Eur J Nutr (2009) 48:22–30
DOI 10.1007/s00394-008-0755-2

ORIGINAL CONTRIBUTION

Bianka Ditscheid
Sylvia Keller
Gerhard Jahreis

Faecal steroid excretion in humans is affected by calcium supplementation and shows gender-specific differences

Received: 24 October 2007
Accepted: 21 October 2008
Published online: 12 November 2008

B. Ditscheid · S. Keller · G. Jahreis (✉)
Dept. of Nutritional Physiology,
Institute of Nutrition
Friedrich Schiller University
Dornburger Straße 24
07743 Jena, Germany
Tel.: +49-3641/949-610
Fax: +49-3641/949-612
E-Mail: gerhard.jahreis@uni-jena.de

Abstract *Background* Previous human studies on the effect of dietary calcium supplementation on faecal excretion of bile acids (BA) and faecal water concentrations of animal neutral sterols (NSt, cholesterol and its metabolites) lack detailed information about single BA and NSt. *Aim of the study* We investigated whether single BA and NSt in faeces and especially in faecal water are affected by calcium supplementation and whether this affects genotoxicity of faecal water. In addition, we differentiated between men and women with regard to the concentrations of BA and NSt in faecal water. *Methods* Thirty-one healthy volunteers consumed a calcium supplemented bread (1.0 g/day) and a placebo bread, respectively, for 4 weeks in a double-blind, randomised cross-over trial. Faeces were collected quantitatively for 5 days in the last week of each period. NSt and BA were analysed by GC–MS. *Results* Due to calcium supplementation faecal concentrations of lithocholic acid (LCA, 14%, $P = 0.008$), deoxycholic acid (DCA, 19%, $P < 0.001$) and 12keto-deoxycholic acid (12keto DCA, 29%, $P = 0.049$) significantly increased whereas BA concentrations in faecal water were only marginally affected. In contrast, concentrations of cholesterol (30%, $P = 0.020$) and its metabolites coprostanol (43%, $P = 0.004$), coprostanone (36%, $P = 0.003$), cholestanol (44%, $P = 0.001$) and cholestenone (32%, $P = 0.038$) in faecal water significantly decreased. Total NSt concentration in faecal water was found to be significantly higher in women compared to men ($P = 0.018$). The genotoxicity of faecal water was neither affected by calcium supplementation nor were there gender-specific differences. *Conclusions* Dietary calcium supplementation diversely affects BA and NSt in faeces and in faecal water but does not influence the genotoxicity of faecal water in healthy adults.

Key words neutral sterols – bile acids – faecal water – genotoxicity – human study

EJN 755

Introduction

The faecal steroid excretion is determined by enterohepatic cycling of steroids, microbial steroid metabolism and potential binding/inactivation of steroids by dietary components. One-forth to one-half of intestinal cholesterol [15] and about 95% of all secreted bile acids (BA) are reabsorbed [3, 24]. The cholesterol that enters the large intestine is largely metabolised by the intestinal microbiota. Thus, faecal animal neutral sterols (NSt) are composed of coprostanol, cholesterol, coprostanone and further microbial degradation products of cholesterol. The BA that escape enterohepatic circulation are also metabolised by the colonic microbiota; up to 95% of the excreted BA in faeces are secondary BA [24].

Regarding the excretion of steroids it has to be differentiated between their total faecal concentrations and the soluble quantities found in faecal water. The soluble fraction and not the total faecal BA concentration determines the toxicity to colonic epithelial cells [33]. While concentrations of BA in faecal water have been measured in several studies [9, 12, 20, 30, 43–45], there are very few studies concerned with the concentrations of total NSt [35] or cholesterol and coprostanol in faecal water [12, 20]. To the best of our knowledge, not one study provides data for cholesterol metabolites other than coprostanol in faecal water whereas data for the concentrations of these metabolites in faeces have been reported [16, 17, 48]. There are also no studies that discriminate between men and women in the determination of NSt and BA in faecal water although there are sexual differences in gut function and physiology [19, 22] and colon cancer risk [8, 22]. Genotoxic activity/DNA damage has been attributed to BA [10, 28] and precipitation of soluble BA might result in decreased genotoxicity. BA have been shown to be precipitated by calcium [12, 13] and NSt are probably also precipitated by calcium [41]. The role of cholesterol metabolites (NSt) in the development of colorectal cancer has not yet been focused on. Since NSt possess the same steroidal backbone like BA there might be similar effects; bacterial metabolites generated in the gut are supposed to augment the susceptibility to colon cancer [25]. In addition, animal studies [14] and human case-control studies [18, 34] reveal an impact of animal neutral sterols on carcinogenesis in the large bowel.

The objective of the present study was to investigate the effects of calcium supplementation on faecal steroid excretion, thereby focusing on the BA profile in faeces and in faecal water and the concentration of cholesterol and its metabolites in faecal water. The resulting effect on faecal water genotoxicity was assessed. It was also differentiated between men and women in respect to the concentrations of these faecal steroids.

Subjects and methods

Subjects, supplement and study design

The study design and characteristics of the subjects have previously been published [6]. Briefly, 31 healthy omnivore volunteers (16 women, 15 men; age 24.3 ± 0.4 years, body mass index 21.7 ± 0.4 kg/m^2) participated in this double-blind, placebo-controlled, cross-over study which was approved by the Ethical Committee of the Friedrich Schiller University of Jena. The participants consumed a bread that was either enriched with calcium (calcium supplement: pentacalcium hydroxy-triphosphate [$Ca_5(PO_4)_3OH$; cfb Germany)] or it was not (placebo bread), each for 4 weeks without a washout period. In the last week of each 4-week period, participants got an energy-adjusted defined diet containing the supplemented bread or the placebo bread, respectively. Simultaneously, faeces were collected quantitatively for 5 days. Thus, all the participants consumed identical food during sample collection.

Sample preparation

Faecal specimens were immediately cooled and transported to the study centre where each specimen was weighed, frozen and stored at −20°C. At the end of the study, faecal samples were thawed over night and homogenised using a commercial blender. The homogenised samples were portioned: One part was used for the preparation of faecal water and the other was freeze-dried. For preparing faecal water, approximately 80 g of homogenised faeces were weighed into centrifugation tubes and were centrifuged at 27,000×*g* for 3.5 h at 20°C. The combined supernatant of each participant was portioned and stored at −80°C until analysis. Lyophilised faeces were ground after gravimetric determination of dry matter and stored at −20°C until analysis.

Analysis of neutral sterols and BA

The concentrations of NSt (cholesterol and its metabolites coprostanol, coprostanone, cholestanol, cholestanone and cholestenone) and BA (iso-lithocholic acid (iso-LCA), lithocholic acid (LCA), iso-deoxycholic acid (iso-DCA), deoxycholic acid (DCA), cholic acid (CA), chenodeoxycholic acid (CDCA) and

12keto-deoxycholic acid (12keto DCA)) were analysed as reported previously [6, 17] with marginal modification for the analysis in faecal water (duplicate samples of 1,000 µl faecal water).

Genotoxicity

Genotoxicity of the faecal water samples was determined in HT-29 cells using the comet assay (single-cell gel electrophoresis) as described by Osswald et al. [27], except that the slides were stained with SYBR-Green (1:1,000, Sigma–Aldrich Chemie GmbH, Deisenhofen, Germany). Fifty images per slide were evaluated randomly. The percentage of DNA (intensity of fluorescence) in the comet tail (tail intensity, TI), the tail length (TL) and the tail moment (TM = TI × TL) were determined.

Statistics

Data analysis was performed using the statistical software package SPSS for Windows V. 11.5 (SPSS Inc., Chicago, Ill, USA). Values were reported as means ± SEM. Differences were considered statistically significant at $P \leq 0.05$, two-tailed. All data were tested for normal distribution using the Kolmogorov Smirnov test. When data were distributed normally, the GLM (General Linear Model) procedure of repeated measurement (between-subject factor: gender, covariate: supplementation sequence) was used to test for significant differences between the study periods. In case of non-normal distribution, data were analysed by a paired Wilcoxon signed rank test. Gender-specific differences were examined using the independent *T* test; in case of non-normal distribution comparisons were made by the Mann–Whitney *U* test. Correlations between parameters were calculated independent of calcium supplementation. Thus, analysis for correlations was based on the values of both periods (calcium and placebo). The indicated correlation coefficients were calculated with Pearson's linear correlation (normally distributed parameters). Spearman's correlation analysis was used to correlate non-normally distributed parameters. Unless otherwise stated, data were normally distributed and parametric tests were used.

Results

Nutrient intake

The nutrient intake of the participants was equal in both periods except for calcium and phosphorus [6]. Calcium intake significantly increased due to the supplementation from 1,193 ± 295 mg/day to 2,204 ± 290 mg/day ($P < 0.001$). The intake of phosphorus also significantly increased (1,528 ± 288 mg/day in the placebo period and 1,998 ± 291 mg/day in the calcium period, $P < 0.001$). Men had significantly higher intakes of energy (placebo period: men 10.49 ± 0.32 MJ/day, women 7.66 ± 0.20 MJ/day, $P < 0.001$; calcium period: men 10.41 ± 0.36 MJ/day, women 7.61 ± 0.18 MJ/day, $P < 0.001$), fat (placebo period: men 35.7 ± 1.24 energy%, women 31.8 ± 0.70 energy%, $P = 0.011$; calcium period: men 35.9 ± 0.85 energy%, women 31.9 ± 0.63 energy%, $P = 0.001$) and cholesterol (placebo period: men 367 ± 14.3 mg/day, women 255 ± 8.26 mg/day, $P < 0.001$; calcium period: men 366 ± 15.6 mg/day, women 262 ± 8.78 mg/day, $P < 0.001$) whereas the intake of carbohydrates (placebo period: men 48.3 ± 1.53 energy%, women 51.9 ± 0.84 energy%, $P = 0.053$; calcium period: men 48.1 ± 0.87 energy%, women 51.7 ± 0.62 energy%, $P = 0.002$) and fibre (placebo period: men 3.80 ± 0.07 g/MJ, women 4.08 ± 0.07 g/MJ, $P = 0.009$; calcium period: men 3.80 ± 0.09 g/MJ, women 3.99 ± 0.05 g/MJ, $P = 0.068$) was lower compared to women. Additionally, compared to women men ingested significantly more calcium (placebo period: men 1,409 ± 65.0 mg/day, women 991 ± 39.7 mg/day, $P < 0.001$) and phosphorus (placebo period: men 1,764 ± 47.8 mg/day, women 1,308 ± 40.3 mg/day, $P < 0.001$). Due to the calcium supplementation calcium (men 2,411 ± 61.7 mg/day, women 2,009 ± 44.5 mg/day, $P < 0.001$) and phosphorus intake (men 2,224 ± 55.6 mg/day, women 1,787 ± 40.9 mg/day, $P < 0.001$) rose significantly in both genders ($P < 0.001$).

General excretion parameters

The molar ratio of excreted calcium to phosphate in faeces which was 20.6:17.9 in the placebo period changed to 44.6:31.3 in the calcium period. The faecal pH value significantly increased from 6.48 ± 0.05 in the placebo period to 6.76 ± 0.06 ($P < 0.001$) in the calcium period; there was no difference between men and women (placebo period: men 6.44 ± 0.09, women 6.52 ± 0.06, $P = 0.487$; calcium period: men 6.71 ± 0.10, women 6.80 ± 0.08, $P = 0.521$).

Neutral sterols

Of the 31 healthy participants six participants were excluded because of an inverse NSt conversion profile (low converters) [6]. The sum of NSt in faecal water

Table 1 Concentrations of neutral sterols in faecal water of healthy adults under defined dietary conditions without (placebo) and with calcium supplementation (calcium)

µmol/l	Defined diet (placebo)		Defined diet + intervention (calcium)		*P* for intervention
	Mean ± SEM	*P* for gender	Mean ± SEM	*P* for gender	
Coprostanol					
Men	213 ± 82.4		156 ± 56.1		NS
Women	546 ± 103	0.018	271 ± 49.8	NS	0.021
All	373 ± 72.5		211 ± 38.7		0.004
Cholesterol					
Men	36.2 ± 13.6		24.9 ± 8.4		NS
Women	92.5 ± 40.1	NS	65.3 ± 22.9	NS	NS
All[a]	63.2 ± 20.9		44.3 ± 12.3		0.020
Coprostanone					
Men	8.05 ± 2.14		5.24 ± 1.40		0.096
Women	23.4 ± 4.65	0.009	14.9 ± 3.21	0.014	0.012
All	15.4 ± 2.9		9.90 ± 1.94		0.003
Cholestanol					
Men	6.67 ± 2.37		3.96 ± 0.94		NS
Women	13.4 ± 2.58	0.067	7.30 ± 1.49	0.066	0.007
All	9.89 ± 1.84		5.57 ± 0.91		0.001
Cholestanone					
Men	0.47 ± 0.08		0.46 ± 0.07		NS
Women	0.83 ± 0.15	0.040	0.69 ± 0.08	0.034	NS
All	0.65 ± 0.09		0.57 ± 0.06		NS
Cholestenone					
Men	2.12 ± 0.75		1.36 ± 0.24		NS
Women	2.61 ± 0.49	NS	1.87 ± 0.28	NS	0.030
All	2.35 ± 0.45		1.61 ± 0.19		0.038
Total NSt					
Men	267 ± 101		192 ± 65.5		NS
Women	679 ± 128	0.018	361 ± 69.8	0.091	0.018
All	465 ± 89.3		273 ± 49.9		0.003

Men $n = 13$, women $n = 12$, all $n = 25$
NSt neutral sterols
[a]Wilcoxon rank-sum test

drastically decreased during calcium supplementation; this decrease was due to decreased concentrations of all measured sterols except for cholestanone (Table 1). There was a significant inverse correlation between the sum of NSt in faecal water and the faecal pH ($r = -0.387$, $P < 0.01$, $n = 49$). Similar significant inverse correlations were also found between all single NSt and faecal pH. Under defined dietary conditions (defined diet) women had significantly higher concentrations of NSt in faecal water compared to men (Table 1). This was not due to single outliers; indeed, the NSt concentration in faecal water was below 250 µmol/l in ten out of 13 men but in only two out of 12 women. Soluble NSt concentrations above 400 µmol/l were found in two out of 13 men but in eight out of 12 women. On the contrary, in faecal dry matter (soluble plus insoluble NSt), the concentrations of coprostanol ($P = 0.991$), cholesterol ($P = 0.349$), coprostanone ($P = 0.245$) and cholestanol ($P = 0.670$) did not differ between men and women. Significantly higher concentrations of the minor compounds cholestanone ($P = 0.010$) and cholestenone ($P = 0.006$) were observed in women but these account for only about 3% of faecal NSt and, thus, can hardly explain the gender-specific differences in soluble NSt.

BA

Due to calcium supplementation there were significant increases in the concentrations of LCA, DCA and 12keto DCA in faeces (Table 2). In contrast, the concentration of soluble BA (BA in faecal water) was not affected by calcium supplementation with 119 ± 13.7 µmol/l in the placebo period and 121 ± 13.5 µmol/l in the calcium period ($P = 0.712$). iso-LCA concentration significantly decreased in the calcium period ($P = 0.015$, Table 3). The concentration of BA in faecal water showed no correlation with faecal pH ($r = 0.115$, $P = 0.376$, $n = 61$). The comparison between men and women revealed no differences except for a significantly higher LCA concentration in the faecal water of women (Table 3).

Table 2 Concentrations of bile acids in faeces of healthy adults under defined dietary conditions without (placebo) and with calcium supplementation (calcium)

µmol/g dry matter	Defined diet (placebo) Mean ± SEM	Defined diet + intervention (calcium) Mean ± SEM	*P* for intervention
iLCA	3.97 ± 0.34	4.18 ± 0.40	NS
LCA	4.72 ± 0.25	5.36 ± 0.31	0.008
iDCA	3.66 ± 0.45	3.69 ± 0.51	NS
DCA	8.25 ± 0.39	9.79 ± 0.50	0.000
CA[a]	0.87 ± 0.22	0.81 ± 0.12	NS
CDCA[a]	0.49 ± 0.06	0.54 ± 0.08	NS
12keto DCA	0.61 ± 0.07	0.79 ± 0.10	0.049

$n = 31$
iLCA iso-lithocholic acid, *LCA* lithocholic acid, *iDCA* iso-deoxycholic acid, *DCA* deoxycholic acid, *CA* cholic acid, *CDCA* chenodeoxycholic acid, *12keto DCA* 12-keto deoxycholic acid
[a]Wilcoxon rank-sum test

Genotoxicity

The amount of faecal water from one female participant was too small and could be used for NSt and BA analysis only. Therefore, genotoxicity was determined in 30 instead of 31 samples. There was no significant effect of calcium supplementation on measurements of genotoxicity (Table 4). However, the tail intensity correlated inversely with the intake of calcium ($r = -0.313$, $P = 0.015$, $n = 60$, Spearman). Genotoxicity of faecal water did not differ between men and women (Table 4).

Discussion

In the present study we could show that—under defined dietary conditions—faecal steroid excretion was significantly affected by the supplementation of calcium and there were gender-specific differences in NSt concentrations of faecal water.

The molar ratio of excreted calcium to phosphate in faeces changed from about 1:1 in the placebo period to 3:2 due to calcium supplementation. This is the precondition for the formation of amorphous calcium phosphate (ACP) [39], which binds and inactivates BA [13, 40]. The present study demonstrates that the increased BA excretion in faeces in consequence of calcium supplementation was due to increased contents of LCA, DCA and 12keto DCA which are all secondary BA. Significant increases in total faecal BA concentration due to calcium supplementation have previously been reported [12, 42]. However, these studies did not provide data for the effect of calcium supplementation on the faecal excretion of single BA. Van der Meer et al. [42] showed that the increased faecal BA and phosphate excretion due to calcium supplementation was completely associated with faecal calcium. Similarly, in the present study, faecal BA and calcium excretion were significantly associated ($r = 0.554$, $P < 0.001$, $n = 62$).

Despite the increased BA content in faeces, there were only marginal changes in BA concentration in faecal water; there was a significant 34% decrease in iso-LCA concentration. Similar results were also shown by Lapré et al. [20]. In contrast, another study found a significant 49% decrease in total BA concentration in faecal water despite a significant increase in total faecal BA excretion [12].

Table 3 Concentrations of bile acids in faecal water of healthy adults under defined dietary conditions without (placebo) and with calcium supplementation (calcium)

µmol/l	Defined diet (placebo)		Defined diet + intervention (calcium)		*P* for intervention
	Mean ± SEM	*P* for gender	Mean ± SEM	*P* for gender	
iLCA					
Men	4.52 ± 1.51		2.94 ± 0.79		0.086
Women	8.25 ± 2.09	NS	4.84 ± 0.93	NS	NS
All	6.45 ± 1.32		3.92 ± 0.63		0.015
LCA					
Men	8.00 ± 1.65		6.23 ± 0.86		NS
Women	14.6 ± 2.56	0.041	12.8 ± 2.61	0.029	NS
All[a]	11.4 ± 1.63		9.60 ± 1.51		NS
iDCA					
Men	19.3 ± 2.12		22.0 ± 3.38		NS
Women	23.1 ± 5.37	NS	21.1 ± 4.38	NS	NS
All	21.3 ± 2.93		21.5 ± 2.75		NS
DCA					
Men	37.7 ± 8.10		42.8 ± 8.16		0.019
Women	53.7 ± 11.0	NS	53.6 ± 9.72	NS	NS
All[a]	46.0 ± 6.95		48.4 ± 6.35		NS
CA					
Men	6.73 ± 1.28		5.82 ± 0.79		NS
Women	10.3 ± 1.72	NS[b]	11.7 ± 4.47	NS[b]	NS[a]
All[a]	8.57 ± 1.12		8.87 ± 2.36		NS
CDCA					
Men	4.31 ± 0.68		4.08 ± 1.10		NS[a]
Women	5.70 ± 1.59	NS[b]	5.71 ± 1.90	NS[b]	NS[a]
All[a]	5.03 ± 0.88		4.92 ± 1.11		NS
12keto DCA					
Men	20.4 ± 3.99		29.1 ± 5.53		0.010
Women	19.3 ± 4.94	NS	18.6 ± 3.42	NS	NS
All	19.8 ± 3.15		23.7 ± 3.30		NS
Total BA					
Men	101 ± 14.1		113 ± 15.0		0.085
Women	135 ± 22.8	NS	128 ± 22.5	NS	NS
All	119 ± 13.7		121 ± 13.5		NS

Men $n = 15$, women $n = 16$, all $n = 31$
iLCA iso-lithocholic acid, *LCA* lithocholic acid, *iDCA* iso-deoxycholic acid, *DCA* deoxycholic acid, *CA* cholic acid, *CDCA* chenodeoxycholic acid, *12keto DCA* 12-keto deoxycholic acid, *BA* bile acids
[a]Wilcoxon rank-sum test
[b]Mann–Whitney *U* test

Table 4 Genotoxicity of faecal water of healthy adults under defined dietary conditions without (placebo) and with calcium supplementation (calcium)

	Defined diet (placebo)		Defined diet + intervention (calcium)		*P* for intervention
	Mean ± SEM	*P* for gender	Mean ± SEM	*P* for gender	
Tail intensity					
Men	5.43 ± 0.62		4.97 ± 0.86		NS
Women	6.00 ± 0.80	NS	4.95 ± 0.32	NS	NS
All	5.71 ± 0.51		4.96 ± 0.45		NS
Tail moment					
Men	1.14 ± 0.15		1.09 ± 0.25		NS
Women	1.26 ± 0.18	NS	0.96 ± 0.08	NS	NS
All	1.20 ± 0.12		1.02 ± 0.13		NS
Tail length					
Men	29.7 ± 0.67		29.8 ± 1.08		NS
Women	28.6 ± 0.88	NS	27.5 ± 0.61	0.069	NS
All	29.2 ± 0.55		28.6 ± 0.65		NS

Men $n = 15$, women $n = 15$, all $n = 30$

Although there was no effect of calcium supplementation on total NSt concentration in faeces in the present study as reported previously [6], the analysis of NSt in faecal water revealed a drastic 41% decrease in the concentration of soluble NSt. This result points to an interaction between NSt and ACP in vivo in humans. For the interaction between ACP and BA, Govers et al. [13] supposed the hydrophobic aggregation of BA monomers on the ACP surface. Possible mechanisms of interaction between ACP and NSt may be the inclusion of NSt into hydrophobic aggregates of BA at the surface of ACP (direct co-precipitation) or a direct interaction between NSt and ACP. An indirect co-precipitation due to decreased solubility of BA as previously hypothesised [41] can be excluded because soluble NSt drastically decreased despite a constant BA concentration in faecal water. Thus, in vitro models are warranted to clarify the mechanisms of interaction between NSt and ACP.

In line with our results, similar decreases were observed in other studies with calcium supplementation [12, 20]. However, in these studies, total NSt were reported as sum of cholesterol and coprostanol; other cholesterol metabolites were not analysed. Govers et al. [12] hypothesised the precipitation of these hydrophobic surfactants by milk calcium in the intestinal lumen; the role of phosphate in this process was not quite clear. The precipitation of coprostanol and cholesterol was accompanied by a significant increase in faecal pH in the previous studies [12, 20] and in the present study as well. This might be explained by the phosphate moiety of the calcium phosphate complexes which increased the faecal buffer capacity [12]. The relation between faecal pH and colon cancer is still disputed. While some authors postulated that an acidic faecal pH is protective (summarised by Lupton et al. [21] and van Faassen et al. [43]) other authors provided contrary arguments [4, 38].

In the present study, the concentrations of cholesterol, coprostanol and further microbial cholesterol metabolites in faecal water significantly decreased. Weisburger et al. [47] claimed that - in contrast to BA - NSt do not promote colon cancer in man and rodents. These conclusions were based on cholesterol metabolites like cholesterol-α-epoxide and cholestane-3,5,6-triol. In human faeces, the major microbial cholesterol metabolite is coprostanol [23]. Through microbial conversion of cholesterol into coprostanol intermediates like coprostanone and 4-cholesten-3-one are formed [31]. Results of studies by Suzuki et al. [37] reveal 4-cholesten-3-one as a component of human faeces with potential (co-) carcinogenic activity. Against this background, the observed drastic decrease in the concentration of NSt in faecal water including 4-cholesten-3-one due to calcium supplementation can be assessed as beneficial.

The importance of calcium in the diet with respect to potential risk factors for colon cancer was shown by Glinghammar et al. [11]. After shifting from a dairy product-rich (high calcium) to a dairy product-free (low calcium) diet, significantly increased cytotoxicity but no effect on genotoxicity of faecal water was observed in 18 healthy adults. In the present study, the supplementation of calcium did also not affect genotoxicity of faecal water. This seems to be very likely due to the absence of an effect on BA concentration in faecal water. Reducing BA concentrations in faecal water would subsequently affect genotoxicity. Increased genotoxicity of the secondary BA LCA and DCA were shown in the comet assay at concentrations above 300 μM [46]. Such concentrations were not found in healthy

individuals [6, 9, 12, 20, 43, 44] but in single individuals with adenomatous polyps of the colon [36]. However, de Kok et al. [4] did not found differences in BA concentration in faecal water between individuals at high, medium and low risk (control subjects) of developing colorectal cancer. In consistence with previous findings [26] genotoxicity of faecal water did not correlate with BA concentration of faecal water (for tail intensity: $r = -0.110$, $P = 0.404$, $n = 60$). In addition, there was also no correlation between the concentrations of NSt in faecal water and the genotoxicity of faecal water (for total NSt in faecal water and tail intensity: $r = 0.204$, $P = 0.165$, $n = 48$; no correlations for single NSt, too). Diets high in fat and cholesterol have been shown to increase the genotoxicity of faecal water [32]. There were significantly higher intakes in energy, fat, protein and cholesterol in the dairy product-rich diet in the study by Glinghammar et al. [11]. However, in the present study, genotoxicity of faecal water did not differ between men and women despite different dietary intakes of energy, fat and cholesterol.

The present study indicates that the influence of NSt on genotoxicity seems to be negligible: neither the drastic decrease in soluble NSt due to calcium supplementation nor the obvious differences in NSt concentrations in faecal water between men and women (see below) are reflected in the genotoxicity of faecal water. Thus, there must be other compounds that contribute to faecal water genotoxicity. It has been shown that the addition of an enzyme (*Escherichia coli* endonuclease III) that specifically detects oxidised pyrimidines significantly increased DNA damage, which points - at least in part - to an oxidative mechanism that is responsible for the genotoxicity of faecal water [46]. The solubility of fecapentaene-12, a potent mutagen in human faeces, is facilitated by CA and DCA as well as mixtures of BA [5] and calcium has been shown to counteract this effect [1, 5].

The analysis of NSt concentrations in faecal water revealed distinct gender-specific differences. In contrast, NSt concentrations in faeces (insoluble plus soluble) did not differ between men and women. Since an increase in calcium intake resulted in a drastic decrease in soluble NSt, the significantly lower NSt concentrations in faecal water of men might be due to the higher calcium intake. A weak but significant correlation was found between calcium intake and total NSt in faecal water ($r = -0.337$, $P = 0.017$, $n = 50$, Spearman). Men ingested more fat and, thus, more fatty acids were precipitated by calcium in the intestine. This hydrophobic precipitate may trap more NSt resulting in lower concentrations of NSt in faecal water of men. But the gender-specific differences might also be caused by factors influencing the solubility of NSt. Correlation analysis showed a relation between faecal pH and the concentration of soluble NSt. However, the faecal pH did not differ between men and women in the present study. BA are known to facilitate the solubility of NSt [41]. The results of the present study show that there were neither significant differences in faecal (insoluble plus soluble) BA concentration nor in (soluble) BA concentration in faecal water between men and women despite for the monohydroxy BA LCA. Another reason might be the concentration of other steroids, e.g. sex hormones, in faeces and faecal water. Based on their chemical structure, sex hormones like testosterone and estradiol are better soluble in water than cholesterol and its metabolites. Serum concentrations of testosterone in men are by approximately ten times higher compared to serum concentrations of sex hormones in women [2, 7, 29]. Thus, it is conceivable, that the faecal water is more enriched with these sex steroids in men compared to women leaving the female faecal water to contain more cholesterol and cholesterol metabolites.

In conclusion, the present study showed distinct effects of calcium supplementation on faecal steroid excretion resulting in significantly higher faecal concentrations of the secondary BA LCA, DCA and 12keto DCA and in a significant decrease in the faecal water concentrations of cholesterol and nearly all its metabolites. It has to be remarked that the calcium intake in the placebo period was relatively high and the observed effects might be more conclusive or different if the calcium intake during placebo was below 1,000 mg. To the best of our knowledge, this is the first study that provided data for the concentrations of the cholesterol metabolites coprostanone, cholestanol, cholestanone and cholestenone in faecal water. Our study further revealed significantly higher concentrations of cholesterol and its metabolites in the faecal water of women. The results indicate that the concentrations of soluble cholesterol and cholesterol metabolites do not affect the genotoxicity of faecal water in healthy men and women. Nevertheless, based on the present knowledge, the observed decreases in soluble NSt due to calcium supplementation may rather be advantageous.

References

1. Block JB, Dietrich MF, Jawaid S, Gilgomez K, Morrison L, Rucker P (1987) Inhibition of fecapentaene-12 and carcinogen induced mutagenesis in the Ames test by carageenans and calcium. Proc Am Assoc Cancer Res 28:103
2. Burger HG (2002) Androgen production in women. Fertil Steril 77(Suppl 4):S3–S5
3. Chiang JYL (2003) Bile acid regulation of hepatic physiology: III. Bile acids and nuclear receptors. Am J Physiol Gastroint Liver Physiol 284:349–356
4. De Kok TMCM, van Faassen A, Glinghammer B, Pachen DMFA, Eng M, Rafter JJ, Baeten CGMI, Engels LGJB, Kleinjans JCS (1999) Bile acid concentrations, cytotoxicity, and pH of faecal water from patients with colorectal adenomas. Dig Dis Sci 44:2218–2225
5. De Kok TMCM, Vaniersel MLPS, Tenhoor F, Kleinjans JCS (1993) *In vitro* study on the effects of faecal composition on fecapentaene kinetics in the large bowel. Mutation Res 302:103–108
6. Ditscheid B, Keller S, Jahreis G (2005) Cholesterol metabolism is affected by calcium phosphate supplementation in humans. J Nutr 135:1678–1682
7. Dorgan JF, Fears TR, McMahon RP, Aronson Friedman L, Patterson BH, Greenhut SF (2002) Measurement of steroid sex hormones in serum: a comparison of radioimmunoassay and mass spectrometry. Steroids 67:151–158
8. dos Santos Silva I, Swerdlow AJ (1993) Sex differences in the risks of hormone-dependent cancers. Am J Epidemiol 138:10–28
9. Geltner Allinger U, Johansson GK, Gustafsson JÅ, Rafter J (1989) Shift from a mixed to a lactovegetarian diet: influence on acidic lipids in faecal water—a potential risk factor for colon cancer. Am J Clin Nutr 50:992–996
10. Glinghammar B, Inoue H, Rafter JJ (2002) Deoxycholic acid causes DNA damage in colonic cells with subsequent induction of caspases, COX-2 promoter activity and the transcription factors NF-kB and AP-1. Carcinogenesis 23:839–845
11. Glinghammar B, Venturi M, Rowland IR, Rafter JJ (1997) Shift from a dairy product-rich to a dairy product-free diet: influence on cytotoxicity and genotoxicity of faecal water–potential risk markers for colon cancer. Am J Clin Nutr 66:1277–1282
12. Govers MJAP, Termont DSML, Lapré JA, Kleibeuker JH, Vonk RJ, van der Meer R (1996) Calcium in milk products precipitates intestinal fatty acids and secondary bile acids and thus inhibits colonic cytotoxicity in humans. Cancer Res 56:3270–3275
13. Govers MJAP, Termont DSML, van Aken GA, van der Meer R (1994) Characterization of the adsorption of conjugated and unconjugated bile acids to insoluble, amorphous calcium phosphate. J Lipid Res 35:741–748
14. Hayashi E, Amuro Y, Endo T, Yamamoto H, Miyamoto M, Kishimoto S (1986) Fecal bile acids and neutral sterols in rats with spontaneous colon cancer. Int J Cancer 37:629–632
15. Hofmann AF (1999) The continuing importance of bile acids in liver and intestinal disease. Arch Intern Med 159:2647–2658
16. Hylla S, Gostner A, Dusel G, Anger H, Bartram HP, Christl SU, Kasper H, Scheppach W (1998) Effects of resistant starch on the colon in healthy volunteers: possible implications for cancer prevention. Am J Clin Nutr 67:136–142
17. Keller S, Jahreis G (2004) Determination of underivatised sterols and bile acid trimethyl silyl ether methyl esters by gas chromatography-mass spectrometry-single ion monitoring in faeces. J Chromatogr B 813:199–207
18. Korpela JT, Adlercreutz H, Turunen MJ (1988) Fecal free and conjugated bile acids and neutral sterols in vegetarians, omnivores, and patients with colorectal cancer. Scand J Gastroenterol 23:277–283
19. Lampe JW, Fredstrom SB, Slavin JL, Potter JD (1993) Sex-differences in colonic function—a randomized trial. Gut 34:531–536
20. Lapré JA, deVries HT, Termont DSML, Kleibeuker JH, deVries EGE, van der Meer R (1993) Mechanism of the protective effect of supplemental dietary calcium on cytolytic activity of faecal water. Cancer Res 53:248–253
21. Lupton JR, Steinbach G, Chang WC, O'Brien BC, Wiese S, Stoltzfus CL, Glober GA, Wargovich MJ, McPherson RS, Winn RJ (1996) Calcium supplementation modifies the relative amounts of bile acids in bile and affects key aspects of human colon physiology. J Nutr 126:1421–1428
22. McMichael AJ, Potter JD (1983) Do intrinsic sex differences in lower alimentary tract physiology influence the sex-specific risks of bowel cancer and other biliary and intestinal diseases? Am J Epidemiol 118:620–627
23. Midtvedt T, Lingaas E, Carlstedt-Duke B, Höverstad T, Midtvedt AC, Saxerholt H, Steinbakk M, Norin KE (1990) Intestinal microbial conversion of cholesterol to coprostanol in man. APMIS 98:839–844
24. Nagengast FM, Grubben MJAL, van Munster IP (1995) Role of bile acids in colorectal carcinogenesis. Eur J Cancer 31A:1067–1070
25. Nair PP (1988) Role of bile acids and neutral sterols in carcinogenesis. Am J Clin Nutr 48:768–774
26. Nordling MM, Glinghammar B, Karlsson PC, de Kok TMCM, Rafter JJ (2003) Effects of cell proliferation, activator protein-1 and genotoxicity by faecal water from patients with colorectal adenomas. Scand J Gastroenterol 38:549–555
27. Osswald K, Becker TW, Grimm M, Jahreis G, Pool-Zobel BL (2000) Inter- and intra-individual variation of faecal water–genotoxicity in human colon cells. Mutation Res 472:59–70
28. Powolny A, Xu J, Loo G (2001) Deoxycholate induces DNA damage and apoptosis in human colon epithelial cells expressing either mutant or wild-type p53. Int J Biochem Cell Biol 33:193–203
29. Price JF, Lee AJ, Fowkes FGR (1997) Steroid sex hormones and peripheral arterial disease in the Edinburgh Artery Study. Steroids 62:789–794
30. Rafter JJ, Child P, Anderson AM, Alder R, Eng V, Bruce WR (1987) Cellular toxicity of faecal water depends on diet. Am J Clin Nutr 45:559–563
31. Ren D, Li L, Schwabacher AW, Young JW, Beitz DC (1996) Mechanism of cholesterol reduction to coprostanol by *Eubacterium coprostanoligenes* ATCC 51222. Steroids 61:33–34
32. Rieger MA, Parlesak A, Pool-Zobel BL, Rechkemmer G, Bode C (1999) A diet high in fat and meat but low in dietary fibre increases the genotoxic potential of 'faecal water'. Carcinogenesis 20:2311–2316
33. Roberton AM (1993) Roles of endogenous substances and bacteria in colorectal cancer. Mutation Res 290:71–78
34. Roy P, Owen RW, Faivre J, Scheppach W, Saldanha MH, Beckly DE, Boutron MC (1999) Faecal neutral sterols and bile acids in patients with adenomas and large bowel cancer: an EPC case-control study. European cancer prevention. Eur J Cancer Prev 8:409–415
35. Stadler J, Stern HS, Yeung KS, McGuire V, Furrer R, Marcon N, Bruce WR (1988) Effect of high fat consumption on cell proliferation activity of colorectal mucosa and on soluble faecal bile acids. Gut 29:1326–1331
36. Stadler J, Yeung KS, Furrer R, Marcon N, Himal HS, Bruce WR (1988) Proliferative activity of rectal mucosa and soluble faecal bile acids in patients with

normal colons and in patients with colonic polyps or cancer. Cancer Lett 38:315–320
37. Suzuki K, Bruce RW, Baptista J, Furrer R, Vaughan DJ, Krepinsky JJ (1986) Characterization of cytotoxic steroids in human faeces and their putative role in the etiology of human colonic cancer. Cancer Lett 33:307–316
38. Ten Bruggencate SJM, Bovee-Oudenhoven IMJ, Lettink-Wissink MLG, Katan MB, Van der Meer R (2004) Dietary fructo-oligosaccharides and inulin decrease resistance of rats to salmonella: protective role of calcium. Gut 53:530–535
39. Termine JD, Posner AS (1970) Calcium phosphate formation in vitro. I. Factors affecting initial phase separation. Arch Biochem Biophys 140:307–317
40. Van der Meer R, de Vries HT (1985) Differential binding of glycine- and taurine-conjugated bile acids to insoluble calcium phosphate. Biochem J 229:265–268
41. Van der Meer R, Lapré JA, Govers MJAP, Kleibeuker JH (1997) Mechanisms of the intestinal effects of dietary fats and milk products on colon carcinogenesis. Cancer Lett 114:75–83
42. Van der Meer R, Welberg JWM, Kuipers F, Kleibeuker JH, Mulder NH, Termont DSML, Vonk RJ, de Vries HT, de Vries EGE (1990) Effect of supplemental dietary calcium on the intestinal association of calcium, phosphate and bile acids. Gastroenterol 99:1653–1659
43. Van Faassen A, Hazen MJ, van den Brandt PA, van den Bogaard AE, Hermus RJJ, Janknegt RA (1993) Bile acids and pH values in total faeces and in faecal water from habitually omnivorous and vegetarian subjects. Am J Clin Nutr 58:917–922
44. Van Gorkom BAP, van der Meer R, Boersma-van Ek W, Termont DSML, de Vries EGE, Kleibeuker JH (2002) Changes in bile acid composition and effect on cytolytic activity of faecal water by ursodeoxycholic acid administration: a placebo-controlled crossover intervention trial in healthy volunteers. Scand J Gastroenterol 37:965–971
45. Van Munster IP, Tangerman A, Nagengast FM (1994) Effect of resistant starch on colonic fermentation, bile acid metabolism, and mucosal proliferation. Dig Dis Sci 39:834–842
46. Venturi M, Hambly RJ, Glinghammar B, Rafter JJ, Rowland IR (1997) Genotoxic activity in human faecal water and the role of bile acids: a study using the alkaline comet assay. Carcinogenesis 18:2353–2359
47. Weisburger JH, Reddy BS, Barnes WS, Wynder EL (1983) Bile acids, but not neutral sterols, are tumor promoters in the colon in man and in rodents. Environ Health Persp 50:101–107
48. Weststrate JA, Ayesh R, Bauer-Plank C, Drewitt PN (1999) Safety evaluation of phytosterol esters. Part 4. Faecal concentrations of bile acids and neutral sterols in healthy normolipidaemic volunteers consuming a controlled diet either with or without a phytosterol ester-enriched margarine. Food Chem Toxicol 37:1063–1071