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New biomarkers of habitual dietary intake in observational studies

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Summary

The role of dietary intake in understanding the relationship between diet, health and disease has become crucial over the past several years. The valid estimation of habitual dietary intake in nutritional epidemiology is still challenging to this day. Identifying new habitual dietary biomarkers based on "omics" data in observational studies is a relatively new concept, which could improve the characterization of nutritional status in study participants and offer a deeper understanding of the effects of food components and diet on the human body, independent of dietary assessment measurement errors. The aim of this dissertation is to emphasize the need to identify new habitual dietary biomarkers in different biosamples in observational studies and to highlight the emerging field of nutritional metabolomics and relevant analytical methods.

This dissertation comprises three manuscripts. As there are several sampling techniques used in identifying dietary biomarkers, the first manuscript gives an overview and suggestions for using alternative sampling techniques to improve the quality of nutritional research in large epidemiological studies. In the second manuscript, associations between habitual meat consumption and plasma concentrations of anserine, carnosine, pi-methylhistidine (Π-MH), tau-methylhistidine (T-MH) and the ratio of tau-methylhistidine to pi-methylhistidine are identified in a cross-sectional study (BVS II). Principally, red meat intake was associated with carnosine, whereas poultry and chicken consumption were related to Π-MH. Plasma anserine concentrations significantly increased with higher processed meat and turkey intake.
The associations in the third manuscript between usual food intake and fecal sterols and bile acids are assessed and analyzed by means of a metabolomics technique in the KORA FF4 study. It has revealed an effect of habitual diet on fecal concentrations of animal sterols but the influence of diet on bile acids was limited in this population study.

In conclusion, this dissertation provides results with novel information as it has shown the association between usual dietary meat intake and plasma concentration of a cross-sectional study and has analyzed fecal concentrations of sterols in feces in a population-based study and related it to habitual dietary intake. However, it may be also important to further investigate the use of combinations of biomarkers of meat intake to specify particular types of meat

consumed, since plasma anserine, carnosine, and Π-MH seem to be markers for overall meat intake rather than specific markers of a certain type of meat. Additional future intervention studies are needed to reveal if the metabolites found in plasma are characteristic for a specific type of meat or not. Further studies are essential for assessing fecal animal sterol levels as biomarkers of diet and to confirm the findings in other populations with different dietary habits. Finally, the findings of this dissertation in combination with findings from the previous literature (and future studies) on associations between dietary intake and dietary biomarkers should be used in identifying dietary patterns (or food items, etc.) related to chronic diseases over the long term in the general population.

Zusammenfassung

In den letzten Jahren ist die Rolle der Ernährung im Verständnis der Beziehung zwischen Ernährung, Gesundheit und Erkrankungen immer wichtiger geworden. Eine valide Schätzung des gewöhnlichen Verzehrs in der Ernährungsepidemiologie ist noch immer eine Herausforderung. Im Rahmen von Beobachtungsstudien ist die Identifizierung neuer Biomarker, die gewöhnlichen Verzehr beschreiben und auf "omics" Daten basieren ein relativ neues Konzept. Dieses könnte die Charakterisierung des Ernährungsstatus der Studienteilnehmer verbessern und gleichzeitig ein tieferes Verständnis der Einflüsse von Ernährungsbestandteilen und Ernährungsgewohnheiten auf den Menschen ermöglichen, unabhängig von Messfehlern bei der Erhebung der Nahrungsmittelzufuhr. Das Ziel dieser Dissertation ist, die Notwendigkeit der Identifizierung solcher Biomarker des gewöhnlichen Verzehrs in verschiedenen Bioproben aus Beobachtungsstudien zu unterstreichen und dieses aufstrebende Gebiet der Ernährungs-Metabolomics und deren analytische Methoden hervorzuheben.

Die Dissertation umfasst drei Manuskripte. Das erste Manuskript gibt einen Überblick zur Identifizierung von Biomarkern der Ernährung in verschiedenen Bioproben sowie Vorschläge für alternative Bioproben, die die Qualität der Ernährungserhebung in großen epidemiologischen Studien verbessern können. Das zweite Manuskript untersucht die Assoziation von üblichem Fleischverzehr und den Plasmakonzentrationen von Anserine, Carnosine, Pi-Methylhistidine (Π-MH), Tau-Methylhistidine (T-MH) und dem Quotienten von Tau-Methylhistidine zu Pi-Methylhistidine in einer Querschnittsstudie (BVS II). Der übliche Verzehr von rotem Fleisch war mit der Carnosinekonzentration assoziiert, wohingegen der übliche Geflügel- und Hähnchenverzehr mit Π-MH assoziiert war. Die Konzentration von Anserine im Plasma stiegen mit vermehrter gewöhnlicher Aufnahmemenge von verarbeitetem Fleisch und Truthahn signifikant an. Im dritten Manuskript werden Assoziationen zwischen dem gewöhnlichen Verzehr und Metaboliten (genauer: Sterolen und Gallensäuren) im Stuhl von Probanden der KORA FF4 Studie untersucht. Es zeigte sich ein Einfluss des üblichen Verzehrs auf die Metabolit-Konzentrationen der tierischen Sterole im Stuhl, wohingegen der

Einfluss auf die Metabolite der Gallensäuren in dieser populationsbasierten Studie begrenzt war.

Zusammengefasst kann man festhalten, dass diese Dissertation im Ergebnis neue Erkenntnisse liefert, da sie zum einen die Assoziation zwischen gewöhnlichem Fleischverzehr und der Biomarker-Konzentration im Plasma in einer Querschnittsstudie und zum anderen die Sterolkonzentrationen im Stuhl in einer populationsbasierten Studie analysiert und jeweils mit dem gewöhnlichen Verzehr in Zusammenhang gebracht hat. Trotzdem ist es von Interesse, auch eine Kombination verschiedener Biomarker für Fleischverzehr zu untersuchen, um zu identifizieren, welche Art von Fleisch verzehrt wurde. Anserine, Carnosine, und Π-MH im Plasma stellen generell Biomarker für Fleischkonsum dar und erlauben keine Rückschlüsse auf die Art des verzehrten Fleisches. Außerdem sind zukünftig Interventionsstudien nötig, die aufdecken könnten, ob bestimmte Metabolite die im Plasma nachgewiesen wurden für den Verzehr von bestimmten Fleischarten charakteristisch sind. Weitere Studien müssten zudem durchgeführt werden, um die Eignung von tierischen Sterolen im Stuhl als Biomarker für üblichen Verzehr zu untersuchen und um die gefundenen Resultate in anderen Bevölkerungsgruppen mit unterschiedlichen Ernährungsgewohnheiten zu bestätigen. Schließlich sollten die Ergebnisse dieser Dissertation und Resultate aus der Literatur und auch zukünftige Studien zur Assoziation von Ernährungszufuhr und Biomarkern der Ernährung als Grundlage dienen um Ernährungsmuster zu identifizieren, die über einen langen Zeitraum mit chronischen Erkrankungen in einer allgemeinen Bevölkerung assoziiert sind.

Abbreviations

ATC	Anatomical therapeutic chemical classification system
24h-DR	24 hours dietary recall
24HFL	24-hour food list
BMI	Body mass index
BVS II	Second Bavarian Food Consumption Survey
CV	Coefficient of variation
CVD	Cardiovascular disease
DBS	Dried blood spots
DifE	German Institute of Human Nutrition Potsdam-Rehbruecke (Deutsches Institut für Ernährungsforschung Potsdam-Rehbrücke)
EDTA	Ethylenediamine tetra-acetic acid
EFPQ	European Food Propensity Questionnaire
EPIC	European Prospective Investigation into Cancer and Nutrition
FFQ	Food frequency questionnaire
GC/MS	Gas chromatography-mass spectrometry
HESI-II	Heated electrospray ionization
KORA	Cooperative health research in the region of Augsburg
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography-mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantitation
MONICA	Multinational Monitoring of Trends and Determinants in Cardiovascular disease
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry

MSM	Multiple Source Method
NCI	National Cancer Institute
NMR	Nuclear magnetic resonance
OGTT	Oral glucose tolerance test
Π-MH	Pi-methylhistidine
RI	Retention index
SOP	Standard operating procedure
T-MH	Tau-methylhistidine
UPLC	Ultra-performance liquid chromatography
WHO	World Health Organizations

Tables and Figures

Tables

Figures

1. Introduction

Assessment methods of habitual dietary intake

The role of dietary intake has been investigated over several years in the context of understanding the relationship between diet, health and disease [1]. To this day, valid estimation of habitual dietary intake in nutritional epidemiology remains a challenge [2].

Each dietary assessment tool, even the most appropriate ones, has its own limitation in assessing dietary intake at the individual level in large cohort studies. Although dietary questionnaires such as food frequency questionnaires (FFQs) and 24 hours dietary recalls (24h-DRs) have been utilized to collect food and nutrient intake data, they are very subjective and liable to different biases, affecting the accurate estimation of dietary intake [3]. These methods rely on memorizing or monitoring dietary intake from the participants leading to under- and over-reporting of certain foods [4]. To increase precision and validity of the currently available dietary assessment methods, a blended dietary assessment approach has been developed that combines the use of two different assessment instruments in one study. Combining the two methods improves the accuracy of the nutritional assessment [2,5,6]. Finding biomarkers that are independent of dietary assessment measurement errors would be advantageous in getting and assessing further information on dietary intake and nutritional status [7]. Dietary intake biomarkers may afford a more proximal measure of nutrient status for disease outcomes and for population-wide nutritional status measures [8]. Furthermore, they may overcome the problem of intra-individual diet variability [9]. However, it must be noted that biomarkers are not a gold standard to validate dietary questionnaires. They are more useful in comparing dietary assessment methods against each other [8]. On the contrary, questionnaires for dietary intake assessment are essential for biomarker evaluation, particularly when dietary supplements are administered, which can lead to an alteration in the correlation between dietary intake and a biological biomarker of a nutrient.

Overall, identification of novel biomarkers is essential to improve nutritional science as suggested in previous reviews of this area [10,11].

Definition of dietary biomarkers

There are several different definitions for the term "biomarker" in literature. One of the WHO definitions is "any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of an outcome or disease" [12]. Since this dissertation covers biomarkers of dietary intake or health effects, the most appropriate definition would be a "chemical or biological test results in an analyzed biological material related to a certain exposure, susceptibility, or biological effect" [13].

Additionally, there are several different classifications for dietary biomarkers. They can be categorized into short-term, medium-term and long-term markers, where short-term biomarkers indicate intake over past hours or days, and medium-term represent intake over weeks or months. Long-term biomarkers observed in epidemiologic studies are focused on the intake of nutrients over months or years to assess habitual dietary intake [7,8].

Jenab et al. [14] divided biomarkers into recovery (exposure), predictive, concentration and replacement biomarkers; these are described in more detail elsewhere [15]. However, Biesalski et al. [13] focused only on their use and categorized them into exposure, effect and susceptibility biomarkers. An exposure biomarker is an intake biomarker that indicates the level of exposure to a diet or food component, while an effect biomarker indicates the human body's functional response to an exposure. A susceptibility biomarker is a measurable indicator such as genetic or acquired host factors that affects the response of an individual to a dietary exposure [11]. Nevertheless, a biomarker can be classified into several different classifications contingent upon the aim of use.

An ideal biomarker should assess nutritional intake more accurately than self-reported methods by being more specific and sensitive for the food it is supposed to monitor. It should validate and evaluate self-reported intake of food items [8]. Additionally, it should not be altered by diseases, by metabolic conditions or lifestyle, or other genetic or environmental factors. It should also be able to detect dietary intake alterations over time. Moreover, it should be inexpensive, non-invasive and applicable across different populations [7,16].

Methods to identify dietary biomarkers

Dietary biomarkers can be discovered by hypothesis-driven or data-driven approaches. In the past, most dietary biomarkers have been identified by hypothesis-driven methods, but today numerous new biomarkers of diet-related health effects are being explored by rapid data-driven approaches in the context of the development of "omics" technologies [10]. Metabolomics offers opportunities in the identification of the relationship between human nutrition and health status. Identifying dietary intake patterns by the use of metabolomics to find molecules that alter between different diets may lead to determining potential diet-disease risk biomarkers [17] and food-specific novel biomarkers [7,18]. Furthermore, identification of new dietary biomarkers of food-specific intake will correspond to the new trend of personalized nutrition and will help in distinguishing individuals and populations according to their different diets, lifestyles and environments [10,11].

Limitations of biomarkers

It should be noted that biomarkers represent many more biological processes than just dietary intake. Unlike dietary questionnaires, biomarker concentrations are altered by many physiological, genetic and environmental factors like digestion, absorption, metabolism, and elimination as well as by physical activity, microbiota composition, drug use and diseases [7].

Furthermore, food is a complex mixture and the absorption and metabolism of each single nutrient may depend on other food components, which makes it difficult to assess the nutrient-metabolism interactions by dietary assessment methods [15].

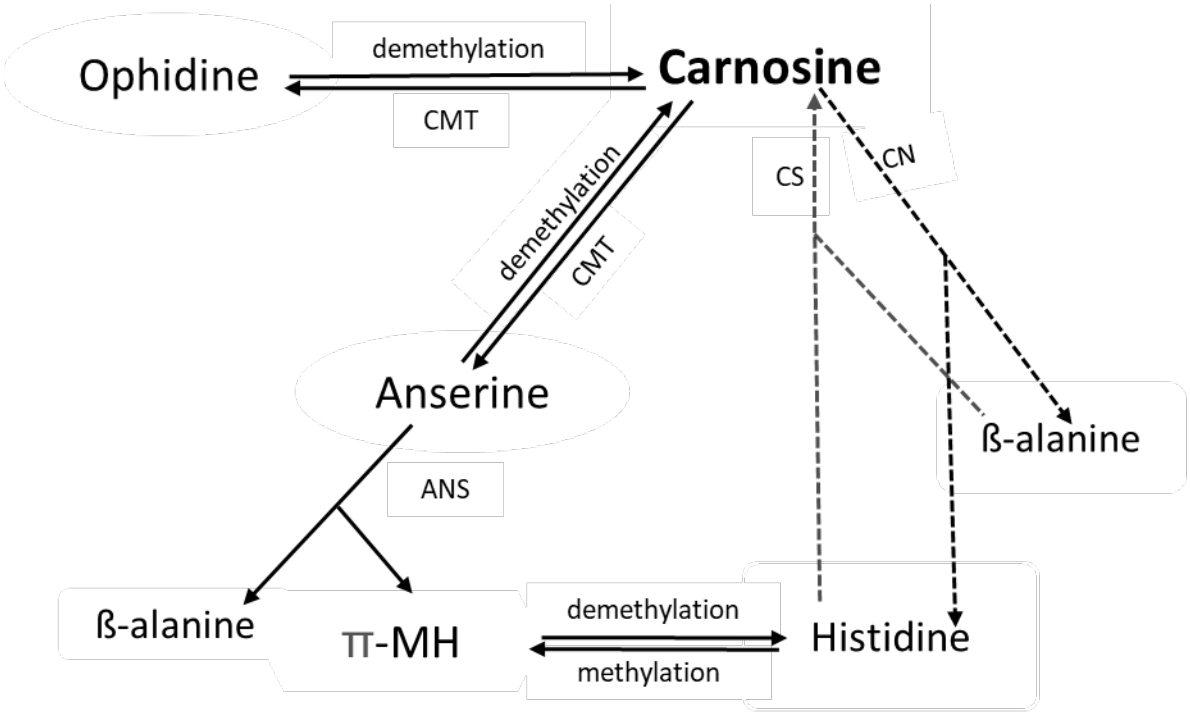
Identifying nutritional status by biomarkers is also affected by the method of bio-specimen collection, storage, laboratory variability and the study design in general [19]. There are only limited types of noninvasive, easily obtainable biospecimens for dietary intake assessment [11].

This dissertation addresses dietary biomarkers of habitual meat intake in plasma samples and the associations between habitual dietary intake and fecal animal and plant sterols, and bile acids.

Biomarkers of meat intake

Meat intake offers a good source of protein in omnivorous populations and has a possible influence on an individual's nutritional and health status. Defining total protein intake or categorizing individuals according to their meat intake is one of the main interests of epidemiological studies. The WHO has provided recommendation for red meat and processed meat intake, as it has been widely proposed in many studies that processed meat consumption increases the risk of colorectal cancer and probably other cancers [20]. Yet significant evidence is still needed to directly link the type of meat consumed to colorectal cancer and other chronic diseases such as diabetes, cardiovascular diseases and total mortality [21-23]. As dietary questionnaires are imprecise and subjective to measurement errors as mentioned above, finding valid and reliable biomarkers of different types of meat intake in biosamples would be helpful in supporting and evaluating existing dietary intake data and dietary recommendations. Biomarkers can be utilized to classify an individual's dietary consumption to improve the assessment of the relationship between diet and chronic disease.

Carnosine [24], anserine [24], pi-methylhistidine (Π-MH) [12], tau-methylhistidine (T-MH) [12], urea [24], creatine [24], creatinine [24], carnitine [24], ophidine [24]and sulphate [24] have been proposed as biomarkers of meat intake in different studies and various biospecimens [24]. They are found in different concentrations in all types of meat [24]; however, some of these biomarkers are catabolites of meat intake that resemble human catabolites of heme and protein. In this case, an ideal meat intake biomarker should specify the mean intake over a longer period of time and should also classify individuals in line with the type and amount of meat consumed in large, population-based studies.
Thus, the association between dietary meat intake and plasma concentrations of anserine, carnosine, Π-MH, T-MH and the ratio of T-MH to Π-MH was assessed in this thesis.



The dipeptide carnosine is synthesized by carnosine synthase (CS) and degraded by carnosinase (CN). The dipeptide anserine is degraded by anserinase (ANS) into ß-alanine and Π-MH. Solid lines represent reactions that either do not occur in humans or poorly understood, whereas dashed lines represent reactions occurring in humans.

Figure 1: Overview of the metabolism of carnosine (Adapted from Boldyrev et al., 2013)

[Boldyrev A.A, Aldini G and Derave W. Physiology and pathophysiology of carnosine. Physiol Rev, 2013. 93(4): p. 1803-45. Copyright 2013 "the American Physiological Society". Adapted with permission.] [25]

Carnosine (beta-alanyl-histidine) is a dipeptide synthesized by carnosine synthase from L-histidine and ß-alanine. It has several naturally occurring derivatives such as anserine (β-alanyl-Nπ-methyl-histidine), ophidine (β-alanyl-Nτ-methyl-histidine), homocarnosine and acetylcarnosine. Anserine and ophidine are the methylated analogs of carnosine. The metabolic pathways linking the related compounds to carnosine are depicted in **Figure 1** (Adapted from Boldyrev et al., 2013) [25]. Π-MH is a degradation product of anserine, whereas T-MH is degraded from actin and myosin, the main proteins found in the striated muscle filaments [24]. Over many years, the distribution of carnosine, anserine and ophidine in various animals species has been studied [25]. Nearly all mammals have carnosine and one of the methylated

carnosine analogs except Homo sapiens. Homo sapiens possess only carnosine. Anserine is found more frequently than ophidine in mammals, whereas ophidine is only observed in high amounts in marine mammals. As this study is concerned with finding dietary biomarkers of meat intake, the focus was placed on anserine, carnosine, T-MH and Π-MH.

Fecal sterols and bile acids as biomarkers of habitual dietary intake

In the third manuscript, animal sterols [26], plant sterols [26,27] and bile acids [28] were assessed in relation to their excreted concentrations in feces, since they are found in different amounts in various foods. They have been proposed as dietary intake biomarkers by several studies [26-30] and have been analyzed in fecal samples to assess their associations with dietary intake and disease. Yet it is still not known if these biomarkers can also indicate habitual dietary intake or not.

Metabolic pathway of cholesterol and bile acids

Generally, cholesterol is excreted either as part of a sterol or in the form of bile acids in feces. Cholesterol is mainly metabolized in the liver into primary bile acids such as cholic- and chenodeoxycholic acids. These are conjugated with taurine and/or glycine, which are eliminated with bile into the intestine, where 95% are reabsorbed and enter the enterohepatic cycle. The other 5% are not absorbed and pass to the large intestine. Unabsorbed bile acids are deamidated or dehydroxylated by colonic microbiota to produce secondary bile acids e.g., deoxycholic acid and lithocholic acid. These compounds are finally excreted in feces [31,32] (See **Figure 2**).

Thus, 9.5% of fecal neutral sterols are excreted as cholesterol itself, whereas coprostanol, a metabolite product of cholesterol, makes up 65% of fecal neutral sterols [33]. A thorough description of the fecal metabolic pathway of cholesterol and bile acids is outlined elsewhere [32].

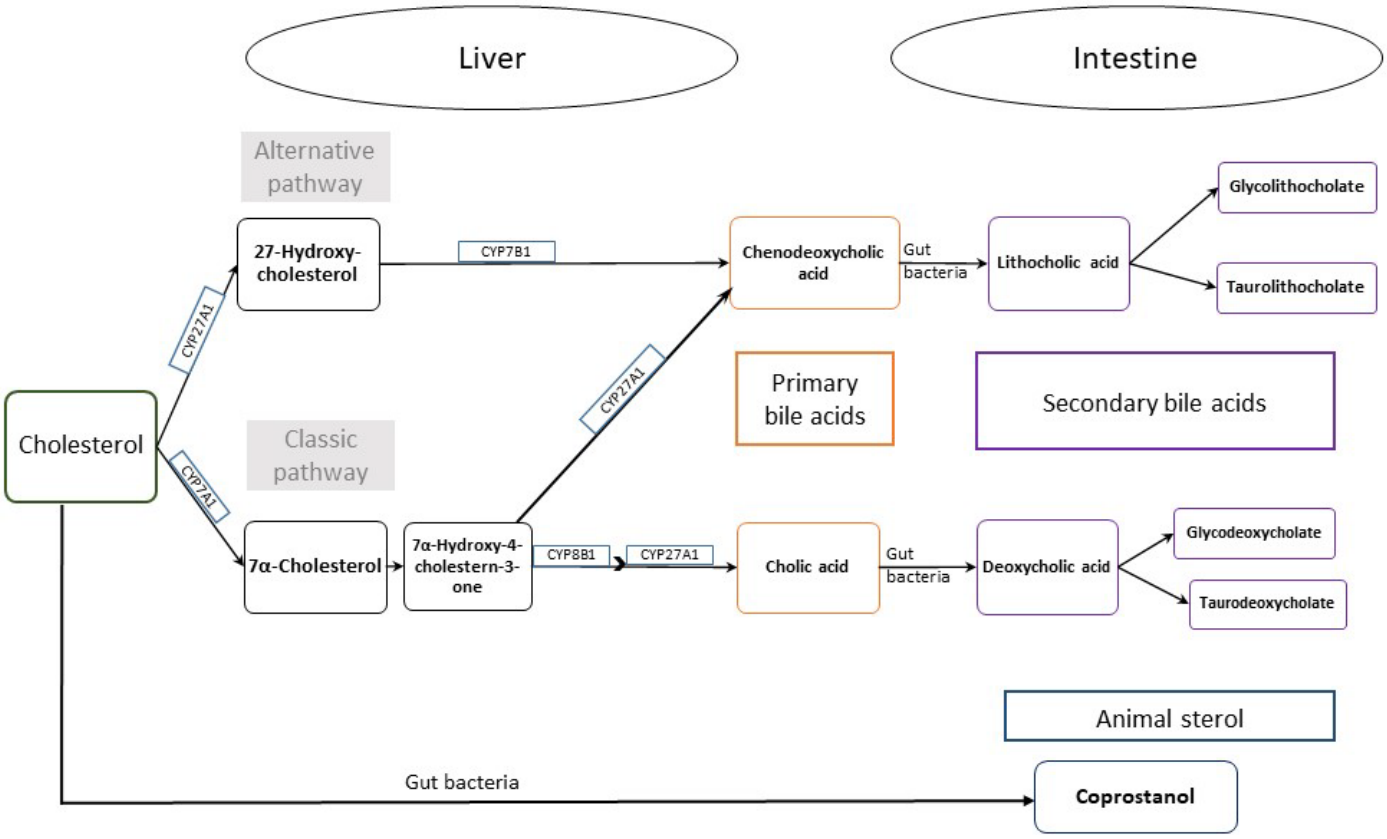


Figure 2: Overview of the metabolic pathway of cholesterol (from Mitry et al., 2019)

[Mitry P, Wawro N, Sharma S et al. (2019) Associations between usual food intake and fecal sterols and bile acids: results from the KORA FF4 study. Br J Nutr, 2019: p. 1-26. Copyright Clearance Center. Reproduced with permission.] [34]

Metabolic pathway of phytosterols

Phytosterols like stigmasterol or ß-sitosterol are naturally occurring compounds that are derived by humans only through dietary intake of plant foods. They are similar to cholesterol in both structure and biological function except that they contain an additional ethyl- or methyl group at the C_{24} position on the sterol side chain [35]. It is necessary to mention that habitual dietary consumption of plant sterols is influenced by population and sex [36-38]. Vegetable oils are very rich in phytosterols, but their serving sizes are small in comparison with the serving sizes of seeds and nuts, grain products, vegetables, and fruits [27].

Dietary intake of plant sterols is mostly rich in campesterol and sitosterol, which are metabolized into methyl- or ethyl- coprostanol and methyl- or ethyl- coprostanone. Less than 5% of dietary levels of phytosterols are absorbed in healthy humans; the remaining 95% enter

the colon [39]. Intestinally-absorbed phytosterols are excreted quicker than cholesterol via the biliary route, which results in a small pool of phytosterols in contrast to cholesterol [40]. The amount of dietary plant sterol intake is proportional to the total amount of plant sterols and plant sterol derivatives excreted in feces [41]. Additionally, plant sterol intake enhances fecal animal sterols excretion.

Aim of this dissertation

Identifying new habitual dietary biomarkers based on "omics" data in observational studies is a relatively new concept, which could improve the characterization of nutritional status in study participants and offer a deeper understanding of the effects of food components and diets on the human body. This concept may be useful for nutritional epidemiology since it aims to observe, independent of measurement errors, biomarkers of dietary intake and nutritional status [11]. It is the aim of this dissertation to emphasize the need to identify new habitual dietary intake biomarkers in different biosamples available from observational studies. Furthermore, it highlights the emerging field of nutritional metabolomics and potential analytical methods. Several meat, animal and plant sterol, and bile acid biomarkers of habitual intake are not well explored or validated in the present literature [7,10,11,24] and may be identified as useful upon better assessment in cross-sectional studies.

Accordingly, the main objectives of this dissertation are:

1. To emphasize the need to identify biomarkers of nutrient intake using alternative sampling techniques to improve the quality of nutritional research in large epidemiological studies; e.g. utilizing fecal samples as a potential matrix for dietary biomarkers. It highlights the emerging field of nutritional metabolomics and the relevant analytical methods.

2. To identify associations between habitual meat consumption and plasma concentrations of anserine, carnosine, pi-methylhistidine (Π-MH), tau-methylhistidine (T-MH) and the ratio of tau-methylhistidine to pi-methylhistidine in a cross-sectional study (BVS II).

3. To assess associations between usual food intake and fecal sterols and bile acids, analyzed by means of a metabolomics technique, in the KORA FF4 study.

Objective 1 resulted in a manuscript with the doctoral candidate as the fourth author. Objectives 2 and 3 resulted in two manuscripts with the doctoral candidate as the first author. All three manuscripts are published in international, peer-reviewed journals ([42], [34] and [16]).
This dissertation is based on these three manuscripts. The term ‘manuscript’ is used in the following for published manuscripts for reasons of simplification.

2. Methods

2.1 Literature research

The first manuscript gives an overview of different sampling techniques to facilitate identifying biomarkers of nutrient intake or nutrients status. For the chapter “feces— a potential matrix for dietary biomarkers”, a literature search was conducted mainly based on original research papers available in PubMed, Google, and Science Direct (ELSEVIER) up to November 2015.

The search strategy addressed the subject of finding biomarkers of dietary intake in fecal samples by metabolite profiling and to what extent the gut microbiota composition is subject to dietary control. Different combinations of the following keywords were used to search for studies that were related to biomarkers of dietary intake in feces and metabolite profiling: “dietary intake”, “nutrient intake”, “short-chain fatty acids”, “branched-chain fatty acids”, “sterols”, “prebiotic”, “dietary polyphenols, “carotenoids”, “diet and stool metabolites” “bile acids”, “fecal metabolites”, “dietary intake and stool metabolites”.

Additionally, an extended search was performed on this topic including information on metabolite profiling in fecal samples and the analytical methods used. To this end, the search terms “metabolite profiling in fecal samples” and “metabolomics techniques for stool metabolites” were included in addition to the above-mentioned keywords. Finally, the search was limited to human studies written in the English language.

2.2 Study population and data collection

The analysis in the second manuscript is based on data from the second Bavarian food consumption survey (BVS II), which was conducted between September 2002 and June 2003 and included a random sample of German-speaking participants of the Bavarian population [43].

The total participation rate was 71% after following a three-step random routine sampling. Participants were selected from 42 communities that were stratified by county and community characteristics. The aim of this cross-sectional study was to investigate the dietary and lifestyle habits of the included 1050 participants aged 13 to 82 years.

Participants who completed at least one 24h-DR within six weeks of recruitment and were at least 18 years of age (n=879) were invited for standardized anthropometric measurements and blood sampling at their closest health office. Sixty-five percent (n=568) of those invited chose to participate.

The second manuscript is based on the results of 294 participants whose plasma sample volume was adequate for lab analysis. A flow diagram of the study population used in the second manuscript is shown in **Figure 3.**

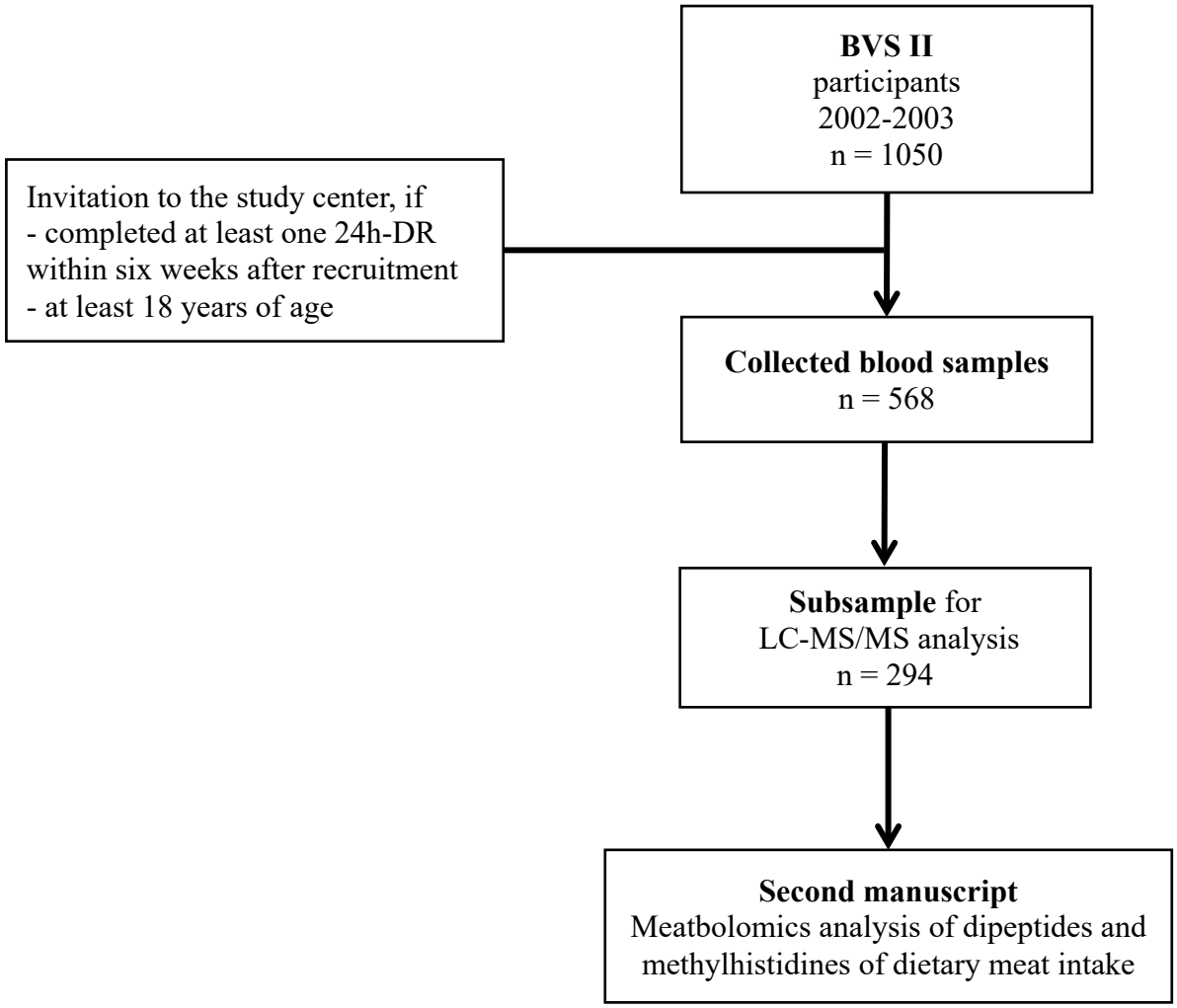


Figure 3: Flow diagram of the BVS II study population used in the second manuscript

The Ethics Committeeof the Bavarian Medical Association (Bayerische Landesärztekammer) approved the BVS II study. The study was in agreement with the criteria laid down in the declaration of Helsinki and written informed consent was obtained from all study participants.

The analysis in the third manuscript is based on data from the Cooperative Health Research in the Region Augsburg (KORA) research platform. It was established in the region of Augsburg to perform population-based surveys and subsequent follow-up studies in the fields of epidemiology, health economics and health care research. It originated as the World Health Organization (WHO) MONICA Project (monitoring trends and determinants in cardiovascular disease) and continued as the KORA cohort since 1996.

The KORA cohort comprised four independent cross-sectional studies (S1-S4). A sum of 6,640 community-dwelling adults aged 25 - 74 years and living in Augsburg were randomly chosen from the local population registries, adopting the same sampling technique used in MONICA [44], and were enrolled in the final KORA S4 health survey. In the final KORA S4 health survey (1999-2001), 4,216 out of 6,640 individuals participated, a 67% participation rate. The KORA FF4 cohort study (2013/2014) is a second follow-up examination, where 2,279 individuals from the original S4 study participated. One of the main objectives of this study is to determine changes in health status and lifestyle habits that occurred over the follow-up period [45]. Further details on the participation response is given elsewhere [46].

The third manuscript included 1,008 participants, for whom habitual food intake estimates were available and the metabolomics analysis was successfully completed.

Stool samples were collected in the KORA FF4 study from 2,279 participants. For the metabolomics analysis, 864 samples were excluded and the remaining 1,415 stool samples were analyzed. Samples were included if participants had not been on antibiotics within the last two months prior to sample collection and the lab ID number was obtainable. Additionally, only stool samples that were not exposed to room temperature for longer than 3 hours overall and which were cooled for less than 48 hours before they were deep-frozen were included. A flow chart of the study population used in the second manuscript is shown in **Figure 4**.

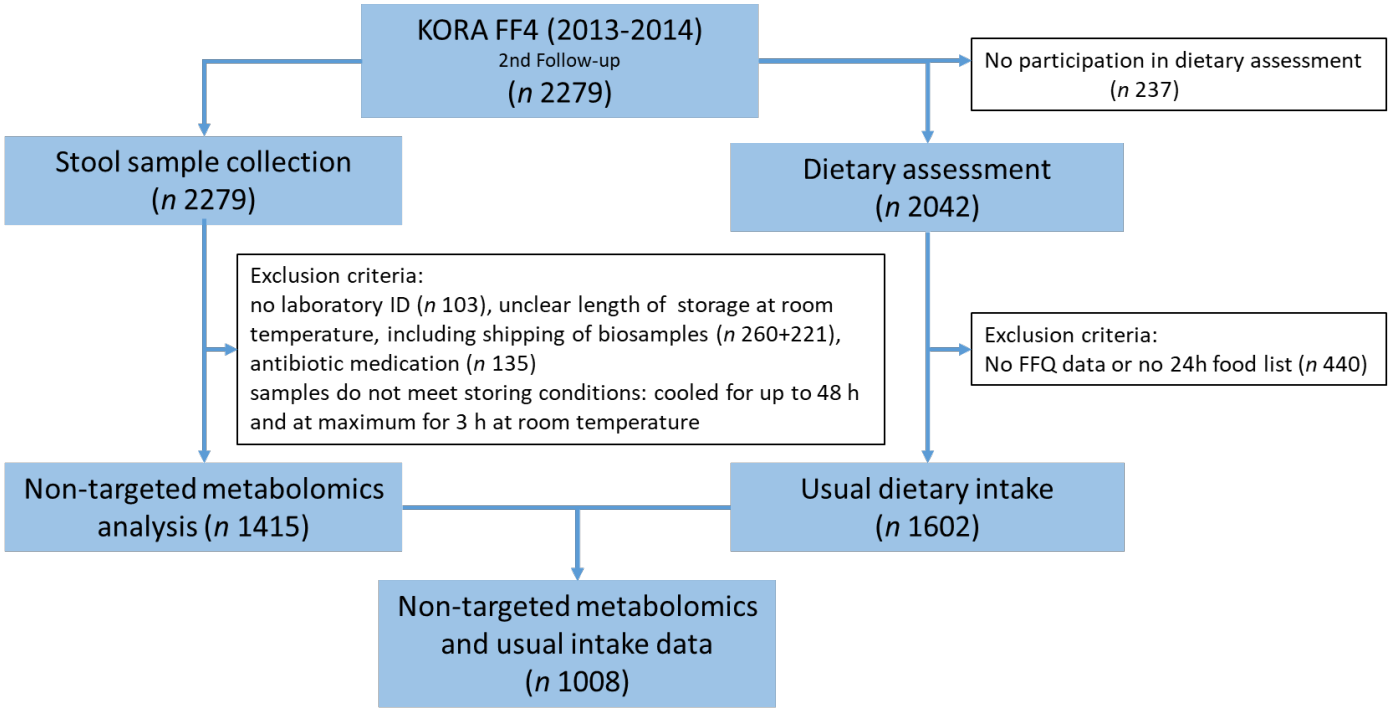


Figure 4: Flow chart of the KORA FF4 study population used in the third manuscript. (Adapted from Mitry et al., 2019)

[Mitry P, Wawro N, Sharma S et al. (2019) Associations between usual food intake and fecal sterols and bile acids: results from the KORA FF4 study. Br J Nutr, 2019: p. 1-26. Copyright Clearance Center. Reproduced with permission.] [34]

In the third manuscript in the KORA FF4 study, baseline information on sociodemographic variables, lifestyle factors, medical history and medication use was collected in an extensive standardized face-to-face interview at the Augsburg study center. Additionally, all participants underwent standardized examinations such as anthropometric measurements that included blood examination, weight and height measurement and the collection of bio-specimens.

KORA studies were approved by the Ethics Committee of the Bavarian Medical Association (Bayerische Landesärztekammer). Written informed consent was obtained from each participant in accordance with institutional requirements and the Declaration of Helsinki principles.

2.3 Biospecimen collection and laboratory analysis

In the second manuscript, plasma samples of the BVS II study were analyzed to identify meat intake biomarkers whereas, in the third manuscript, fecal samples were analyzed to identify sterols and bile acids.

In the second manuscript venous blood was drawn, chilled at 4 °C and stabilized with sodium ethylenediamine tetra-acetic acid (EDTA) (1 g/L). Plasma was separated from red blood cells by centrifugation before being divided in aliquots and stored at -80 °C until lab analyses.
For the quantitative amino acids analyses in plasma, targeted LC-MS/MS measurements were performed based on the method described by Harder et al. [47]. Plasma samples were diluted 51-fold in ice-cold methanol and an internal standard mixture of 16 deuterated amino acids was included, which was obtained from ChromSystems (Gräfelfing, Germany), Cambridge Isotope Laboratories (Tewksbury, MA, USA), and Sigma-Aldrich (Taufkirchen, Germany). After centrifugation of the samples and evaporation of supernatants containing the extracts, amino acids contained in the dried samples were derivatized to their butyl esters, as outlined by Gucciardi et al. [48]. The dried, derivatized samples were reconstituted in a 300 µl methanol/water/formic acid mixture.

Mass spectrometric detection was performed using the QTRAP5500 LC-MS/MS system functioning in positive ESI mode (AB Sciex, Framingham, MA), supplied with a 1200 series binary pump (Agilent, Santa Clara, CA) and attached to an HTC pal autosampler (CTC Analytics, Zwingen, Switzerland). The mass spectrometric detection is described in more detail in the second manuscript [42]. Peak integration and data analysis were obtained utilizing Analyst 1.51 ® software (AB Sciex).

In the KORA FF4 study (third manuscript), participants collected stool samples according to the instructions provided with the equipment on the day of the study center visit or the evening before. Participants were asked to collect probes of different areas of the fecal sample into two sterile plastic collection tubes (one of which already contained a stabilizing agent) that were to

be packed into the provided sterile plastic bag. Subjects were instructed to refrigerate the tubes (4-8 °C) after placing them in a storage box. Participants were requested to complete a questionnaire regarding the time and method of stool collection to confirm that fecal collection was obtained based on the SOP guidelines. Upon the participants' arrival at the study center, fecal samples were directly deep-frozen initially at -20 °C and afterward at -80 °C until further processing.

136 to 143 mg frozen fecal samples were administered 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). Samples were re-suspended at a 12.5:1 (12.µl H_2O for every 1 mg of feces weight) ratio and homogenized in a Precellys 24 homogenizer (PEQLAB Biotechnology GmbH, Germany) equipped with an integrated cooling unit 3 times for 20 s at 6,500 rpm, with 15 s intervals between the homogenization steps. For the non-targeted metabolomics analysis, 100 µl of the homogenate were pipetted onto a 2 ml 96- deep well plate.

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 was applied for all analytical methods. The applied untargeted UPLC-MS/MS analysis method is described in detail in the third manuscript in the Supplementary Material [34].

Metabolon's hardware and software (Metabolon, Inc., North Carolina, USA) was utilized for raw data extraction, peak identification and quality control processing. Compounds were identified by relating to library entries of purified standards or recurrent unknown entities. Identification of the biochemical metabolites were based on three criteria: retention index (RI) 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 derived from a comparison of the ions present in the experimental spectrum to the ions present in the library spectrum.

The flow diagram in **Figure 5** illustrates the number of metabolites which were assessed by metabolon and were included for further analysis in the third manuscript.

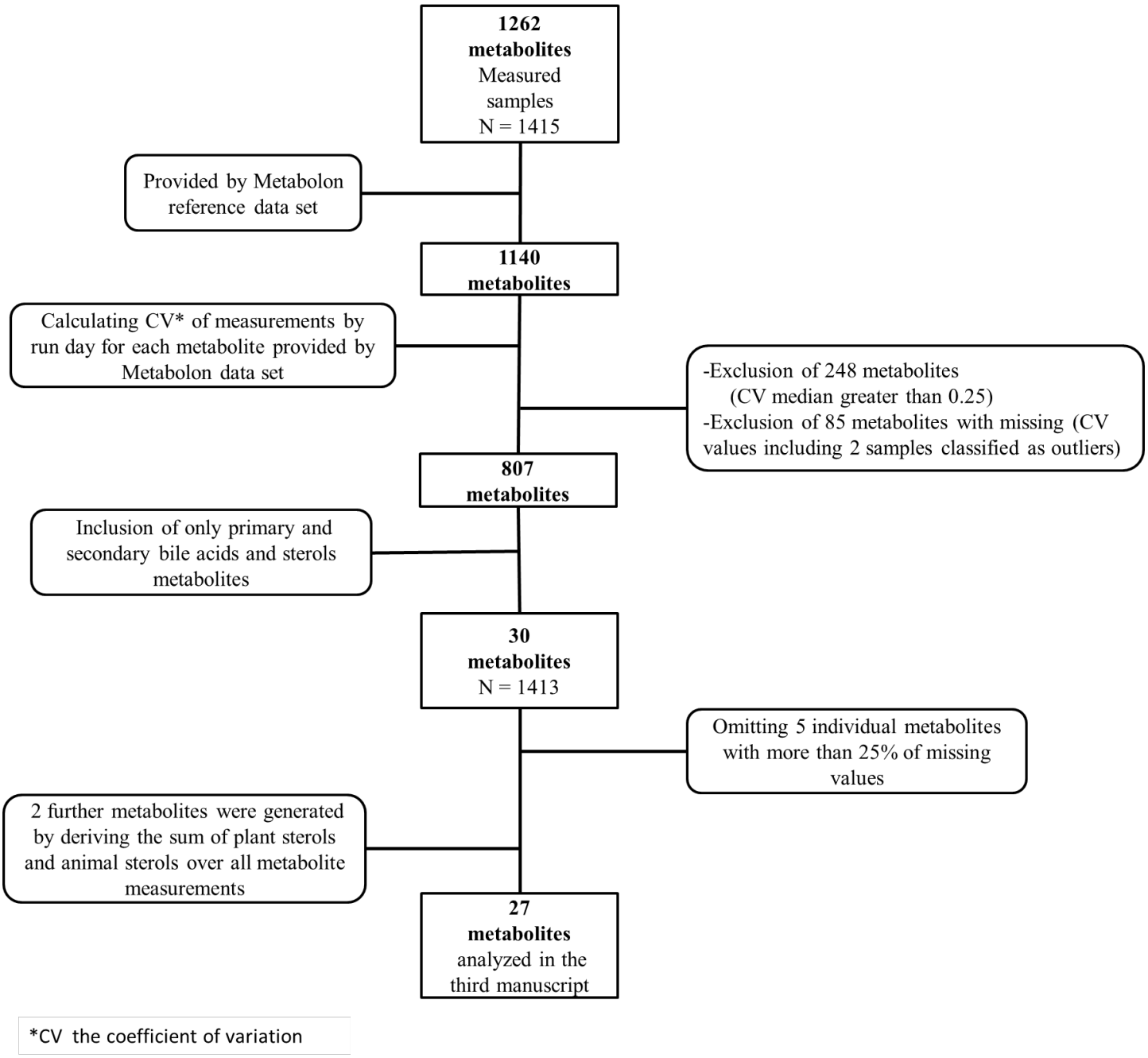


Figure 5: Flow diagram of the KORA FF4 analyzed metabolites

Primary and secondary bile acids and sterol metabolites were chosen from 807 metabolites for the analysis in the third manuscript. Thirty metabolites measured in 1413 subjects were selected for the preprocessed data set and missing values were imputed based on the minimum value per metabolite as we supposed they were below the limit of detection. The sum of all primary bile acids, secondary bile acids, plant sterols and animal sterols was calculated from the imputed data set and included as two additional variables in the analysis.

Furthermore, five individual metabolites with more than 25% of missing values (cholate sulfate, 7-ketolithocholate, glycocholenate sulfate, taurodeoxycholate and ursodeoxycholate sulfate) were omitted from individual analysis. In all, 27 metabolites were available to be analyzed in the third manuscript.

2.4 Assessment of Anthropometric measures

The anthropometric measure BMI was used as a covariable in the analysis of associations between dietary intake and fecal sterol/bile acids excretion (third manuscript). In the second manuscript, BMI was used as a continuous variable in the descriptive statistics of the population and as an adjustment variable in the analysis of associations between meat intake and plasma metabolites.

Weight (kg) in KORA FF4 and BVS II was measured with an electronic scale and standing height (cm) with a stadiometer. These measures were obtained during the physical examination at the study center by trained investigators; participants wore light clothing without shoes. BMI was calculated as weight/height² (kg/m^2) and subjects were classified as underweight (BMI < 18.5 kg/m^2), normal (18.5 kg/m^2 <=BMI < 25 kg/m^2), preobese (25 kg/m^2 <=BMI < 30 kg/m^2) or obese (BMI >= 30 kg/m^2) according to WHO thresholds (2000) (third publication).

2.5 Assessment of sociodemographic and lifestyle variables

Sociodemographic and lifestyle information of participants was used for the characterization of participants in BVS II (second manuscript) and KORA FF4 (third manuscript). Further, they were utilized as adjustment variables in the second manuscript and as covariables in the analysis of associations between diet intake and sterol/bile acids fecal excretion (third manuscript).

The sociodemographic and lifestyle information was determined in the KORA FF4 study through questionnaires and the computer-assisted personal interview, whereas in the BVS II study it was assessed through computer-assisted personal interview and computer-assisted telephone interview.

The second and third manuscripts included sex (men, women), age of participants (years), smoking status (smoker, ex-smoker and never-smoker), physical activity (active, inactive) and education (years) or socioeconomic status (classified based on the educational level, household net income and career position of the principal earner) as sociodemographic and lifestyle variables.

The third manuscript additionally included alcohol consumption for the adjustment of the associations between dietary intake and fecal sterol excretion. Participants were classified into 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 following the recommendations given by the German Nutrition Society[49]. The years of education reported were classified as up to 12 years of education and more than 12 years of education.

The physical activity assessment in KORA FF4 (third manuscript) was categorized into four levels according to the amount of regular leisure-time exercise per week during summer and winter. The four levels are (i) more than 2 h/week regularly, (ii) about 1 h/week regularly, (iii) and about 1h/week irregularly and (iv) almost no or no physical activity. In the second

manuscript, participants recalled their physical activity in the last 24 hours via telephone interview.

2.6 Assessment of chronic metabolic diseases

In the third manuscript, hypertension status (hypertensive or not) and diabetic status (type 2 diabetes or no type 2 diabetes) of the participants was assessed via questionnaire, personal interview or in the physical examination at the study center. These were used in the descriptive analysis of KORA FF4 participants.

The systolic and diastolic blood pressure were measured with an oscillometric digital blood pressure monitor three times at intervals of at least 3 minutes at the right arm of the sitting subject. Participants with a mean blood pressure ≥ 140 mmHg or diastolic ≥ 90 mmHg and/or use of antihypertensive medication (given that they were aware of having hypertension) were specified as hypertensive.

Diabetes was measured by a standard 75 g oral glucose tolerance test (OGTT) performed in the morning (7:00-11:00 a.m) in all subjects who were not previously diagnosed with diabetes and who were fasting overnight for at least eight hours. Diabetes was then specified as a fasting plasma glucose level ≥ 126 mg/dl or a plasma glucose level 2h after intake of 75 g of ≥ 200 mg/dl. For participants with a self-reported diabetes history, the diagnosis was verified by intake of antidiabetic medication and/or by consulting the respective treating physician. Furthermore, the use of lipid-lowering drugs and anti-diabetic drugs was comprised. These were identified by ATC codes C10 and A10. The intake of medications was documented in the study center through a database-supported computer software called IDOM [50]. These medications were used as adjustment variables in the main analysis of the third manuscript.

2.7 Assessment of dietary intake

In the second manuscript, dietary intake data, which were assessed in BVS II participants, were investigated for their association with plasma biomarkers of meat intake in 294 individuals, stratified by age, sex and BMI.

The dietary intake of BVS II participants was estimated by three 24-hour dietary recalls (24h-DR), where two of the 24h-DRs were to be completed on a working day (Monday-Friday) and the third on a weekend day (Saturday or Sunday). Trained study personnel conducted these telephone interviews and participants had to recall their dietary intake from the previous day. The 24h-DR results were grouped to 16 food groups and 21 subgroups according to the EPIC-Soft classification scheme [51]. Dietary intake data were weighted correspondingly to weekday or weekend day to calculate a mean value for every food group or subgroup to present a usual daily intake in g/day. A mean value for every food group or subgroup was obtained from three 24h-DRs. This represents the usual intake of the particular item in g/day. The food groups and subgroups included in this analysis are listed in **Table 1.**

Table 1: Food groups and subgroups selected for the analysis of diet-plasma dipeptides/methylhistidines concentration associations (g/d)

Main groups (g/d)	Subgroups (g/d)			
All meat and meat products	Red meat	Beef	Pork	Beef and Pork
	Poultry	Chicken	Turkey	Processed meat
Fish and Shellfish				
Egg and egg products				
Milk and dairy products				

In the third manuscript, 1008 participants of KORA FF4 were included who completed one FFQ and up to three web-based 24 hours food lists (24 HFL) by the end of November 2014, and for whom the metabolomics analysis was successfully completed. The 24HFL is a closed and structured list of 246 food items that assesses the participants' food and drink consumption over the previous day [2], whereas the FFQ encompasses 148 items and reports the usual frequency of consumption and the usual portion size of the consumed food and drink items during the past 12 months. The FFQ is a validated assessment tool [52] based on the German

version of the multilingual European Food Propensity Questionnaire (EFPQ) [53], and it includes pictograms to help participants in estimating the portion sizes.

Regarding the 24HFL, it does ask which meals were consumed, but it neither assesses which foods were consumed at each meal nor portion sizes. KORA FF4 participants were contacted on two randomly selected days within three months after their first study center visit to complete the 24HFL based on their diet on the previous day. Two 24HFLs were to be completed on a working day and the third one on a weekend day.

To minimize the occurrence of errors in the dietary assessment [54,55], a blended approach that combines two assessment instruments was used to estimate usual dietary intake in the third manuscript. The blended approach is based on the National Cancer Institute (NCI) [56,57] and the Multiple Source Method (MSM) [58].

It is a two-step model approach that separates consumption probability from consumption amount on a consumption day. Details are described elsewhere [6]. Briefly, the consumption probability was estimated first for each food item for each participant with a logistic mixed model including age, sex, BMI, smoking, physical activity, education level, and FFQ data as covariables. In the second step, the consumption amount on consumption days was estimated based on models having the same covariables to link both steps. Since the 24 HFLs do not assess portion sizes, these were calculated based on the data of the 24h-DR of the Bavarian Food Consumption Survey II (BVS II) (same study of the second manuscript). Using linear mixed models, the usual consumption amount of each food item on a consumption day was estimated with the BVS II data. These models were adjusted for age, sex, BMI, smoking, physical activity, and education level. Since consumption amounts were not normally distributed, they were transformed by Box-Cox transformations and predicted amounts were then back-transformed. At last, the usual intake was then derived by multiplying the estimated consumption probability of a certain food item by the estimated amount consumed on a consumption day.

In the third manuscript, the usual dietary intake analysis focuses on 19 main food groups or subgroups, which are listed in **Table 2.**

Table 2: Food groups and subgroups for the analyses of diet-fecal sterol/bile acids excretion associations (g/d)

- Potatoes	- Yogurt	-Processed meat	- Vegetable oil
- Vegetables	- Cheese (including cream cheese)	-Fish and shellfish	- Soy products
- Fruits	- Cereal and cereal products	- Eggs and egg products	- Non-alcoholic drinks
- Nuts, seeds and nut spreads	- Meat and meat products	- Sugar	- Alcoholic drinks
- Milk and dairy products	- Pork	- Butter	

Total caloric intake [kcal/d] was used in the second and third manuscript as a covariable and was derived from the usual intake of the participants, taking into account all available food groups and subgroups.

2.8 Statistical analysis

All statistical analyses in the second manuscript were carried out using the SAS software, version 9.3 of the SAS system for Windows (Copyright © 2002-2010 SAS Institute Inc.), whereas in the third manuscript the statistical software R, version 3.3.2 (R Development Core Team, 2010, http://www.r-project.org) was used. P-values < 0.05 were considered statistically significant.

Descriptive statistics

In the second and third manuscript, the BVS II and KORA FF4 participants were stratified by sex and characterized depending on the respective study aim by socio-demographic and lifestyle variables, anthropometric and dietary intake characteristics, and clinical status.

Due to deviations from the normal distribution, descriptive statistics are presented in both manuscripts as median and interquartile ranges (for continuous data) or absolute numbers (n) and relative frequency (%) (for categorical variables) as appropriate.
In the third manuscript, descriptive characteristics were stratified by sex and t-tests and the Chi^2-test was used for sex-specific differences between characteristics of the population. The Kruskal-Wallis test was used to identify sex-specific differences in usual dietary intake, as intake distributions are typically not normal.

Since the plasma concentrations of the metabolites in the second manuscript were non-normally distributed, the differences of the metabolites across categories of BMI, sex, age, smoking, social class and physical activity were described by presenting the geometric means and 95% CI (confidence interval). Geometric means were obtained by fitting models with PROC GENMOD utilizing log-transformed plasma concentrations and transforming the estimates obtained from the model. BMI was categorized into quartiles and age into three groups (< 40 years, 40-60 years, ≥ 60 years). All bivariate models were adjusted for age, sex and BMI. The p-trend was obtained based on integer scores assigned to the categories of age, sex, BMI, smoking, social class, and physical activity.

In the third manuscript, the percentage of missing values, median, 25%- and 75%-quantiles of all metabolites and metabolite groups was reported. The median and 25%- and 75%-quantiles or absolute numbers and percent of categories for the mentioned variables were provided for all 1,008 participants for which the fecal metabolite data and the habitual dietary intake data were available. Further analyses were completed with log-transformed metabolite data.

T-tests or Kruskal-Wallis tests were used for the categorical variables and correlations were examined for continuous variables. In bivariate analyses, associations or differences in metabolite levels with the covariates were tested and related variables were chosen to be included as adjustment variables in additional regression analysis.

Analysis of dietary intake- biomarkers associations

Different usual dietary intake variables (listed under section 2.7) were analyzed with respect to their association with biomarkers of meat intake in plasma in BVS II (second manuscript) or of animal- and plant sterols, and bile acids in feces in KORA FF4 (third manuscript).
In the second and third manuscript, for each dietary intake variable, regression models with different sets of covariables were fitted.

Geometric means for categories of food intake were obtained in the second manuscript by fitting linear models utilizing log-transformed plasma concentrations. The classification of the food items was done according to the intake data. Some food items were grouped into quartiles ("all meat and meat products", "processed meat", and "milk and dairy products"), but most other food groups could only be divided into no, low and high consumers (e.g. "red meat", "pork", "beef and pork", and "eggs and egg products") by their median value. For a small group of food items, only a distinction into consumers and non-consumers was possible ("beef", "chicken" and "turkey"). Age, sex and BMI were included as adjustment variables. The p-trend was obtained based on integer scores assigned to the categories of food intake. Further non-parametric comparison of the consumption of "all meat and meat products", "milk and dairy products", "fish and shellfish", and "eggs and egg products" across groups defined by the quartiles of biomarkers was carried out. Dunn's post-hoc test was performed if the Kruskal-Wallis test showed significant differences ($p < 0.05$).

For the third manuscript, the usual intake variables were obtainable only for 1,008 subjects for which the stool samples were analyzed. In the regression models described below, adjustment variables age, sex, BMI, smoking status, alcohol consumption, physical activity, years of education, medication, total caloric intake and usual intake of selected groups and subgroups were included. Smoking status, alcohol consumption, physical activity and education level of the participants were assessed and included as categorical variables. Further details are described elsewhere [42]. Further, BMI (kg/m^2) and age of participants (years) were analyzed as continuous variables; as described in detail in (manuscript 3) [34].

Differences in metabolite levels were investigated with respect to medication intake in order to select related variables to be included as adjustment variables in further regression analysis (p-values < 0.05). Differences were tested for continuous variables and for categorical variables; t-tests or Kruskal-Wallis tests were completed.

Regression models were fitted for all 27 metabolites and adjusted for age, sex, BMI, total caloric intake, smoking status, alcohol consumption, physical activity, years of education, diabetes medication, and lipid-lowering medication. The effect of the usual intake of a certain food group on metabolite levels was examined and the effect estimates and p-values were reported. Bonferroni adjustment of the p-values was done to correct for multiple testing by dividing 0.05 by the number of tests conducted (27*19=513, leading to 9.746589e-05).

3. Short description of publications

3.1 Publication 1: Biomarkers for nutrient intake with focus on alternative sampling techniques

Identifying new dietary biomarkers using alternative sampling techniques may help in understanding the relationship between food intake and nutritional status, leading to an improvement in the quality of nutritional status. Since most dietary intake data collected in large epidemiological studies rely on participants memorizing or monitoring food intake, these data are prone to over- and underreporting of certain foods. Utilizing new technology such as the dried blood spots (DBS) sampling technique is now cheaper and able to accurately measure several metabolites in very small biological samples. The DBS sampling technique is also easier to implement and more stable than classical blood sampling. The sample's storage, transport and analysis is also cheaper and more practical.

Diet has an essential role in maintaining the composition, metabolism, and function of the gut microbiota. Identifying dietary biomarkers in feces may help to understand how the composition of the gut microbiota is related to dietary intake. Metabolite profiling in feces allows for the discovery of new biomarkers of gut health and dietary intake. However, metabolite profiling in fecal samples is very difficult due to its physicochemical properties that have an impact on the level of reproducibility and full coverage of metabolite profiling that can be achieved. Additionally, different fecal sample collection methods and storage conditions may influence metabolite concentrations. Therefore, it is essential to utilize more than one analytical method to attain a comprehensive metabolite profile, since fecal metabolite levels can vary widely based on these factors.

The doctoral candidate performed the literature research, drafting, writing and revising of the chapter "Feces— a potential matrix for dietary biomarkers".

[Holen T, Norheim F, Gundersen T E, **Mitry P**, Linseisen J, Iversen P O, Drevon C A. Biomarkers for nutrient intake with focus on alternative sampling techniques. Genes & Nutrition (2016) 11:12 DOI 10.1186/s12263-016-0527-1).] [16]

3.2 Publication 2: Plasma concentrations of anserine, carnosine and pi-methylhistidine as biomarkers of habitual meat consumption

Finding biomarkers of meat intake in plasma could deliver more valid intake estimates in observational epidemiological studies than dietary questionnaires, as the latter are prone to reporting bias. Since habitual dietary intake of meat has been shown to be correlated with human health and risk of chronic diseases, it was the aim of the study to investigate the relationship of habitual red and processed meat, poultry and fish consumption with plasma concentrations of anserine, carnosine, pi-methylhistidine (Π-MH), tau-methylhistidine (T-MH) and the ratio of T-MH to Π-MH in a cross-sectional study.

The plasma metabolite concentrations were measured by applying the ion-pair LC-MS/MS technique. Three 24-h dietary recalls were administered per participant to assess habitual food consumption. For assessing the correlation between plasma metabolite concentrations and meat, fish, eggs and dairy product consumption, geometric means for food categories were obtained by fitting generalized linear models, adjusted for age, sex, and BMI.

In the present study, we observed a correlation between habitual meat intake and plasma concentrations of anserine, carnosine, Π-MH and the ratio of T-MH to Π-MH. Red meat intake was associated with carnosine, whereas poultry and chicken consumption were related to Π-MH.

However, intervention studies are required to determine if the analyzed plasma metabolites are distinctive for a specific type of meat or not.

The doctoral candidate conceived and conducted the literature research, drafting, writing and revising of the manuscript, correspondence to the journal and final approval of the publication.

[**Mitry P**, Wawro N, Rohrmann S, Giesbertz P, Daniel H, Linseisen J: Plasma concentrations of anserine, carnosine and pi-methylhistidine as biomarkers of habitual meat consumption. European journal of clinical nutrition 2018.] [42]

3.3 Publication 3: Associations between usual food intake and fecal sterols and bile acids: results from the KORA FF4 study

Sterols and bile acids in fecal samples have been proposed as biomarkers of dietary intake, but it is still unknown if these markers can be used to characterize long-term habitual dietary intake. The aim of this study was to examine whether habitual dietary intake is associated with fecal concentrations of animal sterols, plant sterols and bile acids in participants of an observational study.

Only stool samples that met the quality criteria and were collected according to the standard protocol were analyzed. The fecal metabolite concentrations were measured by applying a non-targeted UPLC-MS/MS technique. Repeated 24-h food lists and a food frequency questionnaire were administered for assessing the habitual food consumption per participant. Relationships between food intake and log-normalized metabolite concentrations were assessed in multivariable adjusted regression models. To adjust for multiple testing, the Bonferroni correction was utilized. A diet high in "fruits" and "nuts and seeds" was associated with a decreased total fecal cholesterol concentration, whereas a habitual diet high in "meat and meat products" was associated with an increase in animal fecal sterols concentrations. The associations between usual dietary intake and fecal concentrations of bile acids were limited; however, a positive association between glycocholate and fruit consumption was observed. Nevertheless, additional studies are needed to assess fecal animal sterols as biomarkers of diet, and most importantly, the results should be confirmed in other population-based studies with different dietary habits.

The doctoral candidate performed the literature research, drafting, writing and revising of the manuscript, correspondence to the journal and final approval of the publication.

[**Mitry P**, Wawro N, Sharma S, Kriebel J, Artati A, Heier M, Meisinger C, Thorand B, Rathmann W, Grallert H, Peters A, Linseisen J: Associations between usual food intake and fecal sterols and bile acids: results from the KORA FF4 study. British Journal of Nutrition.2019] [34]

4. Discussion

Main findings

The **first manuscript** gives an overview of different sampling techniques to identify biomarkers of nutrient intake. Obtaining accurate measures of exposure to different nutrients or food items on the individual level would help in understanding the relationship between diet, disease and health [59]. The high sensitivity and specificity of new technologies have facilitated the sampling and identification of dietary biomarkers in very small amounts of biosamples. Within the first manuscript, the use of fecal samples in identifying dietary biomarkers that could help in understanding to what extent diet can modify the gut microbial composition and affect human health and disease was outlined [60]. Identifying food metabolites that have been structurally modified by gut microbial activity in fecal samples may further reveal an individual's dietary intake. The review included examples of metabolites identified in fecal samples that can be possibly utilized as dietary biomarkers, such as short-chain fatty acids as metabolites of carbohydrate intake, metabolites of protein fermentation, and metabolites of fatty acids and sterols. Further, it briefly described the different methodological aspects of metabolite profiling in fecal samples using different analytical tools.

Consequently, the aim of the **second manuscript** was to examine associations between usual dietary intake of meat intake and plasma concentrations of anserine, carnosine, Π-MH and T-MH biomarkers in a cross-sectional study [42]. In brief, with higher total meat consumption, plasma anserine, carnosine, and Π-MH concentrations significantly increased. Plasma concentrations of carnosine and Π-MH also increased with higher red meat intake. Additonally, the intake of beef and pork were significantly related to anserine and carnosine plasma concentrations, whereas the plasma anserine concentrations significantly increased with higher processed meat and turkey intake. For Π-MH, a significant association with poultry and chicken consumption was observed. Yet no association between T-MH and any type of meat intake was observed. All analyses were adjusted for age, sex and BMI, as these factors were associated with the biomarkers of meat [42]. This is the first study that examined associations between habitual meat consumption and plasma concentrations of anserine, carnosine, T-MH or Π-MH in a cross-sectional format.

Associations between usual dietary intake and fecal metabolite concentrations of animal and plant sterols and bile acids were analyzed in the **third manuscript**. Before the Bonferroni correction was applied, a significant inverse correlation between the food groups “fruits”, “nuts, seeds and nuts spread”, “milk and dairy products”, “cheese” and “yogurt” and the fecal concentration of animal and plant sterols was determined. For the food groups “potatoes”, “meat and meat products”, “pork”, “processed meat”, “eggs and egg products” and “butter”, a positive correlation with animal sterol and plant sterol fecal concentrations was found. A statistically significant inverse association between “fruit” intake and fecal concentrations of campesterol, cholesterol, and sum of animal sterols was found after applying the Bonferroni correction. Further, a decline in fecal concentrations of these metabolites has been observed by a higher dietary intake of “nuts and seeds”. Additionally, a significant positive association between “meat and meat product” consumption and fecal cholesterol concentration was observed. After correction for multiple testing, only one significant association (a positive association between high fruit intake and glycocholate) could be observed with respect to bile acids.

Biomarkers of meat intake

Assessing plasma concentrations of objective meat biomarkers in observational studies would be useful in enhancing the characterization of nutritional status in participants and would provide a much deeper understanding of the effect of food constituents and diet on human health and disease. A meta-analysis of cohort studies observed an 18% and 22% higher risk of any disease compared to cardiovascular disease (CVD) mortality, respectively, based on intake of processed meat, whereas red meat consumption was associated with a 16% higher risk of CVD mortality. However, no association between white meat intake and CVD mortality was observed [61].

As mentioned previously carnosine, anserine, pi-methylhistidine (Π-MH), tau-methylhistidine (T-MH), urea, creatine, creatinine, carnitine, ophidine and sulfate have already been identified as biomarkers of meat intake [12,24,62]. Trimethylamine-N-oxide (TMAO) has also been proposed as a biomarker of meat intake as trimethylamine, the precursor of TMAO is produced

by gut microbiota from carnitine, phosphatidylcholine (lecithin), choline, and betaine [63-65]. Carnitine and phosphatidylcholine are found in high concentrations in red meat, liver, fish, milk, cheese, and eggs [66]. One study assessed the associations between consumption of red meat, eggs, or dairy products and the concentrations of the circulating TMAO [66]. However, no significant associations between the plasma concentrations of TMAO and the intake of red, processed, or white meat; fish; or eggs have been observed. It should also be mentioned that identifying high concentrations of animal sterols in the stool could be another biomarker for high meat intake [34].

Histidine dipeptides are mainly found at very high concentrations in skeletal muscles, the heart and the central nervous system. Therefore, in humans, carnosine and its derivatives such as anserine are mainly derived from a diet comprised of animal protein such as meat, poultry and/or fish [67]. Yeum et al. indicated that 150 g of beef contains 343.4 mg carnosine and 42.9 mg anserine, whereas 150 g of chicken contains 322 mg carnosine and 660 mg anserine [68]. Another study has reported that carnosine concentrations in pigs, beef, and turkey muscle is higher than in anserine, whereas anserine concentrations in rabbit, salmon, and chicken muscle are much higher than carnosine [69].

As mentioned above, carnosine is synthesized by carnosine synthase from β-alanine and histidine and degraded by carnosinase, also named dipeptidase [70]. There are two isoforms of carnosinase, one named serum carnosinase, which is a highly specific dependent homodynamic dipeptidase and the other a tissue carnosinase that acts as non-specific dipeptidase [70]. Tissue carnosinase hydrolyzes carnosine and anserine rapidly and their metabolites are eliminated in urine [71]. This could be a possible reason for finding very low plasma levels of these metabolites in the second manuscript [42].

Unlike Park et al. [72], Mitry et al. [42] could not find any association between beef intake and carnosine plasma concentrations. This could be due to several reasons described thoroughly in Mitry et al.[42]. However, a study conducted by Yeum et al. is in good accordance with the findings of the second manuscript, stating that carnosine plasma levels were not significantly associated with beef or chicken consumption. Further, it indicated an increase in Π-MH plasma level after poultry and all meat consumption, as reported in other studies [73] [74].

In one intervention study, meat protein intake was predicted by assessing carnosine, T-MH and Π-MH levels in urine. The authors reported that T-MH and Π-MH plasma levels were too low to be measured in plasma due to their rapid excretion in urine [75]. However, in the second manuscript we identified sufficient plasma levels of T-MH and Π-MH. Thus, no association between meat intake and T-MH was found, despite the results of the Cheung et al. study [73], that unlike Π-MH, T-MH does not reflect only meat intake, but also human muscle catabolism and muscle mass. Therefore, after consuming the same diet, a substantial difference in circulating T-MH concentrations was found among subjects [76].

Previously, some studies assessed the concentrations of T-MH and Π-MH eliminated in urine in intervention studies [62,71] or in a population-based study [77] to examine if they can serve as biomarkers of meat consumption. Other studies assessed meat intake biomarkers such as histidine dipeptides or TMAO in plasma or serum samples [66,72], whereas other studies attempted to identify carnosine, anserine, T-MH and /or Π-MH as meat biomarkers in plasma and urine samples as well [68,73,75]. Only two studies [68,73] had comparable analysis methods to the method utilized in the second manuscript; however, the biomarkers of meat intake in the second manuscript were assessed in a cross-sectional study and in plasma samples only.

Fecal animal or plant or sterols or bile acids as biomarkers for dietary intake

A high intake of cholesterol from animal origin, such as from meat and meat products, leads to high concentrations of animal sterols in feces [78,79]. Therefore, the sum of animal sterols in stool can be a promising biomarker of the intake of meat and meat products. Unlike cholesterol, the concentration of fecal bile acids did not increase with high meat intake, which is in line with Hentges et al's study [80]. However, Reddy et al. observed an increase in secondary bile acids following high meat consumption [78]. A higher plant sterol intake may physiologically affect cholesterol absorption and excretion [81,82], and this may explain the inverse association of "fruits and nuts and seeds" intake with animal sterols fecal excretion found in the third manuscript. Plant sterols are known for their plasma cholesterol-lowering properties [26,83-87], as they reduce cholesterol absorption through competition with cholesterol for incorporation into micelles [26]. Several intervention studies observed an

increase in fecal excretion of cholesterol due to the intake of a phytosterol-enriched diet [81,82,88]. However, in the long term, it is still unknown if a diet high in plant sterols is associated with lower fecal animal sterols. Since the third manuscript is the first study that analyzed fecal concentrations of sterols in a population study and related it to habitual dietary intake, the observations are not comparable with results from intervention studies and other studies dealing with the effects of phytosterol consumption. This could be because an average of 4.7 g/d of nuts and seeds were consumed in the KORA FF4 study, which is probably too small of a quantity to observe a phytosterol-based effect on cholesterol excretion. Further, high dietary fiber intake may cause an increased fecal bulk, and this may lead to a lower fecal concentration of sterols and bile acids per g of dry weight [89].

Identifying biomarkers in serum versus urine

Different types of biosamples can be collected in order to assess the nutritional status of a subject or population.

It is proposed that the collection of multiple 24-hour urine samples can reflect the total protein intake of individuals [7,90], although it is impractical in epidemiologic studies [8]. Dietary histidine-dipeptides are mainly eliminated in urine [24]. One study reported 82% of the anserine consumed is directly and quantitatively eliminated as Π-MH in urine [71]. In plasma, anserine and carnosine are quickly hydrolyzed by the enzyme carnosinase and eliminated in urine [68], yet several limitations exist. Comparing a 28-day feeding study with multiple 24-hour urine nitrogen outputs, a correlation of 0.99 was found, whereas a correlation of 0.5 was observed when the time period was reduced to a single observation. However, an 18-day feeding study produced a correlation of 0.95 [90]. Therefore, it is more convenient to collect 24-hour urine samples in intervention studies, as individuals should maintain a controlled constant daily diet, whereas this is not possible in epidemiological studies. Short-term biomarkers such as Π-MH and T-MH have been suggested for use with 24-hour recalls, as they have an elimination half-life of 11.7 and 12.6 hours [62]. Accordingly, a study observed urinary biomarkers of meat intake in 24-hour urine samples and indicated that their study design is not suitable for identifying biomarkers of long-term consumption [76]. It is important to point out that short-term biomarkers are only useful in populations that frequently and not

episodically consume these dietary sources [10]. This may explain why no associations were identified between meat intake and T-MH concentration in the second manuscript, as T-MH is rapidly eliminated and the food groups meat, beef and pork were sporadically consumed in BVS II study.

Blood samples are assumed to be the specimen of choice in epidemiologic studies, as they are accessible and easily stored and handled. Having plasma and serum samples in biobanks facilitate conduction more metabolomics studies [10]. Yet several methods have now been developed to utilize urine spot samples in identifying metabolites of interest in large-scale population studies [8].

Playdon et al. [91] compared metabolite profiling of habitual diet in urine and serum and observed that biomarkers of habitual dietary intake were identifiable in serum as well as in urine. Consequently, it was concluded that the same habitual dietary intake biomarkers can be identified in different types of biosamples in epidemiological studies. Although urine sample collection is less invasive and less expensive to obtain, only water-soluble nutrients pass through urine [8], and samples may need acidification and cold storage promptly after sample collection to avoid the degradation of nutrients. Therefore, metabolite concentrations might vary greatly due to ionic strength, osmolarity, pH and dilution, which may cause urine analysis to result in lower quality data on habitual dietary biomarkers status [92]. It is also important to mention that few other studies compared urine and blood for either the identification of similar biomarkers using metabolomics techniques or to prove their stability and reliability as potential biomarkers of meat intake [68,73-75]. One study observed carnosine and anserine stability in human serum [68], where carnosine rapidly disappeared within 5 minutes with a hydrolysis rate of 170 nmoles ml^{-1} $min^{-1}$and a 3 minute half-life. Although anserine disappeared rapidly, a quantifiable amount could be identified since it disappeared with a slower hydrolysis rate than carnosine (16nmoles ml^{-1} min^{-1}, half-life 30 minutes). The same study did not observe any carnosine in plasma after consumption of beef, chicken, or chicken broth ingestion. Anserine was detectable (between the limit of detection (LOD) and limit of quantitation (LOQ)) after beef consumption. However, after chicken and chicken broth consumption, anserine plasma concentrations were higher than the LOQ. This is in line with the findings presented in the second manuscript.

Parameter selection of fecal samples for identification of biomarkers

Feces is composed of undigested food, gut bacteria and other metabolites of the host's cellular components. This makes it an optimal biospecimen for non-invasive assessment of the whole bowel environment [93]. Studies that obtain fecal samples are important in assessing nutritional issues, e.g., several studies collected fecal samples to assess sterols, bile acids and fiber to identify a correlation between their intake and relevant diseases such as colon cancer [8,94,95].

However, metabolite profiling in feces is considered to be more complex than in any other biosample, since the physicochemical properties of the feces affect the reproducibility and full coverage of the obtained metabolite profile [96]. Nevertheless, lyophilization of fecal samples, extracted by water-methanol mixtures, facilitate the analysis of reproducible metabolite profiles that comprise a variety of compounds. Another limitation of fecal samples is that there are several different techniques of fecal sample collection and storage conditions that may alter the metabolite concentrations, such as instant freezing, freezing after cooling on ice or in refrigerator, or freezing after keeping it at room temperature [97]. For instance, in water extracts, the concentration of glucose and amino acids are higher in frozen samples than in fresh fecal samples [98].Alternatively, other studies immediately combine the collected fecal samples with a stabilizing agent such as RNAlater or Amis Transport Medium [99]. Loftfield et al., found that metabolomic measurements are stable and reproducible when fecal samples are collected with 95% ethanol or a fecal occult blood test (FOBT) [100]. However, standard fecal collection protocols are still required for the identification of dietary biomarkers in feces that can be obtained by optimizing and harmonizing sample collection, storage and processing procedures. Additionally, epidemiological studies also have to consider the day-to-day-variation in fecal samples. Therefore, a study suggested that fecal samples should be collected from each subject on 3-5 different days in order to cover not only differences in food consumption, but also alterations in stool transit time, gut microbiota activity, etc. [101]. The analytical tools used for fecal metabolite profiling, such as NMR, GC-MS, LC-MS, and LC-MS-MS should be chosen according to their coverage, dynamic range, selectivity, accuracy,

precision, and price per sample [102]. As fecal metabolites are highly diverse, more than one analytical method is needed to obtain a comprehensive metabolite profile [103].

Role of gut microbiota and metabolism on stool-derived dietary biomarkers

Epidemiology studies are interested in microbiome analysis in order to understand the interactions and relationships between dietary intake, host metabolism and gut microbiota [104].

The gut microbiota, one of the most densely populated microbial ecosystems in nature, is an extremely active metabolic "organ" that produces metabolites that influence physiological processes in the intestine and elsewhere. Furthermore, metabolites of the gut microbiota interact with the host's metabolic phenotype and may have an impact on human health and disease [104]. Therefore, several studies have examined the bile acid-gut microbiome axis to understand its role in colon cancer risk [105-107]. Other studies were interested in understanding the concept of how the intake of prebiotic alters the composition, metabolism, and function of the gut microbiota in order to enhance the gut and host health [108]. Still, it should be considered that not only dietary intake affects the gut microbiota composition and its metabolic activity, but also the physicochemical properties of foods, nutrient availability, colonic transit time, and age of the host [60,109].

Dietary intake can be identified by the metabolites produced by bacterial energy metabolism [34]. Metabolites such as minor food constituents that have been structurally altered by microbial activity might be identified in fecal samples, and can indicate the consumption of specific foods such as animal sterols and plant sterols. Sterols, bile acids and their metabolites are mainly excreted in feces, and their content and proportions are highly affected by the gut microbial composition and several other factors [110]. Recently, Ridlon et al. reviewed that the host and gut microbiome modulate bile acids. After the host produces a large pool of conjugated hydrophilic bile acids, the microbiota convert bile acids and their conjugates into a smaller pool of unconjugated hydrophobic acids [105]. In contrast, bile acids seem to be a major regulator of the gut microbiota as well. This was observed in a study searching for the relationship between the liver health, gut microbiota composition and fecal bile acid concentrations [111]. Diet also appears to modulate both the gut microbiome and bile acid

levels. A high fat diet may not only increase bile acids concentrations, but also modulate gut microbiota activity, resulting in the production of tumor-promoting substances from bile acids [89,112]. On the contrary, a high fiber diet dilutes the carcinogens and promoters by increasing the bulk of stool [113]. Additionally, it alters the metabolism of carcinogenic compounds.

Role of identification and application of dietary biomarkers in large epidemiological studies

Several calibration and validation methods that apply different references of dietary and biological measurements have been generated to overcome the problem of measurement errors in dietary assessment methods [114-116]. Additionally, an increasing number of metabolomics studies have been conducted over the past few years in the nutrition field [117,118]. Metabolomic techniques help in screening differences in metabolic profiles of specific nutrients or food consumption in intervention studies [10]. However, the food metabolome is complicated and highly variable, as humans consume different foods, comprising more than 25,000 compounds - most of which are then metabolized in the human body [119]. To achieve an accurate measurement of dietary exposure in epidemiologic studies, an accurate assessment method with minimized random and systematic errors is required. The conventional dietary assessment methods like dietary recalls or food frequency questionnaires used in population studies suffer from systematic and random errors as well as from recall bias and difficulty in measuring portion sizes [120]. Consequently, the misclassification of participants according to their dietary intake in epidemiological studies can affect the observed associations between dietary intake and disease outcomes, and may cause essential discrepancies in published findings in the field of nutritional epidemiology [121]. Therefore, the application of dietary biomarkers in nutritional epidemiology as an objective assessment of dietary intake is essential [14]. Few biomarkers of dietary intake have been utilized as surrogate biomarkers in population studies. For example, urinary polyphenols, plasma carotenoids and plasma vitamin C are proposed as biomarkers for fruit and vegetable intake [122,123]; fatty acids (e.g. plasma phythanic acid) and urinary and plasma amino acids are proposed for meat [77,124]; plasma alkylresorcinols for whole grain cereals [125]; and intake of fatty acids as plasma eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) for fish and plasma phythanic acid for dairy products [124,126].

Reliability of dietary biomarkers and its limitations

As discussed before, dietary biomarkers also have their limitations. They not only provide objective data on nutrient intake or status, but they also act as markers of other biological processes in the human body. Biomarkers are prone to variation due to potential interactions with genetic factors, health status, or factors such as age, sex or BMI [127]. Other factors such as diet and lifestyle (i.e. smoking and alcohol) [128] may alter the biomarkers as well. Therefore, these covariables have to be taken into consideration during the analyses by adjustment.

For short-term dietary biomarkers with a half-life of less than 24 hours, a higher intraindividual variability is presumed [129]. Habitual dietary intake measurement errors are likely to occur due to intraindividual variability when the habitual dietary intake in observational studies is to be assessed and small numbers of measurements across subjects are obtainable [10]. Therefore, these short-term biomarkers such as polyphenols, alkylresorcinols and amino acids [130,131] can be beneficial only in populations who consistently and frequently consume these dietary sources. However, long-term biomarkers such as carotenoids [132] and fatty acids [133] have a longer half-life ranging from weeks to months, which makes them appropriate for cohort studies.

Intervention studies are needed to measure the sensitivity and the concentration of dietary biomarkers upon consuming a specific diet. This information is needed before biomarkers can be utilized in population studies. Dietary biomarkers should have sufficient sensitivity and specificity to assess dietary intake. A very low or high concentration of dietary biomarkers is unreliable to assess a dietary intake of a specific food [10]. Further, some biomarkers can be highly specific to a certain food e.g., proline betaine for citrus fruits [134] and lycopene for tomatoes [135], or commonly found in several foods. Examples include vitamin C and carotenoids, which are found in several vegetables and fruits [135], or anserine or carnosine, which are characteristic of an entire food group (meat) [42].

Most biomarkers are still inadequate to meet the gold standard that reflects true intake. In these cases, concentrations of the biomarkers identified in biological samples vary between subjects despite identical dietary intake. Further, individual variations are present not only in

absorption, but also in rates of metabolism and excretion. Yet errors in biomarkers are independent of errors in other dietary assessment methods e.g., dietary questionnaires [136] . Accordingly, two properly selected biomarkers can be utilized to offer relatively unbiased estimates of correlations between dietary estimates and true intakes [137].

Strengths and limitations of the manuscripts

The analytic approaches used in this dissertation are a targeted LC-MS/MS approach in the second manuscript and a non-targeted UPLC-MS/MS approach in the third manuscript. Because of the metabolite diversity and the range of concentrations in which they may appear, there is no single technique capable of measuring the entire metabolome. Therefore, a number of these technologies are employed, although these approaches all have their advantages and limitations [138]. Of the two methods mentioned above, the non-targeted approach provides a broader coverage of known metabolites and allows for the discovery of new target metabolites [139]. Nevertheless, this approach requires a lot of time to process the extensive amounts of raw data produced and faces difficulties in characterizing the bias towards high-abundance molecules [140,141].

Both analytic methods are state-of-the-art with a fairly low limit of detection. Recent studies administered the same analytic method applied in the second manuscript for amino acids quantitation [68,73].

The strength of the second and third manuscripts is that they are the first studies that examined the association between habitual dietary meat intake and plasma carnosine, anserine, Π-MH and T-MH, and between habitual dietary intake and fecal concentration of animal sterols, phytosterols and bile acids in cross-sectional studies administering targeted or untargeted metabolomic technique.

The second manuscript included the results of only 294 participants who completed three 24-h dietary recalls and had a plasma sample volume that was sufficient for lab analysis. Differential bias is unlikely despite the fact that this study is not a random subsample. Due to its relatively good validity and measurement properties, the 24-h dietary recall is the favored assessment method of dietary intake at the population level [142-144]. This is why it was implemented in this study. Weighting factors were applied in order to account for consumption differences due

to the day of the week on which the questionnaire was completed. However, dietary intake as well as information on socio-demographics, lifestyle and disease status were assessed mainly in the second and third manuscript based on self-reports; therefore, misreporting cannot be excluded. Because certain food groups (such as pork and beef) are rarely consumed, the second manuscript was limited in its capacity to examine the effect of these meat subgroups on their related biomarkers. Further, it was not possible to assess short-term biomarkers of rarely consumed foods, as the food may not have been consumed on the day prior to blood collection.

In the third manuscript, dietary intake was comprehensively assessed in KORA FF4 participants using an advanced method combining the information of a FFQ and repeated 24HFLs so that a full range of food items could be examined. Nevertheless, since only habitual intake of food groups is analyzed in observational studies, it was not possible to examine special diets (with specific dietary patterns), which may be high in a specific food item, e.g. plant sterols.

Feces is the biospecimen of choice in identifying sterols, bile acids, and their metabolites, as they are mainly metabolized by the gut microbiota and excreted in feces [32,145]. Feces can also be easily obtained in a non-invasive manner. Yet the collection and storage of fecal samples has its limitations. As opposed to blood samples that are collected in the study center by trained personnel, fecal samples are usually collected by participants at home and kept cooled until their study center visit. Despite the fact that participants were well-instructed about the handling and storage of fecal samples, not all stool samples were correctly stored until being handed over in the study center. Therefore, all samples that were stored at room temperature for more than three hours were excluded. This cut-off was based on a study [100] that compared the concentrations of metabolites in fresh samples and samples stored under different conditions. Still, metabolite degeneration in the selected samples cannot be excluded.

Conclusion and future perspectives

The current Ph.D. project was designed to identify new biomarkers of habitual dietary intake in various biosamples in observational studies that can be used to find associations between dietary intake and chronic disease risk. It also aims to improve nutritional science by utilizing metabolite profiling to identify new dietary biomarkers.

The first manuscript provides an overview of the identification of dietary biomarkers utilizing different biosamples and techniques. Two different cross-sectional studies which differ in bio-specimen collection and analysis method were carried out to specify dietary biomarkers of meat intake in relation to the specific type of meat consumed, and to identify associations between dietary intake and fecal animal and plant sterols and bile acids. Metabolite profiling of stool was performed by non-targeted UPLC-MS/MS analysis. Quantitative measures of carnosine levels and its derivatives were obtained using targeted LC-MS/MS analyses. The analytic methods applied in this dissertation to quantify or identify the biomarkers in plasma and feces are state-of-the-art with a fairly low limit of detection.

This dissertation provides novel results, by demonstrating the association between usual meat intake and plasma concentrations in a cross-sectional study. Additionally, it related the concentrations of sterols in feces to habitual dietary intake in a population-based study. As plasma anserine, carnosine, and Π-MH are associated with meat intake, they seem to be markers for overall meat intake, rather than markers specific to a certain type of meat. Therefore, it may also be important to further investigate the use of combinations of biomarkers of meat intake to specify the particular type of meat consumed. Further, future intervention studies should reveal if the metabolites found in plasma are characteristic for a specific type of meat or not.

The third manuscript results revealed an effect of habitual diet on fecal concentrations of animal sterols, but the influence of diet on bile acids was limited in this study.
Further studies are essential for assessing fecal animal sterols as biomarkers of diet and to confirm the findings in other populations with different dietary habits.
A further question that should be explored is the possible health benefits or risks of a higher or lower fecal animal sterol content in response to dietary habits.

Finally, the findings of this dissertation in combination with findings from the existing literature and future studies should be used in identifying which dietary factors that are related to which diseases in the general population over the long-term. Biomarkers are essential to the objective measurement of nutrients status because the measurement error is a major limitation of conventional dietary assessment methods [7]. Dietary biomarkers require additional research

so that they can be better utilized and interpreted. High-quality dietary intake data, reliable dietary biomarkers, accurate metabolite profiling, and new statistical methods are urgently needed in epidemiological studies to uncover new diet-disease risk associations[14]. Biomarkers of dietary intake should be valid, reproducible, sensitive, and able to detect alterations in intake over time. Further, they should be applicable into the general population [7].

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Appendix

1. **Original Papers**

Publication 1
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Genes & Nutrition

REVIEW Open Access

Biomarkers for nutrient intake with focus on alternative sampling techniques

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Abstract

Biomarkers of nutrient intake or nutrient status are important objective measures of foods/nutrients as one of the most important environmental factors people are exposed to. It is very difficult to obtain accurate data on individual food intake, and there is a large variation of nutrient composition of foods consumed in a population. Thus, it is difficult to obtain precise measures of exposure to different nutrients and thereby be able to understand the relationship between diet, health, and disease. This is the background for investing considerable resources in studying biomarkers of nutrients believed to be important in our foods. Modern technology with high sensitivity and specificity concerning many nutrient biomarkers has allowed an interesting development with analyses of very small amounts of blood or tissue material. In combination with non-professional collection of blood by finger-pricking and collection on filters or sticks, this may make collection of samples and analyses of biomarkers much more available for scientists as well as health professionals and even lay people in particular in relation to the marked trend of self-monitoring of body functions linked to mobile phone technology. Assuming standard operating procedures are used for collection, drying, transport, extraction, and analysis of samples, it turns out that many analytes of nutritional interest can be measured like metabolites, drugs, lipids, vitamins, minerals, and many types of peptides and proteins. The advantage of this alternative sampling technology is that non-professionals can collect, dry, and mail the samples; the samples can often be stored under room temperature in a dry atmosphere, requiring small amounts of blood. Another promising area is the potential relation between the microbiome and biomarkers that may be measured in feces as well as in blood.

Keywords: Dried blood spots (DBS), Biomarkers, Lipidomics, Nutrients, Microbiome, Diet

Background

Reliable knowledge about the relationship between food intake and nutritional status is very important for improving the quality of nutritional research. Most data generated in large epidemiological studies in humans are based on memorizing or monitoring food intake from the participants [1, 2]. These methods are inaccurate and represent challenges concerning under- as well as over-reporting of certain foods [3]. Based on these facts, there is an urgent need for biomarkers of objectively describing both intake and nutritional status. Different omics analyses can be applied on all types of tissues and biological liquids to improve dietary assessments [4]. However, the use of objective biochemical variables is complicated by confounding factors. The amount and composition of a biomarker in tissues or blood depend on multiple processes such as digestion and absorption in the gastrointestinal tract, transport in the blood, uptake, distribution, and metabolism in a variety of cells, and excretion via the kidney and gastrointestinal tract. All these processes involve multiple gene products with polymorphisms potentially creating large individual variations [5]. Moreover, different physiological states like fasting feeding, cold, warm, resting, exercising, sex, menstrual cycle, pregnancy, lactation, and age might have effects on the lipid spectrum. Finally, the nutrient composition of ingested food, endogenous production of different molecules, flux into and out of various compartments in the body, and sampling time points, must be considered when omics data are interpreted. All these considerations make it likely to suggest that the rapid development of biomarker measurements to be discussed in this review will represent an important addition to the information obtain by classical methods

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for registration of food intake simply because the two approaches intend to monitor different variables (food intake with all its inaccuracies) and a resultant of many biological processes (biomarker measurement).

Definition of biomarkers

In the current context, we will apply the concept of biomarkers as a way to characterize objectively nutrient intake and or nutritional status. There is a distinction between nutrient intake and nutritional status as exemplified in the case of vitamin D. The best way to evaluate vitamin D intake objectively would be to measure vitamin D_2 and D_3 in blood. However, the concentration of these vitamins is so low in blood that it is not feasible to measure these molecules by available technology. It turns out that a hydroxylated derivative of vitamin D (25-hydroxy vitamin D, formed in the liver) is a sensitive marker of how much vitamin D is found in the body based on the two main sources, diet and sun exposure over a period of months [6]. Another example of the distinction between intake and status can be polyunsaturated fatty acids (PUFAs). The best way to evaluate intake of especially PUFAs is to isolate the triglyceride-rich chylomicrons in the time interval of 2–6 h after the meal. Although it is difficult to monitor the amount of fatty acids consumed, it is possible to have good estimates on the quality of fatty acids by gas liquid chromatography (GLC) in combination with flame ionization detection (FID) [7]. The status of PUFA in the body is obtained best by having samples of tissues with slower turnover than plasma lipoproteins, like red blood cells, as can be obtained in whole blood samples and thus on dried blood spots (DBS).

The ideal biomarker is:

a) Sensitive and specific for the nutrient or food it is supposed to monitor.
b) Reflecting the period of interest concerning health or disease. Often in biomedicine, the long-term exposure is the most important.
c) Unaffected by diseases or conditions of importance for metabolism of the actual nutrient. An example is plasma concentration of LDL-cholesterol, which is a risk factor for myocardial infarction, at the same time as a myocardial infarct by itself will reduce plasma LDL-cholesterol during the first days after infarction [8].
d) Unaffected by other environmental or genetic factors. Often this is impossible to avoid, but the actual factors should be characterized and adjusted for.
e) Inexpensive and reproducible to measure.

Many variables influence biomarker concentrations

It is important to note that the concentrations of biomarkers in body tissues are influenced by many factors like digestion, absorption, distribution, transport, storage, metabolism, and export, as well as dietary characteristics like matrix differences, physical activity, the microbiome, environmental temperature, the use of drugs, and the presence of diseases. All these phenotypic, genetic, and environmental factors may give other results than what are obtained in traditional dietary studies based on 24 h recalls or food frequency questionnaires (FFQ). Thus, it is essential to be aware that in addition to provide objective data on nutrient intake or status, measurements of biomarkers represent many more biological processes than just food intake.

Hypothesis-driven and data-driven search for biomarkers

The methods used to discover novel biomarkers can be divided into two categories: hypothesis-driven and data-driven [9]. Using the hypothesis-driven approach, prior knowledge might be obtained from food composition databases such as FooDB before methods are developed to measure the biomarker candidates of interest [9]. An example of the hypothesis-driven approach is the identification of pentadecanoic acid as a marker of dairy fat intake. Pentadecanoic acid is a saturated fatty acid with odd numbers of carbon atoms (15:0) and cannot be synthesized in the human body. However, pentadecanoic acid can be synthesized by the bacterial flora of the rumen of ruminants. Wolk et al. showed that the level of pentadecanoic acid in subcutaneous adipose tissue can serve as a marker of long-term milk fat intake [10]. Recently, the plasma phospholipid levels of pentadecanoic acid have been shown to associate with consumption of dairy fat and butter in adults [11]. The plasma or serum level of pentadecanoic acid represents a short-term marker for intake [12] and is inversely associated with type 2 diabetes [13]. The finding of a relationship between the plasma phospholipid levels of the trans-fatty acid elaidic acid (18:1, n-9) and intake of highly processed foods is another example of the hypothesis-driven approach [14]. Elaidic acid is generated during partial hydrogenation of vegetable oils and is used for the formulation of processed foods.

In the data-driven approach, there is no prior knowledge of the biomarkers. This makes the investigators measure as many lipids as possible, with the main limitation being the capacity of the analytical procedure. The recent study of Hanhineva et al. [15], studying the Nordic diet, is a good example of a successful data-driven approach. Using non-targeted LC-MS plasma metabolite profiling, in a randomized controlled trial with 106 participants assigned to three dietary interventions for 12 weeks, they identified several lipid species as potential biomarkers for fatty fish intake. The suggested biomarkers for fish intake included EPA, DHA, lysophosphatidylcholine (22:6 and 20:5), lysophosphatidyl-

ethanolamine (22:6 and 20:5), and the furan fatty acid 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF). CMPF was clearly changed and positively associated with increased consumption of fish, and using a stepwise linear regression model, they observed that plasma CMPF is an even stronger independent marker of fish intake than plasma EPA [15].

DBS technology

With modern technology, it is possible to measure accurately thousands of metabolites, as well as nutrients, in small biological samples. It is also possible to monitor hormones, peptides, and proteins to enhance the quality of nutritional evaluation. We will describe the principles for metabolomics, including lipidomics, in addition to measurements of different nutrient-relevant proteins and minerals.

With enhanced sensitivity for measurements of very small amounts of nutrients and other molecules, it is obvious that we do not need large biological samples but can obtain high-quality measurements based on small samples (microliters) collected by non-professional subjects, who are able to follow simple instructions. This will allow a marked reduction in costs and will make it much easier to collect samples from thousands of subjects, e.g., in remote study-fields.

DBS sampling has been used to screen newborn babies for metabolic diseases for more than 50 years [16]. A spot of blood from a heel stick is applied on a filter paper and allowed to air dry. A circular punch (about 3 mm) is removed, eluted, and analyzed for metabolic markers. More than 50 separate analytes can be measured from a quarter of a blood spot [17, 18], mainly due to adoption within the last decades of the high sensitivity of liquid chromatography (LC) combined with mass spectrometry (MS) (http://vitas.no/services/dried-blood-spots).

Advantages of DBS

As an important part of alternative sampling techniques, we will focus on DBS and similar alternatives because:

a) Sampling can be performed by non-professionals following simple instructions.
b) Sampling can be performed anywhere: in the field, classroom, gym, workplace, and before, during and after sports competitions.
c) DBS is close to being non-invasive.
d) DBS requires much less material (uL compared to mL) due to the improved sensitivity obtained with modern chromatography in combination with use of sensitive detectors like mass spectrometry, fluorescence, and flame ionization [19–21].
e) Sampling is much cheaper than classical blood sampling and does not require participation of health professionals.
f) Sample stability is often very good for DBS but has to be validated for each analyte.
g) Transport of samples is easier, is less expensive, and represents minimum biohazard compared to classical blood/plasma samples.
h) Samples are easy to store in tissue banks due to stability and small volume.
i) There is often no need for laborious blood processing before analyses.

Disadvantages of DBS

Due to the fact that the DBS technology is relatively new, much less experience is accumulated than for classical measurements in plasma or serum:

a) Less information is available on metabolites, nutrients, and proteins in the whole blood compared to classical plasma/serum samples.
b) Measurements of every new analyte collected by DBS have to be validated—i.e., accuracy, reference values, pathological values, and reproducibility should be established before the measurements can be fully interpretable.
c) Whole blood is much more heterogeneous than plasma/serum, including all components of plasma in addition to platelets and several cell types.
d) Some analytes are differentially distributed between plasma and blood cells. Potassium is a classical example of an intracellular mineral with 15–50-fold higher concentration in red blood cells than in plasma [22].
e) Small volumes of blood are available making it difficult to measure analytes with very low blood concentrations.
f) The quality of DBS can be poor because sample donors do not follow instructions concerning sampling, drying, and mailing.
g) Some donors are hesitant to perform their own finger-prick sampling.
h) The exact volume of blood might be difficult to obtain because the blood sample might be unevenly distributed on the filter paper.

Applications of DBS

The DBS technology has been used for clinical and pre-clinical pharmacokinetic studies, taking advantage of smaller samples and simplified sample collection and handling. DBS sampling has also been used for disease surveillance in developing countries [23], at home, in the pharmacy, in the gym, in sports competitions, and in large epidemiological studies [24–28].

Validation of DBS technology

The best way to obtain high-quality measurements is to be aware of the pitfalls in the procedure and carefully standardize all steps in the use of DBS technology [29].

Blood sampling

Cleaning of the finger, earlobe, or heel can be done with soap and clean water before the sampling place is dried. The sampling place (finger or heel most often) should be warmed in hot water (~40 °C) to enhance the capillary blood-flow and make sampling easier. It is also important to increase the pulse pressure by standing up during blood sampling. A safety lancet in the form of a needle or blade is used to penetrate the skin with a depth of 1–2 mm thereby cutting one or several capillary blood vessel. The initial drop of blood is dried off with a clean gauze pad because it may be contaminated with interstitial fluid [30, 31]. The free dripping blood drops are applied on the filter paper and should not be squeezed out blood by milking movements to avoid tissue fluid and hemolysis. Clotting, layering, or supersaturating the filter should be prevented. The predefined circle on the filter should be homogenously and symmetrically filled and both sides of the card/paper must show the same red color. Samples indicating contamination or hemolysis or with insufficient volume collected are not suitable [18, 29, 32] depending on the type of analysis.

In every procedure, we depend on a device to cut into the skin a few millimeters to get access to capillaries. There are many protected types of lancets available, which are released when pressure is applied on the lancet resting on the skin, thus providing blood drops for sticks or filter papers. Proper application of blood to the filter paper requires care to reduce artifacts due to uneven sample coverage; touching the paper, too large or too small blood drop, or too much time between the blood drops can make a sample unsuitable for analysis. These difficulties may limit the ability of non-professionals to self-collect samples from home or a remote location. However, in a large internet-based dietary intervention study named Food4Me, it was possible to collect thousands of samples by and from non-professionals with acceptable quality provided the instructions were properly communicated [33].

Sampling matrix

The filter paper should be standardized in terms of particle retention, pore size, thickness, and weight (grams per square meter). Most filter papers are cellulose filters, and the recommended filters have a CE mark from the European Union (EU). Typically a 1.2-cm-diameter circle holds 30–100 uL per spot. It is important to collect enough blood to fill a circle in one go to obtain an even distribution of blood with an even thickness. One of the authors (TEG) has extensive experience with the standardized Whatman DBS filter paper 903 (Guthrie paper) from General Electric (GE). Since the 1960s and until recently, the only devices available have been different versions of the Guthrie card, a cellulose-based paper card with a clean and reproducible surface allowing even distribution of the blood on the paper. However, in the last 6–8 years, a number of new DBS devices have been developed. Some of these devices are shown in Fig. 1. The newly developed devices include different types of material like strips, sticks, and pens.

The Mitra sticks (Fig. 1) seem to be especially promising because they have a clearly defined volume of blood adsorbed on the gel matrix representing exactly 10.2 uL. The whole collected sample is used in analyses, without relying on punching out a representative area that could contain an amount of blood that is not as expected from the punched area. Another advantage is that it is easy to load the Mitra sticks into a machine for robotic handling of the whole analytical procedure. HemaSpot (Fig. 1) is a cartridge containing an absorbent paper and desiccant. Once blood drops are applied, the cartridge is closed and the desiccant rapidly dries the sample. Thus, long exposure to air during drying, with possible airborne contamination, is avoided.

Strips (Fig. 1) are designed to overcome non-specific binding limitations of classical dried blood spot cards; special low retention absorbing material is used, which in turn, readily releases proteins, enzymes, antibodies, DNA, and nutrient biomarkers. Strips may have an advantage because less samples may be wasted, and drying

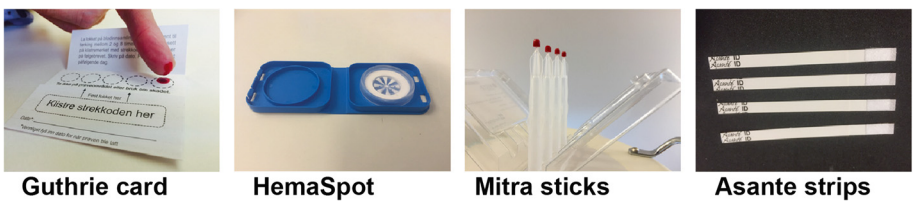

Fig. 1 Different devices for collecting capillary blood samples in small quantities by non-professionals

time is shorter than for classical DBS. In particular, strips have been used for sampling of blood for glucose measurements among subjects with diabetes [34]. Still, strips have for some reason been less used in recent times for new and more demanding analyses.

Pens can be used for finger-pricking, sample collection, and processing, and be integrated with commercially available paper-based assays [35]. This approach ensures safety and can be used by untrained end users in multiple settings. The pen format may provide low cost, high degree of safety, and robotization.

Sample drying

Drying the samples in air is quite important to improve the stability of most metabolites, nutrients, and proteins. Most biological molecules are often stable under dry conditions. Required drying time is 1–4 h at room temperature of filters loaded with blood to make the analytes stable. Glucose can be metabolized in wet samples for weeks after collection of samples, whereas dry samples keep a much more stable glucose concentration. It is important to keep the samples dry also during transport (see below). Current methods require that the blood spotted onto filter papers should be dried in open air for a few hours prior to shipping or storage. This exposes the sample to potential contamination from circulating air and from foreign surfaces. Dried DBS samples in this manner may be stored at room temperature for many weeks, months, or years [36], depending on the analyte stability. However, samples containing unstable compounds should be stored at a lower temperature (≤−80 °C); [37, 38] to enhance the stability. Moreover, the drying rate can be variable based on ambient humidity; a sample will dry much more quickly in an arid atmosphere (e.g., Arizona) than a humid area (e.g., Amazon). Samples have greater stability with rapid drying and storage in low humidity conditions [20].

Packaging, transport, and stability

Once the samples have been dried (usually 2 h at room temperature is sufficient), they should be placed in an airtight small aluminum envelope, with a small amount of dry silica to keep the humidity low during transportation. The small aluminum envelope can be placed in a regular postal envelope and sent by regular mail to the analytical laboratory for advanced analyses. Requirement for all analytes is that the stability should be evaluated during regular mailing/storage for up to 10 days, to make sure that the analyses are performed on high-quality samples.

Sample extraction

For water-soluble analytes, the most common solvent is water, whereas different organic solvents like methanol, isopropanol, and chloroform are used for lipid extraction. For lipidomics of fatty acids, transmethylation is often performed in parallel with the extraction [26]. For extraction of peptides or proteins from DBS, enzyme-linked immunosorbent assays (ELISA) are mostly used with special buffers designed for optimal detection by the actual antibody, whereas a lysis buffer is used as a solvent when HbA1c is measured.

Quality control

Many studies show that DBS sampling is compatible with, and equivalent to, current tests performed with fresh blood samples [39–43]. The accuracy and precision of a DBS LC-MS/MS method should be evaluated using quality control samples prepared at least at three different concentration levels (low, mid, and high), and analyzed along with a set of non-zero calibration standards in three separate validation runs. The lower limit of quantification (LLOQ) of samples must be assessed at least in one of the three validation batches. The intra- and inter-day accuracy, the bias (%) from the nominal concentration values, should be within ±15 % for all quality control samples except the LLOQs, for which a bias of within ±20 % is acceptable. The intra- and inter-day precision, assessed by the standard deviation divided by the mean coefficient of variation (CV%) from the replicate analyses, should be ≤15 % for the results of all quality controls except the LLOQs, for which a ≤20 % CV is considered acceptable [44].

Hematocrit is usually 0.41–0.51 for men and 0.37–0.47 for women [45]. The percentage might be out of the above ranges in certain populations, e.g., 0.28–0.67 for neonates (0–1 year old) and 0.35–0.42 for children (2–12 years old). Capillary blood tends to have a higher hematocrit (e.g., 0.61) than venous blood [46]. With high hematocrit, the viscosity of blood is enhanced and the diffusion of sample in the filter will be reduced, and the layer of blood will be thicker and the concentration of 31 amino acids and acylcarnitines was higher in the samples with highest hematocrit [47]. However, other studies do not show marked effect of hematocrit on 25-hydroxy vitamin D [48] or cyclosporine A [45]. Alternatively, MS signal for each lipid species can be standardized to the summed intensities of selected signals, providing a relative quantitation independent of blood volume and hematocrit level, as demonstrated by DBS studies of 3 months old infants compared with 12 months old children [49, 50].

The volume of the blood spot may also influence analytical results [44]. For every new analyte, there should be performed quality controls where the relationship between DBS area/weight and the amount of blood spotted on the paper should be examined by spotting increasing volumes of blood on the paper, and measuring the areas

of the obtained spots [51], or weighing the obtained spots [52].

Chromatographic effects may also cause skewed distribution of blood and/or analytes on the filter paper. This is another factor that might cause significant differences in the measured analyte concentrations between central and peripheral areas within a spot. Different results have been reported depending on the analytes [47, 53]. During assay method development, it should be assessed whether the same analyte concentration could be measured from punches in different locations of the filter at different concentrations. Analyte classes with successful recovery and analysis from DBS include many metabolites (Table 1).

DBS differs from blood plasma or serum samples mainly with respect to the presence of white and red blood cells. White blood cells make up only 1 % of the blood, whereas the red blood cells can vary between 30 and 70 % of the blood. The red color of blood is caused by the heme-iron complex and will interfere with many of the classical clinical chemistry methods using specific reagents in colored reactions detected by colorimetry. Thus, most analytical methods for DBS rely on separation of the analytes to be measured by means of chromatography, mass spectrometry, or antibody-based extraction. There is also a possibility of forming complexes with color that do not interfere with the heme complex or they may have fluorescent properties.

LC-MS/MS may be used for several types of analyses to measure the concentration of many metabolites like prostaglandins [28]. Solid-phase extraction (SPE) and liquid chromatography/tandem mass spectrometry (LC/MS/MS) may be used for the extraction, separation, and detection of 8-epi-PGF2α in DBS. Li and Tse [44] reviewed several aspects of DBS sampling in combination with LC-MS/MS, in particular focusing on lipid analyses and several lipophilic drugs.

In sandwich ELISA, a primary antibody is immobilized to the bottom of the sample container and the biomarker of interest is bound, whereas other constituents including the red color of heme are washed out. A secondary antibody is added that binds to the biomarkers and provides a chromophore, which can be measured by UV absorbance, fluorescence, or chemiluminescence. Many proteins, and some smaller molecules, can be measured by ELISA, which exhibits better specificity towards proteins than small metabolites like amino acids or drugs [54, 55].

Once a DBS sample has arrived in the laboratory for testing, a small punch (3–6-mm diameter) is taken from the card, eluted in a relevant solvent, and analyzed by a proper analytical method. The blood spot must be examined carefully to ensure that the sample punch is taken from a representative area. Uneven sample coverage due to poor application, variable hematocrit levels, or chromatographic effects may cause variable analytical results. Although the vast majority of analytical methods can be used with DBS, analyses requiring whole cells or volatile analytes are incompatible with DBS.

Table 1 Many types of metabolites, peptides, and proteins can be measured using DBS technology [29]. Examples of analytes measurable by the dried blood spot technique

Analytes class	Typical analytes
Small molecules	Amino acids, drugs, hormones, peptides, lipids, vitamins, minerals
Nucleic acids	DNA, miRNA, mRNA, RNA, virus
Proteins	Hemoglobin, cytokines, adipokines, myokines, thyroglobulin
Drugs	Anitepileptics, chloroquine, cyclosporin, gentamycin, paracetamol

Lipid profiling using classical lipidomics

Lipids include several classes of metabolites defined as substances extracted by organic solvents. They have several structural functions in cell membranes, lipid droplets, and lipoproteins [56]. Moreover, functional roles of lipids include membrane fluidity and microenvironment, signaling via eicosanoids and lipokines, ligands for transcription factors, and interaction with proteins based on hydrophobic as well as covalent bonds, and they often are important energy sources [5]. Whereas most lipids can be synthesized in the body, some fat-soluble vitamins and polyunsaturated fatty acids are essential, such as vitamin A, D, E, and K, and linoleic (omega-6) and alpha-linoleic acids (omega-3). These nutrients have to be obtained from the diet for mammals. One classification system divides the lipids into eight classes: fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids, and polyketides [57]. Lipidomics represent the large-scale study of lipids present in a given cell, tissue, or organism at a defined time-point. It can be used to relate variation in lipid composition in biological samples to consumption of specific lipids, foods, or diets [4, 15, 58, 59].

Dietary lipid biomarkers in tissues, plasma, and sera

A variety of tissues and plasma/sera specimens have been studied in search of biomarkers for intake of dietary lipids [60–62]. Adipose tissue and plasma are the most studied biological samples concerning biomarkers for dietary fatty acid intake, and they are considered the biological samples to choose for the study of relative intake of PUFA [63]. The composition of fatty acids in adipose tissue is to some extent determined by the habitual fatty acid intake [64, 65]. This is due to the slow turnover of fatty acids in the adipose tissue as well as in

red blood cells. The half-life of fatty acids in adipose tissue is estimated to be between 6 months and 2 years [66–68]. Direct measurement of lipid age in subcutaneous fat using a ^{14}C method, showed a mean lipid age of 1.6 years, which is consistent with a half-life of approximately 400 days [66–69]. Whereas the fatty acid composition of stored triglycerides is influenced by diet, the structural lipids in adipose tissue seem to be less influenced because of special functional requirements [64]. The fatty acid pattern of plasma phospholipids and cholesteryl esters is mostly reflecting the dietary intake of the past few weeks [70]. After 14 to 20 h of fasting, the plasma free fatty acids (also called non-esterified fatty acids) composition is dominated by the release of fatty acids from adipose tissue [71]. Thus, the free fatty acid composition of plasma from a fasting individual may serve as a surrogate for the fatty acid composition in adipose tissue. As an example, Leaf et al. [65] found correlation coefficients of 0.94 and 0.83 between adipose tissue and plasma phospholipid fractions of eicosapentaenoic acid (EPA) and docosahexaeonic acid (DHA), respectively. The red blood cells (RBC) may be a useful long-term marker of fatty acid intake, as the RBC turnover is 120 days [72]. Thus, when dietary information is collected to be compared with lipid composition in biological samples, the time frame must be considered [59].

Exogenous fatty acids as biomarkers

Exogenous fatty acids (not de novo synthesized) serve as the best candidates for dietary biomarkers. Although biomarkers representing dietary intake of total fat and saturated fatty acid (SFA) have demonstrated conflicting results [73], PUFA and monounsaturated fatty acids (MUFA) measured in adipose tissue and plasma appear to be more valid [63]. The fact that the total pool of fatty acids in circulation represents both de novo synthesized (endogenous) and dietary (exogenous) fatty acids has made it difficult to find biomarkers for total fat intake [63]. However, one study showed that the combined changes of a group of fatty acids in RBC, plasma phospholipids, and cholesterol esters, in response to a low-fat or moderate-fat diet almost perfectly discerned between the total fat consumptions [72]. The same authors reported systematic increase in many endogenous fatty acids in response to a low-fat diet, despite reduced consumption of these fatty acids [72]. It has also been shown that high carbohydrate diets promote increased de novo synthesis of palmitic acid [74]. A recent study showed that the level of pentadeconic acid (15:0) in plasma and RBC reflected saturated fatty acid intake within an 8 weeks period [75]. Interestingly, there was no change in pentadeconic acid content in adipose tissue triglycerides [75]. Thus, future studies investigating changes in dietary intake of saturated fatty acids for up to 2 months might concentrate on plasma or RBC, as can be obtained in DBS.

Dietary interventions and observational studies

The pattern of dietary PUFAs correlates with the fatty acid pattern in plasma and adipose tissue in dietary interventions as well as observational studies [58, 65, 76]. Already in 1966, Dayton and colleagues showed in a group of 393 institutionalized men, that increasing dietary intake of linoleic acid from 11 % to almost 40 % of total fatty acids, enhanced percentage of linoleic acid in serum as well as adipose tissue [58]. The content of linoleic acid in lipids from adipose tissue increased from 11 to 32 % after 5 years with the diet high in linoleic acid [58]. Supplementing the diet with marine n-3 fatty acids for more than 12 months caused enhanced incorporation of EPA and DHA into adipose tissue fatty acids [65]. In a study comparing Greenland Inuits and white Danes, it was shown that the Inuits had a higher concentration of EPA in plasma, probably reflecting their much higher consumption of very-long-chain n-3 [76]. Andersen and colleagues observed a correlation coefficient of 0.51 and 0.49 between dietary intake of EPA and DHA and corresponding plasma phospholipids, respectively [4]. Finally, the sum of EPA and DHA in RBC membranes is often called the Omega-3 Index. This index has been shown to discern between different dietary intake of EPA and DHA [77]. However, the omega-3 index might not be better than measuring EPA and DHA in plasma phospholipids or whole blood [77].

Fatty acid profiles based on DBS

The search for efficient biomarkers has been hampered by the fact that most studies are relatively small scale. For the last 10 years, the lipid profiling assay developed by Marangoni and coworkers has been extensively tested in field studies [78]. The Marangoni-assay includes drying a blood drop on a filter paper strip containing the antioxidant butylated hydroxytoluene (BTH). The paper strip can be stored, then subjected to transmethylation (HCl and methanol) at high temperature, which will methylate fatty acids for GLC-MS. Large-scale cross-sectional studies with several thousand participants have been performed [79, 80], as have studies under field conditions in Cambodia and Tibet [81, 82] with several supplement interventions and validations (Table 2).

Furthermore, method development has revealed and resolved several methodological challenges [39–42], providing a more robust method for future nutrient analyses. Recently, a breakthrough paper described DBS stabilization by chelators, which seemed to eliminate iron-promoted oxidation of PUFA, resulting in an excellent correlation ($r = 0.97$) between venous blood samples and DBS samples [43].

Table 2 Overview of studies measuring FA lipid profile by dried blood spot technique

Author	Subjects	Objective
Marangoni (2004) [78]	100 (46M, 54F)	Founder paper establishing method
Agostoni (2005) [91]	39 (22M, 17F) + 95 controls	Study infants of smoking mothers
Agostoni (2007) [81]	191 (100M, 91F) + 21 Italian controls	Intervention Cambodian infants (12 months)
Marangoni (2007a) [83]	10 (5M, 5F)	Walnut intervention (3 weeks)
Marangoni (2007b) [86]	108 (47M, 61F) - 10 (5M, 5F)	Cross-sectional study PUFA intervention (21 days)
Agostoni (2008) [90]	106 + 53 controls	Study infants of smoking mothers (follow-up of Agostini (2005) [89])
Risé (2008) [82]	13 (13M, 0F) + 14 Italian controls	Diet and FA profile study of Tibetians
Agostoni (2011)	16 pairs	Study of whole blood FA in infant, cord and mother
Saga (2012) [80]	3476 (1463M, 2013F)	Cross-sectional study of FA profile in Scandinavian population
Risé (2013) [79]	1835 total	Cross-sectional study of PUFA
	- 81 infants	
	- 728 children	
	- 434 adults	
	- 592 elderly	
Hinriksdottir (2015) [92]	52 (19M, 33F) + 25 controls	PUFA enriched fish meal intervention

Small scale nutritional studies by DBS

Four walnuts per day, containing 1.2 g ALA and 4.4 g LA, for 3 weeks, favorably affected the n-3 LC-PUFA status of volunteers ($n = 10$) [83]. Time course of measurements included 2 weeks run-in period and a 2 weeks washout period. However, the high EPA values are in contrast to two other studies with larger doses of walnuts [84, 85].

A cross-sectional study investigated the fatty acid profiles in a drop of blood from a fingertip and correlated with physiological, dietary, and lifestyle parameters in volunteers. A total of 108 healthy volunteers (47 males, 61 females), including 8 pregnant women, were questioned for dietary and lifestyle habits for the last 3 days and blood collected. In addition, 10 volunteers ingested either capsules (350 mg EPA, 300 mg DHA) or 200 g/week of smoked salmon, for 3 weeks [86]. These early studies indicated that the DBS method was suitable for cross-sectional studies as well as supplementation studies.

Population screening by DBS

The Marangoni DBS technology was early used for screening fatty acid profiles under field conditions in less developed countries like Cambodia [81] and Tibet [82]. The effects of supplementation of two micronutrient powders on fatty acid status in Cambodian infants ($n = 204$) were compared in a 12 months intervention [81]. The fatty acid profiles of blood in a Tibetanian population ($n = 13$, Italian controls $n = 14$) were significantly correlated with dietary fatty acid patterns from the same population [82].

In a large cross-sectional study on blood from Italian infants, children, adults, and elderly, different patterns of n-6 and n-3 PUFA levels were observed. Data from four cohorts of Italians ($n = 1835$) were pooled in four age groups: 4 days old, 2–9 years old, adults (40–59 years), and elderly (60–79 ears) [79]. This study showed that the Marangoni-assay could be used in a large cross-sectional study. The study also showed that the DBS assay may allow detection of distinct PUFA profiles in new born infants. Large cross-sectional population studies in Norway and Sweden ($n = 3476$) have shown that food supplements like cod liver oil are in common use especially in middle-aged and older subjects, with marked influence on the fatty acid profiles [80].

Ethics and lipid profiling of children

Samples of relatively small DBS from finger- or heel-pricks represent an important development due to the increased range of experiments that can be performed in an ethical way, like screening of infants [79, 87], very old patients [88] or disadvantaged, and cognitively challenged small school children [89]. Agostoni and coworkers reported a 24 % reduction in DHA (22:6) in children with mothers smoking throughout pregnancy ($n = 159$) [90]. The study was a follow-up of a smaller study observing the same effects ($n = 19$ smokers + 20 first trimester smokers + 95 reference controls) [91]. In a recent cross-sectional study 493 school children, aged 7–9 years,

provided blood fatty acids obtained from finger-prick samples; the results showed that low blood n-3 PUFAs was associated with poor cognitive performance and behavior [89].

Commercial applications of DBS

The ease and flexibility of sampling blood using DBS technology have revealed new commercial applications, such as demonstrating the bioavailability of long-chain n-3 PUFAs in fish oils. To fortify foods with PUFA from marine sources has remained problematic, due to the strong odor and taste. Hinriksdottir et al. added flavor-neutral microencapsulated marine fish oil to meals and compared with meals fortified with liquid fish oil and placebo control meals. Icelandic individuals ($n = 99$) were studied in a 4-week double-blinded dietary intervention in three groups [92], demonstrating that bioavailability of PUFA in encapsulated powder is very similar to meals enriched with liquid fish oil.

The supplement industry has also used DBS technology to demonstrate bioequivalence of different n-3 supplements [40, 93]. In the latter study, the n-3 fatty acid status of 50 young men was determined. In 10 individuals, the effect of supplements was investigated with time course from 2 to 24 h [93]. In times of increased competition for consumers and increased demands for new products, the demonstration of supplement efficacy might be an important competitive advantage.

Cardiac disease studies using DBS

In a 3 g per day, PUFA supplement study of cardiac patients, using DBS technology, no effect on atrial fibrillation was seen after 6 months. The study was a randomized, double-blind, multicenter study including 204 Italian patients [94].

In another Italian study of patients with a recent myocardial infarction, matched case and control pairs ($n = 112$) showed that whole blood n-6 and n-3 PUFA levels were reduced. Using food frequency questionnaire (FFQ) demonstrated for 86 cases and 72 controls significant correlations between reported fatty acid intake and measured fatty acid pattern from DBS [95]. In contrast, another study applying DBS-based analyses showed no difference between fatty acid profiles of patients with arrhythmia without or with myocardial infarction and controls at hospital admission [96].

Methodological challenges and refinement in lipid profiling using DBS technology

High-throughput DBS analyses

DBS technology was originally used for screening of genetic diseases and mailing the samples to core laboratories [97, 98]. Stabilization of PUFAs by butyl hydroxyl toluene (BHT) has been successfully used [99]. Several researchers have increased the throughput of the fatty acid analyses using microwave oven transmethylation [100, 101]. Improved methodology allowed more frequent sampling and time series of fatty acid profiles in a fish oil supplementation study ($n = 16$) over 4 weeks and a washout period over 8 weeks [101]. In a study of soldiers ($n = 287$), fatty acid profiles were reported to be available 1 h after finger-prick sample collection [102].

DBS method challenges and refinement

Some scientists have advocated BF_3 use for transmethylation, but clear superior results using BF_3 have not been demonstrated [100]. The less strict necessity of fasting blood samples is an important issue in self-administrated tests. Stark and coworkers [103] reported excellent stability of blood samples on paper immersed with BHT over 8 weeks at room temperature, although other scientists observed lower stability, in particular for DHA [41].

The importance of chelators

Interestingly, Stark and coworkers [104] demonstrated a striking difference between DBS samples stored with or without BHT at −20 °C. In contrast, storage at room temperature, 4 and −78 °C showed little or no effect, which may suggest that ice crystal damage to membranes and release of chemicals within cells has important consequence for fatty acid stability. Metherel et al. in a follow-up study demonstrated that loss of PUFA probably was due to release of iron from heme in erythrocytes and advocated glycerol addition for freeze-protection of RBC [42].

A recent study reported that adding chelators (such as EDTA) to the DBS papers increased correlations between stored DBS samples and venous blood control samples markedly ($r > 0.97$) [43]. Similar improvement was also observed when ascorbic acid was added to DBS filters and during extraction, to improve the stability of vitamin A [105].

DBS profiling of vitamins A and D

Vitamin A deficiency has long been recognized as a major cause for blindness among children in developing countries and to increased risk of infectious diseases [106, 107]. The use of DBS technology for population screening and monitoring of vitamin A status is often used in developing countries. The National Facility for DBS Technology for Vitamin A Estimation (Hyderabad, India) has carried out a vitamin A symptom study of 8777 pre-school children. A sub-group of 407 children with symptoms had DBS samples analyzed, finding vitamin A deficiency, particularly among rural children 3–5 years of age, and of lower socioeconomic class [108]. In a study in West Bengal, of 9228 children, 590 children had vitamin A deficiency, with higher incidence

among boys than girls, and increasing deficiency with age [109].

A study in Guinea-Bissau of 1102 children (6–24 months of age), using DBS combined with ELISA for retinol-binding protein, observed a high prevalence of "vitamin A deficiency" (defined as RBP concentration equivalent to plasma retinol below 0.7 μmol/L) varying with season, ethnicity, and vaccination status [110]. A higher prevalence of vitamin A deficiency was found in children with infection, which is consistent with a study in Uganda of 661 children (6–59 months of age), demonstrating that infection status (measured by C reactive protein (CRP)) influenced the ELISA values for retinol-binding protein. Another interpretation is that dietary vitamin A deficiency causes reduced immune function.

Commercial offers for measuring vitamin A status are available [111–113]. However, there exists some controversy on the efficacy of sampling and extraction techniques [114]. A recent paper obtained a high correlation ($r = 0.97$) between venous blood and DBS samples from healthy subjects ($n = 24$) using acidic extraction [105]. Similar results were demonstrated 30 years ago, where the loss of vitamin A in serum samples was eliminated when adding ascorbic acid before extraction [115].

Vitamin D is linked to rickets, skeletal deformities, and bone disease. More recently vitamin D deficiency has been suggested to increase risk of many chronic diseases such as certain types of cancer, autoimmune diseases, cardiovascular diseases, and diabetes [116, 117], although intervention studies do not support the results based on observational, epidemiological studies [6].

An early study to optimize DBS technology for neonatal 25-hydroxy vitamin D status was performed by Eyles et al. [118, 119]. In a study of 118 archive samples stored up to 22 years, clear seasonal variations were detected but no annual variation, suggesting that DBS technology is reliable and promising for investigation of archive material [118]. In a follow-up study, neonatal cord serum and matched DBS samples ($n = 100$) were compared, finding them to be highly correlated ($r = 0.85$) [119].

Validity and reliability of the DBS technique was further investigated in plasma and matching DBS samples ($n = 62$) [120]. Commercial kits for DBS for vitamin D are available [121]. The feasibility of self-sampling of blood and saliva on filters was studied in a Norwegian breast cancer screening program ($n = 381$), reporting that postal service transport was efficient and low cost [24].

There are rather few studies using DBS in studies of older individuals [17]. Vitamin D status of seniors (> 60 years old, average age 72 years) was studied in 224 subjects in the ethnically diverse Older Adult Centre in Toronto [122]. No major differences between ethnic groups were found, although women had higher vitamin D status than men. Supplements were identified as the major factors responsible for the uniformly high vitamin D status. The concentration of 25-hydroxy vitamin D in blood has also been found to correlate negatively with cortical thinning in the brain during normal aging [26].

The vitamin D status in older adults in Toronto [122] contrasted starkly with a study of vitamin D status in young adults ($n = 351$), which showed that subjects with South Asian and East Asian ancestry had substantially lower 25-hydroxy vitamin D concentrations than subjects with European ancestry [123]. However, vitamin D status in 185 pairs of adolescent twins (average age 16 years) was found to be highly heritable (0.86) [124]. In a global perspective, the socioeconomic factors of vitamin D status evaluated by DBS sampling was emphasized in a cross-sectional study in rural Nepal, where 280 healthy children (12–60 months of age) were screened, reporting widespread (> 90 %) vitamin D deficiency [25].

Water-soluble vitamins

B-vitamins

DBS has been used quite successfully for measurements of folate [125], 5-methyltetrahydrofolic acid [126], as well as a sensitive marker of vitamin B_{12} deficiency, methyl malonic acid (MMA) [127]. Scolamiero et al. [128] screened 35,000 newborns over 6 years using DBS. Those showing altered propionyl carnitine (C3), 10 % of the subjects, underwent second-tier testing of MMA, finding 7 cases of acquired vitamin B12 deficiency. Algorithms, combining input for several analytes and genetic disease models, from very large data sets, have been developed. For example, Weisfeld-Adams et al. [129] reported on screening of 1,006,325 infants in New York from 2005 to 2008, in which 10 cases of confirmed cblC mutations causing vitamin B_{12} metabolism disorder were found. DBS data were retrospectively studied to validate the algorithm [129].

C-vitamin

Vitamin C, or a range of carotenoids and flavonoids, has been widely used as general biomarkers of intake of fruit and vegetables [9, 130]. We have not found any studies using DBS to measure vitamin C.

Amino acids, proteins, minerals, and trace elements

Biomarkers of protein intake

An assessment of protein intake has been used extensively to determine nutritional status in subjects at risk of undernutrition, as well as among various patient groups, e.g., those with chronic renal disease, obesity, or in need of energy restrictions [131, 132]. The classical way to evaluate protein status has been to study nitrogen

balance, in particular the urinary output of nitrogen. Bingham [133] reviewed the use of urine nitrogen as a biomarker for dietary protein intake and concluded that the method was reliable and inexpensive. However, it is a tedious and inaccurate procedure as it involves at least one, but preferably several, 24 h samplings of urine, and the study subjects should be in nitrogen balance. Moreover, measurement of urinary nitrogen tends to underestimate protein intake at high levels and overestimate at low protein intakes.

Measurements of single protein molecules have been used to assay whole protein intake and protein status. Among the most widely studied are prealbumin and albumin, which are produced endogenously by the liver. Recent data have discredited the use of albumin, in particular, as biomarker for protein intake, as it seems to be markedly affected by coexistent morbidities, especially in cancer and inflammatory disorders, as reviewed by Lee et al. [134]. Creatinine, creatine, and transferrin are other candidate biomarkers for protein intake, but clinical studies have not shown reproducible results [135, 136].

Meat is among the protein rich foods, and several biomarkers have been used to determine protein intake following meat consumption. Cross et al. [135] performed a randomized crossover study feeding 17 adults with various types of red meat for 15 days. Based on urinary excretion, they concluded that 1-methylhistidine and 3-methylhistidine were good biomarkers, which is in line with the reviews provided by Dragsted [136] and Scalbert et al. [9].

Petzke and Lemke [137] used a different approach to estimate protein intake, namely by determining hair isotope compositions. They studied if additional meat intake (200 g pork fillet/day) or omission of meat and meat products had an impact on ^{15}N and ^{13}C within 4 weeks in hair and plasma of young women. They concluded that hair protein ^{15}N and ^{13}C abundances take more than 4 weeks to show animal protein intake, in these women with a habitual daily protein consumption of more than 1 g per kg body weight. Stable isotope ratio analysis at natural abundance in human hair protein offers a non-invasive method to reveal information about long-term nutritional exposure to specific nutrients, including proteins [137]. However, the use of isotopes in hair as biomarkers of protein intake requires more testing, in particular in randomized intervention studies.

Biomarkers of amino acid intake

Among the about 100 amino acids found in nature, 20 of them serve as building blocks and metabolites used for energy and in signaling pathways in humans, 8 of which are essential and have to be supplied in the diet. Traditionally blood and urinary concentrations of amino acids have been used as biomarkers of their intake. Recent methodological advances have also made it possible to assess local amino acid contents in hair [138] and locally in various organs, e.g., in neuronal tissues [139]. The advent of DBS to collect and store blood samples has opened opportunities to assess biomarkers in vulnerable populations, like premature infants and in populations in developing countries where access to freezers is limited. High-performance liquid chromatography and tandem mass spectrometry can be used to assess the amino acid concentrations from DBS and with satisfactory results [140].

Historically, the importance of amino acids has mostly been related to disorders due to deficiencies in amino acid metabolism such as maple syrup disease and phenylketonuria. However, there is increased focus on the use of amino acids including the claimed benefit of branched amino acids (leucine and isoleucine) to enhance physical performance [141], as a risk factor for cardiovascular disease (L-arginine) [142], and to improve cognitive function (tyrosine) [143]. Given the multitude of functions amino acids, more studies are warranted to delineate how well blood concentrations of amino acids and urine reflect subcellular levels of different amino acids.

Iron

Iron is part of the heme molecule, which is an integral component of hemoglobin. In addition, iron is an important constituent of enzymes such as in the mitochondrial respiratory chain (cytochromes). According to the World Health Organization iron deficiency anemia still ranks among the top 5 causes of years lost to disability globally (http://www.who.int/maternal_child_adolescent/epidemiology/adolescence/en/) and continues to be a problem among adolescent girls living in developed regions [144].

The body stores of iron can be used as a proxy of long-term iron intake and can be determined in various ways. The classical way of estimating body iron content is the assessment of iron in bone marrow using light microscopy, although this approach yields only semi-quantitative estimates. To evaluate iron overloading, in particular in patients receiving frequent blood transfusions, imaging techniques like magnetic resonance imaging (MRI) have been used [145]. However, these are tedious procedures, and hence blood biomarkers are much more frequently used. Serum levels of ferritin are in most cases a reliable estimate of body iron stores, although it is affected by a range of concomitant disorders, in particular inflammations. Serum levels of ferritin also increase with age. Complementary to ferritin is the ratio between serum levels of iron and its transport protein transferrin, denoted iron-, or transferrin saturation.

This is also affected by individual health status and pregnancy. It is debated whether serum ferritin levels and transferrin saturation levels correlate [146]. To circumvent these pitfalls, the use of serum levels of soluble transferrin receptor has gained increasing attention, as this is mostly independent of coexistent disease. Cook et al. [147] found excellent correlation between whole body iron content (measured according to known ferritin and hemoglobin levels after phlebotomy in healthy subjects), and the ratio of soluble transferrin receptor to ferritin; the latter two biomarkers being measured by ELISA.

Ferritin as well as transferrin receptor can also be measured using DBS with accuracy comparable to whole plasma values [148]; this facilitates measurements of iron status in remote areas where anemia is frequent. For example, the use of DBS to measure soluble transferrin receptor and hemoglobin was successfully applied among pastoral women of fertile age residing in rural North-Kenya [149].

Selenium

This trace element is mostly found in enzymes involved in the human antioxidant defense system. Deficiency of selenium has been linked to many conditions including cardiovascular diseases and different forms of cancer. Many grain-based foods contain selenium, although its availability depends on the concentration of selenium in the soil. Vacchina et al. [150] recently described a method to assess selenium using mass spectrometry following acidic digestion of the DBS. In addition to plasma selenium, selenoprotein 1 and glutathione peroxidase activity are responsive to changes in selenium intake. However, their use as biomarkers for selenium intake is limited by inconsistent response to selenium intake [151] and might be explained in part by ethnic differences [152]. Moreover, Ashton et al. [151] concluded that there was insufficient evidence to assess the usefulness of other biomarkers of selenium status, including urinary selenium, plasma triiodothyroxine/thyroxine ratio, plasma thyroxine, plasma total homocysteine, hair and toenail selenium, erythrocyte, and muscle glutathione peroxidase activity. Currently, no biomarker is available reliably mirroring (i) variable selenium intake and (ii) selenium intake in different subpopulations.

Zinc

Zinc exerts several functions, including stabilization of membranes, co-factor of transcription proteins, and as part of metalloproteinases. The plasma/serum levels of zinc and the erythrocyte zinc content have traditionally been the most common ways to evaluate zinc intake. The WHO report from 2007 on intake required to prevent zinc deficiency recommend serum levels of zinc as a biochemical marker of zinc status [153]. However, results are conflicting regarding their sensitivity to low and high zinc intake [154]; thus, new biomarkers for zinc intake are needed. Recently, Reed et al. [155] used a chicken model (*Gallus gallus*) to propose the erythrocyte linoleic acid/dihomo-γ-linolenic acid ratio as a sensitive biomarker of alterations in zinc intake. This was based on previous findings that this broiler chicken is sensitive to dietary intake of zinc [156]. In addition, a similar membrane fatty acid composition has been reported in mammals, which makes it possible to take advantage of a link between the ratio of these two essential fatty acids and mineral intake to evaluate zinc status [157]. The DBS method developed by Vacchina et al. [150] can also be used to assess zinc.

Magnesium

Magnesium is an important component of bone and plays a role in energy metabolism and protein and nucleic acid synthesis and is a co-factor for many proteins and hormones. Magnesium is mostly located intracellularly in spite of the fact that measurements of serum and urine levels of magnesium are usually performed to evaluate magnesium intake. Notably, ethnic variations and concomitant intake of other trace elements like sodium and calcium may affect these measurements [158]. Another less studied biomarker of magnesium intake is the content of magnesium in erythrocytes [159]. Witkowski et al. [160] performed a systematic review of analyses of 20 biomarkers of magnesium intake and concluded that the serum or plasma magnesium concentrations, erythrocyte concentration, and urinary magnesium excretion responded to dietary manipulation and could be used as biomarkers. We have not been able to find any scientific articles published concerning measurement of magnesium applying DBS technology although preliminary data show that whole blood analyses are feasible.

Chromium

Chromium is important for the metabolism of glucose, protein, and lipid by virtue of its action as co-factor for several enzymes. To evaluate exposure to chromium in foods and liquid intakes, measurements of both plasma and hair have been used. Sazakli et al. [161] performed a population-based cross-sectional study of chromium exposure and intake in Greece, a country with higher than WHO-recommended levels of chromium in the drinking water. Both the plasma concentrations and the hair levels of chromium were associated with intake in different Greek regions. Urinary chromium may not be a valid biomarker for chromium intake [162]. There is a need for better biomarkers of chromium intake [163]. We have not been able to find any scientific articles

published concerning measurement of chromium applying DBS technology although it should be feasible.

Fluoride

Fluoride is associated with dental enamel and bone density. Little is known about useful biomarkers of assessing fluoride intake. Rugg-Gunn et al. [164] concluded in their review that: "While fluoride concentrations in plasma, saliva, and urine have some ability to predict fluoride exposure, present data are insufficient to recommend utilizing fluoride concentrations in these body fluids as biomarkers of contemporary fluoride exposure for individuals. Daily fluoride excretion in urine can be considered a useful biomarker of contemporary fluoride exposure for groups of people." We have not been able to find any scientific articles published concerning measurement of fluoride applying DBS technology although it should be feasible.

Mercury

Measuring mercury intake is important to control for the toxic effects of this trace element, in particular concerning the developing nervous system. The concentration of mercury in plasma as well as its content in hair has been used as biomarkers for mercury exposure, and they are apparently well inter-correlated [165]. Blood samples can be assayed using DBS [166]. The urinary excretion of mercury offers some promise as a biomarker of mercury intake [167].

Cadmium

Similar to mercury, undesirably high intakes of cadmium may lead to toxic effects. Often the plasma concentration (e.g., as in DBS) is used as a biomarker of cadmium intake [150, 166]. Interestingly, Piasek et al. [168] reported a potential usefulness of cadmium content in the placenta for evaluating cadmium exposure during pregnancy.

Iodine

Iodine is essential for adequate thyroid function. To assess iodine intake, direct measurements of urine iodine as well as of iodine incorporated into thyroid-derived molecules (e.g., thyroxine, thyroid-stimulating-hormone and thyroglobulin) have been used as functional markers [169]. The systematic meta-analysis by Ristic-Medic et al. [169] supported the use of all of these biomarkers for evaluating iodine intake, although to a varying degree, urine excretion being the better [170]. Moreover, the analysis of thyroglobulin in DBS has emerged as a putative biomarker alternative for iodine intake [171].

Feces—a potential matrix for dietary biomarkers

It has been generally acknowledged that the gut microbial ecosystem may influence human physiology and health [172]. The understanding to what extent the intestinal microbic composition is subject to dietary control, and to integrate these data with functional metabolic signatures and biomarkers is of utmost interest [173]. The gut microbiota can be recognized as a highly active metabolic organ because it affords metabolites affecting physiological processes in the intestine and beyond. Thus, gut microbiome metabolites interfere with the metabolic phenotype of the host and consequently may affect health and disease risk [174].

Diet and stool metabolites

Diet plays a pivotal role in shaping the human gut microbiota (composition and metabolism), one of the most densely populated microbial ecosystems in nature. As a prominent example, prebiotics are used to modulate composition, metabolism, and function of the gut microbiota to improve the gut and host health [175]. However, a number of additional factors, such as physicochemical food properties, nutrient availability, colonic transit time, and age of the host, may modulate the effect of diet on the composition and metabolic activity of the colonic microbiota [172, 173]. Metabolites due to bacterial energy metabolism may reflect dietary intake, such as short chain fatty acids (SCFA) as a result of carbohydrate metabolism, metabolites of fatty acids and lipid bioconversion, and metabolites of protein fermentation. Minor food constituents structurally modified by microbial activity might be detectable in feces and could be a characteristic for the consumption of certain foods, especially plant foods (bioconversion of secondary plant products). Thus, the hypothesis that metabolites detectable in fecal samples may reflect dietary intake is valid, although not well elaborated yet.

Short chain fatty acids

Carbohydrates that are not digested in the small intestine are fermented by colonic microbiota and produce SCFA, e.g., butyrate, propionate, acetate, and longer-chain fatty acids [176, 177]. The SCFA fecal concentration alters in different stages of life; e.g., the change from breast-feeding to solid food or a higher butyrate production at higher age as a consequence of an increase in *bacteroides* [178]. Furthermore, the relative proportions of SCFA differ from one person to another and they are specifically sensitive to the type of fermented carbohydrate [179, 180]. However, the current evidence for a distinct dietary substrate identification based on SCFA analyses in feces is not conclusive [172]. Also, lactate and succinate are intermediate metabolites of bacterial carbohydrate metabolism. However, a direct link to specific dietary carbohydrates has not been established; rather, they may be useful markers of gut health [172, 181].

Prebiotics

Many studies administering prebiotics as inulin, oligofructose, and fructooligosaccharides (FOS) have reached a significant increase in fecal *bifidobacteria* counts [182]. A double-blind placebo-controlled crossover study has shown that the numbers of fecal *bifidobacteria* and *lactobacilli* have significantly increased after administering very-long-chain inulin (VLI), derived from globe artichoke, as compared to placebo [183]. Also oral administration of a similar dose (similar to inulin dose) of acacia gum increased *bifidobacteria* and *lactobacilli* production [184]. Thus, an increase in fecal *bifidobacteria* and *lactobacilli* excretion reflect prebiotics intake. Another study has shown that administering 10, 15, or 20 g/day of a sugar-free digestion-resistant dextrin for 20 days led to increased number in the fecal *lactobacilli* and *bifidobacteria* and a decrease in *clostridium perfringens* [185]. However, it should be considered that there are different methods and no standard protocols for assessing microbial proportions or numbers or activity in fecal samples [186].

Branched chain fatty acids

Branched chain fatty acids (BCFA), ammonia, amines, phenols, cresols, indoles, hydrogen sulfides (highly volatile), etc. are metabolites arising from protein fermentation [187]. There is a considerable inter-individual variation in the urinary excretion of p-cresol and phenols probably reflecting its production in colon [172]. In addition, fecal concentrations of isobutyrate, 2-methylbutyrate and isovalerate, metabolites of the bacterial fermentation of valine, isoleucine, and leucine [188] decreased after intake of prebiotics [189–191]. Thus, such metabolites could be evaluated for its use as biomarkers of dietary intake. So far, they are used only as more general markers for bacterial protein fermentation [192].

Dietary polyphenols

Most dietary polyphenols (e.g., flavonoids, anthocyanins, phenolic acids, stilbenes, lignans, tannins) pass the small intestine without absorption. Polyphenols have been described to modulate composition of the gut microbiome and probably contribute to gut and host health [193, 194]. In addition, polyphenols are bio-transformed into derivatives that may become bioavailable for the host. Studies using metabolomics techniques have shown that numerous microbial metabolites of polyphenols can be detected in feces and hence may be key markers for colonic bacterial composition and activity [195]. They may also be valid markers of food intake. As an example, serum concentrations of enterolactone and equol are measured as markers of plant lignan intake and microbial metabolism of daidzein (mainly derived from soy food), respectively. Using feces as the analytic matrix, the chances to get more information about diet (and microbial activity) would be high.

Sterols

Secretions from the gastrointestinal tract constitute a substantial portion of feces, and the bile is a major contributor. Bile acids, cholesterol, coprostanol, and their metabolites are subject to the enterohepatic circulation. Their content and proportions (e.g., primary versus secondary bile acids) are strongly influenced by dietary fat (saturated fat) intake, concomitant carbohydrate intake and the gut microbial composition [196].

Fiber and resistant starch supplementation or changing to a lacto-vegetarian diet resulted in decreased fecal bile acid concentrations, particularly secondary bile acids [197, 198]. Cholesterol is transformed to coprostanol, which represents about 60 % of the sterol content and is considered the major 5ß-stanol in human feces [199]. Plant phytosterols, e.g., campesterol and sitosterol, are reduced by enteric bacteria into 5ß-stigmastanol and 5-ß campestanol. Hence, 5ß-stigmastanol and 5-ß campestanol may be used as fecal biomarkers for dietary phytosterol intake [200]. The relative proportions of animal- and plant-derived stanols in feces may reflect dietary preferences [200, 201]. Thus, the possibility of applying various metabolites of bile acids and steroids in feces as biomarkers of current diet should be further explored.

Provitamin A carotenoids

Non-absorbed provitamin A carotenoids are mainly excreted in feces. In addition, absorbed provitamin A carotenoids are partially excreted through bile and pancreatic secretions in feces. Thus, carotenoids can be used as fecal biomarkers for provitamin A (e.g., ß-carotene) intake or for estimating ß-carotene bioavailability or for establishing ß-carotene net balance in the body [202]; e.g., Van Lieshout et al. [202] estimated the difference in the bioavailability of ß-carotene between pumpkin and spinach based on measuring carotenoids concentration in feces and serum.

Future prospect

The use of metabolomics techniques for analyses of fecal samples allows identification of new biomarkers of gut health, as well as understanding the interrelationship between the human gut microbiome activity and host metabolism [203]. Moreover, it provides the possibility to establish new markers of dietary (food) intake. A successful strategy might be to utilize valid and reproducible metabolomics data to discover metabolite patterns in feces that are associated with diet (rather than looking for single compounds only). Thus, dietary intervention studies may allow identification of fecal metabolite pattern, which might be reproduced in population-based

studies (e.g., cross-sectional and cohort studies). Up to now, only some intervention studies have used metabolomics techniques to evaluate the effect of synbiotics in humans [204–206].

Methodological aspects

Metabolite profiling in fecal samples is much more complicated as compared to other biospecimen. The physicochemical properties of the feces influence the reproducibility and full coverage of metabolite profiling attained [206]. However, lyophilized feces samples extracted by water methanol mixtures, allow for the analysis of metabolite profiles that are reproducible and are composed of various compounds. There are different options for fecal sample collection and storage conditions that may impact on the metabolite concentrations, e.g., immediate freezing versus cooling on ice or in the refrigerator before freezing versus storage at room temperature before freezing [207]. For example, in water extracts from frozen fecal samples, the concentration of amino acids and glucose is higher than that in water extracts of fresh fecal samples [208]. Another option is immediate mixing of the collected fecal sample with stabilizing solutions such as RNAlater or Amis Transport Medium [209]. A requirement for use of fecal samples for dietary biomarker identification is a high sample quality; however, optimization and harmonization of sample collection, storage, and processing procedures are yet to be established.

Analytical tools such as NMR, GC-MS, LC-MS, and LC-MS-MS have been applied as metabolomics techniques for the analyses of stool metabolites. Up till now, mass spectrometry techniques have some advantages in characterizing human metabolomes due to their high sensitivity and selectivity [210]. NMR spectroscopy is widely used in metabolite profiling due to its non-destructive sample handling and its ability to quantify compounds at very low concentrations. Furthermore, NMR spectroscopy gives information on the structure of the compounds, which is useful when unknown compounds have to be identified [211, 212]. The heterogeneity of dietary compounds and of their formed metabolites after ingestion is still a big challenge, as many of these compounds are not yet defined [213]. Due to the diversity of fecal metabolites, more than one method is necessary to achieve a comprehensive metabolite profile [214]. Important measures for selecting an appropriate analytical method have to be considered such as coverage, dynamic range, selectivity, accuracy, precision, and price per sample [210].

Conclusions

Biomarkers of nutrient intake or nutrient status are important objective measures of one of the most important environmental factors people are exposed to, namely food. It is very difficult to obtain accurate data on individual food intake, and there is a large variation in nutrient composition of foods. This is the background for studying more objective biomarkers of nutrient intake. Modern technology with high sensitivity and specificity concerning many nutrient-relevant biomarkers has allowed an interesting development of non-professional collection of small amounts of blood by finger-pricking and collection on filters or sticks. With proper collection, drying, transport, extraction, and analysis of the samples, many analytes of nutritional interest can be measured such as metabolites, lipids, vitamins, minerals, and many types of peptides and proteins. The advantage of this alternative sampling technology is that non-professionals can collect, dry, and mail the samples; the samples can often be stored at room temperature in a dry atmosphere; small amounts of blood are required for analyses in professional laboratories with modern analytical methodology. However, it should be noticed that chemical measurements of nutrient biomarkers are hampered by many confounding factors like variation in food matrices, difference in digestion, absorption, transport, distribution, activation, and catabolism. These facts make it obvious that we do not get comparable data from personal registration and objective biomarker measurements. Thus, food registration as well as biomarker measurements will most likely complement each other in future decades of nutritional sciences. Another promising area of potential biological interest is the biology of the microbiome in association with biomarkers. Interesting perspectives are also related to the marked trend of self-monitoring of body functions linked to mobile phone technology.

Competing interests

Thomas E. Gundersen, PhD, is CEO of the contract laboratory Vitas AS (www.vitas.no), where he also is a stock owner.
Christian A. Drevon, MD, PhD, is a founder, stock owner, board member, and consultant for Vitas AS.

Authors' contributions

TH, FN, TEG, PM, JL, POI, and CAD wrote the review. CAD conceived of the review. CAD, FN, and TH participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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ARTICLE

Epidemiology

Plasma concentrations of anserine, carnosine and pi-methylhistidine as biomarkers of habitual meat consumption

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Abstract

Background/Objectives Dietary intake of red and processed meat has been associated with disease risk. Since dietary intake assessment methods are prone to measurement errors, identifying biomarkers of meat intake in bio-samples could provide more valid intake estimates. We examined associations of habitual red and processed meat, poultry, fish, and dairy products consumption with plasma concentrations of anserine, carnosine, pi-methylhistidine (Π-MH), tau-methylhistidine (T-MH), and the ratio of T-MH to Π-MH in a cross-sectional study.

Subjects/Methods Plasma anserine, carnosine, Π-MH, and T-MH concentrations were measured using ion-pair LC–MS/MS in 294 participants in the second Bavarian Food Consumption Survey (BVS II). Habitual food consumption was assessed using three 24-h dietary recalls. Associations between plasma metabolites concentrations and meat, fish, eggs, and dairy products consumption were assessed by fitting generalized linear model, adjusted for age, sex, and BMI.

Results Total meat intake was associated with plasma concentrations of anserine, carnosine, Π-MH and, the ratio of T-MH to Π-MH. Red meat intake was related to carnosine (p-trend = 0.0028) and Π-MH plasma levels (p-trend = 0.0493). Poultry (p-trend = 0.0006) and chicken (p-trend = 0.0003) intake were associated with Π-MH. The highest anserine concentrations were observed in individuals consuming processed meat or turkey. For T-MH we did not observe any association with meat intake.

Conclusions Our results indicate an association between habitual meat consumption and plasma concentrations of anserine, carnosine, Π-MH and the ratio of T-MH to Π-MH. Intervention studies should clarify whether the analyzed plasma metabolites are indicative for a specific type of meat before proposing them as biomarkers of habitual meat intake in epidemiologic studies.

Introduction

The consumption of total meat, red meat, processed meat, poultry, and fish has been associated with human health and disease [1, 2]. In many populations, meat is an important source of dietary protein and substantially contributes to the supply with several essential nutrients, such as iron or zinc [3]. In the past decade a lot of evidence has been obtained showing that effects on chronic disease risk depend on the type of meat consumed. While a high consumption of red meat and processed meat is associated with a higher risk of, e.g., type-2 diabetes, cardiovascular diseases, some types of

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cancer, and even total mortality, people consuming predominantly poultry or fish are not at higher risk. In contrast to that a high consumption of fish even decreases the risk of several chronic diseases [4–7].

In observational epidemiological studies, dietary questionnaires such as food frequency questionnaires and dietary recalls are utilized to estimate habitual daily intake of food [8]. However, dietary questionnaires are prone to different types of bias leading to inaccurate estimates of dietary intake [9], resulting in misclassification of participants and impairment of risk estimates of diet–disease relationships [10]. Hence, identifying new biomarkers of meat intake in bio-samples provides the chance to get additional dietary intake information that is not affected by the same measurement error [10–12]. Biomarkers can complement the dietary intake information and help to evaluate critically the available dietary intake data. They can be used to classify a person's dietary intake and in turn will enhance the assessment of the relationship between diet and chronic disease [13]. Ideally, such metabolites can aid in categorizing participants according to the type and amount of meat consumed.

Several studies have analyzed many single compounds in body fluid samples regarding their correlation with meat and fish intake [3, 11]. Carnosine [3], anserine [3], pi-methylhistidine (Π-MH) [12], tau-methylhistidine (T-MH) [12], and several other metabolic compounds have been suggested as candidate biomarkers of dietary meat intake, as they are found in various amounts in all different types of meat [3]. A recent short-term intervention study confirmed that urinary concentrations of some of the above-mentioned compounds are fairly good biomarkers of meat intake [14]. It remains unclear, however, whether these markers can also be used to characterize an individual's habitual dietary meat intake, the information required in observational (cohort) studies for testing diet–disease associations. Since epidemiological studies usually collect blood samples but often lack (24-h) urinary samples, biomarker measurement in plasma or serum samples can be done in most existing studies.

Thus, in the present study we investigate whether habitual meat, fish, egg, and dairy products consumption is associated with plasma concentrations of anserine, carnosine, Π-MH, T-MH or the ratio of T-MH to Π-MH in a cross-sectional study. Instead of using the terms 1- and 3-methylhistidine we used the terms tau-methylhistidine (T-MH) and pi-methylhistidine (Π-MH), respectively, according to N^{T} and N^{Π} atoms in the imidazole ring, following the most common IUPAC nomenclature [15] to avoid confusion about the numbering of atoms in the imidazole ring of histidine. The methylhistidine structure derived from anserine metabolism is termed as Π-MH, whereas the other methylhistidine structure is named as T-MH.

Methods

Study participants

The second Bavarian Food Consumption Survey (BVS II), conducted between September 2002 and June 2003, included 1050 participants aged 13–82 years. The overall participation rate was 70%. The study was designed as a representative cross-sectional study of the German-speaking Bavarian population to describe their dietary and lifestyle habits. In face-to-face interviews, demographics, lifestyle factors, and medical history of the participants were assessed [16]. The BVS II study was approved by the Ethics Committee of the Bavarian Medical Association (Bayerische Landesärztekammer). Written informed consent was obtained from each participant in accordance with institutional requirements and the declaration of Helsinki principles.

Dietary data assessment

The standardized PC-guided program EPIC-Soft [17, 18] was utilized to assess three 24-h dietary recalls per subject. Trained study personnel conducted telephone interviews to complete two dietary recalls on weekdays and one recall on a weekend for each participant. Intake data were weighted for weekday and weekend to calculate the average daily food intake. Food items were summarized into 16 food groups and 21 subgroups according to the EPIC-Soft classification scheme [17].

Men and women who completed at least one 24-h dietary recall and were at least 18 years of age, participated in blood sampling and anthropometry assessment in the nearest health office of each participant. Blood samples were stabilized with sodium ethylenediamine tetra-acetic acid (EDTA) (1 g/L), centrifuged and separated from blood cells, as described elsewhere in detail [19]. Plasma aliquots were stored at −80 °C until lab analysis.

Potential meat intake biomarkers were measured in 294 plasma samples as only for those participants sufficient plasma volume for the lab analysis was available.

Targeted LC–MS/MS amino acid measurements

Quantitative amino acid analyses were performed using targeted LC–MS/MS based on the method described by Harder et al. [20]. Briefly, plasma samples (10 µl) were dissolved in 500 µl ice-cold methanol containing an internal standard mixture of 16 deuterated amino acids, derived from ChromSystems (Gräfelfing, Germany), Cambridge Isotope Laboratories (Tewksbury, MA, USA), and Sigma-Aldrich (Taufkirchen, Germany). Samples were centrifuged (10 min, 10 °C, 4000 × *g*) and supernatants containing the extract were evaporated to dryness under nitrogen. In the

second step, amino acids in the dried samples were derivatized to their butyl esters, as described by Gucciardi et al. [21]. Briefly, a mixture of 95% n-butanol and 5% acetylchloride (v/v) was added to the dried samples. Subsequently, the samples were incubated at 60 °C for 15 min at 600 rpm (Eppendorf Thermomixer Comfort; Eppendorf, Hamburg, Germany). The derivatized samples were dried and reconstituted in a 300 µl mixture of methanol/water/formic acid (70/30/0.1% v/v).

The analysis was performed on a triple quadrupole QTRAP 5500 LC–MS/MS system operating in positive ESI mode (AB Sciex, Framingham, MA) equipped with a 1200 series binary pump (Agilent, Santa Clara, CA) and coupled to an HTC pal autosampler (CTC Analytics, Zwingen, Switzerland). Chromatographic separation was achieved using a Zorbax Eclipse XDB-C18 column (length 150 mm, internal diameter 3.0 mm, particle size 3.5 µm; Agilent). Eluent A consisted of 0.1% formic acid, 2.5 mM ammonium acetic acid, and 0.005% heptafluorobutyric acid in water. Eluent B consisted of 0.1% formic acid, 2.5 mM ammonium acetic acid, and 0.005% heptafluorobutyric acid in acetonitrile. Analytes were measured in scheduled multiple reaction monitoring (sMRM). For absolute quantification, a 10-point calibration was performed, using a mixture containing all amino acids in the measurement (A9906 amino acid standards, Sigma-Aldrich, Taufkirchen, Germany). Data analysis was done using Analyst 1.5.1® software (AB Sciex).

Intra-day and inter-day precisions for Π-MH and T-MH were evaluated using ClinCheck® control plasma samples (Recipe, München, Germany). Since these control plasma samples do not contain measurable concentrations of anserine and carnosine, we additionally obtained human plasma samples from individuals collected within a time frame of 24 h after meat intake, and evaluated the intra-day and inter-day precision for anserine and carnosine in these samples. Except for low levels of anserine, coefficients of variation for the precision were within 20% for the analytes studied here (Supplementary Table 1). Accuracies were between 77 and 119% for low levels and between 87 and 104% for high levels. Except for low levels of anserine, sample peaks showed signal-to-noise values above 9, defined as lower limit of quantitation.

Statistical methods

From three 24-h dietary recalls a weighted (for weekday and weekend day) mean value for every food group or subgroup was derived representing the usual intake in g/day of the respective item. The following food groups and subgroups were used in the present study: “all meat and meat products”, “red meat” (sum of beef, pork, and other rarely consumed fresh meat, as wild, lamb etc.), “beef”, “pork”, “beef and pork”, “poultry” (sum of chicken, turkey, ducks, goose, etc.), “chicken”, “turkey”, “processed meat”, “fish and shellfish”, “egg and egg products”, “milk and dairy products”.

For the descriptive analysis, BMI (kg/m^2), age of participants (years) and total energy intake (kcal/d) were analyzed as continuous variables. Smoking status was described as “smoker”, “ex-smoker” and “non-smoker”. Socioeconomic status was assessed by household net income, educational level of the one who was interviewed and career position of the principal earner. It was categorized into low, low-medium, medium, medium-high, and high, based on the sum score derived from the single variables. The descriptive analysis of main characteristics of the study population was conducted separately for men and women. We report median and interquartile ranges or absolute numbers and relative frequency as appropriate.

To describe differences of non-normally distributed plasma concentrations of anserine, carnosine, Π-MH, T-MH, and the ratio of T-MH to Π-MH across categories of sex, age, BMI, smoking, social class and physical activity, we report geometric means and 95% CI instead of arithmetic means and standard deviations. Geometric means were obtained by fitting models with PROC GENMOD using log-transformed plasma concentrations and transforming the estimates derived from the model. For this purpose, BMI was categorized into quartiles and age into three groups (“<40 years”, “40–60 years”, “≥60 years”). All bivariate models were adjusted for (continuous) age and sex. When examining BMI, we additionally adjusted for total energy intake. When smoking was examined, (continuous) BMI was added as adjustment variable. Physical activity was categorized as either “active” or “inactive” (no regular sports activity). The *p*-trend was derived based on integer scores assigned to the categories of sex, age, BMI, smoking, social class, and physical activity.

Further, we examined the relationship of plasma concentrations of anserine, carnosine, Π-MH, T-MH, and the ratio of T-MH to Π-MH with food intake: geometric means for categories of food intake were derived by fitting linear models using log-transformed plasma concentrations. The categorization of the food items was done with respect to the intake data. The food groups “all meat and meat products”, “processed meat”, and “milk and dairy products” were each grouped in quartiles whereas”red meat”, “pork”, “beef and pork”, “poultry”, “fish and shellfish”, and “eggs and egg products” were grouped into three groups defined by non-consumers and the consumers divided by their median value. For a small group of food items (“beef”, “chicken”, and “turkey”) only consumers and non-consumers were discriminated. As adjustment variables, only age, sex, and BMI were included, as for smoking, social class and physical activity no significant associations

were detected. The *p*-trend was derived based on integer scores assigned to the categories of food intake.

In addition, we performed a non-parametric comparison of the consumption of "all meat and meat products", "milk and dairy products", "fish and shellfish", and "eggs and egg products" across groups defined by the quartiles of all biomarkers. When the Kruskal-Wallis test indicated overall significant differences ($p < 0.05$), Dunn's post-hoc test was subsequently carried out. These analyses were restricted to the consumers of the respective food groups.

All statistical analyses were performed using SAS software, Version 9.3 of the SAS System for Windows (Copyright © 2002-2010 SAS Institute Inc.).

Results

The present study included 101 (34.4%) men and 193 (65.7%) women. The mean age of the participants was 54 years in men and 44 years in women. On average, men had a higher BMI and a higher energy intake compared with women. Further baseline characteristics of the study participants are shown in Table 1, stratified by sex. Mean intake of milk and dairy products was higher in women (170 g/d) compared with men (106 g/d), whereas total meat intake in men (157 g/d) was much higher as compared with women (91 g/d). Descriptive data on all analyzed dietary intake of food groups and subgroups in gram per day are presented in Table 1. Plasma biomarker concentrations range from 0.0771 µM for anserine in men and 0.0544 µM in women to 15.90 µM for T-MH in men and 12.33 µM women, respectively.

Anserine, Π-MH, and the ratio of T-MH to Π-MH plasma concentrations did not differ by sex. In contrast, carnosine and T-MH concentrations were higher in men as compared to women (Table 2). Plasma concentrations of anserine, T-MH and Π-MH were associated with BMI whereas only T-MH was significantly associated with age. Consequently, all following analyses were adjusted for sex, age, and BMI. Smoking, social class and physical activity were not associated with any potential meat intake marker, and thus not further considered.

Plasma anserine, carnosine, and Π-MH concentrations significantly increased with higher total meat intake whereas the ratio of T-MH to Π-MH plasma concentrations decreased (Table 3). Red meat intake was significantly positively related to plasma concentration of carnosine and Π-MH. Beef or pork consumption did not show any significant association, whereas the sum of beef and pork intake significantly affected anserine and carnosine plasma concentrations. With higher processed meat consumption, plasma anserine concentrations significantly increased. Furthermore, Π-MH and the ratio of T-MH to Π-MH plasma concentrations differed by intake of total poultry and of chicken. In contrast, consumption of turkey was positively associated with plasma anserine concentrations. The potential meat biomarkers were, in general,

Table 1 Dietary and lifestyle characteristics and plasma biomarker concentrations by sex

	Male		Female	
n (%)	101	(34.4)	193	(65.7)
Age (years)	54	(41, 63)	44	(36, 57)
Food consumption (g/d)				
Milk and dairy products	105.8	(57.8, 195.9)	170.1	(80.6, 289.7)
Milk	20.6	(0, 99.3)	57.8	(3.7, 144.3)
Cheese	27.3	(8.6, 48.6)	23.2	(9.8, 38.9)
Total meat	157.4	(97.1, 223.9)	91.1	(47, 135.6)
Red meat	31.7	(0, 89.2)	25.4	(0, 55.7)
Beef	0	(0, 0)	0	(0, 0)
Pork	0	(0, 66.1)	0	(0, 37.7)
Beef & pork	23.6	(0, 78)	13.9	(0, 48.9)
Poultry	0	(0, 15)	0	(0, 10.5)
Processed meat	79.4	(40.7, 120.7)	38.1	(14.9, 68.3)
Fish & shellfish,	0	(0, 23.8)	0	(0, 17.8)
Eggs & egg products	0	(0, 17.4)	0	(0, 17.1)
Total energy intake (kcal/d)	2298	(2003, 2643)	1798	(1499, 2073)
BMI (kg/m^2)	26.4	(24.3, 28.8)	24.6	(22.4, 28.3)
Smoking status, *n* (%)				
Never	41	(40.6)	124	(64.6)
Former	33	(32.7)	38	(19.8)
Current	27	(26.7)	30	(15.6)
Social class, *n* (%)				
Lower	10	(9.9)	29	(15)
Lower-middle	23	(22.8)	48	(24.9)
Middle	32	(31.7)	63	(32.6)
Upper-middle	17	(16.8)	41	(21.2)
Upper	19	(18.8)	12	(6.2)
Plasma biomarker concentrations (µM)				
Anserine	0.0771	(0.018, 0.295)	0.0544	(0.014, 0.254)
Carnosine	0.0153	(0.011, 0.022)	0.0103	(0.007, 0.015)
Π -MH	1.940	(1.090, 4.120)	1.540	(0.750, 3.990)
T-MH	15.90	(13.20, 18.37)	12.33	(10.13, 15.53)
T-MH to Π -MH	2.165	(1.315, 2.708)	2.230	(1.226, 2.822)

Values are *n* (%) or median (IQR)

Table 2 Geometric means of plasma concentrations of anserine, carnosine, T-MH, Π-MH, and Π-MH to T-MH ratio by sex, age, BMI, smoking, social class, and physical activity

	Anserine (μM)		Carnosine (μM)		Π-MH (μM)		T-MH (μM)		T-MH to Π-MH (μM)	
	Geom. mean	95% CI	Geom. mean	95% CI	Geom. mean	95% CI	Geom. mean	95% CI	Geom. mean	95% CI
Sex										
Men	0.081	(0.053, 0.123)	0.015	(0.013, 0.018)	2.17	(1.7, 2.8)	15.08	(14.02, 16.22)	6.94	(5.44, 8.85)
Women	0.061	(0.046, 0.081)	0.010	(0.009, 0.011)	1.89	(1.6, 2.3)	12.98	(12.34, 13.64)	6.86	(5.79, 8.12)
P-trend		0.300		**0.0002**		0.4054		**0.017**		0.9423
Age (y)										
<40	0.096	(0.064, 0.144)	0.014	(0.012, 0.016)	2.29	(1.78, 2.94)	13.59	(12.64, 14.60)	5.93	(4.66, 7.55)
≥40 to <60	0.065	(0.045, 0.094)	0.012	(0.010, 0.014)	1.69	(1.36, 2.11)	13.57	(12.75, 14.46)	8.01	(6.49, 9.89)
≥60	0.056	(0.036, 0.086)	0.012	(0.010, 0.014)	2.34	(1.80, 3.05)	15.3	(14.17, 16.51)	6.53	(5.06, 8.42)
P-trend		0.0781		0.1424		0.998		**0.0395**		0.5362
BMI (kg/m^2)										
<22.9	0.054	(0.034, 0.087)	0.011	(0.009, 0.013)	1.71	(1.28, 2.29)	13.28	(12.22, 14.42)	7.76	(5.86, 10.27)
≥22.9 to <25.4	0.053	(0.034, 0.083)	0.012	(0.010, 0.015)	1.67	(1.27, 2.19)	13.90	(12.87, 15.02)	8.34	(6.42, 10.84)
≥25.4 to <28.5	0.056	(0.036, 0.087)	0.013	(0.011, 0.015)	2.5	(1.92, 3.26)	13.18	(12.22, 14.21)	5.27	(4.09, 6.81)
≥28.5	0.164	(0.103, 0.260)	0.013	(0.012, 0.016)	2.44	(1.83, 3.24)	16.03	(14.78, 17.37)	6.57	(4.99, 8.64)
P-trend		**0.0022**		0.0803		**0.0264**		**0.0086**		0.1271
Smoking										
Never	0.073	(0.053, 0.101)	0.013	(0.012, 0.015)	2.08	(1.71, 2.53)	14.17	(13.4, 14.98)	6.81	(5.63, 8.23)
Former	0.063	(0.04, 0.098)	0.011	(0.0096, 0.013)	2.15	(1.64, 2.83)	13.38	(12.37, 14.47)	6.22	(4.78, 8.1)
Current	0.075	(0.045, 0.125)	0.013	(0.011, 0.015)	1.88	(1.37, 2.57)	14.42	(13.18, 15.77)	7.67	(5.67, 10.38)
P-trend		0.9169		0.6173		0.6818		0.9633		0.6801
Social class										
Lower	0.130	(0.07, 0.244)	0.012	(0.009, 0.015)	2.64	(1.79, 3.88)	15.19	(13.61, 16.96)	5.75	(3.97, 8.33)
Lower middle	0.067	(0.043, 0.105)	0.013	(0.011, 0.015)	2.02	(1.52, 2.67)	14.13	(13.03, 15.32)	7.01	(5.34, 9.19)
Middle	0.056	(0.037, 0.083)	0.013	(0.011, 0.015)	1.86	(1.46, 2.36)	13.52	(12.62, 14.49)	7.28	(5.77, 9.18)
Upper middle	0.076	(0.046, 0.125)	0.012	(0.01, 0.015)	1.97	(1.45, 2.69)	14.26	(13.05, 15.59)	7.23	(5.36, 9.76)
Upper	0.074	(0.037, 0.145)	0.012	(0.01, 0.016)	2.28	(1.51, 3.45)	13.73	(12.2, 15.46)	6.02	(4.04, 8.97)
P-trend		0.383		0.8615		0.5954		0.3377		0.7905
Physical activity										
Inactive	0.079	(0.052, 0.119)	0.014	(0.012, 0.016)	2.28	(1.77, 2.94)	14.24	(13.23, 15.32)	6.25	(4.89, 7.99)
Active	0.068	(0.089, 0.052)	0.012	(0.011, 0.013)	1.97	(1.67, 2.32)	13.94	(13.29, 14.62)	7.09	(6.05, 8.32)
P-trend		0.5536		0.1014		0.3303		0.6271		0.3868

Bold numbers indicate significant results (p-value < 0.05)

Table 3 Geometric means of plasma concentrations of anserine, carnosine, T-MH, Π-MH, and Π-MH to T-MH ratio by consumption of meat, milk & dairy products, eggs and fish

Intake (g/d)	Anserine (μM)		Carnosine (μM)		Π-MH (μM)		T-MH (μM)		T-MH to Π-MH (μM)	
	Geom. mean[a]	95% CI	Geom. Mean[a]	95% CI	Geom. Mean[a]	95% CI	Geom. Mean[a]	95% CI	Geom. Mean[a]	95% CI
All meat										
<54.7	0.033	(0.021, 0.053)	0.011	(0.009, 0.013)	1.55	(1.17, 2.06)	13.55	(12.45, 14.72)	8.73	(6.65, 11.47)
≥54.7 to <116.4	0.075	(0.048, 0.117)	0.012	(0.01, 0.014)	1.73	(1.31, 2.27)	13.88	(12.82, 15.03)	8.04	(6.17, 10.47)
≥116.4 to <165.2	0.072	(0.046, 0.112)	0.011	(0.009, 0.013)	2.21	(1.68, 2.91)	14.12	(13.03, 15.29)	6.38	(4.89, 8.31)
≥165.2	0.115	(0.075, 0.178)	0.016	(0.014, 0.019)	2.71	(2.07, 3.55)	14.43	(13.34, 15.61)	5.33	(4.11, 6.91)
P-trend[b]	**0.0005**		**0.0046**		**0.003**		0.277		**0.0059**	
Red meat										
0	0.04	(0.059, 0.086)	0.011	(0.001, 0.013)	1.8	(1.45, 2.28)	13.50	(12.64, 14.42)	7.44	(5.97, 9.27)
>0 to <54	0.042	(0.063, 0.094)	0.012	(0.010, 0.014)	1.86	(1.44, 2.39)	14.22	(13.22, 15.29)	7.66	(6.01, 9.76)
≥54	0.065	(0.096, 0.142)	0.015	(0.013, 0.017)	2.53	(2.00, 3.2)	14.43	(13.48, 15.44)	5.7	(4.54, 7.15)
P-trend[b]	0.0804		**0.0028**		**0.0493**		0.1632		0.1052	
Beef										
No	0.063	(0,049, 0.082)	0.012	(0.011, 0.013)	2.06	(1.76, 2.42)	14.02	(13.40, 14.69)	6.80	(5.83, 7.93)
Yes	0.105	(0.066, 0.166)	0.014	(0.012, 0.017)	2.01	(1.52, 2.66)	14.03	(12.94, 15.21)	6.99	(5.32, 9.17)
P-trend[b]	0.0552		0.1057		0.8679		0.9954		0.8616	
Pork										
0	0.060	(0.044, 0.081)	0.012	(0.011, 0.013)	1.85	(1.54, 2.23)	13.57	(12.87, 14.30)	7.33	(6.14, 8.76)
>0 to <47.1	0.012	(0.065, 0.210)	0.014	(0.012, 0.017)	2.05	(1.52, 2.76)	15.16	(13.91, 16.52)	7.405	(5.55, 9.89)
≥47.1	0.075	(0.042, 0.133)	0.013	(0.011, 0.016)	2.60	(1.96, 3.44)	14.24	(13.13, 15.43)	5.48	(4.18, 7.19)
P-trend[b]	0.1131		0.1621		0.0528		0.164		0.1115	
Beef-pork										
0	0.055	(0.039, 0.078)	0.011	(0.01, 0.013)	1.89	(1.53, 2.33)	13.5	(12.72, 14.34)	7.15	(5.84, 8.74)
>0 to <52.6	0.07	(0.046, 0.108)	0.013	(0.011, 0.015)	1.98	(1.52, 2.59)	14.35	(13.29, 15.48)	7.24	(5.60, 9.36)
≥52.6	0.104	(0.069, 0.157)	0.015	(0.012, 0.017)	2.37	(1.84, 3.05)	14.53	(13.51, 15.62)	6.12	(4.80, 7.81)
P-trend[b]	**0.0215**		**0.0081**		0.1843		0.1101		0.3675	
Poultry										
0	0.064	(0.049, 0.084)	0.011	(0.012, 0.013)	1.77	(1.50, 2.07)	13.91	(13.27, 14.58)	7.88	(6.75, 9.2)
>0 to <44.2	0.117	(0.065, 0.210)	0.013	(0,016, 0.019)	2.88	(2.01, 4.12)	14.61	(13.16, 16.23)	5.08	(3.6, 7.17)
≥44.2	0.075	(0.042, 0.133)	0.011	(0.014, 0.017)	3.15	(2.21, 4.48)	14.09	(12.70, 15.62)	4.48	(3.19, 6.29)
P-trend[b]	0.3129		0.0509		**0.0006**		0.6325		**0.0006**	
Chicken										
No	0.069	(0.054, 0.089)	0.012	(0.011, 0.013)	1.85	(1.59, 2.15)	13.96	(13.36, 14.59)	7.56	(6.54, 8.73)
Yes	0.084	(0.049, 0.144)	0.015	(0.012, 0.018)	3.52	(2.54, 4.86)	14.35	(13.05, 15.78)	4.08	(2.99, 5.58)

Table 3 (continued)

Intake (g/d)	Anserine (μM)		Carnosine (μM)		Π-MH (μM)		T-MH (μM)		T-MH to Π-MH (μM)	
	Geom. mean[a]	95% CI	Geom. Mean[a]	95% CI	Geom. Mean[a]	95% CI	Geom. Mean[a]	95% CI	Geom. Mean[a]	95% CI
P-trend[b]	0.5027		0.583		**0.0003**		0.6014		**0.0003**	
Turkey										
No	0.065	(0.051, 0.083)	0.012	(0.011, 0.0135)	1.95	(1.68, 2.27)	13.97	(13.38, 14.6)	7.16	(6.19, 8.28)
Yes	0.127	(0.069, 0.237)	0.014	(0.011, 0.017)	2.83	(1.93, 4.15)	14.38	(12.87, 16.06)	5.07	(3.51, 7.33)
P-trend[b]	**0.0473**		0.4025		0.0747		0.6377		0.0875	
Processed meat										
<21.3	0.036	(0.226, 0.057)	0.012	(0.010, 0.143)	1.98	(1.49, 2.64)	14.05	(12.95, 15.26)	7.09	(5.39, 9.33)
≥21.3 to <50	0.087	(0.055, 0.138)	0.012	(0.010, 0.015)	2.19	(1.65, 2.9)	13.80	(12.72, 14.97)	6.31	(4.81, 8.28)
≥50 to <89.6	0.064	(0.042, 0.099)	0.013	(0.011, 0.015)	1.74	(1.33, 2.28)	13.91	(12.87, 15.04)	7.98	(6.15, 10.35)
≥89.6	0.111	(0.072, 0.17)	0.013	(0.011, 0.0154)	2.31	(1.77, 3.02)	14.29	(13.22, 15.44)	6.19	(4.78, 8.01)
P-trend[b]	**0.0025**		0.4969		0.7127		0.772		0.7673	
Fish										
0	0.079	(0.060, 0.010)	0.013	(0.011, 0.014)	1.99	(1.67, 2.34)	13.95	(13.29, 14.64)	7.05	(5.99, 8.29)
>0 to <35.6	0.077	(0.045, 0.135)	0.014	(0.011, 0.017)	1.87	(1.33, 2.62)	14.28	(12.95, 15.75)	7.64	(5.52, 10.59)
≥35.6	0.042	(0.024, 0.073)	0.011	(0.009, 0.014)	2.60	(1.86, 3.65)	14.10	(12.79, 15.55)	5.42	(3.92, 7.51)
P-trend[b]	0.0705		0.604		0.2358		0.7549		0.2554	
Eggs										
0	0.071	(0.058, 0.096)	0.013	(0.011, 0.014)	2.16	(1.80, 2.60)	14.17	(13.44, 14.94)	6.57	(5.5, 7.84)
>0 to <19.3	0.068	(0.043, 0.016)	0.012	(0.011, 0.015)	1.66	(1.26, 2.19)	13.57	(12.53, 14.70)	8.17	(6.26, 10.66)
≥19.3	0.076	(0.045, 0.127)	0.019	(0.01, 0.014)	2.29	(1.68, 3.11)	14.17	(12.96, 15.49)	6.20	(4.61, 8.35)
P-trend[b]	0.9052		0.5272		0.8721		0.8008		0.9268	
All milk and dairy products										
<71.6	0.114	(0.074, 0.177)	0.014	(0.012, 0.017)	2.37	(1.81, 3.10)	13.50	(12.49, 14.58)	5.70	(4.40, 7.39)
≥71.6 to <149.8	0.059	(0.038, 0.091)	0.012	(0.010, 0.014)	1.80	(1.37, 2.35)	14.79	(13.69, 15.97)	8.23	(6.35, 10.65)
≥149.8 to <270	0.076	(0.049, 0.118)	0.011	(0.01, 0.013)	1.98	(1.50, 2.60)	14.02	(12.96, 15.17)	7.10	(5.45, 9.24)
≥270	0.048	(0.031, 0.076)	0.010	(0.012, 0.015)	2.10	(1.58, 2.78)	13.81	(12.75, 14.98)	6.60	(5.04, 8.64)
P-trend[b]	**0.0248**		0.1501		0.6537		0.9056		0.6162	

Bold numbers indicate significant results (p-value < 0.05)

Geom. Mean Geometric means, *95% CI* 95% Confidence Interval

[a]adjusted for age, gender, and BMI

[b]Tests for trend were conducted using integer scores for categories of food intake in the linear regression model

Table 4 Comparison of consumption of all meat, milk and dairy products, fish and shellfish and eggs and egg products by plasma biomarkers (in quartiles, based on all available measurements)

	All meat		Milk and dairy products		Fish		Eggs	
	N	Mean rank	*N*	Mean rank	*N*	Mean rank	*N*	Mean rank
Anserine (μM)								
Q1: <0.0152	72	108.22[a]	78	162.97	33	48.44	32	66.81
Q2: ≥0.0152 to <0.0598	70	159.64[b]	70	144.43	21	47.36	30	67.12
Q3: ≥0.0598 to <0.272	72	141.78[a,b]	69	127.81	19	53.29	36	61.24
Q4: ≥0.272	72	164.81[b]	72	142.56	21	40.93	29	61.10
		$\mathbf{p = 0.0001}$		$p = 0.0861$		$p = 0.5489$		$p = 0.8523$
Carnosine (μM)								
Q1: <0.008	74	104.81[a]	78	158.03[a]	26	54.71	31	65.55
Q2: ≥0.008 to <0.012	68	141.60[b]	70	154.57[a,b]	22	46.05	35	65.97
Q3: ≥0.012 to <0.018	75	147.88[b,c]	69	145.13[a,b]	27	39.98	30	60.07
Q4: ≥0.018	69	182.11[c]	72	121.03[b]	19	50.00	31	64.03
		$\mathbf{p < 0.0001}$		$\mathbf{p = 0.0380}$		$p = 0.2517$		$p = 0.9189$
Π-MH (μM)								
Q1: <0.9	71	120.37[a]	75	159.77	25	41.74	30	56.30
Q2: ≥0.9 to <1.6	69	146.12[a,b]	70	141.34	22	48.00	37	61.38
Q3: ≥1.6 to <4.1	73	142.12[a,b]	73	140.04	21	50.93	32	74.73
Q4: ≥4.1	73	164.90[b]	71	138.11	26	49.85	28	63.45
		$\mathbf{p = 0.0145}$		$p = 0.3595$		$p = 0.6489$		$p = 0.2360$
T-MH (μM)								
Q1: <10.8	70	117.78[a]	71	153.89	19	49.55	35	65.69
Q2: ≥10.8 to <13.6	69	129.33[a,c]	73	159.58	29	42.69	29	52.55
Q3: ≥13.6 to <16.9	74	154.85[b,c]	73	136.36	21	40.69	34	69.00
Q4: 1His≥16.9	73	170.05[b]	72	130.21	25	57.24	29	67.55
		$\mathbf{p = 0.0005}$		$p = 0.1087$		$p = 0.1382$		$p = 0.2847$
T-MH to Π-MH (μM)								
Q1: <1.248	73	163.82[a]	71	141.89	27	49.57	30	60.02[a,b]
Q2: ≥1.248 to <2.166	72	135.97[a,b]	73	141.45	22	50.68	32	77.98[a]
Q3: ≥2.166 to <2.767	72	150.90[a,b]	72	145.29	23	44.74	38	64.97[a,b]
Q4: ≥2.767	69	122.14[b]	73	151.28	22	44.66	27	50.48[b]
		$\mathbf{p = 0.0171}$		$p = 0.8866$		$p = 0.8182$		$\mathbf{p = 0.0339}$

Mean ranks and and *p*-value of Kruskal–Wallis test are reported. Letters indicate groups identified by Dunn's post-hoc test (alpha = 0.05). Only consumers are included in the respective analysis. All group sizes are reported

Q Quartiles

Bold numbers indicate significant results (*p*-value < 0.05)

not related to the intake of eggs, fish or dairy food. However, there was a significant inverse association between plasma anserine concentrations and dairy food consumption. Such an inverse trend did not reach statistical significance in the case of fish consumption.

The Kruskal-Wallis test revealed significant differences of the consumption of "all meat and meat products" across quartiles for all biomarkers investigated (Table 4). The post-hoc test showed group differences among the lower and higher quartile groups. The consumption of "milk and dairy products" was only significantly different across carnosine quartiles, whereas consumption of "eggs and egg products" differed only across quartiles of the T-MH to Π-MH ratio. These results give reassurance to the findings provided in Table 3.

Discussion

In the present study, we examined associations of the usual dietary intake and biomarkers measured in plasma. We observed a statistically significant association between total

meat intake and plasma concentrations of anserine, carnosine, Π-MH and the ratio of T-MH to Π-MH. Red meat intake was significantly related to carnosine and Π-MH plasma levels. Plasma concentrations of Π-MH were significantly related to poultry and chicken intake. The highest mean anserine concentrations were found in individuals consuming processed meat or turkey. For T-MH, we did not observe any association with meat intake.

Red meat, poultry, and fish contain significant amounts of carnosine and its derivatives. Therefore, diet is the main source of carnosine and its derivatives in men [22]. It has been reported 150 g of beef contains 343.4 mg carnosine and 150 g of chicken contains 322 mg carnosine [23]; however, information about absorption and bioavailability of histidine-dipeptides in human is still inadequate. The dietary histidine–dipeptides carnosine and anserine are rapidly hydrolyzed by carnosinase in plasma and excreted in urine. In total 85–90% of dietary anserine intake is eliminated as Π-MH in urine in human [24]. T-MH is a degradation product of actin and myosin, which are the main proteins found in the striated muscle filaments. T-MH is liberated from these proteins in the gut after meat intake [3] and excreted mainly in urine. T-MH reflects muscle catabolism and muscle mass. A considerable variation in circulating T-MH was observed after consuming the same diet between participants [25]. On the other hand, Π-MH is a potential good biomarker for meat intake because it is not affected by muscle mass and activity [26]. To take this potential influence into account, we assessed the bivariate associations of plasma concentrations of our biomarkers with physical activity. No significant associations were found in our study. All analysis were adjusted for BMI, which can at least partly correct for muscle mass, as we found significant associations with our biomarkers investigated. Therefore, we believe that our detected association with meat intake reveals that Π-MH is in fact a marker for meat intake.

Several studies attempted to identify objective biomarkers of meat intake in urine [12, 24, 27, 28] and fewer studies examined plasma or serum samples for meat biomarkers [14, 23, 28–30]. Most studies were intervention studies, e.g., [23, 28, 30] and had comparable analysis methods [23, 28]. To the best of our knowledge, no study has yet examined the association between usual dietary meat intake and plasma carnosine, anserine, T-MH or Π-MH concentrations in a cross-sectional study.

Both anserine and carnosine content is highly variable in different types of meat [3]. Both compounds are likely to serve as generic indicators of total meat intake rather than specific quantitative biomarkers as they cannot differentiate between the types of meat that have been consumed. However, some studies suggest that anserine concentrations in urine can be used as a specific biomarker for chicken intake [28, 31]. Yeum et al. [23] analyzed serum anserine concentrations and found anserine serum levels as a marker for chicken intake rather than beef intake. They reported that anserine concentrations in plasma were well detectable, and concluded that the methyl group lowers the affinity of the histidine dipeptides for the carnosinase enzyme, enhancing its blood (serum) stability. However, we did not find any association between chicken intake and anserine plasma level. This could be due to the short elimination half-life of dipeptides [14], as anserine should be a specific biomarker for chicken consumption like Π-MH. The reported half-life of anserine is 32 min [23], though others indicate half-life of 4 h [3]. Further, our finding of an inverse relationship of the consumption of milk and dairy products and fish with anserine concentrations most likely represents a substitution effect, i.e., a lower meat intake in individuals with a high consumption of dairy food or fish.

We did not observe any association between carnosine plasma concentration and beef consumption in contrast to the findings of Park et al. [30]. The peak plasma concentration of carnosine was reached after 2.5 h of beef consumption, and after 5.5 h of beef consumption carnosine was not detectable anymore [30]. Since we aimed to capture usual dietary intake habits, we did not measure carnosine plasma concentration directly after beef consumption and therefore, most likely, we missed the carnosine peak. Other reasons that can lead to carnosine disappearance in blood are the rapid uptake of dipeptides into tissues or hydrolysis of carnosine by carnosinase [31]. Because the carnosine plasma concentration depends on the amount of carnosine consumed [30, 31], our sample is likely to show on average low carnosine levels as beef is not regularly consumed in Bavaria, compared with other types of meat [32]. Carnosine has been detected in urine after fish consumption showing an association between carnosine urine concentration and fish intake [24]. However, we did not observe any significant association of carnosine plasma levels and fish consumption.

In line with our results, Yeum et al. [23] reported that carnosine plasma levels were not significantly associated with beef or chicken consumption. They suppose that the carnosine metabolic pathway differs according to the dietary source in humans. For example, beef may have compounds such as carnosine inhibitor or peptides that compete with carnosine in the hydrolytic process, which may stabilize carnosine in serum.

In plasma, Π-MH has been found to be the best marker for chicken intake and Π-MH levels are increased as well after consumption of red meat and processed meat [28, 33]. This is in good accordance with our findings since we observed additional associations with poultry and all meat food group, although no association with consumption of processed meat was found. Associations of meat and chicken consumptions and Π-MH levels have been found in urine as well [25, 28]. Π-MH is a short-term biomarker especially for chicken intake rather than describing the

usual intake [28]. The use of T-MH as a biomarker for meat intake has been investigated in urine as well as in plasma. Cross et al. found a significant increase of T-MH levels in urine after red meat consumption [25], however Dragsted argued that T-MH is not a marker of a specific kind of meat [3]. Furthermore, Cheung et al. did not observe any association neither in urine nor in plasma [28]. This is in line with our result of no significant association of dietary intake and T-MH plasma levels. One study reported that 1-MH and 3-MH half-lives were 11.7 and 12.6 h [12].

Urine analyses are less accurate compared to analyses in plasma samples because human muscle catabolism consistently contributes to urinary excretion of T-MH [3]. Fasting T-MH plasma levels are low as after T-MH intake from meat, dipeptides are rapidly excreted in urine [14]. Further, a reason for not obtaining any significant T-MH plasma concentration could be that the frequency of red meat, beef or pork consumption by participants in the BVS II was possibly too low to show a stable association with plasma concentrations of T-MH. The median intake of beef, pork or even poultry was 0 g/day.

Strength and limitations

The analytic method used to quantify anserine, carnosine, Π-MH and T-MH in plasma samples is state of the art with a fairly low limit of detection, which has also been applied in other recent studies [23, 28]. Our results are based on 294 plasma samples available of the BVS II study participants who completed three 24-h dietary recalls. Although this is not a random subsample of the BVS II study participants, a differential bias seems unlikely.

Three 24-h dietary recalls are efficient to determine dietary intake at the population level. The use of 24-h dietary recalls is the preferred dietary assessment method, since several studies reported its good relative validity and measurement properties [34–36]. We applied weighting factors to account for consumption differences depending on the day of the week. Yet, some food subgroups such as pork and beef are rarely consumed. For that reason, this study had a limited capacity to assess statistically the particular effect of specific meat subgroups consumption on the related meat intake biomarkers in contrary to the food group of total meat consumption. As we did not assess dietary intake on the day or the day before blood collection, we cannot assess short-term biomarkers of intake of rarely consumed foods.

Conclusion

In conclusion, results of this study indicate an association between meat consumption and plasma concentration of anserine, carnosine, Π-MH and the ratio of T-MH to Π-MH. For epidemiologic studies that did not collect urine samples, these could be valid biomarkers for meat intake. Further investigations of the use of these biomarker combinations as indicators of specific types of meat consumed are needed. In addition, intervention studies should clarify whether the analyzed plasma metabolites are indicative for a specific type of meat.

Data availability

The datasets generated and/or analyzed during the current study are not publicly available due ethical considerations (informed consent) but are available from the corresponding author on reasonable request.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Associations between usual food intake and faecal sterols and bile acids: results from the Cooperative Health Research in the Augsburg Region (KORA FF4) study

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Abstract

Animal sterols, plant sterols and bile acids in stool samples have been suggested as biomarkers of dietary intake. It is still unknown whether they also reflect long-term habitual dietary intake and can be used in aetiological research. In a subgroup of the Cooperative Health Research in the Augsburg Region (KORA FF4) study, habitual dietary intake was estimated based on repeated 24-h food list and a FFQ. Stool samples were collected according to a standard operating procedure and those meeting the quality criteria were extracted and analysed 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 faecal animal and plant sterols and bile acids concentrations and dietary intake data were available. In adjusted regression models, the associations between food intake and log-normalised metabolite concentrations were analysed. Bonferroni correction was used to account for multiple testing. In this population-based sample, associations between habitual dietary intake and faecal concentrations of animal sterols were identified, while the impact of usual diet on bile acids was limited. A habitual diet high in 'fruits' and 'nuts and seeds' is associated with lower animal faecal sterols concentrations, whereas a diet high in 'meat and meat products' is positively related to faecal concentrations of animal sterols. A positive association between glycocholate and fruit consumption was found. Further studies are necessary for evaluation of faecal animal sterols as biomarkers of diet. The findings need to be confirmed in other populations with diverse dietary habits.

Key words: **Dietary intake: Faeces: Sterols: Bile acids: KORA FF4**

Several studies have analysed individual compounds in faecal samples regarding their association with dietary intake and diseases[1–3]. Faecal 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 metabolised 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 (Fig. 1, adapted from Kaddurah *et al.*[7]). Cholic and chenodeoxycholic acids as the major primary bile acids are synthesised in the liver from cholesterol by side chain oxidation and

Abbreviations: 24HFL, 24-h food list; KORA FF4, Cooperative Health Research in the Augsburg Region.

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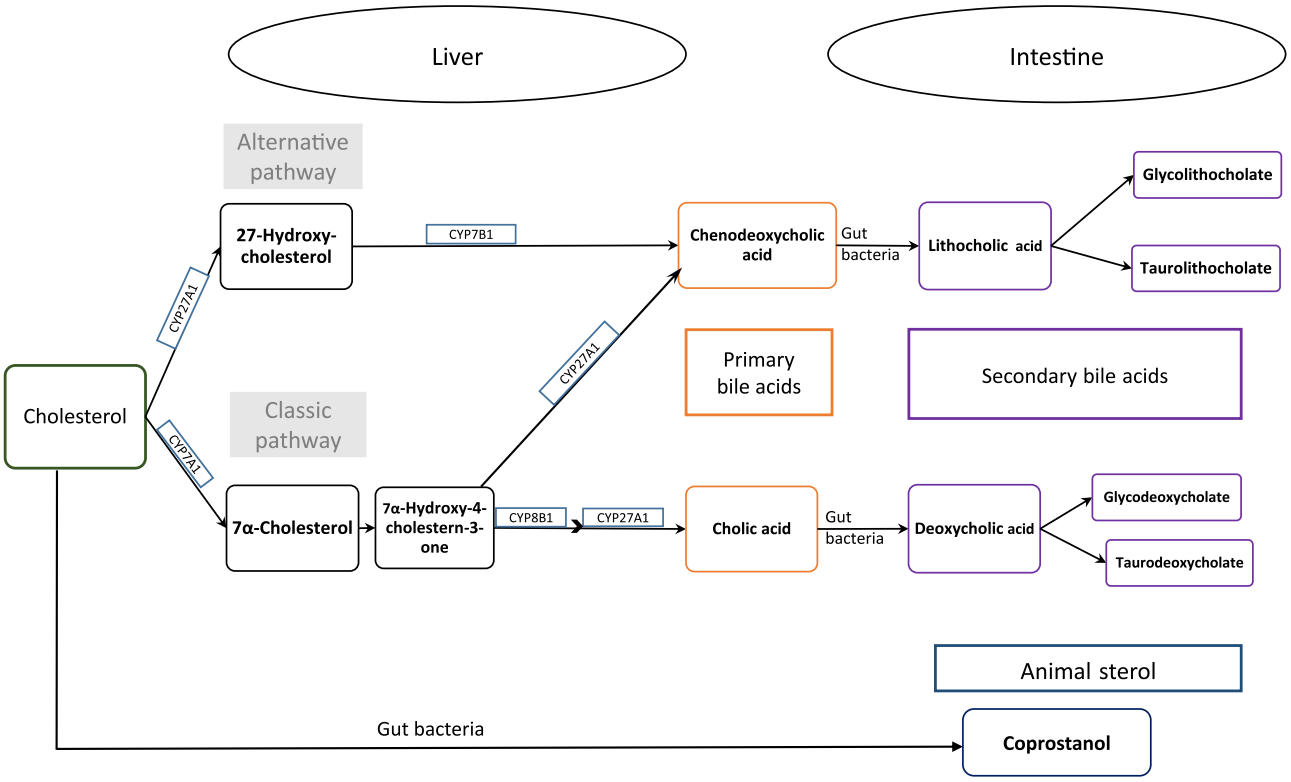

Fig. 1. Cholesterol metabolism pathway. Primary bile acids are produced in the liver by endogenous enzymes in the liver and metabolised into secondary bile acids by intestinal microbiota. Cholesterol is also metabolised to coprostanol by intestinal bacteria with a microbial steroid 5β-reductase enzyme (adapted from Kaddurah-Daouk *et al.* 2011[7]).

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 faeces either directly or in the form of bile acids. Ferezou *et al.*[6] described already in the late 1970s that 9·5 % of faecal neutral sterols is cholesterol itself. Among the direct cholesterol metabolites produced in the gut, coprostanol (next to cholestanol and coprostanone) contributes 65 % of faecal 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 faeces. A detailed description of the metabolic pathway of cholesterol and bile acids, its absorption and excretion in stool is given elsewhere[8].

Phytosterols, such as stigmasterol or β-sitosterol, are naturally occurring compounds in plant foods and are similar to cholesterol in both structure and biological 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[10–12]. 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[13], where coprostanol and coprostanone are formed from cholesterol. Sitosterol and campesterol are metabolised to methyl or ethyl coprostanol and methyl or ethyl coprostanone. Absorbed phytosterols from the intestine are excreted faster than cholesterol via biliary secretion, leading to a small pool size of phytosterols compared with animal sterols[14]. According to Gylling & Miettinen[15], the sum of plant sterols and its derivatives excreted in faeces is proportional to dietary plant sterol intake. Furthermore, the amount of plant sterols consumed does influence the faecal sterol concentrations, since plant sterols enhance cholesterol excretion[5].

In the present study, we investigate whether habitual dietary intake, meaning long-term intake, is associated with faecal concentrations of animal sterols, plant sterols and bile acids in participants of the observational, population-based Cooperative Health Research in the Augsburg Region (KORA FF4) study. Such biomarkers of dietary intake would be very helpful in characterising 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[16]. Of the 4216 participants aged 25–74 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, 14 years. 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.

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

The flow chart in Fig. 2 illustrates samples sizes and the inclusion and exclusion criteria applied. Stool samples were collected from all 2279 participants in the KORA FF4 study. From these, 1415 stool samples were analysed. Samples were excluded if participants had been on antibiotics within the last two months before sample collection, no laboratory ID number was available, or if storage conditions of the sample were unclear. Furthermore, due to financial constraints, only samples with the best storage conditions were chosen. The stool samples analysed were not exposed to room temperature longer than 3 h overall and were cooled for less than 48 h before they were deep-frozen. Finally, the metabolomics analysis was 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-h food lists (24HFL) and a web-based 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[17]. Briefly, the 24HFL is a closed and structured list of food items used to identify which food items and drinks were consumed over the past day. It neither assesses meals nor portion sizes. For each food item, either yes or no must be answered regarding consumption during the past 24 h. Freese *et al.*[17] describe that the 246 food items were chosen such that at least 75 % of the variation in nutrient intake was covered. It is important to note that in our study the 24HFL was used in a blended approach. The stand-alone use of the 24HFL was not validated.

The results of the combination of two assessment instruments for usual intake estimation were compared with the isolated use of these instruments. The results clearly demonstrated that the combined use of at least two 24-h recalls and an FFQ gave more valid results on food intake as compared with the use of 24-h recalls or an FFQ alone[18].

The FFQ included 148 food items to record food consumption frequencies and amounts over the past 12 months. It is based on the German version of the multilingual European Food Propensity Questionnaire (EFPQ)[19] and has been validated[20]. Participants used pictograms to estimate portion size. The frequency of food item consumption was assessed in specified categories (never, once a month or less, two or three times a month, one to two times a week, three to four times a week, five to six

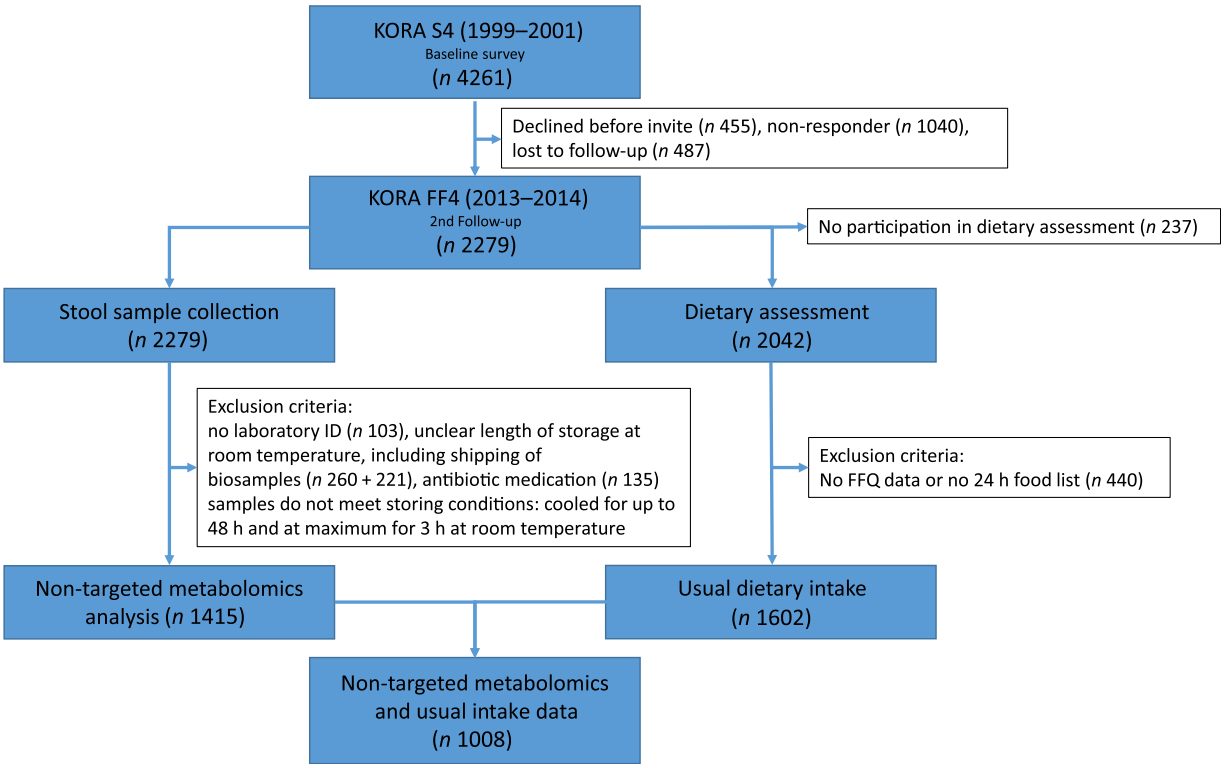

Fig. 2. Flow diagram illustrating the sample sizes and exclusion criteria of metabolite measurements and usual dietary intake in the Cooperative Health Research in the Augsburg Region (KORA FF4) study.

times a week, one time per d, two times per d, three times per d, and more than three times per d).

Information on sociodemographic variables and lifestyle factors was collected in an extensive, standardised, face-to-face interview at the study centre. Furthermore, all participants underwent anthropometric assessment that included weight and height measurement.

Stool sample collection, preprocessing and non-targeted metabolomics analysis

Together with the appointment letter, participants were provided with instructions and the necessary equipment to collect faecal samples on the day of their study centre 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 stabilising 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 into the study centre where the faecal samples were deep-frozen at −20°C immediately and later stored at −80°C until processing. Using the stool collection questionnaire, storage conditions of the samples were assessed, as these have great impact on the quality of the sample. For our analysis, the native samples without stabilising agent were used. Details on the following laboratory measurements are given in the Supplementary material.

Sample weight correction was achieved by dividing the raw measurement (area counts) by the sample weight. Each participant gave one stool sample from 1 d, so no intra-individual variation was captured. No further technical adjustment was performed, as inspection of data showed consistent performance across all run days. We term these preprocessed metabolite data as metabolite concentrations throughout the paper.

From all finally available 807 metabolites, primary and secondary bile acids and sterols were selected for the analysis. The final preprocessed data set included thirty 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. Five individual metabolites with more than 25 % of missing values (cholate sulphate, 7-ketolithocholate, glycocholenate sulphate, taurodeoxycholate and ursodeoxycholate sulphate) were excluded from the analysis. Based on the imputed data set, for plant sterols and animal sterols, the sum over all metabolite measurements of the respective group was derived as a further variable to be analysed, generating two further metabolite variables. In total, this results in $30 - 5 + 2 = 27$ metabolites to be analysed.

Usual dietary intake

From each participant up to three 24HFL and a FFQ were available to estimate usual, meaning long-term, intake by combining the results of these two instruments. From these two instruments, the usual intake was derived as described in detail in the Supplementary material. We restricted the analysis to the following nineteen 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', 'soya 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 were analysed. When including usual intake values in the regression models described below, each food item was scaled by its standard deviation. The total energy intake (kJ/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 categorised as 'no or low alcohol consumption' (<5 g/d for men and <2 g/d for women), 'moderate alcohol consumption' (≥5–<20 g/d for men and ≥2–<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 d, estimated from the 24HFL and FFQ as described before[21]. 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 1 h/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 categorised as 'up to 12 years of education' and 'more than 12 years of education'. Use of lipid-lowering medication and antidiabetic medications were included. These were identified by ATC codes C10 and A10.

For the descriptive analysis, BMI, computed as weight/height^2 (kg/m^2), was additionally categorised as underweight (BMI < 18·5 kg/m^2), normal (18·5 kg/m^2 ≤ BMI <25 kg/m^2), preobese (25 ≤ BMI < 30 kg/m^2) or obese (BMI ≥ 30 kg/m^2); 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 (6·99 mmol/l) or plasma glucose level 2 h after intake of 75 g of glucose ≥200 mg/dl (11·1 mmol/l)). For thirty-one participants, the diabetes status was missing (OGTT information missing or no validation possible), and for two 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

Table 1. Clinical and lifestyle characteristics of the study population, by sex (*n* 1008)
(Numbers and percentages; medians and 25%-quantiles, 75%-quantiles)

	Male		Female		
	n	%	*n*	%	*P**
Sex	513	50·9	495	49·1	
Age, years					0·018
Median	60		58		
25 %-quantile, 75 %-quantile	50, 70		48, 66		
BMI (kg/m^2)					0·027
Median	27·32		26·24		
25 %-quantile, 75 %-quantile	24·96, 30·41		23·49, 29·96		
BMI†					<0·001
Underweight	0	0	3	0·6	
Normal	130	25·3	187	37·8	
Preobese	242	47·2	181	36·6	
Obese	141	37·5	124	25·0	
Smoking status					<0·001
Never	211	41·1	287	58·0	
Former	225	43·9	145	29·3	
Current	77	15·0	63	12·7	
Physical activity‡					0·013
Regular, >2 h/week	150	29·2	133	26·9	
Regular, 1 h/week	149	29·0	189	38·2	
Irregular, 1 h/week	72	14·0	67	13·5	
Almost no/no physical activity	142	27·7	106	21·4	
Alcohol consumption§					<0·001
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					0·008
≤12 years	297	57·9	328	66·3	
>12 years	216	42·1	167	33·7	
Type 2 diabetes					<0·001
Yes	79	15·8	53	11·1	
No	420	84·2	425	88·9	
Missing	14		18		
Hypertension					<0·001
Yes	231	45·1	162	32·8	
No	281	54·9	332	67·2	
Missing	1		1		

* *t* Test for continuous variables, χ^2 test for categorical variables.
† Underweight, BMI < 18·5 kg/m^2; normal-weight, BMI < 25 kg/m^2; preobese, BMI 25–29·99 kg/m^2; obese, BMI ≥ 30 kg/m^2. For the χ^2 test underweight participants were discarded.
‡ 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–<20 g/d for men and ≥2–<10 g/d for women; heavy alcohol consumption ≥20 g/d for men and ≥10 g/d for women.

education, medication, total energy intake and usual intake of selected food groups and subgroups. Age (years) and BMI were included as continuous variables in the analyses.

The descriptive analysis provided information about percentage of missing values, medians, and 25 %- and 75 %-quantiles of all metabolites and metabolite groups. Median and 25 %- and 75 %-quantiles or absolute numbers and percentage 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. Sex differences between characteristics of the population were examined by *t* tests and χ^2 tests. To detect sex-specific differences in the usual dietary intake, we chose the Kruskal–Wallis test due to typically skewed intake distributions.

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 twenty-seven metabolite variables, 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, lipid-lowering medication and total energy intake. We report effect estimates and *P* values. Bonferroni adjustment of the *P* values was done by dividing 5 % by the number of tests conducted ($27 \times 19 = 513$, leading to $9{\cdot}746589 \times 10^{-5}$).

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

Table 2. Dietary characteristics of the study population, by sex (*n* 1008)
(Medians and 25%-quantile, 75%-quantile)

	Male		Female		
	Median	25%-quantile, 75%-quantile	Median	25%-quantile, 75%-quantile	*P**
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	<0·001
Vegetables	148·7	121·3, 185·6	182·6	146·8, 224·4	<0·001
Fruits	138·5	80·7, 205·3	147·5	100·2, 208·3	0·026
Nuts, seeds, nut spread	4·7	3·3, 9·3	3·8	2·5, 9·05	<0·001
Milk and dairy products	149·6	99·2, 229·0	202·8	136·5, 274·7	<0·001
Yogurt	18·6	11·8, 54·0	39·3	17·9, 79·5	<0·001
Cheese (including cream cheese)	27·9	19·3, 38·5	25·7	18·3, 36·8	0·041
Cereals and cereal products	187·8	162·6, 219·4	137·9	120·4, 163·6	<0·001
Meat and meat products	140·2	116·9, 161·9	85·2	72·65, 99·75	<0·001
Pork	21·5	17·7, 30·1	13·9	11·25, 19·05	<0·001
Processed meat	60·1	43·7, 75·3	31·2	24·5, 40·45	<0·001
Fish and shellfish	18·5	13·2, 26·9	15	11·1, 22·2	<0·001
Eggs and egg products	14·8	10·7, 21·9	13·2	9·9, 17·7	<0·001
Sugar	39·5	27·7, 50·7	33·6	24·6, 42·9	<0·001
Butter	16·5	8·6, 21·9	12·5	7·15, 15·45	<0·001
Vegetable oil	5·7	3·8, 8·5	5·2	3·45,7·65	0·006
Soya products	0·1	0, 0·1	0·1	0, 0·2	0·626
Non-alcoholic drinks	1501	1312·0, 180·0	1582	1416·0, 1783·0	<0·001
Alcoholic drinks	283·6	100·7, 564·7	37·3	25·1, 82·7	<0·001
Usual nutrient intake (g/d)					
Protein	76·59	69·46, 85·84	61·27	54·12, 68·31	<0·001
Carbohydrate	216·2	189·1, 248·6	173·2	149·8, 201·9	<0·001
Ethanol	13·53	5·53, 25·33	2·42	1·64, 5·28	<0·001
Fat	86·55	77·15, 97·91	67·49	60·0, 74·91	<0·001
SFA	38·95	34·24, 43·87	30·89	27·12, 34·45	<0·001
Unsaturated fatty acids	30·73	27·65, 34·99	23·33	20·89, 26·23	<0·001
PUFA	10·98	9·58, 12·9	8·61	7·69, 10·09	<0·001
n-3 fatty acids	1·63	1·42, 1·92	1··31	1·14, 1·52	<0·001
n-6 fatty acids	9·29	8·02, 10·99	7·25	6·47, 8·50	<0·001
Cholesterol	0·32	0·28, 0·37	0·25	0·22, 0·28	<0·001

* Kruskal–Wallis test (*P* value is 5 %).

Results

The present study was comprised of 513 men and 495 women with a mean age of 60 years and 58 years, respectively. On average, men had a higher BMI and a higher energy intake as compared with 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. These differences in food intake are naturally reflected in the nutrient intake. Higher amounts of alcohol, carbohydrate, fat and protein in men are due to higher intake of alcoholic drinks, cereal products and meat and meat products.

Faecal metabolite concentrations after minimum imputation are described in Table 3. We report the medians, 25 %- and 75 %-quantiles of the data and the percentages of imputed values.

Table 4 shows the correlations between metabolites. The top 10 % of correlations are highlighted, as are the 10 % of lowest correlations. It is noteworthy that both sitostanol and coprostanol consistently show the weakest correlations with most bile acids.

Tables 5 and 6 show the significant findings when analysing 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 faecal 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 faecal concentrations. An exception arises with 'cereals and cereal products' intake. This food group intake was inversely correlated with cholesterol faecal concentration, whereas positively associated with sitostanol faecal concentration. After Bonferroni correction, 'fruit' intake was significantly inversely associated with the faecal concentration of campesterol, cholesterol and the sum of animal sterols. With higher 'nuts and seeds' consumption, faecal total cholesterol concentration significantly decreased. Furthermore, consumption of 'meat and meat products' was significantly positively related to the faecal concentration of cholesterol. Partial R^2 values show, in general, a small contribution of the dietary intake in the models fitted. Nonetheless, the

Table 3. Description of metabolite concentrations in faecal samples of the study participants (after imputation) (*n* 1008) (Medians and 25%-quantiles, 75%-quantiles)

	n imputed (%)	Median	25%-quantile	75%-quantile
Primary bile acids				
Cholate	3	0·0469	0·0134	0·1899
Glycochenodeoxycholate	6	0·0479	0·0188	0·1215
Glycocholate	1	0·0525	0·0195	0·1490
Secondary bile acids				
12-Dehydrocholate	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
Dehydrolithocholate	1	0·0514	0·0241	0·0896
Deoxycholate	2	0·0504	0·0170	0·1067
Glycodoxycholate	9	0·0426	0·0165	0·1123
Glycolithocholate sulphate	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
Ursocholate	1	0·0495	0·0247	0·1786
Ursodeoxycholate	2	0·0466	0·0205	0·1172
Plant sterols				
β-Sitosterol	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
Animal sterols				
Cholesterol	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	

impact is highest for those associations with the smallest *P* values.

To find out if our detected inverse association of fruit intake and animal sterols is confounded by meat and meat products intake, we adjusted the fruit intake models for this potential confounder. The sum of animal sterols was still significantly associated with fruit intake at the Bonferroni-corrected significant level. The association of cholesterol and fruit intake was just above the significant level (results not shown). Overall, the intake of 'fruits', 'nuts and seeds', 'yogurt', 'cheese', 'butter', and 'soya products' was mainly inversely associated with selected faecal primary and secondary bile acid concentrations (Table 6). However, some bile acids were also positively associated with 'fruits' and 'yogurt' consumption (e.g. glycocholate, glycolithocholate sulphate). 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 faecal 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 faecal 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 faecal concentrations of campesterol, cholesterol and sum of animal sterols. A higher consumption of 'nuts and seeds' was also associated with lower faecal concentrations of these metabolites. Additionally, a significant positive association between 'meat and meat products' consumption and faecal 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 faeces. 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 are not surprising[22,23]. 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 faecal bacterial enzymes, although faecal secondary bile acid

Table 4. Correlation matrix of metabolites (*n* 1008)

	β-Sitosterol	Campesterol	Ergosterol	Sitostanol	Stigmasterol	Cholesterol	Coprostanol	Cholate	Glycochenodeoxycholate	Glycocholate	12-Dehydrocholate	3b-Hydroxy-5-cholenoic acid	6-Oxolithocholate	7,12-Diketolithocholate	7-Ketodeoxycholate	Dehydrolithocholate	Deoxycholate	Glycodoxycholate	Glycolithocholate sulphate	Glycoursodeoxycholate	Hyocholate	Isoursodeoxycholate	Lithocholate	Ursocholate	Ursodeoxycholate
β-Sitosterol	1·00*	0·88*	0·07	−0·03	0·75*	0·66*	−0·17†	0·16	0·11	0·12	0·19	0·26	0·15	0·22	0·19	0·04	0·32	0·13	0·10	0·16	0·35	0·28	0·33	0·20	0·30
Campesterol	0·88*	1·00*	0·07	−0·04	0·66*	0·71*	−0·05	0·15	0·09	0·10	0·18	0·28	0·15	0·22	0·19	0·06	0·40	0·13	0·10	0·14	0·39	0·30	0·40	0·20	0·32
Ergosterol	0·07	0·07	1·00*	0·02	0·03	0·06	0·01	0·00	0·12	0·03	0·01	0·04	0·02	0·00	0·00	0·07	0·01	0·11	0·08	0·03	0·02	0·01	0·04	0·01	0·01
Sitostanol	−0·03	−0·04	0·02	1·00*	0·02	−0·33†	0·64*	−0·18†	−0·11†	−0·11†	−0·18†	−0·07	0·14	−0·13†	−0·15†	0·26	−0·08	−0·12†	−0·13†	−0·14†	−0·19†	−0·19†	0·05	−0·16†	−0·20†
Stigmasterol	0·75*	0·66*	0·03	0·02	1·00*	0·51	0·02	0·08	0·07	0·09	0·11	0·16	0·10	0·17	0·12	0·11	0·21	0·10	0·03	0·14	0·23	0·18	0·25	0·11	0·18
Cholesterol	0·66*	0·71*	0·06	−0·33†	0·51	1·00*	−0·26†	0·23	0·10	0·11	0·29	0·34	0·06	0·27	0·23	−0·01	0·40	0·15	0·14	0·17	0·48	0·38	0·36	0·25	0·41
Coprostanol	−0·17†	−0·05	0·01	0·64*	0·02	−0·26†	1·00*	−0·16†	−0·10†	−0·09	−0·13†	−0·03	0·17	−0·07	−0·13†	0·29	0·03	−0·08	−0·10†	−0·11†	−0·16†	−0·16†	0·18	−0·14†	−0·15†
Cholate	0·16	0·15	0·00	−0·18†	0·08	0·23	−0·16†	1·00*	0·31	0·24	0·57	0·25	−0·10	0·29	0·51	−0·15†	0·25	0·37	0·49	0·34	0·51	0·51	0·06	0·55	0·62*
Glycochenodeoxycholate	0·11	0·09	0·12	−0·11†	0·07	0·10	−0·10†	0·31	1·00*	0·58	0·13	0·07	−0·04	0·12	0·17	0·00	0·15	0·72*	0·57	0·50	0·18	0·21	0·12	0·15	0·20
Glycocholate	0·12	0·10	0·03	−0·11†	0·09	0·11	−0·09	0·24	0·58	1·00*	0·16	0·07	−0·06	0·12	0·18	−0·03	0·09	0·52	0·32	0·69*	0·16	0·21	0·03	0·17	0·22
12-Dehydrocholate	0·19	0·18	0·01	−0·18†	0·11	0·29	−0·13†	0·57	0·13	0·16	1·00*	0·36	0·01	0·66*	0·60	−0·09	0·22	0·23	0·18	0·23	0·48	0·52	0·12	0·45	0·59
3b-Hydroxy-5-cholenoic acid	0·26	0·28	0·04	−0·07	0·16	0·34	−0·03	0·25	0·07	0·07	0·36	1·00*	0·10	0·19	0·18	0·14	0·44	0·14	0·15	0·10	0·29	0·34	0·47	0·28	0·37
6-Oxolithocholate	0·15	0·15	0·02	0·14	0·10	0·06	0·17	−0·10	−0·04	−0·06	0·01	0·10	1·00*	0·08	−0·06	0·33	0·06	−0·04	−0·03	−0·05	0·06	−0·03	0·24	−0·07	−0·02
7,12-Diketolithocholate	0·22	0·22	0·00	−0·13†	0·17	0·27	−0·07	0·29	0·12	0·12	0·66*	0·19	0·08	1·00*	0·65*	0·04	0·15	0·14	0·07	0·26	0·40	0·46	0·13	0·31	0·43
7-Ketodeoxycholate	0·19	0·19	0·00	−0·15†	0·12	0·23	−0·13†	0·51	0·17	0·18	0·60	0·18	−0·06	0·65*	1·00*	−0·10	0·15	0·13	0·10	0·26	0·38	0·67*	0·04	0·63*	0·58
Dehydrolithocholate	0·04	0·06	0·07	0·26	0·11	−0·01	0·29	−0·15†	0·00	−0·03	−0·09	0·14	0·33	0·04	−0·10	1·00*	0·02	0·01	−0·04	−0·06	−0·11†	−0·12†	0·31	−0·12†	−0·12†
Deoxycholate	0·32	0·40	0·01	−0·08	0·21	0·40	0·03	0·25	0·15	0·09	0·22	0·44	0·06	0·15	0·15	0·02	1·00*	0·27	0·22	0·15	0·34	0·39	0·78*	0·18	0·45
Glycodoxycholate	0·13	0·13	0·11	−0·12†	0·10	0·15	−0·08	0·37	0·72*	0·52	0·23	0·14	−0·04	0·14	0·3	0·01	0·27	1·00*	0·69*	0·51	0·23	0·19	0·16	0·11	0·27
Glycolithocholate sulphate	0·10	0·10	0·08	−0·13†	0·03	0·14	−0·10†	0·49	0·57	0·32	0·18	0·15	−0·03	0·07	0·10	−0·04	0·22	0·69*	1·00*	0·30	0·22	0·17	0·12	0·15	0·24
Glycoursodeoxycholate	0·16	0·14	0·03	−0·14†	0·14	0·17	−0·11†	0·34	0·50	0·69*	0·23	0·10	−0·05	0·26	0·26	−0·06	0·15	0·51	0·30	1·00*	0·32	0·38	0·07	0·20	0·35
Hyocholate	0·35	0·39	0·02	−0·19†	0·23	0·48	−0·16†	0·51	0·18	0·16	0·48	0·29	0·06	0·40	0·38	−0·11†	0·34	0·23	0·22	0·32	1·00*	0·61	0·21	0·48	0·69*
Isoursodeoxycholate	0·28	0·30	0·01	−0·19†	0·18	0·38	−0·16†	0·51	0·21	0·21	0·52	0·34	−0·03	0·46	0·67*	−0·12†	0·39	0·19	0·17	0·38	0·61	1·00*	0·25	0·83*	0·89*
Lithocholate	0·33	0·40	0·04	0·05	0·25	0·36	0·18	0·06	0·12	0·03	0·12	0·47	0·24	0·13	0·04	0·31	0·78*	0·16	0·12	0·07	0·21	0·25	1·00*	0·09	0·30
Ursocholate	0·20	0·20	0·01	−0·16†	0·11	0·25	−0·14†	0·55	0·15	0·17	0·45	0·28	−0·07	0·31	0·63*	−0·12†	0·18	0·11	0·15	0·20	0·48	0·83*	0·09	1·00*	0·75*
Ursodeoxycholate	0·30	0·32	0·01	−0·20†	0·18	0·41	−0·15†	0·62*	0·20	0·22	0·59	0·37	−0·02	0·43	0·58	−0·12†	0·45	0·27	0·24	0·35	0·69*	0·89*	0·30	0·75*	1·00*

* Highest 10 % of correlations.
† Lowest 10 % of correlations.

Table 5. Regression coefficient estimates, standard errors of estimates, *P* and partial R^2 modelling associations of faecal sterols with habitual intake (only associations with *P* <5 % are reported†; *n* 1008)

Food group (g/d)	Metabolite	Estimate	SE	*P*	Median of concentration	Partial R^2
Potatoes	Campesterol	0·120	0·040	0·002892	0·0500	0·0089
Potatoes	Sum of plant sterols	0·085	0·033	0·009016	0·2989	0·0069
Potatoes	Stigmasterol	0·089	0·038	0·018518	0·0476	0·0056
Potatoes	β-Sitosterol	0·086	0·037	0·018881	0·0511	0·0056
Potatoes	Cholesterol	0·108	0·048	0·023228	0·0522	0·0052
Fruits	Sum of animal sterols	−0·131	0·028	0·000003*	0·1320	0·0216
Fruits	Campesterol	−0·160	0·036	0·000007*	0·0500	0·0202
Fruits	Cholesterol	−0·180	0·042	0·000021*	0·0522	0·0181
Fruits	Sum of plant sterols	−0·111	0·029	0·000132	0·2989	0·0147
Fruits	Stigmasterol	−0·121	0·034	0·000336	0·0476	0·0129
Fruits	Ergosterol	−0·173	0·054	0·001509	0·0508	0·0101
Fruits	β-Sitosterol	−0·102	0·032	0·001618	0·0511	0·0100
Nuts, seed and nut spread	Sum of animal sterols	−0·112	0·026	0·000024*	0·1320	0·0179
Nuts, seed and nut spread	Ergosterol	−0·192	0·051	0·000179	0·0508	0·0141
Nuts, seed and nut spread	Sum of plant sterols	−0·090	0·027	0·000971	0·2989	0·0109
Nuts, seed and nut spread	Campesterol	−0·097	0·034	0·004115	0·0500	0·0083
Nuts, seed and nut spread	Cholesterol	−0·096	0·040	0·016798	0·0522	0·0058
Nuts, seed and nut spread	Coprostanol	−0·110	0·048	0·020992	0·0492	0·0054
Milk and dairy products	Sum of animal sterols	−0·065	0·028	0·021292	0·1320	0·0053
Milk and dairy products	Sum of plant sterols	−0·062	0·029	0·031410	0·2989	0·0047
Milk and dairy products	Ergosterol	−0·116	0·054	0·032991	0·0508	0·0046
Yogurt	Sum of animal sterols	−0·078	0·026	0·002554	0·1320	0·0092
Cheese	Sum of animal sterols	−0·094	0·027	0·000509	0·1320	0·0121
Cheese	Cholesterol	−0·129	0·040	0·001432	0·0522	0·0102
Cheese	Stigmasterol	−0·088	0·032	0·006093	0·0476	0·0076
Cheese	β-Sitosterol	−0·079	0·031	0·010856	0·0511	0·0065
Cheese	Campesterol	−0·079	0·034	0·020562	0·0500	0·0054
Cheese	Sum of plant sterols	−0·055	0·028	0·045793	0·2989	0·0040
Cereals and cereal products	Cholesterol	−0·134	0·062	0·031550	0·0522	0·0047
Cereals and cereal products	Sitostanol	0·161	0·078	0·037869	0·0490	0·0043
Meat and meat products	Cholesterol	0·244	0·062	0·000081*	0·0522	0·0156
Meat and meat products	Campesterol	0·189	0·052	0·000301	0·0500	0·0131
Meat and meat products	Sum of animal sterols	0·136	0·041	0·000949	0·1320	0·0110
Meat and meat products	β-Sitosterol	0·122	0·047	0·010114	0·0511	0·0067
Meat and meat products	Sum of plant sterols	0·100	0·042	0·018348	0·2989	0·0056
Pork	Sum of animal sterols	0·084	0·031	0·005991	0·1320	0·0076
Pork	Cholesterol	0·119	0·046	0·009913	0·0522	0·0067
Pork	Campesterol	0·082	0·039	0·035595	0·0500	0·0045
Pork	Sum of plant sterols	0·065	0·031	0·040136	0·2989	0·0042
Processed meat	Cholesterol	0·173	0·052	0·001015	0·0522	0·0109
Processed meat	Sum of animal sterols	0·102	0·035	0·003689	0·1320	0·0085
Processed meat	Campesterol	0·125	0·044	0·004922	0·0500	0·0080
Eggs and egg products	Sum of animal sterols	0·079	0·026	0·002129	0·1320	0·0095
Eggs and egg products	Cholesterol	0·091	0·039	0·018491	0·0522	0·0056
Butter	Ergosterol	0·147	0·054	0·006293	0·0508	0·0075
Butter	Sum of animal sterols	0·061	0·028	0·029641	0·1320	0·0048

* *P* indicating significant associations after Bonferroni correction ($P < 9{\cdot}746589 \times 10^{-5}$).
† 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 energy intake.

excretion is increased[22]. However, Hentges *et al.*[24] did not observe increasing concentrations of bile acids in faeces 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 faeces is not as straight forward. At least in part, it may be explainable by the higher plant sterol intake and its physiological consequences on cholesterol absorption and excretion[25,26], which shall be discussed in the following paragraph.

Plant sterols have a plasma cholesterol-lowering property as described in numerous studies[3,27–31]. 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)[13,32]. Weststrate *et al.*[25] analysed faecal concentrations of sterols and bile acids and found a significant increase in faecal neutral sterols after consumption of phytosterol-enriched margarine. Also Racette *et al.*[26] concluded that phytosterols act as bioactive compounds that lead to increased cholesterol excretion in faeces. In an intervention study, eighteen participants followed a low-phytosterol diet and received beverages supplemented with 0, 400 or 2000 mg phytosterols/d for 4 weeks each, with 1 week washout period. They reported that consuming dietary

Table 6. Regression coefficient estimates, standard errors of estimates, *P* and partial R^2 modelling associations of faecal bile acids with habitual dietary intake (only associations with *P* <5 % are reported†; *n* 1008)

Food group (g/d)	Metabolite	Estimate	SE	*P*	Median of concentration	Partial R^2
Potatoes	Deoxycholate	0·143	0·058	0·014425	0·0504	0·006031
Potatoes	Lithocholate	0·082	0·037	0·024920	0·0504	0·005070
Vegetables	12-Dehydrocholate	0·223	0·091	0·014203	0·0320	0·006058
Fruits	Glycocholate	0·237	0·059	0·000058*	0·0525	0·013907
Fruits	Hyocholate	−0·199	0·055	0·000326	0·0454	0·013128
Fruits	7,12-Diketolithocholate	−0·189	0·071	0·007827	0·0338	0·005920
Fruits	Ursocholate	−0·153	0·061	0·012232	0·0495	0·007134
Fruits	Glycolithocholate sulfate	0·152	0·061	0·013571	0·0446	0·006044
Fruits	Isoursodeoxycholate	−0·123	0·051	0·016015	0·0491	0·006119
Fruits	Glycochenodeoxycholate	0·129	0·055	0·018184	0·0479	0·000002
Fruits	Deoxycholate	−0·117	0·052	0·024726	0·0504	0·004882
Nuts, seed and nut spread	Hyocholate	−0·144	0·052	0·005792	0·0454	0·006880
Nuts, seed and nut spread	Lithocholate	−0·080	0·031	0·008960	0·0504	0·005328
Nuts, seed and nut spread	Dehydrolithocholate	−0·093	0·040	0·021496	0·0514	0·004603
Nuts, seed and nut spread	Ursodeoxycholate	−0·108	0·050	0·032633	0·0466	0·007062
Milk and dairy products	Glycocholate	0·156	0·059	0·008093	0·0525	0·004675
Milk and dairy products	Dehydrolithocholate	0·093	0·043	0·031300	0·0514	0·008118
Yoghurt	Glycocholate	0·154	0·054	0·004511	0·0525	0·004310
Yoghurt	Ursocholate	−0·116	0·056	0·038704	0·0495	0·004308
Yoghurt	Isoursodeoxycholate	−0·097	0·047	0·038749	0·0491	0·004252
Yoghurt	Glycolithocholate sulphate	0·116	0·056	0·040044	0·0446	0··006643
Cheese	Lithocholate	−0·080	0·031	0·010226	0·0504	0·004301
Cheese	Deoxycholate	−0·103	0·050	0·038898	0·0504	0·004206
Cheese	Ursodeoxycholate	−0·105	0·051	0·041136	0·0466	0·004063
Meat and meat products	Hyocholate	0·162	0·081	0·044747	0·0454	0·003970
Meat and meat products	Ursocholate	0·177	0·089	0·047247	0·0495	0·010524
Pork	6-Oxolithocholate	0·193	0·059	0·001214	0·0435	0·009596
Pork	Lithocholate	0·109	0·035	0·002009	0·0504	0·003984
Processed meat	Hyocholate	0·137	0·069	0·046860	0·0454	0·012058
Eggs and egg products	Deoxycholate	0·164	0·047	0·000531	0·0504	0·008699
Eggs and egg products	Lithocholate	0·087	0·030	0·003280	0·0504	0·008213
Eggs and egg products	Ursodeoxycholate	0·139	0·049	0·004283	0·0466	0·005262
Eggs and egg products	Isoursodeoxycholate	0·106	0·046	0·022321	0·0491	0·004382
Eggs and egg products	Hyocholate	0·105	0·050	0·037117	0·0454	0·008743
Butter	Glycolithocholate sulfate	−0·179	0·061	0·003202	0·0446	0·005174
Vegetable oil	7,12-Diketolithocholate	0·149	0·066	0·023479	0·0338	0·005802
Soya products	Cholate	−0·170	0·071	0·016418	0·0469	0·006031

* *P* indicating significant associations after Bonferroni correction ($P < 9{\cdot}746589 \times 10^{-5}$).
† Models were adjusted for age, sex, BMI, smoking status, alcohol consumption, physical activity, years of education, medication use and total energy intake.

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 faecal excretion of cholesterol through the intake of a phytosterol-rich diet[33].

In the long-term, however, it is not clear whether a diet relatively high in plant sterols is associated with lower faecal animal sterols. Jaceldo-Siegl *et al.*[34] examined dietary intake and plasma concentration of plant sterols and cholesterol across five 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 d was observed[35]. However, the daily intake of plant sterols following a regular diet ranges between 160 and 400 mg/d[34], which is lower than the required concentration to achieve a higher excretion rate of sterols in faeces[26].

Our results provide novel information, since no other study has analysed faecal concentrations of sterols in faeces 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 faecal animal sterols. In our study, on average 4·7 g/d 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 fibre intake. As high dietary fibre intake leads to increased faecal bulk, and this may result in lower faecal concentration of sterols and bile acids per g of dry weight[36].

Diets that are rich in fruits are linked to a decreased colorectal cancer incidence. Regarding bile acids, it is noteworthy that dietary fibres from vegetables and fruits can bind to the secondary bile acid lithocholate and enhance its faecal excretion[37,38]. This may explain our finding of a positive association between fruit consumption and glycocholate excretion,

although for all other bile acids we found (non-significant) inverse associations.

Also, the amount of vegetable oils – rich in phytosterols – consumed in our population sample (on average, 5·7 g/d) is likely not high enough to result in a significant effect on faecal sterols. However, intervention studies did observe an effect of replacing butter consumption with vegetable oils and found a significant increase in faecal excretion of sterols and bile acids[39]. In another study, the faecal sterols concentration increased from 30 mg/g to about 50 mg/g dry weight after enriching margarine with 8·6 g vegetable oils[25]. However, we only observed a correlation between vegetable oils intake and faecal 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 faecal 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 faecal samples[26,33] or plasma only[34]. Some studies examined faecal samples only in (short- to medium-term) intervention studies[1,25,26,33].

Our study is of observational nature and stool samples were collected only once per subject. To consider day-to-day variation, collecting faecal samples on 3–5 d from each participant was recommended by Setchell *et al.*[40]. 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 centre; rather, it is collected at home and thus has to be stored until the study centre visit. Although correct handling and storage was communicated to all participants, not all stool samples were stored cooled until handed over in the study centre. In a prestudy, comparisons were made concerning metabolite concentrations in fresh samples and samples stored under different conditions. Due to the results of this study and in accordance with Loftfield *et al.*[41], samples with storage at room temperature of more than 3 h were excluded from the analysis. Nonetheless, we cannot rule out that metabolite degeneration took place in the selected samples.

Faeces can be easily accessed in a non-invasive manner and make it possible to study the diet–gut microbiota–host interaction via the analysis of unabsorbed metabolites[42]. Further, sterols and bile acids are mainly metabolised by the gut microbiota and excreted in faeces[8]. Therefore, it is more reasonable to estimate especially sterol in faeces rather than in any other biospecimen. Several sterol metabolites have previously been identified in human faeces[43]. Phytosterols may be metabolised into C_{21}-bile acids in the liver and not to the common C_{24}-bile acids in mammals[13]. Since only the common C_{24}-bile acids were detected in our study and not the C_{21}-bile acids, we could not observe the total metabolite excretion of plant sterols in faeces.

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 analysed 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 faeces. Daily intake of plant sterols ranges from 160 to 400 mg in different populations (see Jaceldo-Siegl *et al.*[34]). However, to observe significant reduction of plasma LDL-cholesterol concentrations and to obtain cardiovascular health benefits, adults should consume 2 g/d[44], a dose not attainable by habitual diet without supplementation.

In conclusion, the results of this study conducted in participants from the general population indicate an effect of habitual diet on faecal 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 faeces. As expected, a diet high in 'meat and meat products' leads to higher concentrations of animal sterols in faeces. Further studies are necessary for evaluation of faecal 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 faecal animal sterol content in response to dietary habits needs further discussion.

Supplementary material

To view supplementary material for this article, please visit https://doi.org/10.1017/S000711451900103X

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P. M. drafted the manuscript and interpreted the results, N. W. conducted the statistical analysis, S. S., J. K. and A. A. prepared, analysed and processed the samples, J. L. and P. M. formulated the research question and designed the study; B. T., C. M., A. P., H. G., M. H., J. A. and J. L. conducted research and/or provided essential materials; All authors read, critically commented on and then approved the final manuscript.

There were no conflicts of interest.

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