 2) show that the experimental group excreted about 20 mmol = 800 mg less Ca per day during the trial than the control group. Moreover the initial data for Ca are equal between both groups.

TABLE 5

Mean (SD) of baseline values and changes in variables of faecal water

Variable of faecal water	Control diet (n=13)		Ca-restricted diet (n=13)	
	baseline	change	baseline	change
Water (ml/24 g)	6.9 (2.8)	1.0 (2.3)	9.0 (3.2)	- 1.2 (3.1)*
pH	6.0 (1.8)	-0.1 (0.6)	6.2 (0.3)	- 0.0 (0.4)
Bile acids (μmol/l)	281 (228)	28 (134)	340 (216)	- 35 (107)
Deoxycholic acid (μmol/l)	69 (60)	23 (68)	98 (65)	- 1.4 (73)
Long chain fatty acids (mmol/l)	1.5 (1.9)	0.2 (2.2)	4.2 (6.0)	- 3.0 (5.6)#
Cytotoxicity (%)	51 (41)	21 (49)	74 (35)	- 16 (36)#
Calcium (mmol/l)	11.1 (6.7)	3.1 (7.3)	15.5 (6.1)	- 1.9 (9.5)

* p<0.1

p<0.05

TABLE 6

Spearman-correlation coefficients of baseline values and changes in cytotoxicity of faecal water with other faecal water variables

	cytotoxicity (%)	changes in cytotoxicity (%)
pH	- 0.63*	
Long chain fatty acids (mmol/l)	0.74#	
Linoleic acid (mmol/l)	0.79#	
Oleic acid (mmol/l)	0.71#	
Palmitic acid (mmol/l)	0.73#	
Stearic acid (mmol/l)	0.53*	
Bile acids (μmol/l)	0.58*	
Mono + dihydroxy bile acids (μmol/l)	0.65#	
Keto ^a +trihydroxy bile acids (μmol/l)	0.04	
Changes in long chain fatty acids		0.60*
Changes in bile acids		0.31

^a 7-K-DC, 12-K-LC, 7-K-LC

* p<0.01

p<0.001

The urinary excretion of urea and sodium tended to decrease in the experimental group (Table 3) although the changes were not statistically significant. These urinary markers of dietary intake of protein and sodium respectively (20), show that there was no difference in initial intake of these dietary constituents between both groups.

TABLE 7

Mean (SD) baseline values and changes in men and women

Men	Control diet (n=9)		Ca-restricted diet (n=7)	
	baseline	change	baseline	change
Ca in 24 h urine +				
Ca in 24 h faeces (mmol/d)	31.9 (15.8)	3.0 (33.4)	38.8 (24.5)	-30.3 (27.5)*
Urea (mmol/d)	352 (102)	-1 (81)	312 (122)	-16 (152)
Sodium (mmol/d)	147 (57)	15 (35)	178 (78)	-34 (105)
Ca in 24 h urine (mmol/d)	6.3 (1.3)	-0.8 (1.4)	6.9 (2.5)	-1.8 (2.8)
Age (yrs)	49 (11)		44 (10)	
Weight (kg)	80.1(12.2)		78.5 (8.0)	
Body mass index (kg/m ²)	27.6 (3.7)		25.1 (1.7)	
<hr/>				
Women	Control diet (n=3)		Ca-restricted diet (n=5)	
	baseline	change	baseline	change
Ca in 24 h urine +				
Ca in 24 h faeces (mmol/d)	20.5 (9.1)	2.1 (2.1)	17.1 (2.0)	1.9 (6.8)
Urea (mmol/d)	255 (75)	-47 (33.2)	413 (227)	-62 (99)
Sodium (mmol/d)	181 (13.2)	-68 (20)	148 (8.4) [#]	-9 (47)
Ca in 24 h urine (mmol/d)	5.6 (2.0)	1.8 (4.4)	5.6 (1.9)	1.0 (2.3)
Age (yrs)	57 (16)		43 (7)	
Weight (kg)	70.0 (7.4)		70.2 (14.3)	
Body mass index (kg/m ²)	25.0 (4.5)		24.9 (3.0)	

* p < 0.1

[#] p<0.05

The excretion of Ca with urine decreased, but there was no significant difference between the Ca-restricted and the control group. Moreover the variation in shifts was extremely high.

The faecal excretion of LCFA seemed to decrease on the Ca-restricted diet ($p < 0.05$; Mann Whitney-test) (Table 4).

Neither the concentration of BA nor that of LCFA in faecal water raised in the experimental group. Both decreased in comparison with the control group, although the difference was only significant for LCFA (Table 5). The

FIGURE 1

Faecal bulk = $24.2 + 7.0$ (urinary + faecal excretion). $SE \beta = 1.7$; $r^2 = 0.46$

FIGURE 2

Change of faecal bulk = $-35.1 + 6.8$ (change of urinary + faecal Ca-excretion). $SE \beta = 1.1$; $r^2 = 0.65$

pH of faecal water did not change due to the Ca-restricted diet., nor did the concentration of Ca in faecal water. The cytotoxicity of faecal water decreased significantly in the experimental group ($p < 0.05$).

Sixty-one percent of the variance in baseline values of faecal bulk could be explained by sex, fibre intake and intake of Ca calculated from the urinary +

faecal excretion of Ca: $^{10}\log$ faecal bulk (g/d) = 0.37 - 0.06 sex (1=man; 2=woman) + 0.51 $^{10}\log$ fibre intake (g/d) + 0.87 $^{10}\log$ (Ca-excretion in faeces + urinary Ca-excretion) (mmol/d). The increase of faecal bulk by increasing intake of Ca was most prominent above 25 mmol/d (= 1000 mg/d) (Figure 1). Also seventy-one percent of the variance in change of faecal bulk could be explained by the change in intake of fibre and Ca (see also Figure 2).

The baseline values of cytotoxicity of faecal water was significantly correlated with those of pH, concentration of LCFA and concentration of BA in faecal water. The correlation was highest with the LCFA. The sum of the concentrations of the secondary BA (the hydrophobic ones) was correlated more strongly with cytotoxicity than total BA in faecal water. The correlation was lowest for the sum of the hydrophilic BA. The change in cytotoxicity was correlated significantly with the change in LCFA (Table 6).

Discussion

The excretion data of Ca, Mg and phosphorous and biomarkers of dietary intake like urea indicated no difference in dietary intake of both groups. The difference in dietary intake data may be caused by an underreporting of the intake of e.g. milkproducts by the control group. This group happened to contain three time more subjects with a body mass index higher than 26, including two with an index higher than 30. Such persons are more likely to under report their dietary intake (H. Boeing, personal communication).

The Ca-restricted diet did not significantly lower Ca in urine. Moreover the variability in the change was extremely high: the Ca-excretion of some patients in the experimental group even increased. When males and females were separated (Table 7), it seemed that the Ca-excretion in urine and the Ca-balance increased in women and decreased in man while in both the intake of Ca, protein and salt decreased. Exclusion of two postmenopausal women did not change the results. A lowering effect of a 4 days severe Ca-restriction (250 mg/d) has been detected in male, hypercalciuric renal stone forming subjects (21). A Ca-restriction to 400 mg/d and moderate sodium restriction during 4 days lowered the Ca-excretion in hypercalcuria type II patients (22). The small effect in the male patients of our study might be caused by an increase of calcitriol in serum when subjects are maintained on a low Ca-diet for at least 10 days (23).

The lower excretion of oxalate will have been caused by the omitting of protein from dairy products and the lower intake of carbohydrates during the Ca-restricted diet. The expected higher excretion of oxalate due to an enhanced solubility of oxalate in the intestine and therefore an increased absorp-

tion of oxalate, may play a minor role when the subjects are consuming already a diet moderately restricted in oxalate (24). On the other hand the effect of a lower intake of protein, fat and carbohydrates may be stronger than the lowered intake of Ca (25).

The Ca-restricted diet did not influence the urinary excretion of citrate. It was already suggested (26), that dairy protein did not lower the citrate excretion, although dairy protein is a form of animal protein. Dairy products may contain constituents that lead to a lower acidification in the nephron and therefore to less lowering of the citrate excretion. This hypothesis is supported by our data on sulfate in 24 h urine (Table 3) which were not changed by the experimental diet.

The diminishing concentration of BA and LCFA in faecal water will most probably be caused by the lower intake of fat that accompanies the Ca-restriction in these patients due to omission of dairy products. Based on the regression equation derived from our previous cross-sectional study (27) a decrease of about 90 $\mu\text{mol/l}$ could be predicted by the decrease in saturated fat. The expected enhancing effect of the lower intake of Ca as derived from the data of van der Meer and Govers (28) would be about 40 $\mu\text{mol/l}$. Therefore the decreasing effect of our Ca-restricted diet is predominating.

The enhancing effect of Ca on BA in faecal water may be explained by the increase of faecal bulk due to an increase of Ca-intake above 1000 mg/d. This effect has been reported before for humans and rats (29,30) and may be caused by a higher excretion of fatty acids due to their reduced absorption. In our previous cross-sectional study (27), the concentration of the main BA in faecal water, DC, was lower in subjects having higher faecal bulks.

The cytotoxicity of faecal water diminished significantly on the diet. This agrees with the diminution of the concentration of BA and LCFA in faecal water. The higher correlation between cytotoxicity and LCFA than between cytotoxicity and BA has been reported before (31).

A negative correlation between the cytotoxicity and the pH of faecal water has not been reported before. It occurred also after the dietary intervention, although the correlation was lower ($r = -0.35$; $p < 0.1$). It is not caused by a correlation between Ca in faecal water and cytotoxicity, because this correlation was positive (Spearman correlation coefficient of 0.14). It may be caused by the unionized form of DC, which will be present at higher concentrations at this pH, because the pKa of DC varies between 5 and 6.6 (32). Van der Meer et al (33) have shown that hydrophobic BA are more cytolytic than the

hydrophilic ones. Unionized BA are more hydrophobic than the ionized ones. The *in vitro* inhibiting effect of a lower pH on the damaging effect of 5 mM DC to the rat colon reported by Rafter et al (34), may be due to a decreased amount of DC in solution. This effect may not be relevant for the physiological situation because DC concentrations in faecal water are only about 0.1 mM. The lysis of erythrocytes by faecal water correlates very well with those for CaCo-2 cells derived from a human colon adenocarcinoma cell line (35) and with hyperproliferation of colonic cells in rats (8).

Lapr et al (36) found higher concentrations of Ca in faecal water after supplementation with Ca-carbonate from 1200 to about 3000 mg/d, but Govers et al (16) found no significant increase after supplementation with Ca from milk products from 800 to about 1800 mg/d. Therefore in normal dietary situations the concentration of Ca in faecal water is probably not influenced by dietary intake of Ca. Although a low concentration of Ca in faecal water may enhance CRC (37), no epidemiological or animal studies have been performed on this subject.

Correlations between dietary variables and faecal biomarkers were similar to those reported by us before for omnivores and vegetarians (28), except the correlation between Ca-intake and faecal pH and that between DC in faecal water and intake of saturated fat. In this study, the correlation between the pH of faecal water and Ca-intake was very low (0.09), but the correlation with Ca in faeces was statistically significant (Pearson correlation coefficient of 0.52, $p < 0.05$). Baseline data of daily faecal wet weight and defaecation frequency were significantly higher in men than in women. Also, 26% of the variance in defaecation frequency was explained by sex and the intake of dietary fibre ($P < 0.05$). The variance in the concentration of DC in faecal water could not be explained significantly by dietary factors. The reasons may be the lower validity of the food frequency questionnaire compared to the dietary history method and the lower variability in faecal DC and saturated fat intake in the urolithiasis patients compared to the omnivores and vegetarians.

The decrease in intake of animal protein and saturated fat will have been caused by the omitting of dairy products like milk and cheese due to the restriction of Ca. The subjects most probably did not substitute their milkproducts. This may be due to their high dietary intake: the intake of protein, carbohydrates and energy were respectively 8 g, 50 g and 1350 kJ higher than the age and sex controlled values of healthy subjects in The Netherlands (38). This is reflected in the high body mass indices of about half

of the patients. The lower intake of carbohydrates during the diet may be due to a lower intake of sweetened milkproducts like chocolate milk and puddings.

The therapeutic regimen of drinking more than 2.5 l water per day was not followed by all patients as can be derived from their volume of daily urine. The difference between intake and excretion might be about 0.5 - 1 l (1) . On the Ca-restricted diet three patients drank much less than on their normal diet. Most probably they didn't compensate for their lower intake of milk and milkproducts.

In stead of omitting dairy products, which are valuable sources of nutrients like vitamin B₂, potassium and fluid, the diet should be restricted to 0.5 g/kg ideal body weight of animal protein and 3100 mg sodium (a prudent diet). This would lower the Ca-excretion in urine about 2 mmol/d (39,40), would not lower the intake of fluid, would favor bone absorption due to its high ratio Ca:protein, would lower the concentration of BA in faeces (41) and can be checked easily by measuring urea and sodium in 24 h urine. On the other hand if dietary regimens are not followed, medical therapy by thiazide + potassium citrate + sodium restriction varied with cellulose phosphate + magnesium+oxalate restriction is possible (42) . However these therapies are much more complicated than a prudent + high fluid diet.

In conclusion, our diet of hypercalciuria type II patients will be a small benefit for male patients and will not enhance their risk for CRC because it lowers the excretion of Ca and oxalate in urine and does not enhance the concentration of BA and/or LCFA in faecal water. Also it does not affect the pH of faecal water and its cytotoxicity. For women, it may be the opposite and for them a diet restricted in animal protein and table salt may be more effective against urolithiasis, safer for their bones and of equal protection against CRC.

Acknowledgements

Urologists, clinical chemists and dietitians of the Carolus hospital, the Groot Ziekengasthuis and the Willem-Alexander hospital 's- Hertogenbosch, St. Maartensgasthuis, Venlo, St. Elisabeth hospital, Tilburg, and Sophia hospital, Zwolle are thanked for their cooperation. S de Kort, urologist in the Carolus hospital in 's-Hertogenbosch is acknowledged for his computerized screening program, ms. Denise Termont of the Netherlands Institute for Dairy Research, Ede, for the determination of faecal cytotoxicity, Mrs. Sigrid Bokkerink and Mrs. Esther Debie, dietitians for comparing the dietary habits of the patients with those of the general Dutch population and Mrs. Saskia Houterman for analyzing the sulphate concentration in urine. The patients

who volunteered in the study are gratefully acknowledged for their cooperation. The study was supported financially by the Dutch Dairy Foundation on Nutrition and Health.

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General discussion and conclusions

8.1 Introduction

In this thesis we reported about the effect of habitual dietary pattern (omnivorous, vegetarian, vegan and Ca-restricted) on the faecal markers of effective dose related to CRC risk that were known in 1990: bulk, pH, defaecation frequency, secondary BA, LCFA and butyrate, one of the main SCFA. Our studies were based on the hypothesis that meat, dietary fibre and Ca are related to CRC risk and that biomarkers of the biologically effective dose occur in the faecal stream.

The faecal markers that uptill now are most strongly related to CRC risk and most sensitive to dietary modulation, are secondary BA and daily faecal bulk. A low concentration of secondary BA and a high faecal bulk, which are the result of a diet low in saturated fat, high in dietary fibre and resistant starch, may prevent hyperproliferation of colorectal mucosal cells. A low proliferation activity means a decreased DNA replication, and therefore a lower chance that mutations are fixed and will lead to neoplastic cells (1).

Other potential faecal markers of the biologically effective dose are the concentration of LCFA in faeces and/or faecal water and the concentration of Ca and/or Mg in faecal water (this thesis), the activity of the bacterial enzyme β -glucuronidase in faeces (2), the concentration of diacylglycerol in faeces (3) and the in vitro production of butyrate (4).

In this chapter the results of our studies with widely different designs (analytical chemistry, metabolic, epidemiological correlation, animal and human experimental, clinical trial) will be discussed in relation to:

1. the results of other studies of dietary effects on faecal markers of exposure
2. new knowledge on the relation between these and other faecal markers and CRC risk
3. new data about dietary and environmental factors and CRC risk.

The significance of the results for CRC risk of an omnivorous, vegetarian and ovo-lacto-vegetarian diet and of the Ca-restricted diet of urolithiasis patients is discussed.

At the end, a model is proposed how diet may influence CRC risk by constituents of the faecal stream and how patients with Ca-oxalate stones should be metabolically diagnosed and treated by dietary measures.

8.2 Analysis of faecal bile acids

The validity and reproducibility of the analysis of faecal BA has been studied in detail. The developed GLC-method is accurate and valid for the main BA in human faeces (chapter 2). New techniques like capillary GLC prompted us to modify the method, which could measure also the 3 β -isomers of the main BA in human faeces (chapter 3). For the measurement of the low concentrations of BA in faecal water, another capillary GLC column and other derivatives of the BA have to be used. Also enzymatic deconjugation and solvolysis were omitted because in healthy subjects without gastrointestinal pathology only a minor amount of faecal BA occur as conjugates or sulphates (chapter 4). Compared to other published methods from laboratories in Munich (5), Helsinki (6) and Wageningen (7) our method of chapter 4, which was described in detail in De Kok et al (8), gives identical results for the main BA in human faeces (Table 1).

TABLE 1

Concentrations (mmol/g dry weight) of BA in a pool sample of freeze-dried faeces from 40 healthy subjects (9)

	Wageningen	Helsinki	Munich	Maastricht
Lithocholic acid	4.1	4.5	3.8	4.3
Deoxycholic acid	5.3	10.4	6.2	5.0
Chenodeoxycholic acid	0.2	0.5	0.4	0.2
Cholic acid	0.3	0.9	0.6	0.3

The remarkably higher BA concentrations found by the Helsinki laboratory may relate to their habit to consider all peaks in the gaschromatogram that appear after LC as BA. However, analysis by GLC-mass spectrometry performed by Glatz and coworkers revealed that this faecal pool contains appreciable amounts of compounds that can not be identified as BA (Glatz and Stellaard, personal communication).

Simplified methods like the enzymatic method (10), the one which measures BA and LCFA in one GLC run (11) or a recently published method using high performance liquid chromatography (12), do not measure all individual BA correctly. The 3 β -isomers of the BA are not measured in the enzymatic method and are not reported by the other methods. Moreover, the other methods do not report data about 7- and 12-keto BA. LC and DC and their 3 β -isomers and the 7- and the 12-keto BA are called secondary BA, because they are produced by the bacteria of the large intestine from the primary BA chenodeoxycholic and cholic acid. These primary BA are formed in the liver from cholesterol.

8.3 Faecal bulk and faecal bile acids

In our metabolic ward study with an omnivorous, lacto-ovo-vegetarian and vegan diet (chapter 3), the concentration of the main faecal BA, DC, was reduced by a large amount of dietary fibre and enhanced by a high amount of Ca in the diet. Dietary fibre increased the daily faecal bulk (13), and therefore lowered the concentration of DC. Ca will have increased the excretion of DC by gastrointestinal binding to insoluble Ca (14). There was a positive correlation between faecal bulk and Ca-intake in the study with habitual omnivores and vegetarians: $r = 0.28$; $P > 0.1$ (chapter 4) and in the trial with urolithiasis patients (chapter 7): $r = 0.68$; $P < 0.01$. Moreover, in this study the change in Ca-intake correlated significantly with the change in faecal bulk. An increase of the Ca-intake above 1000 mg/day increased faecal bulk in rats and humans (15,16). It may be caused by a higher excretion of Ca and of fatty acids due to their reduced absorption. Faecal bulk is also increased by intake of 'resistant starch' (17) and intake of an inhibitor of starch digestion (18). The larger faecal bulk of males compared to females was confirmed in our studies (chapter 4 and 7).

Faecal bulk was negatively associated with CRC-incidence in a correlation study in England. A bulk higher than 200 g/day would decrease the CRC risk 3 times to values like that of Japan. Daily stool weight is highly correlated with transit time (and defaecation frequency) until 150 g faeces/day (19).

The intake of dietary fibre, especially from vegetables, is consistently and negatively correlated with the risk of CRC in retrospective studies (19-22). No clear association between fibre or vegetable intake and colon cancer risk in

men was found in a prospective epidemiological study (23), but in a prospective study in women, the group with the highest animal fat and lowest crude fibre intake had the highest risk of colon cancer (24).

The ratio CDC to ILC + LC (primary to secondary BA) was significantly higher on the vegan diet than on the omnivorous diet (chapter 3), which was probably caused by the pH 6.7 of three-fold diluted faeces. This ratio did not differ between habitual omnivores and vegetarians (chapter 4). The ratio primary to secondary BA decreased after subtotal colectomy in familial adenomatous polyposis patients. It was attributed to the absence of the colon with his bacterial flora and the pH 5.5 of two-fold diluted faeces (25). The dehydroxylation enzyme which converts primary to secondary BA, is inhibited *in vitro* at a pH lower than 5.5 (26). At more usual conditions like a lacto-ovo-vegetarian (chapter 4) or a Ca-restricted diet (chapter 7), no significant correlation between faecal pH and the ratio primary to secondary BA in faeces was found.

Neither the concentration of faecal BA nor the ratio hydrophobic (mono+dihydroxy) to hydrophilic (keto+trihydroxy) BA (26) differed between habitual omnivores and vegetarians. However the concentration of BA in the aqueous part of the faeces was lower in the vegetarians. Moreover defaecation frequency and faecal bulk were higher in this population. Thirty percent of the variance in the concentration of DC in faecal water was explained by the intake of saturated fat, while twenty-four percent could be explained by the intake of cholesterol. Twenty percent of the variance in faecal bulk could be explained by the intake of dietary fibre, especially from fruit and vegetables. Also, faecal bulk was higher in men than in women (chapter 4).

In our study in rats and humans with dietary Ca-binders (chapter 5 and 6), neither the concentrations of BA and LCFA in caecal and faecal water respectively, nor the ratio hydrophobic to hydrophilic BA or the pH were influenced by a lower availability of Ca and Mg. On the other hand, the concentrations of BA and LCFA in total faeces decreased. The results may be explained by the lowering of the bioavailability of Ca below 1000 mg/day: there are indications from literature (15,16) that Ca-supplementation above 1000 mg/day increases faecal bulk. In our cross-sectional study (chapter 4) no relation was found between faecal pH and Ca-intake below 1000 mg/day. A higher faecal bulk will decrease the concentration of BA in faecal water as can be derived from our study with the habitual omnivores and vegetarians (chapter 4). This may be the result of binding of BA to solid material such as calcium ions. Moreover, the fat content of the diet during the trial with Calciisorb[®] was much lower than the amount for which Newmark et al. (27)

proposed their hypothesis, that a diet high in Ca and Mg may lower CRC risk by making BA and LCFA less water-soluble. This hypothesis may be only valid at high intakes of saturated fat, which lead to high faecal concentrations of BA and LCFA. Such an effect has been found for Ca in rats (28).

The Ca-restricted diet of hypercalciuria type II patients lowered the concentrations of BA in faecal water and also the cytotoxicity of faecal water, but faecal pH did not change (chapter 7). This may be due to the lower intake of saturated fat that unintentionally accompanied the Ca-restriction in these patients due to omission of dairy products. Based on the regression equation derived from our previous cross-sectional study (chapter 4), a decrease of about 90 $\mu\text{mol/l}$ BA could be predicted by the decrease in saturated fat. The expected enhancing effect of the lower intake of Ca as derived from the data of van der Meer and Govers (29) would be about 40 $\mu\text{mol/l}$. Therefore the lowering effect of the diet is predominating. The ratio hydrophobic to hydrophilic BA did not change after Ca-restriction, as would be expected by the data of Lapré et al (30). They found a decrease in the ratio hydrophobic to hydrophilic BA in faecal water after Ca-carbonate supplementation of volunteers to 3000 mg/day but no lower concentration of BA in faecal water. This was ascribed to the low baseline values of BA in faecal water. Govers and van der Meer (29) supplemented volunteers with Ca from milk products to 1800 mg/day and found a lower concentration of BA in faecal water. This result may have been caused by the higher daily faecal weight after Ca-supplementation.

In a large randomized trial with men, no changes in the concentration of BA in faecal water were detected after supplementation with 3000 mg Ca-carbonate, but a statistically significant increase in the concentration of DC in faecal water (31). However, their method of isolating faecal water is disputable, because they dilute faeces 1:1 with water before centrifuging. In this way, an equilibrium between the concentrations of BA in the solid and in the aqueous phase of the faeces may be disturbed.

A recent, small study in which was corrected for factors influencing BA excretion like cholecystectomy and liver function, and in which faeces were sampled during 5 days, showed that faecal BA were statistically significant higher in patients with adenomatous polyps in the large intestine than in controls. Also CRC patients had higher concentrations than controls although this difference was not statistically significant (32). Adenomatous polyps are considered as precursors of CRC (33). In a randomized trial of a low-fat, high-fibre diet to prevent the recurrence of colorectal polyps, the rate of neoplastic polyp recurrence increased with the concentration of BA in faeces (34). In an *in vitro* study using colon cancer cell lines, the cytotoxicity

of the BA at concentrations occurring in faecal water, increased with the hydrophobicity of the BA and the time of exposure (35). The mechanism underlying the cytotoxicity of BA is most probably the activation of protein kinase C, directly or indirectly by stimulating the formation of diacylglycerol (36). Protein kinase C is a key enzyme in cell proliferation (37).

Not all epidemiological studies found an increased CRC risk after a diet high in saturated (animal) fat. Most studies found a positive relation with red meat (23) and eggs (38). The mode of cooking red meat may be an additional risk factor, because during the process of cooking or frying at high temperatures, heterocyclic amines are formed. These substances are activated to carcinogenic compounds by the cytochrome P450 system of liver and intestine and transported by the blood to different organs. The metabolic activation is increased in rapid metabolizing phenotypes and by smoking (39). Eggs may be a risk factor because these food products contain unsaturated fatty acids and cholesterol, which combination has been shown to increase faecal bile acid excretion in humans (40). Moreover, the lecithin in eggs is a powerful inducer of the gallbladder contraction, thereby increasing the faecal excretion of BA.

8.4 Defaecation frequency

The role of defaecation frequency in CRC-risk is still unknown. In the Dutch cohort study on diet and cancer (41) no relation between defaecation frequency asked by questionnaire and CRC-incidence was found in males (Bausch-Goldbohm, unpublished results). The validity of asking for defaecation frequency was tested in our Ca-restriction study: the Spearman correlation coefficient between asked and registered defaecation frequency was 0.84 ($P < 0.01$). In the studies of chapter 4 and 7, defaecation frequency was higher in man than in women. Would defaecation frequency play a role in CRC risk of women?

In our study with habitual omnivores and vegetarians (chapter 4) 35% of the variance in defaecation frequency was explained by sex and the intake of dietary fibre, especially fibre from fruits and vegetables. In the Ca-restriction study, 26% of the variance was explained by sex and the intake of dietary fibre.

8.5 Faecal pH

Faecal pH was lowered by a vegan diet (chapter 3). Thirty percent of the variance in faecal pH was explained by the intake of Ca (chapter 4). A higher explanation was obtained when the Ca-concentration in faeces and the concentration of one of the main short chain fatty acids (SCFA), butyrate, in faecal water was put into the regression equation ($r^2 = 0.47$): a lower concentration of Ca and a higher concentration of butyrate lead to a lower pH. A significant positive correlation between Ca in faeces and faecal pH was also found in the Ca-restriction study (chapter 7): $r = 0.45$. Moreover in that study, a high pH of faecal water was correlated with a low cytotoxicity of faecal water (Spearman correlation coefficient = -0.63 , $p < 0.01$). The cytotoxicity of faecal water for erythrocytes was highly correlated with that for cells derived from a human colon adenocarcinoma cell line (42) and with hyperproliferation of colonic cells of rats (43). The positive correlation between Ca-intake and faecal pH has been explained by the increase in faecal phosphate after Ca-supplementation, which may increase the buffer capacity of faeces (16).

Earlier studies have reported that faecal pH is higher in patients with CRC than in controls (chapter 1). However a more recent study showed no difference for age matched groups (44). They found higher faecal pH's in older subjects. Also no difference was found in a case-control study measuring gastrointestinal pH by a pH sensitive radiotelemetry capsule (45).

In literature (17) a positive association between pH and percentage solubilized BA has been suggested. We did not find such a relation in our studies (chapter 4 and 7). We propose a CRC protecting effect of a high faecal pH: at a higher pH the secondary BA and the LCFA are more dissociated and therefore less hydrophobic. Both compounds were more cytolytic when their hydrophobicity increased (42).

8.6 Calcium and magnesium in faecal water

In our study in animals and humans with dietary Ca-binders (chapter 5 and 6), binding of dietary Ca by Calcisorb® leads to lower concentrations of Ca and Mg in caecal and faecal water respectively. The concentration of Ca or Mg in faecal water may be related to CRC-risk (46,47). Both ions have a direct inhibitory effect on colonic cell proliferation. The concentration of Ca in faecal water was not lowered on the Ca-restricted diet (chapter 7). Lapré et al (30) found higher concentrations of Ca in faecal water after supplementa-

tion with Ca-carbonate from 1200 to about 3000 mg/day, but Govers et al (16) found no significant increase after supplementation with Ca from milk products from 800 to about 1800 mg/day.

No epidemiological or animal studies have been performed on the CRC risk of low concentrations of Ca and Mg in faecal water.

8.7 Long chain fatty acids in faecal water

The concentrations of LCFA in faecal water were not influenced in animals and humans by a lower availability of Ca and Mg (chapter 5 and 6). On the other hand, faecal excretion values of LCFA were lower after binding Ca and Mg. The hypothesis of Newmark (28) that a diet high in Ca and Mg lowers CRC risk by making LCFA less water-soluble, may be only valid for Ca at high intakes of saturated fat, which lead to high faecal concentrations of LCFA as in the studies of Lapré and Govers, where the intake of saturated fat was 120 g/day, while the intake in our human study was only 85 g/day.

The Ca-restricted diet of hypercalciuria type II patients does lower the concentration of LCFA in faecal water and also the cytotoxicity of faecal water (chapter 7). The concentration of LCFA in faecal water was highly correlated with the cytotoxicity of faecal water for erythrocytes ($r=0.74$). Moreover the correlation between changes in both variables was 0.60.

The relation between faecal LCFA and CRC-risk has not been reported after the study of Sperry et al (48). They are suspected of increasing CRC because like BA, they activate protein kinase C directly or through the activation of phospholipase D (49).

8.8 Dietary treatment of urolithiasis patients

Risk factors for Ca-oxalate stones are the enhanced excretion of oxalate, Ca, urate, the decreased excretion of the inhibitors citrate and Mg as well as low urinary volume. The excretion of oxalate is the most important risk factor (50).

From our intervention study it appears that a diet restricted in Ca to 500 mg/day has a small benefit for hypercalciuric urolithiasis patients, because it

lowers the excretion of oxalate, most probably due to the lower content of protein and carbohydrates of this diet. This also lowered the concentration of BA and LCFA in faecal water and its cytotoxicity.

The expected lowering of the Ca-excretion in urine by the Ca-restricted diet occurred slightly and only in men. The reasons for this effect may be:

1. Women react in a different way from men on Ca-restriction. Studies reported in literature on the effects of a Ca-restricted diet on Ca-excretion have been performed mainly in men (chapter 7)
2. The lowering effect reported in literature was measured after 4 days, while only after 10 days of Ca-restriction the concentration of calcitriol in serum will increase (51) and this will increase the Ca-excretion with urine (52)
3. Not all patients in our intervention study were true hypercalciuria type II patients because their diagnosis was based on a Ca-excretion > 5 mmol/day and a lowered Ca-excretion after a diet restricted in Ca, sodium and oxalate. Some may be more sensitive for the effect of sodium on Ca-excretion (53), others may be normal calciuretic and respond too slightly on a Ca-restriction to 400 mg/day (54). At our Dept. of Clinical Chemistry, normal values of urinary Ca-excretion are 1.4 - 7.5 mmol/day.

Therefore the following scheme (see Figure 1) for diagnosis and dietary treatment of patients with Ca-oxalate stones should be studied for its effectiveness and feasibility. Firstly, stone composition should be analyzed preferentially by IR-spectroscopy or X-ray diffractometry to distinguish the different kinds of stones (55). **Patients with a primary Ca-oxalate stone** are screened in the following way:

1. Medical history including medications, clinical chemical analysis of 24 h urine for volume, creatinine, urate, oxalate, glyoxylate, citrate, urea, Na, K, Mg, fasting urine for pH and fasting serum for Ca, phosphate, parathyroid hormone 1-84, a dietary anamnesis and antropometric data on height and body weight. A pyelogram is made to detect anatomical abnormalities.
2. Creatinine excretion lower than 20 mg/kg body weight means wrong sampling of 24 h urine (56). A new sample of 24 h urine has to be collected.
3. Hyperuricosuria (> 450 $\mu\text{mol/d}$) and urinary pH < 5.5 in the absence of excessive intestinal alkali loss or animal protein intake, will be caused by gouty diathesis. The concentration of uric acid in serum will also be increased (57). If pH is normal, the uricosuria maybe due to a high intake of meat or fish products, beer or coffee (58). These dietary habits can be detected from the dietary and urinary data: urea-excretion > 250 mmol/d

FIGURE 1
Proposed metabolic screening of Ca-oxalate stone patients

means an intake of animal protein higher than 50 g/day (S. Houterman, personal communication). A prudent diet (see below), restricted a.o. in animal protein will be the dietary therapy. With gouty diathesis or if the diet is inadequate, allopurinol or potassium citrate should be given.

4. Hyperoxaluria ($>450 \mu\text{mol/d}$) with a high concentration of urinary glyoxylate means a pyridoxine deficiency (primary hyperoxaluria type I). These patients should be treated with pyridoxine and if not effective with citrate (59). Other hyperoxalurias may be caused by bowel disturbances (60) or a high intake of animal protein or oxalate-rich products. Bowel disturbances should be treated with Ca-supplementation (1.5 g/d) and a diet with minimal amounts of oxalate. The other hyperoxalurias can be corrected by dietary measures.
5. Hypocitraturia (citrate $< 2.1 \text{ mmol/d}$) may be due to a high intake of animal protein or salt (61). The therapy should be a prudent diet (see below).
6. Hypercalciuria (Ca $> 5 \text{ mmol/d}$) (57) with serum phosphate $> 0.8 \text{ mmol/l}$ means a renal leak of phosphate. Orthophosphate is the therapy. If serum Ca $> 2.6 \text{ mmol/l}$ and serum parathyroid hormone $1-84 > 58 \text{ pg/ml}$ the diagnosis is primary hyperparathyroidism. They can be treated with parathyroid surgery.

The other types of hypercalciuria should be checked by their dietary intake of protein, Na, K, Ca, carbohydrates, mono- and disaccharides, oxalate-rich products, energy, fluid and Mg-rich products (62). The data on dietary intake of the first 4 components can be confirmed by urinary data on urea, Na, K, Ca, while the intake of fluid must correlate with the amount of daily urine. A high intake of carbohydrates, mono- and disaccharides and energy may be reflected in a body mass index > 25 . If there are large deviations from normal values and/or eating pattern shows large meals in the evening (63), a prudent diet (see below) equally spread over the day with a high intake of fluid ($> 2.5 \text{ l}$ urine per day,) are prescribed. The patients can control their intake of fluid by measuring the specific gravity of urine ($< 0.012 \text{ g/l}$) by dip-sticks (64,65).

Recurrent stone patients (a stone within an interval of 2 yrs), patients with stones in both kidneys and patients of age $< 18 \text{ yrs}$ should be studied for the causes of their hypercalciuria, hyperoxaluria, hyperuricosuria, hypocitraturia and/or hypomagnesuria on a Ca-rich and if necessary on a Ca-low diet (66,67):

1. They consume during one week a constant, prudent diet: 30 g animal protein, 1000 mg K, 3100 mg Na, 120 g mono- and disaccharides, 30 energy of fat, 300 mg Mg, at least 1.5 l fluid, no more than 4 glasses of beer, no more than 3 cups of coffee/day. Moreover, this experimental diet should contain no oxalate-rich products like spinach or rhubarb. The

Ca-content of the diet will be about 400 mg. The compliance to the diet can be derived from data on urinary urea, K and Na. The other amount of Ca (600 mg) is standardized and can be checked by giving Ca-gluconate-tablets (6 per day) and counting pills that are not used. Comparing data of 24 h urine before and after this week for urea, Ca, Na, K, phosphate, uric acid, Mg, oxalate and citrate will detect a dietary origin of these abnormalities. They can be treated with a prudent diet, that is palatable and also active against other chronic diseases like cardiovascular diseases and several forms of cancer.

2. Patients who show no decrease in Ca-excretion on the diet mentioned in 1., should consume the constant, prudent diet for one week containing only the basal 400 mg Ca. If normalization of Ca occurs, while the urinary excretions of Na, Mg, K and urea are constant, these patients have an hyperabsorption of dietary Ca and should be treated with a Ca- and oxalate-restricted diet (hypercalciuria type II). Further research is needed to form a group of patients that is at high risk for osteopenia on this diet.
3. Patients whose Ca-excretion does not respond to the Ca-restricted diet should be treated with thiazide + K-citrate + Na-restricted diet (150 mmol/d) and high fluid intake divided over day and night. Further research is needed to split these patients into groups in which mainly the bones, the kidney or the intestine are involved.

Conclusions

Based on the results of the studies described in this thesis and on the literature described and discussed above, the model for the role of faecal constituents in the pathogenesis of CRC have to be adapted (Figure 2). The faecal constituents influence the proliferation of colonic epithelial cells. Increased cell proliferation is associated with a higher expression of mutations.

The influences of several dietary factors on the faecal constituents are shown.

Meat and meatproducts are rich in saturated fat, while eggs are rich in cholesterol. Both are supportive for higher concentrations of BA and LCFA in faeces and faecal water. Vegetables and grains are rich in dietary fibre and resistant starch, leading to a higher faecal bulk and a lower faecal pH. A high faecal bulk diminishes the concentration of BA and LCFA in faeces and faecal water. Dietary fibre and resistant starch will diminish faecal pH by increasing the butyrate production in the colon. A low pH will increase the amount of LCFA and undissociated BA in the colonic contents, which will increase the hyperproliferation of epithelial cells. However, butyrate will lower this hyper-

FIGURE 2

Proposed model for the role of diet and faecal markers in colorectal carcinogenesis

proliferation. Dietary Ca above 1000 mg/day may decrease the amount of solubilized BA and LCFA and the ratio hydrophobic to hydrophilic BA in faecal water.

A diet rich in dietary fibre and resistant starch and restricted in saturated fat may diminish the risk for CRC. Vegan diets may protect against CRC, in spite of their resulting low faecal pH, due to their low content of saturated fat and their high dietary fibre content. Lacto-ovo-vegetarian diets which may not be that high in dietary fibre, may be protective because of their lower amount of saturated fat and their higher amount of Ca than the vegan and the omnivorous diet. A prudent diet which is rich in dietary fibre from grains and fruits and vegetables, sufficient in milkproducts and fluids and moderate in meat, eggs, sugar and table salt may exert a similar protective effect as the vegetarian diets.

A diet restricted in Ca in which the omitted milkproducts are not substituted by products rich in animal protein like meat and eggs, may be of a small benefit for male urolithiasis patients genetically predisposed to Ca-oxalate stones, without increasing the risk of CRC. However in such women, that diet may be ineffective and may even raise the risk of osteoporosis. A prudent diet as described by the Dutch guidelines for a healthy diet (see above), should fulfill all requirements at the same time with regard to the risk of urolithiasis, osteoporosis and CRC.

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Samenvatting

De invloed van verschillende voedingen op kenmerken van de ontlasting die samenhangen met het risico op dikke darmkanker. Onderzoek met vegetariërs en niersteenpatiënten.

Dikke darmkanker (colon- en rectumkanker) is de op één na meest voorkomende vorm van kanker bij vrouwen en de op twee na meest voorkomende bij mannen in de meeste Westerse landen. De behandeling d.m.v. operatie, medicijnen en/of bestraling kan slechts bereiken dat 5 jaar na behandeling nog 50% van de patiënten in leven is. Daarom is het gewenst ook aandacht te schenken aan de preventie van dikke darmkanker.

Het lijkt erop dat het vóórkomen van dikke darmkanker beïnvloed wordt door omgevingsfactoren, o.a. voedingsgewoonten. Het risico is ook genetisch bepaald, maar groepen mensen met voedings- en andere leefgewoonten die sterk verschillen van de gemiddelde bevolking zoals Zevende dags adventisten, Mormonen en vegetariërs, hebben een lagere sterfte tengevolge van dikke darmkanker.

Onderzoek naar het ontstaan van dikke darmkanker heeft laten zien, dat deze ziekte ontstaat door veranderingen in delen van het DNA die betrokken zijn bij de vorming van kanker. De tijd tussen de eerste verandering en het optreden van de ziekte is tenminste 20 jaar. Daarom is er gezocht naar biologische kenmerken van dit risico die naar voren komen in die 20 jaar. Omdat de dikke darm in contact staat met het bloed en met de darminhoud, zouden kenmerken van de omgevingsfactoren die dikke darmkanker veroorzaken, in deze delen van het lichaam te vinden moeten zijn. Resten van de darminhoud komen terecht in de ontlasting en onderzoek van de ontlasting zal dan ook informatie moeten kunnen opleveren over risico's voor het ontstaan van dikke darmkanker.

In **hoofdstuk 1** zijn de onderzoeksgegevens die beschreven zijn in de literatuur voor het begin van ons laatste onderzoek (1990), samengevat. Op grond hiervan gingen we ervan uit dat een voeding met veel vlees, weinig voedingsvezel en weinig calcium samenhangt met een hoog risico op dikke darmkanker. Kenmerken van de ontlasting die hoogst waarschijnlijk samenhangen met het risico op dikke darm kanker en die veranderen onder invloed van voedingsgewoonten, zijn:

- de hoeveelheid ontlasting per dag
- de concentraties van de secundaire galzuren deoxycholzuur en lithocholzuur en van vetzuren zoals stearinezuur in de gehele ontlasting en in het waterige deel van de ontlasting
- de zuurgraad van de ontlasting.

Het doel van de onderzoeken in dit proefschrift was na te gaan of gebruikelijke voedingsgewoonten die verschillen in de dagelijkse hoeveelheid vlees, voedingsvezel en calcium, zoals een omnivore, een lacto-ovo-vegetarische en een veganistische voeding en het calcium-beperkte dieet van niersteenpatiënten, invloed hebben op de ontlastingskenmerken die samenhangen met het risico op dikke darmkanker. Als de effecten in dezelfde richting wijzen als de gegevens over de invloed van voedingsgewoonten op het risico voor dikke darmkanker, dan kunnen in epidemiologische onderzoeken de biologische kenmerken gebruikt worden als factor ter voorspelling van het risico op ziekte en hoeft niet gewacht te worden tot zich een tumor ontwikkeld heeft. Als de gegevens over de ontlastingskenmerken passen bij een biologische verklaring voor de veronderstellingen over voeding als oorzaak van het ontstaan van dikke darmkanker, dan worden die veronderstellingen ondersteund.

Allereerst hebben we een nauwkeurige en juiste methode ontwikkeld voor de bepaling van de concentratie van de meest voorkomende galzuren in de menselijke ontlasting, deoxycholzuur en lithocholzuur (**hoofdstuk 2**). Met de hierop gebaseerde methoden die in onze verdere onderzoeken werden gebruikt, zijn getallen voor beide galzuren werden verkregen die overeenkwamen met de resultaten uit andere laboratoria. Bovendien kunnen de in lagere hoeveelheden voorkomende galzuren zoals de 3β -isomeren van deoxy- en lithocholzuur en de 7- en 12-keto-galzuren bepaald worden. Deoxy-, lithocholzuur en hun 3β -isomeren en de keto-verbindingen worden ook wel de secundaire galzuren genoemd, omdat ze gevormd worden door de bacteriën in de dikke darm uit de de in de lever gevormde primaire galzuren cholzuur en chenodeoxycholzuur.

Hoofdstuk 3 beschrijft de resultaten van een streng gecontroleerd onderzoek met jonge, mannelijke vrijwilligers, die drie typen voedingen na elkaar gebruikten. Een lacto-ovo-vegetarische voeding (vegetarisch met melk en eieren) en een veganistische voeding (alleen plantaardige producten) verlaagden de concentraties van deoxy- en lithocholzuur in de totale ontlasting en verhoogden de hoeveelheid ontlasting per dag en het aantal keren ontlasting per week. Deze effecten werden hoogst waarschijnlijk veroorzaakt door de grotere hoeveelheid voedingsvezel in vergelijking met de gemengde voeding

(vlees,eieren,melk- en plantaardige produkten). De concentratie van de som van deoxy- en lithocholzuur in de totale ontlasting was hoger tijdens de lacto-ovo-vegetarische voeding dan tijdens de veganistische voeding. Dit werd in de discussie toegeschreven aan de grotere hoeveelheid calcium in de lacto-ovo-vegetarische voeding. De zuurgraad van de ontlasting was lager tijdens de veganistische voeding dan tijdens de twee andere voedingen.

Uit andere epidemiologische onderzoeken komt vaak naar voren, dat een hogere inname van voedingsvezel gepaard gaat met een lager risico op dikke darmkanker. Groepen mensen met een dagelijkse hoeveelheid ontlasting van meer dan 200 gram hadden een 3 maal zo laag risico op dikke darmkanker. In een klein onderzoek bleek dat patiënten met adenomateuze poliepen in de dikke darm, een voorstadium van dikke darmkanker, hogere concentraties van de secundaire galzuren in de ontlasting hadden dan gezonde controles. Ook bleken patiënten met ontstekingen van de dikke darm die een hoge concentratie hadden van deoxycholzuur in de ontlasting, later meer kans te hebben een dikke darmtumor te ontwikkelen dan soortgelijke patiënten met een lage concentratie.

M.a.w. de concentratie van secundaire galzuren in de ontlasting zou in epidemiologisch onderzoek gebruikt kunnen worden als factor ter voorspelling van het risico op dikke darmkanker. De betekenis van de hoeveelheid ontlasting per dag, het aantal keren ontlasting per week en de zuurgraad van de ontlasting moet nog nader onderzocht worden in prospectieve epidemiologische onderzoeken alvorens ze te gebruiken als biologisch kenmerk van het risico op dikke darmkanker. Onze resultaten ondersteunen de veronderstelling dat een hogere inname van voedingsvezel beschermt tegen het ontstaan van dikke darmkanker.

In hoofdstuk 4 staan de resultaten beschreven van een onderzoek bij mannen en vrouwen tussen de 59 en 67 jaar die gedurende lange tijd gewoon waren een lacto-ovo-vegetarische voeding of een omnivore voeding (mengsel van vlees,eieren, melk en plantaardige produkten) te gebruiken. De lacto-ovo-vegetariërs hadden geen lagere concentraties van galzuren in de totale ontlasting maar wel een lagere concentratie in het waterige deel van de ontlasting. Dit deel wordt verkregen na centrifugatie op hoge snelheid en wordt verondersteld in contact te staan met de cellen van de darmwand. De hoeveelheid ontlasting per dag en het aantal keren ontlasting per week was hoger bij de vegetariërs dan bij de omnivoren en hoger bij mannen dan bij vrouwen. De zuurgraad van de ontlasting verschilde niet tussen de omnivoren en de vegetariërs. In hoofdstuk 8 is beschreven, dat dertig procent van de variatie in de concentratie van deoxycholzuur in het waterige deel van de ontlasting verklaard kon worden door de hoeveelheid verzadigd vet die per dag werd gegeten, terwijl 24 % van de variatie verklaard kon worden uit de inname van

cholesterol. Daarnaast kon nog twintig procent van deze variatie verklaard worden door geslacht en hoeveelheid ontlasting per dag. Ook kon twintig procent van de variatie in de hoeveelheid ontlasting per dag en het aantal keren ontlasting per week verklaard worden door de hoeveelheid voedingsvezel, vooral uit groente en fruit. De helft van de variatie in de zuurgraad kon verklaard worden door de concentratie van calcium in de ontlasting en de concentratie van één van de belangrijkste vluchtige vetzuren, boterzuur, in het waterige deel van de ontlasting.

Niet alle epidemiologische onderzoeken laten zien dat de inname van verzadigd vet gepaard gaat met een verhoogd risico op dikke darmkanker. Maar het veel eten van voedingsmiddelen als rood vlees en eieren, hangt wel samen met een verhoogd risico. Het verband tussen de concentratie van secundaire galzuren in het waterige deel van de ontlasting en het risico voor dikke darmkanker, is nog niet onderzocht in grootschalige epidemiologische onderzoeken.

Uit onderzoek door anderen was gebleken dat calcium-supplementatie de concentratie van galzuren in het waterige deel van de ontlasting kon verlagen. Bovendien hadden epidemiologische en experimentele onderzoeken laten zien, dat een hoge calcium-inname beschermend zou kunnen zijn voor het ontstaan van dikke darmkanker. De effecten van calcium-beperking op biologische kenmerken voor het risico op dikke darmkanker waren nog niet onderzocht.

In hoofdstuk 5 is een onderzoek beschreven met ratten die een stof kregen toegediend om calcium en magnesium uit de voeding niet beschikbaar te maken voor absorptie in de dunne darm: ethyleen diamine tetra azijnzuur of te wel EDTA. In hoofdstuk 6 is beschreven wat er gebeurde als vrijwillige mannen een medicijn innamen, dat door een bepaalde groep niersteenpatiënten wordt gebruikt om te voorkomen dat er te veel calcium uit de voeding wordt opgenomen in de dunne darm: Calcisorb®. In beide gevallen werd verwacht, dat de concentraties van galzuren en vetzuren in het waterige deel van de darminhoud, respectievelijk ontlasting, zouden stijgen. Er werden echter geen verschuivingen gevonden in de concentraties. Dit is mogelijk veroorzaakt door de verlaging van de beschikbaarheid van calcium tot beneden de 1000 mg/dag. Uit de literatuur zijn aanwijzingen verkregen, dat calcium-supplementatie tot ruim boven de 1000 mg/dag de hoeveelheid ontlasting per dag verhoogt. Deze hogere hoeveelheid faeces per dag verlaagt waarschijnlijk de hoeveelheid galzuren in het waterige deel van de ontlasting, zoals af te leiden is uit ons onderzoek met de habituele omnivoren en vegetariërs.

De concentraties van calcium en magnesium in het waterige deel van de darminhoud, respectievelijk ontlasting, waren lager na de interventie. De

mogelijke rol van deze lagere concentraties voor het ontstaan van dikke darmkanker moet nog nader onderzocht worden in dierexperimentele en epidemiologische onderzoeken, omdat beide mineralen ook een direct verlagend effect hebben op celdeling.

Het laatste onderzoek dat beschreven is in **hoofdstuk 7**, betrof de nadelige gevolgen van het calcium-beperkte dieet van een groep niersteenpatiënten (hypercalciurie type II) voor de kenmerken van de ontlasting die samenhangen met het risico op dikke darmkanker. Dit dieet wordt voorgeschreven, omdat deze patiënten te veel calcium absorberen in de dunne darm en daardoor te veel calcium uitscheiden in de urine. Ook hier werd een stijging van de concentraties van gal- en vetzuren in het waterige deel van de ontlasting verwacht. Echter deze concentraties daalden evenals de toxiciteit van het waterige deel van de ontlasting in een test met rode bloedcellen. Deze effecten werden hoogst waarschijnlijk veroorzaakt door de lagere inname van (verzadigd) vet en cholesterol die gepaard ging met de calcium-beperking door het weglaten van melkprodukten. De toxiciteit voor rode bloedcellen hing het sterkst samen met de concentratie van vetzuren in het waterige deel van de ontlasting. De toxiciteit van het waterige deel van de ontlasting voor rode bloedcellen is gebleken goed overeen te komen met de toxiciteit voor epitheelcellen van de dikke darm en met de mate van celdeling in de dikke darm bij ratten. Vetzuren zouden het risico op dikke darmkanker kunnen verhogen, omdat ze evenals galzuren het enzym proteïne kinase C activeren. Dit enzym speelt een cruciale rol bij het delen van cellen. Het verband tussen de concentratie van vetzuren in het waterige deel van de ontlasting en het risico op dikke darmkanker moet nog onderzocht worden in dierexperimentele en epidemiologische onderzoeken. De pH van het faecale water daalde niet, maar aan het begin van het onderzoek gingen hoge waarden gepaard met grote hoeveelheid calcium in de ontlasting. Ook bleken hoge pH waarden samen te gaan met lage waarden van de toxiciteit voor rode bloedcellen. Hoge waarden van de hoeveelheid ontlasting hingen samen met een hoge inname van calcium, gemeten door het optellen van de dagelijkse uitscheiding van calcium met de urine en de ontlasting, vooral boven 1000 mg calcium per dag. Bovendien kon 65 % van de variatie in de verandering van de hoeveelheid ontlasting per dag door het calcium-beperkte dieet verklaard worden door de verandering in calcium-inname.

In het onderzoek uit hoofdstuk 7 werden ook de effecten van het dieet op risicofactoren voor de vorming van calciumoxalaat-nierstenen gecheckt. De uitscheiding van calcium in de urine daalde iets en alleen bij mannen. De uitscheiding van oxalaat daalde, hoewel niet statistisch significant. De citraatuitscheiding veranderde niet.

Op grond van de afwezigheid van het verwachte effect van het calcium-beperkte dieet op de risicofactoren voor de vorming van calcium-oxalaat nierstenen en recente gegevens uit andere onderzoeken, wordt in **hoofdstuk 8** een nieuw schema voorgesteld voor de diagnose en therapie van niersteenpatiënten met calciumoxalaat-stenen, dat nog getest moet worden op efficiëntie en uitvoerbaarheid. Patiënten met een eerste niersteen worden onderzocht met medische, klinisch-chemische en antropometrische methoden. Als er geen metabole afwijkingen worden gevonden, wordt een gezonde voeding volgens de Nederlandse richtlijnen voorgeschreven, bestaande uit normalisering van de hoeveelheid dierlijk eiwit, eieren, suiker en keukenzout, voldoende calcium en vocht en rijk aan groente en fruit. Van patiënten met terugkerende calciumoxalaat-stenen wordt 24 uren urine geanalyseerd voor en na een streng gecontroleerd dieet volgens de Nederlandse voedingsrichtlijnen en een vaste hoeveelheid calcium in tabletvorm. Patiënten bij wie de risicofactoren in de urine zoals calcium, oxalaat, uraat, citraat, magnesium dalen en stoffen in de urine die indicatoren zijn voor de het voorgeschreven dieet zoals ureum en natrium, ook dalen, krijgen een gezonde voeding aanbevolen. Patiënten bij wie de calcium-uitscheiding in de urine niet daalt op het dieet met calcium, maar wel op het dieet zonder de calcium-tabletten, worden gediagnosticeerd als patiënten met hypercalciurie type II. Alleen deze groep krijgt een calcium-en oxalaatbeperkt en vochtrijk dieet. Verder onderzoek is nog nodig om na te gaan welke van deze patiënten een verhoogd risico op botontkalking hebben tengevolge van dit dieet.

Tenslotte wordt in **hoofdstuk 8** een aangepast model gepresenteerd voor de rol van ontlastingskenmerken bij het ontstaan van dikke darmkanker en de invloed van voedingsbestanddelen op deze kenmerken. Bestanddelen van de ontlasting zoals vetzuren en hydrofobe galzuren in het waterige deel van de ontlasting kunnen de deling van epitheelcellen van de dikke darm verhogen. Hierdoor komen veranderingen in het DNA van deze cellen eerder tot uiting. Er zijn sterke aanwijzingen dat een voeding bestaande uit alleen plantaardige producten (veganistisch) beschermend zal zijn tegen het ontstaan van dikke darmkanker door zijn laag gehalte aan verzadigd vet en cholesterol en zijn hoog gehalte aan voedingsvezel en langzaam verteerbaar zetmeel. Hierdoor zullen de concentraties van hydrofobe galzuren en van vetzuren in het waterige deel van de ontlasting laag zijn. Bovendien zal de grote hoeveelheid voedingsvezel en langzaam verteerbaar zetmeel de productie van boterzuur in de dikke darm verhogen, waardoor de deling van de epitheelcellen van de dikke darm geremd wordt. Lacto-ovo-vegetarische voeding die niet zo'n lage hoeveelheid verzadigd vet bevat als een veganistische voeding, zal beschermen tegen dikke darmkanker omdat ze een grote hoeveelheid voedingsvezel, langzaam verteerbaar zetmeel en calcium bevat. De voedingsvezel en het calcium

zullen de hoeveelheid ontlasting per dag verhogen en daardoor de concentraties van hydrofobe galzuren en vezuren in het waterige deel van de ontlasting verlagen. Bovendien zal het calcium de zuurgraad van de ontlasting verhogen, waardoor de hoeveelheid ongedissocieerde secundaire galzuren zal afnemen. Juist deze ongedissocieerde galzuren zijn hydrofoob en daardoor toxisch voor cellen. Omnivore voeding die meestal weinig voedingsvezel, soms weinig calcium en vaak veel (verzadigd) vet en cholesterol uit vlees en eieren bevat, zal het risico op dikke darmkanker verhogen.

Het risico op niersteenvorming bij mannen die erfelijk belast zijn met een verhoogd risico op calciumoxalaat-stenen, kan mogelijk iets verlaagd worden. Het risico op dikke darmkanker zal niet stijgen als de melkprodukten niet vervangen worden door voedingsmiddelen rijk in dierlijk eiwit, zoals vlees en eieren, of door andere voedingsmiddelen. Echter voor vrouwen is dit diet waarschijnlijk niet effectief en verhoogt het mogelijk hun risico op botontkalking. Een voeding zoals beschreven in de Nederlandse richtlijnen voor gezonde voeding, zal voldoen aan de aanbevelingen voor verlaging van het risico op herhaalde vorming van calciumoxalaat-nierstenen en tegelijk aan die voor de vermindering van het risico op botontkalking en dikke darmkanker.

Dankwoord

Velen hebben bijgedragen aan het tot stand komen van dit proefschrift door hun medewerking, adviezen en/of interesse. Ik denk hierbij vooral aan mijn ex- en huidige collega's uit Nijmegen, Zeist en Maastricht, familieleden en vrienden. De onderstaande personen wil ik met naam noemen.

Prof. Janknegt, u gaf mij het vertrouwen het onderzoek rond de niersteen-patienten te leiden van subsidieaanvraag tot eindrapportage.

Ruud Hermus, jouw begeleiding op het gebied van voedingsonderzoek en je kritische houding ten opzichte van mijn onderzoeksactiviteiten hebben mij veel geleerd.

Roelof van de Meer, jouw kennis van en ideeën over de chemie en fysiologie van galzuren en darminhoud waren heel stimulerend voor mij.

Ton van den Bogaard, jouw enthousiasme en inzet voor gal- en vetzuren in relatie tot het ontstaan van dikke darmkanker en jouw kennis van de faculteit Geneeskunde van de Rijksuniversiteit Limburg hebben het mogelijk gemaakt mijn onderzoeksactiviteiten in Nijmegen en Zeist met succes voort te zetten in Maastricht.

Sandra Bausch-Goldbohm, Piet van den Brandt en Pieter van 't Veer, jullie kameraadschap vanaf onze TNO-tijd en jullie deskundigheid op het gebied van de epidemiologie zal ik nooit vergeten.

Wim van Dokkum, jij leerde me hoe je moet omgaan met proefpersonen.

Fokko Nagengast en Toon Huijbregts, onze gezamenlijke publikatie was het resultaat van een heel prettige samenwerking.

Paul Borm, jouw interesse in moleculaire epidemiologie heeft me naar Limburg doen verhuizen en daar verder doen gaan met mijn ingeslagen onderzoeksrichting.

Jan Glatz, jouw ervaring met het meten van galzuren in ontlasting konden we goed gebruiken in Maastricht. Ook een deel van je onderzoeksresultaten mocht ik gebruiken voor dit proefschrift.

Chanin Boekhoudt, samen met jou heb ik eraan gewerkt zoveel mogelijk patienten in het onderzoek te krijgen. Dit lukte dankzij de medewerking van

de (assistent)-urologen in het Academisch Ziekenhuis Maastricht en de volgende urologen in de andere deelnemende ziekenhuizen: V.W.J. de Kort, P.J.N. Kil, L.M.H. Schreinemachers, J.C. Schepers en J.J.M. Kums. Ook dr. H.K. Kreutzer, klinisch-chemicus, wist nog een aantal patienten. San de Kort, zonder jouw gecomputeriseerde beslisboom voor het diagnosticeren van niersteenpatiënten was het onderzoek niet mogelijk geweest.

Carolien Jacobs en Lisette van den Tweel, jullie waren bereid naast onze PBI-kanten ook mijn urologische klanten te woord te staan.

Phil Geerlings, jij zorgde in mijn urologische periode voor de continuïteit in het berekenen van de voedingsgegevens.

Judith Beeser, als collega vanaf 'Arbeidsgeneeskunde' was jij bereid mijn Engelse schrijfstijl te verbeteren.

Geertjan van Zonneveld, bedankt voor je meedenken bij en het uitvoeren van de illustraties voor dit proefschrift.

Mijn paranimfen, Liesbeth van der Ploeg en Math Hazen hebben mij ook tijdens de opzet, uitvoering en rapportage van het onderzoek in Maastricht ter zijde gestaan.

Last but not least dank ik mijn partner, Bert Hoogveld, voor zijn interesse in en waardering voor mijn werk.

Curriculum Vitae

Arendina (Diny) van Faassen werd geboren op 7 maart 1953 te Slagharen. In 1970 haalde zij het h.b.s.-B diploma aan het christelijk atheneum "Jan van Arkel" te Hardenberg. In 1973 verkreeg zij het kandidaatsdiploma scheikunde met wiskunde en natuurkunde (S₁) aan de Rijksuniversiteit Groningen. Het doctoraalexamen scheikunde deed zij in 1978 en bestond uit het hoofdvak Biochemie en het bijvak Humane Voeding. Gedurende een jaar verrichtte ze molekulair-biologisch onderzoek in het Laboratorium voor Fysiologische Chemie van de Rijksuniversiteit Groningen.

Van 1980 tot 1983 was ze fellow van de Nederlandse Kankerbestrijding - het Koningin Wilhelmina Fonds. Ze werkte achtereenvolgens op de afdeling Gastro-enterologie van het Academisch ziekenhuis Sint Radboud in Nijmegen en de afdelingen Voeding en Toxicologie van TNO-Voeding in Zeist. Ze volgde cursussen epidemiologie en statistiek aan de Rijksuniversiteit Leiden en Landbouwwuniversiteit Wageningen.

Bij TNO-Voeding verrichtte ze van 1984 tot half 1986 epidemiologisch onderzoek naar de relatie tussen voeding en het ontstaan van kanker. Daarna deed ze gedurende twee jaar arbeids- en milieuhygiënisch onderzoek bij de vakgroep Arbeidsgeneeskunde, Milieugezondheidskunde en Toxicologie van de Rijksuniversiteit Limburg. Na een half jaar gewerkt te hebben als zelfstandig adviseur op het gebied van kwaliteitszorg, trad ze in 1989 in dienst bij het Projektbureau voor Industrie en Milieu (P.B.I. b.v.) te Maastricht als adviseur Kwaliteits-, Arbo- en Milieuzorg.

Vanaf 1990 werkt ze 1 dag per week bij de afdeling Urologie van het Academisch ziekenhuis Maastricht. Tevens is ze momenteel 3,5 dag per week werkzaam als arbeidshygiënist en biologisch veiligheidsfunctionaris bij de Rijksuniversiteit Limburg en het Academisch Ziekenhuis Maastricht. In 1993 werd ze erkend als epidemioloog A en in 1994 werd ze geregistreerd als arbeidshygiënist.

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