**Associations between usual food intake and fecal sterols and bile acids: results from the KORA FF4 study**

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**Running head:** Dietary intake, fecal sterols and bile acids

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**Abstract**

Animal sterols, plant sterols, and bile acids in stool samples have been suggested as biomarkers of dietary intake. However, it is still unknown whether they also reflect long-term habitual dietary intake and can be used in etiological research. In a subgroup of the KORA FF4 study, habitual dietary intake was estimated based on repeated 24-hour food lists and a food frequency questionnaire. Stool samples were collected according to a standard protocol and those meeting the quality criteria were extracted and analyzed by means of a metabolomics technique. The present study is based on data from 513 men and 495 women with a mean age of 60 and 58 years, respectively, for which fecal animal and plant sterols as well as bile acids concentrations and usual dietary intake data were available. In multi-variable adjusted regression models, the associations between food intake and log-normalized metabolite concentrations were analyzed. Bonferroni correction was used to account for multiple testing. In this population-based sample, strong associations between habitual dietary intake and fecal concentrations of animal sterols were identified, while the impact of the usual diet on bile acids was limited. A habitual diet high in “fruits” and “nuts and seeds” is associated with lower animal fecal sterols concentrations, whereas a diet high in “meat and meat products” is related to higher concentrations of animal sterols in feces. Further studies are necessary for evaluation of fecal animal sterols as biomarkers of diet. The findings especially need to be confirmed in other populations with diverse dietary habits.

**Introduction**

Several studies have analyzed individual compounds in fecal samples regarding their association with dietary intake and diseases (1-3). Fecal animal sterols (4), plant sterols (3; 4) and bile acids (2) have been suggested as biomarkers for dietary intake as they can be modified by changes in actual diet (2; 4-6). However, it is still unknown whether they also reflect long-term habitual dietary intake.

Exogenous or endogenous cholesterol is metabolized mainly in the liver, and cholesterol and primary bile acids are released via bile secretion into the gut, and undergo further metabolism by the intestinal microbiota to form coprostanol and secondary bile acids (**Figure 1**, adapted from Kaddurah et al. (7)). Cholic and chenodeoxycholic acids as the major primary bile acids are synthesized in the liver from cholesterol by side chain oxidation and hydroxylation, conjugated with taurine and/or glycine, and secreted into the small intestine, where 95% of them get reabsorbed (enterohepatic cycle). The other 5% reach the large intestine and undergo metabolic transformation by the gut microbiota, forming secondary bile acids, e.g., deoxycholic acid and lithocholic acid and further metabolites of these acids (see e.g., Gerard (8)).

Thus, cholesterol is excreted in feces either directly or in the form of bile acids. Ferezou et al. (6) described already in the late 1970s that 9.5% of fecal neutral sterols is cholesterol itself. Among the direct cholesterol metabolites produced in the gut, coprostanol (next to cholestanol and coprostanone) contributes 65% of fecal neutral sterols (6). From the 5% of bile acids that reaches the large intestine, metabolites formed by the activity of the gut microbiota are finally excreted in feces. A detailed description of the metabolic pathway of cholesterol and bile acids, its absorption and excretion in stool is given elsewhere Gerard (8).

Phytosterols, such as stigmasterol or ß-sitosterol, are naturally occurring compounds in plant foods and are similar to cholesterol in both structure and biologic function. They have an additional ethyl- or methyl group at the side chain(9), and in humans they are obtained only through dietary sources. Thus, dietary intake of plant sterols depends on food consumption habits and differs by population and sex (10). Vegetable oils are rich in phytosterols; however the serving size of oils is small as compared to the serving sizes of seeds and nuts, grain products, vegetables, and fruits (5). Campesterol and sitosterol are the most abundant phytosterols in the human diet, unlike their saturated counterparts, campestanol and sitostanol. About 95% of dietary phytosterols enter the colon (11) and undergo bacterial conversion to form coprostanol and coprostanone. Absorbed phytosterols from the intestine are excreted faster than cholesterol via biliary secretion, leading to a small pool size of phytosterols compared to animal sterols (12). According to Gylling et al. (13) the sum of plant sterols and its derivatives excreted in feces is proportional to dietary plant sterol intake. Furthermore, the amount of plant sterols consumed does influence the fecal sterol concentrations, since plant sterols enhance cholesterol excretion.(5)

In the present study, we investigate whether habitual dietary intake is associated with fecal concentrations of animal sterols, plant sterols and bile acids in participants of the observational, population-based KORA FF4 study. Such biomarkers of dietary intake would be very helpful in characterizing key features of a subject’s habitual diet as well as their effects on metabolism and health.

**Materials and Methods**

**Study population and design**

The KORA FF4 study (2013-2014) is the second follow-up of KORA S4 (1999-2001), a population-based health survey conducted in the region of Augsburg, Germany (14). Of the 4216 participants aged 25 to 74 years in KORA S4, 2279 individuals participated in the KORA FF4 survey. The KORA FF4 study was designed to determine changes in lifestyle habits and health status that developed over the follow-up period of, on average, fourteen years.

Study participants were invited to the study center to complete a face-to-face interview (including questions on medication), to undergo physical examinations and anthropometric measurements, and to collect bio-specimens.

From those 2279 participants in the KORA FF4 study, 1415 stool samples collected according to the SOP (e.g., samples were excluded if participants had been on antibiotics within one week before sample collection). Further, these stool samples were not exposed to room temperature longer than 3 hours overall and their metabolomics analysis was successfully conducted. The present analysis comprises a subset of 1008 participants (513 men and 495 women) of KORA FF4 for which in addition to the metabolomics data, estimates of habitual food intake were available. Further details on the collection, preprocessing and analysis of stool samples are described below.

Participants were encouraged to complete at least two web-based 24-hour food lists (24HFLs) and a web-based food frequency questionnaire (FFQ). However, paper-based questionnaires were available upon request. The closed 24HFL encompassed 246 food items used to assess which foods and drinks were consumed over the previous day. A detailed description of the 24HFL has been given elsewhere (15). The FFQ included 148 food items to record food consumption frequencies and amounts over the past 12 months. Information on sociodemographic variables and lifestyle factors was collected in an extensive, standardized, face-to-face interview at the study center. Furthermore, all participants underwent anthropometric measurements that included weight and height measurement. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Ethics Committee of the Bavarian Medical Association (Bayerische Landesärztekammer). Written informed consent was obtained from all participants.

**Stool sample collection, preprocessing and non-targeted metabolomics analysis**

Together with the appointment letter, participants were provided with an instruction and the necessary equipment to collect fecal samples on the day of their study center visit or the evening before. Patients were instructed that probes from different areas of the stool samples should be filled directly into two sterile plastic collection tubes. One of the tubes was prefilled with a stabilizing agent (for DNA or RNA analysis). The filled tubes should be packed in a sterile plastic bag and put into a box for storage in the refrigerator (4-8 °C). A stool collection questionnaire had to be filled out, providing information about the time of collection, description of the stool sample and problems experienced (if any). Participants handed over the stool box upon entry of the study center where the fecal samples were deep-frozen at -20°C immediately and later stored at -80°C until processing.

Frozen human stool samples in a weight range of 136 to 143 mg were weighed and were placed into 2 ml homogenization tubes containing ceramic beads with a diameter of 1.4 mm (Precellys Ceramic Kit 1.4 mm, 50x 2,0 ml tubes, Peqlab). Water with a ratio of 12.5µl/mg stool was added into the tubes. The samples were then homogenized in Precellys 24 homogenizer (PEQLAB Biotechnology GmbH, Germany) equipped with an integrated cooling unit for 3 times 20 s at 6,500 rpm, with 15 s intervals between the homogenization steps. After homogenization, 450 µl stool homogenate were transferred into 0.5 ml Eppendorf tube for stool dry mass determination, and 100 µl of the homogenate were pipetted onto a 2 ml 96- deep well plate for non-targeted metabolomics analysis.

In addition to samples from this study, a human reference plasma sample (Seralab, West Sussex, UK) and another reference of human stool (Seralab, West Sussex, UK) were pipetted into 1 and 6 wells of the 96- deep well plate, and were extracted as samples of the study. These samples served as technical replicates throughout the data set to assess process variability. In addition to those samples, 100 μL of water was extracted the same way and placed in 6 wells of 96-well plate to serve as process blanks.

Protein was precipitated and the metabolites in the stool homogenates were extracted with 475 µL methanol, containing 4 recovery standards to monitor the extraction efficiency. After centrifugation, the supernatant was split into 4 aliquots of 100 µL each onto two 96-well microplates. Two for analysis by 2 separate reverse phase (RP)/UPLC-MS/MS methods with positive ion mode electrospray ionization (ESI), 1 for analysis by (RP)/UPLC-MS/MS with negative ion mode ESI, and 1 for analysis by (HILIC)/UPLC-MS/MS with negative ion mode ESI. Sample extracts were dried on a TurboVap 96 (Zymark, Sotax, Lörrach, Germany). To minimize human error, liquid handling was performed on an automated MicroLab STAR® robot (Hamilton Bonaduz AG, Bonaduz, Switzerland).

All analytical methods utilized a Waters ACQUITY ultra-performance liquid chromatography (UPLC) and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution. Prior to the UPLC-MS/MS runs, the dried extract samples were reconstituted with 80 µL of solvents compatible with each of the 4 methods. Each reconstitution solvent contained a series of standards at fixed concentrations to ensure injection and chromatographic consistency. One aliquot was analyzed using acidic positive ion conditions, chromatographically optimized for more hydrophilic compounds. In this method, the extract was gradient eluted from a C18 column (Waters UPLC BEH C18-2.1 x 100 mm, 1.7 µm) using water and methanol, containing 0.05% perfluoropentanoic acid (PFPA) and 0.1% formic acid (FA). Another aliquot was also analyzed using acidic positive ion conditions; however, it was chromatographically optimized for more hydrophobic compounds. In this method, the extract was gradient eluted from the same afore mentioned C18 column using methanol, acetonitrile, water, 0.05% PFPA and 0.01% FA and was operated at an overall higher organic content. Another aliquot was analyzed using basic negative ion optimized conditions using a separate, dedicated C18 column. The basic extracts were gradient eluted from the column using methanol and water, but with 6.5 mM Ammonium Bicarbonate at a pH of 8. The fourth aliquot was analyzed via negative ionization following elution from a HILIC column (Waters UPLC BEH Amide 2.1x150 mm, 1.7 µm) using a gradient consisting of water and acetonitrile with 10 mM ammonium formate, pH 10.8. The MS analysis alternated between MS and data-dependent MSn scans using dynamic exclusion. The scan range varied slightly between methods but covered 70-1000 m/z.

Raw data was extracted, peak-identified and QC processed using Metabolon’s hardware and software (Metabolon, Inc., North Carolina, USA). Compounds were identified by comparison to library entries of purified standards or recurrent unknown entities based on 3 criteria: retention index within a narrow RI window of the proposed identification, accurate mass match to the library +/- 10 ppm, and the MS/MS forward and reverse scores between the experimental data and authentic standards. The MS/MS scores are based on a comparison of the ions present in the experimental spectrum to the ions present in the library spectrum. While there may be similarities between these molecules based on one of these factors, the use of all three data points can be utilized to distinguish and differentiate biochemicals.

Overall,1262 metabolites were measured in samples from 1415 participants, whereof 1140 metabolites were in the reference data set provided by Metabolon. For every metabolite in the reference data set, we computed the coefficient of variation (CV) of measurements by run day. The median CV over run days was used as a measure of the variability of the measurement process. A median CV greater than 0.25 was used as cut-off for reliable measurements for a given metabolite, leading to the exclusion of 248 metabolites (16). We also excluded a metabolite unless the CV could be computed for at least two run days. In particular, metabolites that had only missing values in the reference data were excluded. Overall, 85 metabolites were excluded due to missing CV values. Two samples were excluded as they were classified as outliers. An outlier is a metabolite-sample pair where the distance of the log10-transformed metabolite measurement from the mean of the metabolite is greater than 4 times the standard deviation of the metabolite.

Sample weight correction was achieved by dividing the measurement by the sample weight. No further technical adjustment was performed, as inspection of data showed consistent performance across all rundays.

From all finally available 807 metabolites, primary and secondary bile acids and sterols were selected for the analysis. The final preprocessed data set included 29 metabolites measured in 1413 participants. Missing values were imputed by the minimum (preprocessed) value per metabolite, as we assumed that they were not missing due to technical reason but rather being below the limit of detection. Based on the imputed data set, for primary bile acids, secondary bile acids, plant sterols and animal sterols the sum over all metabolite measurements of the respective group was derived as a further variable to be analyzed, generating four further metabolite variables. Five individual metabolites with more than 25% of missing values (cholate sulfate, 7-ketolithocholate, glycocholenate sulfate, taurodeoxycholate and ursodeoxycholate sulfate) were excluded from individual analysis (but included in the sum variables).

**Dietary intake**

From each participant up to three 24-hour food lists (24HFL) and a food frequency questionnaire (FFQ) were available to estimate usual intake by combining the results of these two instruments. This involved a two-step procedure, where the consumption probability and the amount of consumption on consumption days were estimated separately. Both models included the same covariates, thereby linking the two parts. Consumption amounts on a consumption day were estimated based on the data of the Bavarian Food Consumption Survey II. The usual intake was then derived as the product of the probability of consuming a certain food and the usual amount consumed on a consumption day. We restricted the analysis to the following 19 main food groups or subgroups: “potatoes”, “vegetables”, “fruits”, “nuts, seeds and nut spread “, “milk and dairy products”, “yoghurt”, “cheese (including cream cheese)”, “cereal and cereal products”, “meat and meat products”, “pork”, “processed meat”, “fish and shellfish”, “eggs and egg products”, “sugar”, “butter”, “vegetable oil”, “soy products”, “non-alcoholic drinks” and “alcoholic drinks”. These variables describing the usual intake were only available for a subsample of 1008 participants for which the stool samples have been analyzed. When including usual intake values in the regression models described below, each food item was scaled by its standard deviation. The total caloric intake [kcal/d] was derived from the usual intake of the participants, taking into account all available food groups and subgroups.

**Other covariables**

Smoking status was assessed as “ex-smoker”, “never smoker” and “current smoker”. Following the recommendations given by the German Nutrition Society, alcohol consumption was categorized in “no or low alcohol consumption” (<5 g/d for men and <2 g/d for women), “moderate alcohol consumption” (≥5 to <20 g/d for men and ≥2 to <10 g/d for women) and “heavy alcohol consumption” (≥20 g/d for men and ≥10 g/d for women) based on the usual alcohol intake per day, estimated from the food lists and FFQ as described before (17). Physical activity was assessed in four categories, describing high to no activity. In detail, these were “more than 2 h/week regularly”, “about 1 h/week regularly”, “and about 1h/week irregularly” and “almost no or no physical activity”, regarding physical activity during leisure time in summer and winter. The years of education reported were categorized as “up to 12 years of education” and “more than 12 years of education”. Use of lipid lowering medication and anti-diabetic medications were included. These were identified by ATC codes C10 and A10.

For the descriptive analysis, BMI was additionally categorized as underweight, normal, pre-obese or obese; hypertensive status (actual hypertensive or not) and diabetes status (type 2 diabetes or no type 2 diabetes) have also been included. Actual hypertension status was assessed by blood pressure measurements (systolic >=140 mmHg or diastolic >=90 mmHg) and/or use of antihypertensive medication, given that the subjects were aware of having hypertension. Diabetic participants include persons with known, validated diabetes as well as those who were newly detected by an oral glucose tolerance test (OGTT) (fasting plasma glucose level >=126 mg/dl or plasma glucose level 2h after intake of 75 g of glucose >= 200 mg/dl). For 31 participants the diabetes status was missing (OGTT information missing or no validation possible) and for 2 participants the hypertensive status was missing.

**Statistical analysis**

Variables included in the statistical analyses were age, sex, BMI, smoking status, alcohol consumption, physical activity, years of education, medication, total caloric intake and usual intake of selected food groups and subgroups. Age (years) and BMI (kg/m2) were included as continuous variables in the analyses.

The descriptive analysis provided information about percentage of missing values, median, and 25%- and 75%-quantiles of all metabolites and metabolite groups. Median and 25%- and 75%-quantiles or absolute numbers and percent of categories, whatever appropriate for the variables age, food consumption, total energy intake, BMI, smoking status, physical activity, alcohol consumption, years of education, diabetes and hypertension were given for all n=1008 participants that had metabolite measurements and dietary intake data available. All further analyses were performed with log-transformed metabolite data.

To select relevant variables to be included as adjustment variables in further regression analysis, we examined associations or differences in metabolite levels with the covariates in bivariate analyses. For continuous variables, correlations were examined; for categorical variables, t-tests or Kruskal-Wallis tests were performed.

In the main analysis, regression models were fitted for each of the 28 metabolites, investigating the effect of the usual intake of a certain food group, adjusted for age, sex, BMI, smoking status, alcohol consumption, physical activity, years of education, diabetes medication, and lipid-lowering medication. We report effect estimates and p-values. Bonferroni-adjustment of the p-values was done by dividing 5% by the number of tests conducted (28\*19=532, leading to 9.398496e-05).

All statistical analyses were performed using the statistical software R, version 3.3.2 (R Development Core Team, 2010, http://www.r-project.org)

**Results**

The present study was comprised of 513 men and 495 women with a mean age of 60 and 58 years, respectively. On average, men had a higher BMI and a higher energy intake as compared to women. Most of the participants were non-smokers at the time of assessment and physically active. Further baseline characteristics of the study participants are given in **Table 1**, stratified by sex.

**Table 2** lists descriptive data on dietary intake, given as food group and nutrient intake, and stratified by sex. As expected, mean intake of vegetables, fruit and dairy products was higher in women than in men. In contrast, men had higher consumption of cereals, meat and meat products, and alcoholic beverages.

Fecal metabolite concentrations after minimum imputation are described in **Table 3**. The five metabolites cholate sulfate, 7-ketolithocholate, glycocholenate-sulfate, taurodeoxycholate and ursodeoxycholate sulfate had more than 25 % of values below detection limits (non-detectable) and were excluded from further analysis.

**Tables 4** and **5** show the significant findings when analyzing the association of food group intake with the log-transformed concentrations of sterols and bile acids. In general, the food groups “fruits”, “nuts, seeds and nuts spread”, “milk and dairy products”, “cheese” and “yogurt” were inversely correlated with the fecal concentration of animal and plant sterols, while “potatoes”, “meat and meat products”, “pork”, “processed meat”, “eggs and egg products” and “butter” were positively correlated with animal sterol and plant sterols fecal concentrations. An exception arises with “cereals and cereal products” intake. This food group intake was inversely correlated with cholesterol fecal concentration, whereas positively associated with sitostanol fecal concentration. After Bonferroni correction, “fruit” intake was significantly inversely associated with the fecal concentration of campesterol, cholesterol, and the sum of animal sterols. With higher “nuts and seeds” consumption, fecal total cholesterol concentration significantly decreased. Furthermore, consumption of “meat and meat products” was significantly positively related to the fecal concentration of cholesterol.

Overall, the intake of “fruits”, “nuts and seeds”, “yogurt”, “cheese”, “butter”, and “soy products” was mainly inversely associated with selected fecal primary and secondary bile acid concentrations (**Table 5**). However, some bile acids were also positively associated with “fruits” and “yogurt” consumption (e.g., glycocholate, glycolithocholate sulfate). Additionally, glychochenodeoxycholate was positively associated with “fruit” intake. The dietary intake of “potatoes”, “vegetables”, “meat and meat products”, “pork”, “processed meat”, “eggs and egg products” and “vegetable oils” was positively related to fecal concentrations of bile acids. Among the associations examined in primary and secondary bile acids, only the secondary bile acid glycocholate showed significant positive association with fruit consumption after Bonferroni correction. No other primary and secondary bile acids were significantly associated with habitual food consumption after adjustment for multiple testing.

**Discussion**

In the present study, we examined associations between habitual dietary intake and fecal concentrations of sterols and bile acids measured by a metabolomics technique. After correction for multiple testing, we observed a statistically significant inverse association between “fruit” intake and fecal concentrations of campesterol, cholesterol and sum of animal sterols. A higher consumption of “nuts and seeds” was also associated with lower fecal concentrations of these metabolites. Additionally, a strong significant positive association between “meat and meat products” consumption and fecal cholesterol concentration was found. Concerning the association of bile acids and habitual dietary intake, only one result remained significant after Bonferroni correction: a high fruit consumption was associated with a high glycocholate concentration in feces. All other identified associations were no longer significant after Bonferroni correction.

Due to the high cholesterol content in foods of animal origin, especially in meat and meat products, the findings concerning higher animal sterols in stool when following a high meat diet is not surprising. The identified relationship is strong and thus the sum of animal sterols is a promising biomarker of dietary intake of “meat and meat products”.

Concerning bile acids, it is reported in the literature that consuming a high-fat, high-beef diet does not alter the activity of fecal bacterial enzymes, although fecal secondary bile acid excretion is increased (18). However, Hentges et al. (19) did not observe increasing concentrations of bile acids in feces of subjects following a high-meat diet. This is in line with our findings of a non-significant increase in bile acid excretion with relatively high meat consumption.

The explanation for the findings of an inverse association of “fruits” and “nuts and seeds” intake with animal sterols in feces is not as straight forward. At least in part, it may be explainable by the higher plant sterol intake and its physiologic consequences on cholesterol absorption and excretion, which shall be discussed in the following.

Plant sterols have a plasma cholesterol lowering property as described in numerous studies (3; 20). It is commonly accepted that this effect is mediated by reducing cholesterol absorption through competition of plant sterols with cholesterol for incorporation into micelles (3). Further, the intestinal absorption rate of cholesterol (40-60%) is much higher than of plant sterols (15% or less) (11; 21). Weststrate et al. (22) analyzed fecal concentrations of sterols and bile acids and found a significant increase in fecal neutral sterols after consumption of phytosterol-enriched margarine. Also Racette et al. (23) concluded that phytosterols act as bioactive compounds that lead to increased cholesterol excretion in feces. In an intervention study, 18 participants followed a low-phytosterol diet and received beverages supplemented with 0, 400, or 2000 mg phytosterols/day for 4 weeks each, with 1 week washout period. They reported that consuming dietary phytosterols in moderate or high doses could alter the cholesterol metabolism in human body. The cholesterol excreted was mainly from biliary cholesterol and a smaller proportion from dietary cholesterol. Another intervention study has also observed an increase in fecal excretion of cholesterol though the intake of a phytosterol-rich diet (24).

In the long-term, however, it is not clear whether a diet relatively high in plant sterols is associated with lower fecal animal sterols. Jaceldo-Siegl et al. (25) examined dietary intake and plasma concentration of plant sterols and cholesterol acrossfive different dietary patterns. Dietary phytosterols were highest in the diet of vegan subjects and lowest among non-vegetarians, whereas total cholesterol consumption was highest among non-vegetarians and lowest in vegans. However, the plasma concentrations of plant sterols and animal sterols did not vary across different diets. In addition, an alteration in the intestinal cholesterol absorption by consuming 0.7- 0.9 g/ plant sterols per day was observed (26). However, the daily intake of plant sterols following a regular diet ranges between 160-400 mg/day (25), which is lower than the required concentration to achieve a higher excretion rate of sterols in feces (23).

Our results provide novel information, since no other study has analyzed fecal concentrations of sterols in feces in a population-based study and related it to habitual dietary intake. However, our observations are not consistent with results from intervention studies or other previous projects dealing with the effect of phytosterol intake, as we reported an inverse correlation between fruits and nuts consumption and fecal animal sterols. In our study, on average 4.7 g/day of nuts and seeds were consumed, which is a very small amount, and likely too small for a phytosterol-based effect on cholesterol excretion.

Another aspect explaining the inverse association of fruit consumption and sterols in our study is that fruit intake contributes to the total dietary fiber intake. As high dietary fiber intake leads to increased fecal bulk, and this may result in lower fecal concentration of sterols and bile acids per gram of dry weight (27).

Also, the amount of vegetable oils - rich in phytosterols - consumed in our population sample (on average, 5.7 g/day) is likely not high enough to result in a significant effect on fecal sterols. However, intervention studies did observe an effect of replacing butter consumption with vegetable oils and found a significant increase in fecal excretion of sterols and bile acids (28). In another study, the fecal sterols concentration increased from 30 mg/g to about 50 mg/g dry weight after enriching margarine with 8.6g vegetable oils (22). However, we only observed a correlation between vegetable oils intake and fecal excretion of the secondary bile acid 7,12-diketolithocholate.

**Strengths and limitations**

To the best of our knowledge, the present study provides for the first time data on the association between habitual food intake and fecal concentration of animal sterols, phytosterols and bile acids in a cross-sectional study applying metabolomics techniques. Several studies measured animal and plant sterols in blood and fecal samples (23; 24) or plasma only (25). Some studies examined fecal samples only in (short- to medium-term) intervention studies (1; 22-24).

Our study is of observational nature and stool samples were collected only once per subject. To consider day-to-day variation, collecting fecal samples on 3-5 days from each participant was recommended by Setchell et al. (29). This would allow integrating not only day-to-day variation in food consumption, but also differences in stool transit time, gut microbiota activity, etc.

Unlike blood, stool samples usually cannot be collected in the study center; rather, it is collected at home and thus has to be stored until the study center visit. Although correct handling and storage was communicated to all participants, not all stool samples were stored cooled until handed over in the study center. In a pre-study, comparisons were made concerning metabolite concentrations in fresh samples and samples stored under different conditions. Due to the results of this study, samples with storage at room temperature of more than 3 hours were excluded from the analysis.

Furthermore, it is to mention that our study sample was limited to 1008 participants out of 2279 subjects, since all subjects without data on dietary intake and/or satisfying storage conditions of their stool samples were excluded.

Several sterol metabolites have previously been identified in human feces (30). Phytosterols may be metabolized into C21-bile acids in the liver and not to the common C24- bile acids in mammals (11). Since we have measured only the frequent C24- bile acids and not the C21-bile acids, we did not observe the total metabolite excretion of plant sterols in feces.

In our study, no extremely high amounts in the consumption of certain food groups were observed. Unlike the procedures in short-term intervention studies, we analyzed estimates of usual dietary intake of food groups and not a specific diet (high in a specific food) on the day before stool sampling. Plant sterols are derived only from diet, and if they are not consumed regularly in high amounts, it is unlikely to find high concentration of phytosterols in feces. Daily intake of plant sterols ranges from 160 to 400 mg in different populations (see Jaceldo-Siegl et al.(25)). However, to observe significant reduction of plasma LDL- cholesterol concentrations and to obtain cardiovascular health benefits, adults should consume 2 g/day (31), a dose not attainable by habitual diet without supplementation.

In conclusion, the results of this study conducted in participants from the general population indicate a strong effect of habitual diet on fecal concentrations of animal sterols, while the impact of diet on bile acids is limited. A diet high in “fruits” and “nuts and seeds” is associated with lower concentrations of animal sterols in feces. As expected, a diet high in “meat and meat products” leads to higher concentrations of animal sterols in feces. Further studies are necessary for evaluation of fecal animal sterols as biomarkers of diet. Our findings especially need to be confirmed in other populations with diverse dietary habits. Also, the question of possible health benefits or risks of a higher or lower fecal animal sterol content in response to dietary habits needs further discussion.

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**Conflict of interest**

None

**Authors’ contribution**

PM drafted the manuscript and interpreted the results, NW conducted the statistical analysis, SS, JK and AA prepared, analysed and preprocessed biosamples, JL and PM formulated the research question and designed the study; BT, CM, AP, WR, HG, MH and JL conducted research and/or provided essential materials*;* All authors read, critically commented on, and then approved the final manuscript.

**Figure 1.** Cholesterol metabolism pathway

Primary bile acids are produced in the liver by endogenous enzymes in the liver and metabolized into secondary bile acids by intestinal microbiota. Cholesterol is also metabolized to coprostanol by intestinal bacteria with a microbial steroid 5ß-reductase enzyme. (Adapted from Kaddurah-Daouk et al., 2011(7))

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| **Table 1.** Clinical andlifestyle characteristics of the study population, by sex. Values are n (%) or median (25%-quantile, 75%-quantile). N=1008, |
|   | Male | Female |
| **Sex, n (%)** | 513 | (50.9 %) | 495 | (49.1 %) |
| **Age, years** | 60 | (50, 70) | 58 | (48, 66) |
| **BMI, kg/m2** | 27.32 | (24.96, 30.41) | 26.24 | (23.49, 29.96) |
| **BMI\*, n (%)** |  |  |  |  |
|  underweight | 0 | (0 %) | 3 | (0.6 %) |
|  normal | 130 | (25.3 %) | 187 | (37.8 %) |
|  pre-obese | 242 | (47.2 %) | 181 | (36.6 %) |
|  obese | 141 | (37.5 %) | 124 | (25.0 %) |
| **Smoking status, n (%)** |  |  |  |  |
| Never  | 211 | (41.1 %) | 287 | (58.0 %) |
| Former  | 225 | (43.9 %) | 145 | (29.3 %) |
| Current  | 77 | (15.0 %) | 63 | (12.7 %) |
| **Physical activity\*\*, n (%)** |  |  |  |  |
| regular, >2h/week | 150 | (29.2 %) | 133 | (26.9 %) |
| regular, 1h/week | 149 | (29.0 %) | 189 | (38.2 %) |
| irregular, 1h/week | 72 | (14.0 %) | 67 | (13.5 %) |
| almost no/no physical activity | 142 | (27.7 %) | 106 | (21.4 %) |
| **Alcohol consumption\*\*\*, n (%)** |  |  |  |  |
| no to low consumption | 115 | (22.4 %) | 190 | (38.4 %) |
| moderate consumption | 207 | (40.4 %) | 258 | (52.1 %) |
| heavy consumption | 191 | (37.2 %) | 47 | (9.5 %) |
| **Years of education, n (%)** |  |  |  |  |
| ≤12 years | 297 | (57.9 %) | 328 | (66.3 %) |
| > 12 Years | 216 | (42.1 %) | 167 | (33.7 %) |
| **Type 2 diabetes, n (%)** |  |  |  |  |
| yes | 79 | (15.8 %) | 53 | (11.1 %) |
| no  | 420 | (84.2 %) | 425 | (88.9 %) |
| **Hypertension, n (%)** |  |  |  |  |
| yes | 231 | (45.1 %) | 162 | (32.8 %) |
| no | 281 | (54.9 %) | 332 | (67.2 %) |
|  |  |  |  |  |

\* underweight, BMI <18.5 kg/m2; normal-weight, BMI <25 kg/m²; pre-obese, BMI 25-29.99 kg/m²; obese, BMI ≥30 kg/m².

\*\* physical activity during leisure time in both seasons

\*\*\* no or low alcohol consumption, <5 g/d for men and <2 g/d for women; moderate alcohol consumption, ≥5 to <20 g/d for men and ≥2 to <10 g/d for women; heavy alcohol consumption ≥20 g/d for men and ≥10 g/d for women

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| **Table 2.** Dietary characteristics of the study population, by sex. Values are median (25%-quantile, 75%-quantile). N=1008 |
|   | **Male** | **Female** |
| **Total energy intake, kJ/d** | 8782.2 | (7849.2, 9790.6) | 6640 | (5974.8, 7539.6) |
| **Usual food consumption, g/d** |  |  |  |  |
| Potatoes | 60.7 | (50.0, 76.1) | 50.8 | (41.0, 64.0) |
| Vegetables | 148.7 | (121.3, 185.6) | 182.6 | (146.8, 224.4) |
| Fruits | 138.5 | (80.7, 205.3) | 147.5 | (100.2, 208.3) |
| Nuts, seeds, nut spread | 4.7 | (3.3, 9.3) | 3.8 | (2.5, 9.05) |
| Milk and dairy products | 149.6 | (99.2, 229.0) | 202.8 | (136.5, 274.7) |
| Yoghurt | 18.6 | (11.8, 54.0) | 39.3 | (17.9, 79.5) |
| Cheese (including cream cheese) | 27.9 | (19.3, 38.5) | 25.7 | (18.3, 36.8) |
| Cereals and cereal products | 187.8 | (162.6, 219.4) | 137.9 | (120.4, 163.6) |
| Meat and meat products | 140.2 | (116.9, 161.9) | 85.20 | (72.65, 99.75) |
| Pork | 21.5 | (17.7, 30.1) | 13.9 | (11.25, 19.05) |
| Processed meat | 60.1 | (43.7, 75.3) | 31.2 | (24.5, 40.45) |
| Fish & shellfish | 18.50 | (13.2, 26.9) | 15.0 | (11.1, 22.2) |
| Eggs & egg products | 14.80 | (10.7, 21.9) | 13.20 | (9.9, 17.7) |
| Sugar | 39.5 | (27.7, 50.7) | 33.6 | (24.6, 42.9) |
| Butter | 16.5 | (8.6, 21.9) | 12.5 | (7.15, 15.45) |
| Vegetable oil | 5.7 | (3.8, 8.5) | 5.2 | (3.45,7.65) |
| Soy products | 0.1 | (0, 0.1) | 0.1 | (0, 0.2) |
| Non-alcoholic drinks | 1501.0 | (1312.0, 180.0) | 1582.0 | (1416.0, 1783.0) |
| Alcoholic drinks | 283.6 | (100.7, 564.7) | 37.3 | (25.1, 82.7) |
| **Usual nutrient intake, g/d** |  |  |  |  |
| Protein | 76.59 | (69.46, 85.84) | 61.27 | (54.12, 68.31) |
| Carbohydrate | 216.2 | (189.1, 248.6) | 173.2 | (149.8, 201.9) |
| Ethanol | 13.53 | (5.53, 25.33) | 2.42 | (1.64, 5.28) |
| Fat | 86.55 | (77.15, 97.91) | 67.49 | (60.0, 74.91) |
| Saturated fatty acids | 38.95 | (34.24, 43.87) | 30.89 | (27.12, 34.45) |
| Unsaturated fatty acids | 30.73 | (27.65, 34.99) | 23.33 | (20.89, 26.23) |
| Polyunsaturated fatty acids | 10.98 | (9.58, 12.9) | 8.61 | (7.69, 10.09) |
| Omega 3 fatty acids | 1.63 | (1.42, 1.92) | 1.31 | (1.14, 1.52) |
| Omega 6 fatty acids | 9.29 | (8.02, 10.99) | 7.25 | (6.47, 8.50) |
| Cholesterol | 0.32 | (0.28, 0.37) | 0.25 | (0.22, 0.28) |

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| **Table 3.** Description of metabolite concentrations in fecal samples of the study participants (after imputation). N=1008 |  |
|  |  | **n imputed (%)**  | **Median** | **25%-quantile**  | **75%-quantile**  |
| Cholate | primary bile acids | 3% | 0.0469 | 0.0134 | 0.1899 |
| Cholate sulfate | 54% | 0.0009 | 0.0009 | 0.0495 |
| Glycochenodeoxycholate | 6% | 0.0479 | 0.0188 | 0.1215 |
| Glycocholate | 1% | 0.0525 | 0.0195 | 0.1490 |
| Sum of primary bile acids |   | 0.2141 | 0.0802 | 0.6966 |
| 12-dehydrocholate | secondary bile acids | 17% | 0.0320 | 0.0086 | 0.1532 |
| 3b-hydroxy-5-cholenoic acid | 12% | 0.0459 | 0.0215 | 0.0810 |
| 6-oxolithocholate | 15% | 0.0435 | 0.0175 | 0.0811 |
| 7,12-diketolithocholate | 22% | 0.0338 | 0.0070 | 0.0930 |
| 7-ketodeoxycholate | 12% | 0.0409 | 0.0127 | 0.1781 |
| 7-ketolithocholate | 55% | 0.0003 | 0.0003 | 0.0459 |
| Dehydrolithocholate | 1% | 0.0514 | 0.0241 | 0.0896 |
| Deoxycholate | 2% | 0.0504 | 0.0170 | 0.1067 |
| Glychocholenate sulfate | 31% | 0.0215 | 0.0016 | 0.0885 |
| Glycodoxycholate | 9% | 0.0426 | 0.0165 | 0.1123 |
| Glycolithocholate sulfate | 7% | 0.0446 | 0.0163 | 0.1324 |
| Glycoursodeoxycholate | 19% | 0.0385 | 0.0117 | 0.1026 |
| Hyocholate | 10% | 0.0454 | 0.0215 | 0.0920 |
| Isoursodeoxycholate | 1% | 0.0491 | 0.0218 | 0.1300 |
| Lithocholate | 1% | 0.0504 | 0.0293 | 0.0813 |
| Taurodeoxycholate | 33% | 0.0221 | 0.0013 | 0.0878 |
| Ursocholate | 1% | 0.0495 | 0.0247 | 0.1786 |
| Ursodeoxycholate | 2% | 0.0466 | 0.0205 | 0.1172 |
| Ursodeoxycholate sulfate | 45% | 0.0035 | 0.0002 | 0.0754 |
| Sum of secondary bile acids |   | 1.0923 | 0.6025 | 3.2327 |
| Beta-sitosterol | plant sterols | 1% | 0.0511 | 0.0306 | 0.1002 |
| Campesterol | 1% | 0.0500 | 0.0280 | 0.1006 |
| Ergosterol | 5% | 0.0508 | 0.0218 | 0.1189 |
| Sitostanol | 8% | 0.0490 | 0.0224 | 0.0742 |
| Stigmasterol | 4% | 0.0476 | 0.0308 | 0.0755 |
| Sum of plant sterols |   | 0.2989 | 0.1920 | 0.4786 |
| Cholesterol | animal sterols | 0% | 0.0522 | 0.0241 | 0.1384 |
| Coprostanol | 5% | 0.0492 | 0.0249 | 0.0823 |
| Sum of animal sterols |   | 0.1320 | 0.0828 | 0.2159 |

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| **Table 4.** Regression coefficient estimates, standard error of estimates and p-value modelling associations of fecal sterols with habitual dietary intake. Only associations with p-values <5% are reported †. N=1008 |
| **Food group [g/d]** | **Metabolite**  | **Estimate** | **Std. Error** | **p-value** | **Median of concentration** |
| Potatoes | Campesterol | 0.120 | 0.040 | 0.002892 | 0.0500 |
| Potatoes | Sum of plant sterols | 0.085 | 0.033 | 0.009016 | 0.2989 |
| Potatoes | Stigmasterol | 0.089 | 0.038 | 0.018518 | 0.0476 |
| Potatoes | Beta-sitosterol | 0.086 | 0.037 | 0.018881 | 0.0511 |
| Potatoes | Cholesterol | 0.108 | 0.048 | 0.023228 | 0.0522 |
| Fruits  | Sum of animal sterols | -0.131 | 0.028 | 0.000003\* | 0.1320 |
| Fruits  | Campesterol | -0.160 | 0.036 | 0.000007\* | 0.0500 |
| Fruits  | Cholesterol | -0.180 | 0.042 | 0.000021\* | 0.0522 |
| Fruits  | Sum of plant sterols | -0.111 | 0.029 | 0.000132 | 0.2989 |
| Fruits  | Stigmasterol | -0.121 | 0.034 | 0.000336 | 0.0476 |
| Fruits  | Ergosterol | -0.173 | 0.054 | 0.001509 | 0.0508 |
| Fruits  | Beta-sitosterol | -0.102 | 0.032 | 0.001618 | 0.0511 |
| Nuts, seed and nut spread | Sum of animal sterols | -0.112 | 0.026 | 0.000024\* | 0.1320 |
| Nuts, seed and nut spread | Ergosterol | -0.192 | 0.051 | 0.000179 | 0.0508 |
| Nuts, seed and nut spread | Sum of plant sterols | -0.090 | 0.027 | 0.000971 | 0.2989 |
| Nuts, seed and nut spread | Campesterol | -0.097 | 0.034 | 0.004115 | 0.0500 |
| Nuts, seed and nut spread | Cholesterol | -0.096 | 0.040 | 0.016798 | 0.0522 |
| Nuts, seed and nut spread | Coprostanol | -0.110 | 0.048 | 0.020992 | 0.0492 |
| Milk and dairy products | Sum of animal sterols | -0.065 | 0.028 | 0.021292 | 0.1320 |
| Milk and dairy products | Sum of plant sterols | -0.062 | 0.029 | 0.031410 | 0.2989 |
| Milk and dairy products | Ergosterol | -0.116 | 0.054 | 0.032991 | 0.0508 |
| Yoghurt  | Sum of animal sterols | -0.078 | 0.026 | 0.002554 | 0.1320 |
| Cheese  | Sum of animal sterols | -0.094 | 0.027 | 0.000509 | 0.1320 |
| Cheese  | Cholesterol | -0.129 | 0.040 | 0.001432 | 0.0522 |
| Cheese  | Stigmasterol | -0.088 | 0.032 | 0.006093 | 0.0476 |
| Cheese  | Beta-sitosterol | -0.079 | 0.031 | 0.010856 | 0.0511 |
| Cheese  | Campesterol | -0.079 | 0.034 | 0.020562 | 0.0500 |
| Cheese  | Sum of plant sterols | -0.055 | 0.028 | 0.045793 | 0.2989 |
| Cereals and cereal products | Cholesterol | -0.134 | 0.062 | 0.031550 | 0.0522 |
| Cereals and cereal products | Sitostanol | 0.161 | 0.078 | 0.037869 | 0.0490 |
| Meat and meat products | Cholesterol | 0.244 | 0.062 | 0.000081\* | 0.0522 |
| Meat and meat products | Campesterol | 0.189 | 0.052 | 0.000301 | 0.0500 |
| Meat and meat products | Sum of animal sterols | 0.136 | 0.041 | 0.000949 | 0.1320 |
| Meat and meat products | Beta-sitosterol | 0.122 | 0.047 | 0.010114 | 0.0511 |
| Meat and meat products | Sum of plant sterols | 0.100 | 0.042 | 0.018348 | 0.2989 |
| Pork  | Sum of animal sterols | 0.084 | 0.031 | 0.005991 | 0.1320 |
| Pork  | Cholesterol | 0.119 | 0.046 | 0.009913 | 0.0522 |
| Pork  | Campesterol | 0.082 | 0.039 | 0.035595 | 0.0500 |
| Pork  | Sum of plant sterols | 0.065 | 0.031 | 0.040136 | 0.2989 |
| Processed Meat  | Cholesterol | 0.173 | 0.052 | 0.001015 | 0.0522 |
| Processed Meat  | Sum of animal sterols | 0.102 | 0.035 | 0.003689 | 0.1320 |
| Processed Meat  | Campesterol | 0.125 | 0.044 | 0.004922 | 0.0500 |
| Eggs and Egg products | Sum of animal sterols | 0.079 | 0.026 | 0.002129 | 0.1320 |
| Eggs and Egg products | Cholesterol | 0.091 | 0.039 | 0.018491 | 0.0522 |
| Butter  | Ergosterol | 0.147 | 0.054 | 0.006293 | 0.0508 |
| Butter  | Sum of animal sterols | 0.061 | 0.028 | 0.029641 | 0.1320 |

\* P-values indicating significant associations after Bonferroni correction (p< 9.398496e-05).

†Models were adjusted for age, sex, BMI, smoking status, alcohol consumption, physical activity, years of education, use of lipid-lowering and antidiabetic medication and total caloric intake

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| **Table 5.** Regression coefficient estimates, standard error of estimates and p-value modelling associations of fecal bile acids with habitual dietary intake. Only associations with p-values <5% are reported.† (N=1008) |
| **Food group [g/d]** | **Metabolite**  | **Estimate** | **Std. Error** | **p-value** | **Median of concentration** |
| Potatoes | Deoxycholate | 0.143 | 0.058 | 0.014425 | 0.0504 |
| Potatoes | Lithocholate | 0.082 | 0.037 | 0.024920 | 0.0504 |
| Vegetables | 12-dehydrocholate | 0.223 | 0.091 | 0.014203 | 0.0320 |
| Fruits  | Glycocholate | 0.237 | 0.059 | 0.000058\* | 0.0525 |
| Fruits  | Hyocholate | -0.199 | 0.055 | 0.000326 | 0.0454 |
| Fruits  | 7,12-diketolithocholate | -0.189 | 0.071 | 0.007827 | 0.0338 |
| Fruits  | Ursocholate | -0.153 | 0.061 | 0.012232 | 0.0495 |
| Fruits  | Glycolithocholate sulfate | 0.152 | 0.061 | 0.013571 | 0.0446 |
| Fruits  | Isoursodeoxycholate | -0.123 | 0.051 | 0.016015 | 0.0491 |
| Fruits  | Glycochenodeoxycholate | 0.129 | 0.055 | 0.018184 | 0.0479 |
| Fruits  | Deoxycholate | -0.117 | 0.052 | 0.024726 | 0.0504 |
| Fruits  | Cholate sulfate | -0.180 | 0.084 | 0.032303 | 0.0009 |
| Nuts, seed and nut spread | Hyocholate | -0.144 | 0.052 | 0.005792 | 0.0454 |
| Nuts, seed and nut spread | Lithocholate | -0.080 | 0.031 | 0.008960 | 0.0504 |
| Nuts, seed and nut spread | Dehydrolithocholate | -0.093 | 0.040 | 0.021496 | 0.0514 |
| Nuts, seed and nut spread | Ursodeoxycholate | -0.108 | 0.050 | 0.032633 | 0.0466 |
| Milk and dairy products | Glycocholate | 0.156 | 0.059 | 0.008093 | 0.0525 |
| Milk and dairy products | Dehydrolithocholate | 0.093 | 0.043 | 0.031300 | 0.0514 |
| Yoghurt  | Glycocholate | 0.154 | 0.054 | 0.004511 | 0.0525 |
| Yoghurt  | Ursocholate | -0.116 | 0.056 | 0.038704 | 0.0495 |
| Yoghurt  | Isoursodeoxycholate | -0.097 | 0.047 | 0.038749 | 0.0491 |
| Yoghurt  | Glycolithocholate sulfate | 0.116 | 0.056 | 0.040044 | 0.0446 |
| Cheese | Lithocholate | -0.080 | 0.031 | 0.010226 | 0.0504 |
| Cheese | Deoxycholate | -0.103 | 0.050 | 0.038898 | 0.0504 |
| Cheese | Ursodeoxycholate | -0.105 | 0.051 | 0.041136 | 0.0466 |
| Meat and meat products | Hyocholate | 0.162 | 0.081 | 0.044747 | 0.0454 |
| Meat and meat products | Ursocholate | 0.177 | 0.089 | 0.047247 | 0.0495 |
| Pork | 6-oxolithocholate | 0.193 | 0.059 | 0.001214 | 0.0435 |
| Pork | Lithocholate | 0.109 | 0.035 | 0.002009 | 0.0504 |
| Processed meat  | Hyocholate | 0.137 | 0.069 | 0.046860 | 0.0454 |
| Eggs and Egg products | Deoxycholate | 0.164 | 0.047 | 0.000531 | 0.0504 |
| Eggs and Egg products | Lithocholate | 0.087 | 0.030 | 0.003280 | 0.0504 |
| Eggs and Egg products | Ursodeoxycholate | 0.139 | 0.049 | 0.004283 | 0.0466 |
| Eggs and Egg products | Isoursodeoxycholate | 0.106 | 0.046 | 0.022321 | 0.0491 |
| Eggs and Egg products | Hyocholate | 0.105 | 0.050 | 0.037117 | 0.0454 |
| Butter  | Glycolithocholate sulfate | -0.179 | 0.061 | 0.003202 | 0.0446 |
| Vegetable oil | 7,12-diketolithocholate | 0.149 | 0.066 | 0.023479 | 0.0338 |
| Soy products | Cholate | -0.170 | 0.071 | 0.016418 | 0.0469 |

\*P-values indicating significant associations after Bonferroni correction (p< 9.398496e-05).

†Models were adjusted for age, sex, BMI, smoking status, alcohol consumption, physical activity, years of education, medication use and total caloric intake

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