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The complex human urinary sugar profile: determinants revealed in the cross-sectional KarMeN study

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ABSTRACT

Background: Although sugars and sugar derivatives are an important class of metabolites involved in many physiologic processes, there is limited knowledge on their occurrence and pattern in biofluids.

Objective: Our aim was to obtain a comprehensive urinary sugar profile of healthy participants and to demonstrate the wide applicability and usefulness of this sugar profiling approach for nutritional as well as clinical studies.

Design: In the cross-sectional KarMeN study, the 24-h urine samples of 301 healthy participants on an unrestricted diet, assessed via a 24-h recall, were analyzed by a newly developed semitargeted gas chromatography–mass spectrometry (GC-MS) profiling method that enables the detection of known and unknown sugar compounds. Statistical analyses were performed with respect to associations of sex and diet with the urinary sugar profile.

Results: In total, 40 known and 15 unknown sugar compounds were detected in human urine, ranging from mono- and disaccharides, polyols, and sugar acids to currently unknown sugar-like compounds. A number of rarely analyzed sugars were found in urine samples. Maltose was found in statistically higher concentrations in the urine of women compared with men and was also associated with menopausal status. Further, a number of individual sugar compounds associated with the consumption of specific foods, such as avocado, or food groups, such as alcoholic beverages and dairy products, were identified.

Conclusions: We here provide data on the complex nature of the sugar profile in human urine, of which some compounds may have the potential to serve as dietary markers or early disease biomarkers. Thus, comprehensive urinary sugar profiling not only has the potential to increase our knowledge of host sugar metabolism, but can also reveal new dietary markers after consumption of individual food items, and may lead to the identification of early disease biomarkers in the future. The KarMeN study was registered at drks.de as DRKS00004890. *Am J Clin Nutr* 2018;108:502–516.

Keywords: urinary sugar profile, monosaccharide, disaccharide, polyol, sugar acid, GC-MS, dietary marker, sex, human urine, KarMeN study

INTRODUCTION

A variety of structurally different sugar compounds is present in the human body and even more so in our diet. We use the terms "sugar compounds or sugars" to refer to the following substance classes: mono- and disaccharides, as well as derived compounds thereof like polyols and sugar acids. Currently, sugar compounds are usually analyzed in urine samples with a focus on individual substance classes and, to date, most studies in this area have been performed with only a very limited number of volunteers (summarized in **Supplemental Table 1**). Combining the results from these studies revealed a quite complex urinary sugar profile consisting of many different sugar compounds. This is surprising because most recent studies have investigated the role of sugar compounds in human body fluids and focused mainly on common and well-known sugar compounds. Sugars and sugar derivatives in urine reflect the sugar compounds consumed within the diet as well as from endogenous sources. Of note is that absolute sugar concentrations in urine are very low because numerous sugars are efficiently reabsorbed in kidney tubular cells. Nevertheless, sugar compounds in human urine appear to be suitable dietary markers and, in the future, may even serve as early disease biomarkers, but knowledge on all this is highly limited.

In a few studies, specific sugar compounds were described as dietary markers for individual food items with examples such as *chiro*- and *scyllo*-inositol for citrus fruit in serum (1),

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Supplemental Tables 1–4 and Supplemental Figures 1 and 2 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/ajcn/.

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Abbreviations used: CART, classification and regression tree; GC-MS, gas chromatography–mass spectrometry; KarMeN, Karlsruhe Metabolomics and Nutrition; QC, quality control.

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threonate and threitol for fruit and vegetables in serum (2, 3), and some C4 and C5 sugar compounds for broccoli consumption in urine (4). Other studies suggested that a combination of urinary fructose and sucrose may reflect total sugar intake (5–7), which is especially relevant in view of associations between sugar intake and negative health outcomes such as an increased risk for cardiovascular disease mortality (8, 9).

Because sugar compounds are involved in a variety of disease pathways, urinary sugars could also serve as biomarkers in the health-disease trajectory. Abnormal concentrations of urinary sugar compounds have been described for conditions such as diabetes mellitus (10–14), uremia (12, 15), invasive candidiasis (16), enzyme deficiencies like galactosemia (17, 18), pentosuria (19), or other inborn errors of metabolism (20–25). It thus seems reasonable to state that "there is more than glucose to look at" (26, 27).

With respect to the more commonly analyzed sugars, such as fructose and sucrose, there is a need to understand which factors determine their urinary excretion (28). Even less is known about the origin, metabolism, and functions of polyols (10, 23, 25). In a recent study, a new pathway for erythritol production from glucose was described and erythritol excretion was demonstrated to be associated with weight gain (29). This study highlights our current limited knowledge on sugars and sugar derivatives in the human body beyond glucose and fructose. To bridge this knowledge gap especially from the physiologic and pathophysiologic point of view, new analytical methods offering comprehensive detection of a wide range of major and many minor sugar compounds for nutritional and clinical research are thus urgently needed.

Here, we present a semitargeted gas chromatography–mass spectrometry (GC-MS) profiling method for the detection of >50 known and unknown sugar compounds in human urine and its application to 24-h urine samples derived from the observational KarMeN (Karlsruhe Metabolomics and Nutrition) study with 301 healthy participants (30).

METHODS

Study design and subjects

The cross-sectional KarMeN study was performed at the Max Rubner-Institut in Karlsruhe, Germany, between 2011 and 2013. Details on the study design and examination procedures were previously described by Bub et al. (30). Briefly, a total of 312 healthy participants aged between 18 and 80 y, who gave their written informed consent and were willing and able to perform all examinations, were recruited. Participants were excluded if they had a history of prevalent or chronic disease, were smokers, or took any medication, hormones, or dietary supplements. Women who were pregnant or breastfeeding were also excluded. Eleven participants who completed the study had to be excluded for other reasons, such as diseases requiring treatment, cardiac complications, voluntary dropout, cancer history, and acute cold with medication (30). Thus, a total of 301 participants remained for statistical analysis, 172 of whom were men and 129 were women. The local ethics committee approved the study and it was in accordance with the 1964 Helsinki declaration and its later amendments. The study was registered at the German Clinical Study Register (DRKS00004890) and has the WHO universal trial number U1111-1141-7051.

Participants were asked for a 24-h urine collection. Throughout the collection, bottles were kept in cool bags with cooling units. At the study center, the volume of the received 24-h urine samples was recorded, 2 × 14 mL were centrifuged at 1850 × *g* at 4°C for 10 min and then separated into aliquots. Samples were initially frozen at −20°C for 1 d and then cryopreserved at −196°C until analysis. A quality control (QC) sample was prepared by pooling 24-h urine samples from KarMeN participants. Osmolality was assessed via freezing-point depression of 24-h urine samples with the use of a micro-osmometer (Advanced Miro-Osmometer model 3MO, Advanced Instruments, Norwood, MA).

For the day of the 24-h urine sample collection, trained study personnel assessed the food consumption of each participant (in grams per day) in a personal interview through the use of a 24-h dietary recall with the software EPIC-Soft (developed by the International Agency for Research on Cancer (IARC) in Lyon) (31, 32), now renamed as GloboDiet. The amount of different foods consumed per day was assessed with the use of a picture booklet providing photographs of portion sizes for various foods as well as household measures and standard portions. For further analysis, the reported foods were summarized into food group variables (see **Supplemental Table 2**). Additionally, based on the German Nutrient Database "Bundeslebensmittelschlüssel" (BLS, version 3.02) (33), the total energy intake (in kcal per day) and intake of nutrients were calculated.

Semitargeted GC-MS sugar profiling

A Shimadzu GCMS QP2010 Ultra instrument was used in Scan-/SIM (selected ion monitoring)-mode to achieve high selectivity and sufficient sensitivity while at the same time being able to detect a priori unknown sugar compounds. Additionally, some abundant nonsugar compounds could be analyzed via this method. **Table 1** and **Supplemental Table 3** list all compounds that were detectable via this method, including the target and reference ions used for integration. The structural similarity of sugar compounds enables the usage of only a few selected masses for selective relative quantitation in the urine matrix (see Supplemental Table 3 and **Supplemental Figure 1**). Analytical details regarding chemicals, sample preparation, instrument, method, and data processing parameters were described by Rist et al. (34). Briefly, 24-h urine samples were diluted according to osmolality (60 mosmol/kg), 40 µL were evaporated and then derivatized via a 2-step procedure with 15 µL methoxylaminhydrochloride solution (20 mg/mL in pyridine; 30 min, 70°C, 1000 rpm) and 50 µL *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide + 1% trimethylchlorosilane reagent (1 h, 75°C) before analysis. To remove slight drift and offset effects, the raw signal intensities were corrected through the use of QC sample-based local linear regression functions (35).

Statistics

For all statistical analyses, the software JMP (version 13, SAS Institute Inc., Cary, NC, 1989–2007) was used.

Association of the urinary sugar profile with sex

A matrix with all known and unknown sugar compounds and the information on sex and age of the KarMeN participants was used to build a decision tree with the CART (classification and regression tree) algorithm. This approach was used to

TABLE 1
Summary of all sugar compounds that were included in the statistical analysis (columns 1–6) and additional analytes (columns 7–9)

Polyols	Monosaccharides[1]	Disaccharides[1]	Sugar acids[1]	Other	Unknown sugar-like	Amino acids	Organic acids	Others
meso-Erythritol	Xylose	Disaccharide U26	Erythronic acid	Levoglucosan	Unknown U03	Serine	Tartaric acid	Creatinine-enol
Threitol	Arabinose	Sucrose	Threonic acid	Ethyl-β-glucuronide	Unknown U04	Threonine	Isocitric acid	Unknown U11
Polyol U02	Ribose	Lactose	Sugar acid U01		Unknown U05	Cysteine	Hippuric acid	Unknown U16
Xylitol	Fucose	Maltose	Sugar acid U06		Unknown U12	Phenylalanine	Quinic acid	
Arabitol	Psicose	Disaccharide U27	Xylonic acid		Unknown U24	Lysine		
Ribitol	Fructose	Disaccharide U28	Ribonic acid		Unknown U25	Tyrosine		
1-Deoxysorbitol	Allose	Disaccharide U29	Sugar acid U09					
Fucitol	Galactose		Arabonic acid					
Mannitol	Glucose		Glucuronic acid					
Sorbitol	Mannose		Mannonic acid					
Galactitol	Mannoheptulose		Galactonic acid					
chiro-Inositol	Sedoheptulose		Gluconic acid					
scyllo-Inositol	Monosaccharide U21							
myo-Inositol								
Perseitol								

[1]Generally, 2 derivatives are formed for reducing sugar compounds during methoximation and silylation. For reasons of readability, the chemically exact denomination of compounds was deliberately omitted and only the first of 2 derivatives are listed.

uncover associations between the urinary sugar profile and sex. Advantages of the CART algorithm are its ability to cope with missing (not detected) values and its ability to handle categoric and numeric values in parallel. Not detected values (usually the results of signals below detection limits) were treated by the algorithm as a separate level of the variable. Concerning differences in the sugar profiles between men and women, the focus in this work was primarily on sugar compounds that were detected in <75% of the KarMeN participants, and thus, are potentially more sex-specific (in a qualitative sense). Age was included as an additional continuous variable after the first split, thus allowing the observation of associations between age and sugar compounds. Splitting was only allowed when -$\log_{10}$ (*P* values) (calculated by JMP) were significant after Bonferroni correction, meaning a -$\log_{10}$ (*P* value) >3.1206.

After CART analysis, the nonparametric Wilcoxon test was generally used to test for significant differences between men and women for the 2 most important metabolites as well as to distinguish between the maltose excretion of pre- and postmenopausal women.

Association of the urinary sugar profile with diet

To assess associations of diet with the human urinary sugar profile, an exploratory correlation analysis was performed with the use of the variables derived from the 24-h dietary recall (food and nutrient intake) with detected urinary sugar compounds (listed in Supplemental Tables 2 and 3, respectively). In a first step (selection of interesting correlations), Spearman rank correlation coefficients were determined by the pairwise method (threshold $\rho < -0.30$ or $\rho > 0.30$) and evaluated in conjunction with scatter plots. In a second step, participants were divided into groups based on consumption of certain food items for promising correlations. A Wilcoxon test was performed to ascertain significance for these groups. If <100 participants consumed a particular food or nutrient, an equally large group of nonconsumers was randomly selected. If >100 participants were consumers, tertiles were built and a Wilcoxon test for the first against the third tertile performed.

Sugar screening in plant materials from fruit and vegetables

To assess the plausibility and specificity of some of the potential dietary markers for food consumption, a screening of sugar compounds in a range of fruit and vegetable varieties was performed with the use of the same GC-MS profiling as for the urine samples. The aim was to screen as many fruit and vegetables as possible, but not to perform a comprehensive evaluation. Thus, only 1 pooled sample for each fruit and vegetable variety was measured.

Sample preparation for fruit and vegetables

Fruit and vegetables were bought from regional producers directly, weekly markets, or supermarkets. Overall, a total of 75 fruit and vegetable varieties (see **Table 2**) were purchased and, if possible, they were seasonally and regionally produced. The edible plant material of 5–20 fruits or vegetables (depending on fruit or vegetable size) was pooled into 1 sample, frozen in liquid nitrogen, and then coarsely ground and freeze dried for ≥3 d. The dried material was ground to a fine powder with a ball mill (Retsch MM400, Haan, Germany) for 20–60 s (depending on the consistence of the plant material) at 30 Hz and then stored at −80°C until analysis. For each sample, 20 ± 0.1 mg of freeze-dried powder was weighed out and then after addition of 20 µL of internal standard solution (pinitol and phenyl-β-glucopyranoside in water, each 2 mmol/L) extracted twice with 750 µL methanol for 10 min at 35°C and 1400 rpm. The collected supernatant was mixed and then centrifuged for 5 min at 4°C and 16,100 × *g*. After which, 20 µL of supernatant was evaporated and then derivatized with the use of the same 2-step procedure as for the urine samples, except that 40 µL of methoxylaminhydrochloride solution in pyridine (25 mg/mL) and 96 µL of *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide + 1% trimethylchlorosilane were used.

Semitargeted GCMS sugar profiling and data processing

The method for the measurement of the fruit and vegetable samples was the same as for the urine samples, except that the Rxi-5Sil-MS column was slightly shorter (54 m + 4 m precolumn), and as a result, time frames for SIM had to be adjusted (34). Each day, 30 fruit and vegetable samples, six 24-h urine QC samples, and a solvent blank were prepared and analyzed. Data processing was performed in the same way as for the 24-h urine samples (34).

RESULTS

Analytical performance of the semitargeted GC-MS sugar profiling method

Our newly developed and validated semitargeted GC-MS sugar profiling method (see **Supplemental Tables** 3 and **4** and **Supplemental Figures** 1 and **2**) enables the sensitive detection and relative quantification of 55 major and minor sugar compounds (see **Figure 1** and Table 1) encompassing mono- and disaccharides, polyols, sugar acids, and as yet not identified sugar compounds (see Supplemental Figure 1).

The assignment of the analyzed known and unknown sugar compounds into the different substance classes is shown in Figure 1. If desired, some amino and organic acids can also be analyzed with the method described here and this leads to a total number of 68 integrated analytes (see Table 1 and Supplemental Table 3). All sugar compounds detected via the semitargeted GC-MS method and their signal intensity ranges are listed in **Figure 2**.

Our method is also characterized by very good long-term repeatability and intermediate precision (see Supplemental Table 4 and Supplemental Figure 2) as proven by measurement series comprising overall 456 runs (312 study samples plus 144 QC samples). Thus, the method is suitable for long-term measurement series of human biofluids in large study cohorts.

Sugar profiling in participants of the KarMeN study

Sugar profile of human urine and biological variability

To determine metabolite-specific differences in the interindividual, i.e., biological variability, the CVs of the measured sugar compounds across all 301 KarMeN participants were

TABLE 2
Summary of a sugar screening in 75 fruit and vegetable varieties[1]

	Psicose	Mannoheptulose[2]	Perseitol[2]	Mannitol[3]	Galactose	Threitol	Xylose	Polyol U02
Eggplant	—	5	—	tr	Middle	—	Low	tr
Avocado	—	100	100	tr	tr	—	tr	tr
Leaf spinach	tr	13	—	tr	Low	—	tr	tr
Cauliflower	—	1	—	tr	High	—	Middle	tr
Common bean	tr	6	—	<0.1	Low	—	Low	Low
Broccoli	—	5	—	tr	Low	—	Low	Low
Iceberg lettuce	—	3	—	<0.1	Low	—	Low	Low
Peas	tr	2	—	<0.1	Low	—	tr	Low
Lamb's lettuce	Low	27	—	tr	Middle	—	Low	High
Cucumber	—	4	—	<0.1	Middle	—	tr	Low
Carrot	—	29	—	2.6	Low	—	tr	Low
Potato	—	5	—	<0.1	Low	—	—	—
Garlic	—	—	—	tr	tr	—	—	tr
Kohlrabi	—	tr	—	tr	Low	—	tr	tr
Garden lettuce	—	11	—	tr	Low	—	Low	Middle
Red cabbage	—	tr	—	—	Middle	—	tr	tr
Pointed cabbage	—	tr	—	—	Low	—	tr	tr
White cabbage	—	tr	—	—	Low	—	tr	tr
Pumpkin	—	tr	—	<0.1	Middle	tr	tr	Low
Leek	—	tr	—	—	Low	—	tr	tr
Striped lentil	—	—	—	tr	tr	—	—	tr
Black lentil	—	—	—	tr	tr	—	—	—
Lentil, "Perla"	—	—	—	tr	tr	—	—	—
Corn	—	—	—	tr	Low	tr	tr	—
Green bell pepper	—	6	—	<0.1	High	—	Low	tr
Red bell pepper	—	7	—	<0.1	High	—	Low	tr
Hot pepper	—	22	—	<0.1	High	—	Low	tr
Button mushroom	—	1	<1	100	tr	—	—	tr
Shiitake	—	—	<1	77.4	tr	tr	—	—
Small radish	—	2	—	<0.1	Low	—	Low	tr
Radish	—	—	—	tr	Low	—	tr	tr
Beetroot	—	—	—	—	Middle	—	tr	tr
Pointed pepper	—	12	—	<0.1	Middle	—	tr	—
Soy	—	—	—	<0.1	Low	—	tr	tr
Green asparagus	—	—	—	<0.1	Low	—	Low	tr
White asparagus	—	—	—	<0.1	Low	—	Low	tr
Grape tomato	—	6	—	<0.1	Low	—	Low	—
Tomato, "Matina"	—	11	—	<0.1	High	—	Low	tr
Tomato, "Resi"	—	7	—	<0.1	High	—	tr	tr
Zucchini	—	—	—	<0.1	High	—	Low	Low
Onion	—	tr	—	tr	Low	—	tr	—
Pineapple	—	—	—	—	Middle	—	tr	—
Apple	—	6	—	0.1	Low	—	High	tr
Apricot	—	4	—	<0.1	Low	—	Low	tr
Banana	—	—	—	tr	Low	—	—	—
Pear	—	5	—	<0.1	Low	tr	Low	tr
Blackberry	—	4	—	<0.1	Low	—	Low	—
Clementine	—	—	—	tr	Low	—	Low	—
Strawberry, "Asia"	—	4	—	—	Low	—	High	tr
Strawberry, "Elsanta"	—	2	—	tr	Low	—	High	tr
Pomegranate	—	7	—	18.1	Low	tr	tr	tr
Grapefruit	—	tr	—	tr	Low	—	tr	—
Blueberry	—	10	—	tr	Low	—	Low	—
Raspberry	—	4	—	tr	Low	—	Middle	—
Honeydew melon	—	tr	—	<0.1	High	tr	tr	Low
Red currants	—	6	—	tr	Low	—	Low	—
Black currants	—	14	—	<0.1	Low	—	Low	—
Sour cherry	—	7	—	<0.1	Low	tr	Low	tr
Sweet cherry	—	1	—	<0.1	Low	—	Low	tr
Kiwi fruit	—	5	—	<0.1	Middle	—	tr	—
Mango	—	4	—	tr	Low	—	tr	—
Small yellow plums	—	6	—	<0.1	Low	—	Low	—

(*Continued*)

TABLE 2 (*Continued*)

	Psicose	Mannoheptulose[2]	Perseitol[2]	Mannitol[3]	Galactose	Threitol	Xylose	Polyol U02
Nectarine	—	4	—	<0.1	Low	—	Low	—
Orange	—	tr	—	—	Low	—	Low	—
Papaya	—	tr	—	<0.1	Low	tr	Low	tr
Passion fruit	—	2	—	tr	Low	tr	tr	—
Yellow peach	—	5	—	<0.1	Low	—	Middle	tr
White peach	—	4	—	<0.1	Low	—	Low	tr
Physalis	—	7	—	<0.1	Low	—	tr	—
Gooseberry	—	3	—	—	Low	—	Low	—
Red table grapes	—	4	—	—	Low	—	tr	tr
White table grapes	—	5	—	—	Low	tr	tr	tr
Lemon	—	—	—	tr	Low	—	tr	—
Plum	—	5	—	<0.1	Low	—	Low	tr
Fig	—	4	—	tr	Middle	—	tr	tr

[1]High, fruit or vegetables with signal intensities in the highest tertile of the signal intensities for this analyte; Low, fruit or vegetables with signal intensities in the lowest tertile of the signal intensities for this analyte; Middle, fruit or vegetables with signal intensities in the middle tertile of the signal intensities for this analyte; tr, trace analytes (analytes below a signal intensity of 8000).

[2]Ratios of mannoheptulose and perseitol signal intensities for the fruit and vegetables compared with avocado signal intensity.

[3]Ratios of mannitol signal intensities for the fruit and vegetables compared with button mushroom signal intensity.

determined. Some sugar compounds were excreted with a narrow concentration range, for example glucuronic acid with a CV of 29.8%, whereas others showed a huge biological variability, such as lactose with a CV of 294.5% (see Figure 2). In addition, the relative frequency of occurrence of individual sugar compounds in the 24-h urine samples of KarMeN participants is listed in Supplemental Table 3. To further assess factors underlying the huge biological variability, analyses focused on sex as a determinant and on dietary intake reconstructed from dietary intake measures.

Association of the urinary sugar profile with sex

To identify sugar compounds associated with sex, a decision tree using the CART algorithm was built (see **Figure 3**). In Table 3, possible candidates for a split are given for the first 3 nodes and for leaves. Evidently, the most relevant sugar compound separating men and women was maltose, which was detected in 78.3% of women but only in 35.5% of men. In addition, the urinary maltose concentration was significantly higher in women ($P < 0.0001$, see Figure 3). Other important metabolites were gluconic acid, fructose, and an unknown sugar compound, which were found in >75% of the study samples and recently discussed by Rist et al. (34). In the second node, where age was included as an additional potential splitting candidate, the 3 top determinants for separating men and women were age, gluconic acid, and sedoheptulose. Interestingly, splitting on the basis of sedoheptulose would have been similar to splitting on age as a result of the close association between age and sedoheptulose concentration in 24-h urine samples (34). The second split was done based on age as a top determinant, thereby indicating a close association between urinary maltose, sex, and age. The cut point for age was 45 y, thus suggesting that sugar excretion patterns change with menopause in women (see Figure 3). Gluconic acid was the only possible candidate metabolite for the third and last split (see Figure 3 and Table 3); however, to prevent overfitting, no further splitting was done. Boxplots of the 2 most important sugar compounds that separate men and women and the interaction between maltose excretion, sex, and age (menopausal status) are shown in Figure 3.

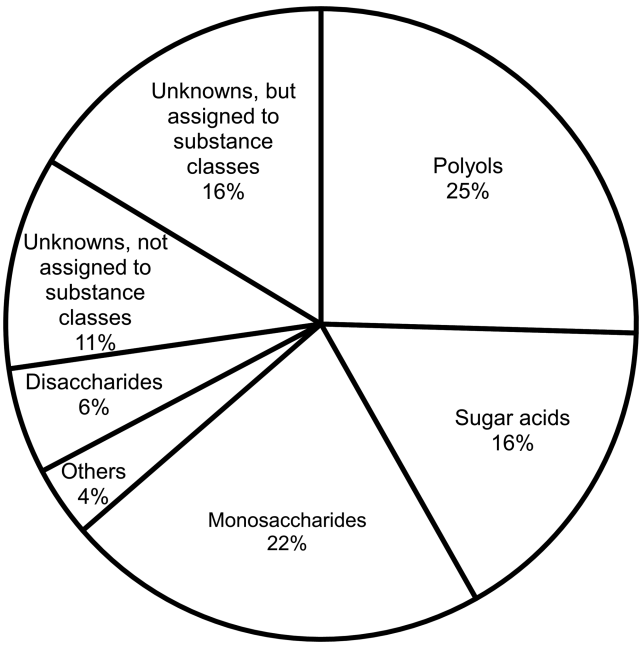

FIGURE 1 Classification assignment of sugar compounds into different substance classes.

Association of the urinary sugar profile with diet

A correlation analysis was performed based on 24-h urinary sugar profiles with the food consumption and nutrient intake data and a heat map generated on the basis of the Spearman rank correlation coefficients (**Figure 4**). The Spearman rank correlation coefficients with $\rho > 0.30$ are listed in **Table 4**; no correlations with $\rho < -0.30$ were observed. Significant correlations were observed for *1)* avocado consumption with perseitol, *2)* dairy product consumption with galactose and lactose, *3)* alcoholic beverage consumption with xylitol and ethyl-β-glucuronide, *4)* mushroom consumption with mannitol,

Sugar compounds	Median	Minimum	Maximum	CV
Mannoheptulose	1.25E+05	3.66E+04	2.45E+07	665.2
Perseitol	1.42E+03	3.65E+02	2.32E+06	653.8
Ethyl-β-glucuronide	4.18E+05	1.79E+05	6.55E+06	328.4
Lactose	9.53E+05	4.18E+05	7.81E+06	294.5
Unknown U25	2.94E+06	1.77E+05	8.50E+05	255.0
Sucrose	4.61E+04	8.49E+03	3.36E+07	227.2
Disaccharide U28	4.55E+04	7.54E+03	2.97E+05	224.9
meso-Erythritol	9.79E+04	4.49E+04	1.66E+07	186.6
Xylitol	2.91E+04	6.06E+03	2.30E+06	181.4
Maltose	1.29E+05	1.09E+04	2.75E+05	162.4
Psicose	7.31E+04	5.44E+03	5.09E+06	162.4
Levoglucosan	4.22E+05	1.07E+05	2.48E+06	146.6
chiro-Inositol	2.32E+05	4.80E+04	7.23E+05	146.5
1-Deoxy-sorbitol	6.76E+05	1.51E+05	1.18E+06	127.2
myo-Inositol	2.34E+04	1.76E+03	5.06E+06	119.4
Mannitol	1.90E+05	4.13E+04	4.45E+07	118.7
Unknown U04	1.11E+04	9.15E+02	2.73E+05	109.0
Fructose	1.23E+04	2.46E+03	2.35E+06	100.4
Sorbitol	6.36E+04	4.30E+03	3.39E+06	92.2
Galactose	5.55E+05	1.44E+04	7.32E+05	83.3
Disaccharide U29	9.08E+04	1.34E+04	1.34E+05	80.9
Galactonic acid	1.04E+05	2.54E+04	3.80E+05	79.9
Polyol U02	3.08E+03	5.40E+02	2.81E+05	79.5
Xylose	1.40E+05	8.91E+03	4.07E+05	77.7
Allose	1.86E+05	1.29E+04	1.63E+06	73.8
Mannose	5.86E+05	2.08E+05	4.02E+05	69.9
Fucose	9.62E+04	1.00E+04	5.69E+06	60.2
Galactitol	9.09E+05	6.68E+04	1.22E+06	58.9
Sugar acid U01	3.60E+05	1.61E+05	3.09E+05	56.0
Disaccharide U26	2.46E+04	1.28E+03	2.63E+05	55.7
Sugar acid U06	3.68E+05	3.60E+04	5.20E+04	54.4
Threitol	3.94E+05	8.53E+04	5.13E+05	54.3
scyllo-Inositol	2.68E+05	3.20E+04	2.37E+06	52.6
Sugar acid U09	2.18E+04	2.22E+03	4.20E+05	51.3
Disaccharide U27	1.11E+04	1.93E+03	9.72E+05	51.0
Unknown U24	2.59E+04	3.03E+03	1.39E+06	50.9
Xylonic acid	4.84E+05	2.24E+04	3.90E+05	50.4
Unknown U12	2.41E+05	4.89E+04	1.45E+05	49.6
Arabinose	4.92E+04	1.99E+03	1.50E+06	46.0
Fucitol	1.31E+04	4.37E+03	7.82E+04	45.5
Sedoheptulose	1.70E+05	3.36E+03	2.53E+05	45.5
Gluconic acid	2.58E+05	6.13E+04	3.47E+05	42.4
Arabonic acid	8.15E+04	2.67E+04	1.03E+06	41.0
Arabitol	2.05E+04	7.54E+03	1.24E+06	38.2
Glucose	1.14E+05	8.81E+03	3.16E+06	36.4
Ribitol	8.70E+04	5.31E+03	2.85E+05	35.0
Unknown U03	7.63E+05	2.40E+05	1.38E+05	34.5
Threonic acid	5.67E+04	7.21E+02	1.14E+06	34.3
Mannonic acid	3.53E+04	4.86E+02	1.50E+05	31.9
Ribonic acid	7.41E+04	2.76E+04	1.88E+05	30.3
Monosaccharide U21	9.80E+04	1.55E+04	4.88E+04	30.0
Glucuronic acid	8.62E+03	1.18E+03	2.03E+06	29.8
Erythronic acid	2.72E+05	3.42E+04	2.03E+06	28.6
Unknown U05	4.85E+03	1.22E+03	1.41E+06	28.5
Ribose	1.20E+05	3.91E+04	2.20E+05	25.6

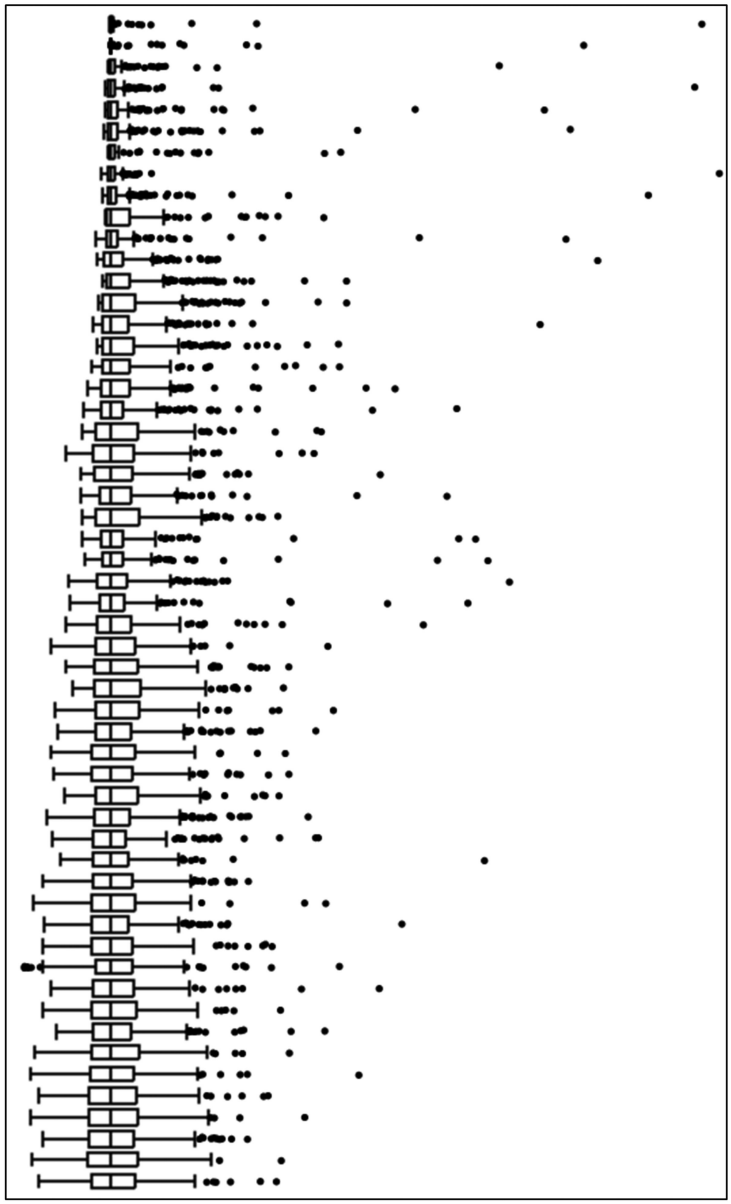

FIGURE 2 Interindividual/biological variability of the sugar profile in human urine from 301 participants of the KarMeN study. Sugar compound intensities were median-centered and unit variance scaled to allow for a comparison of the biological variance. Whiskers of box plots indicate ± 1.5 IQR. Median, minimum, maximum, and CV refer to peak area and enable a relative comparison of signal intensity. For reasons of simplicity, only the first of 2 derivatives was listed.

5) fruit consumption with threitol, xylose, and an unknown polyol, *6)* citrus fruit juice and fruit drink consumption with *chiro*-inositol and galactonic acid, and *7)* sucrose intake with fructose and sucrose (see Table 4 and Figure 4). In the case of avocado, in addition to perseitol, mannoheptulose presented itself as a potential dietary marker although the correlation coefficient was slightly below our threshold of 0.30 ($\rho = 0.2704$; see Figure 4). To verify this observation despite the low number of avocado consumers ($n = 9$), Spearman rank correlation coefficients were calculated for the avocado consumers and 18 randomly chosen nonconsumers ($n = 27$) (mannoheptulose: $\rho = 0.7748$, perseitol: $\rho = 0.8713$; see **Figure 5**). For some of these potential dietary markers box and scatter plots as well as their origin and potential confounders or other interferences are shown in Figure 5. A second line of evidence that those metabolites may be potential dietary markers for distinct foods/products was provided by analyzing the sugar profiles of 75 selected fruit and vegetable varieties (see Table 2).

DISCUSSION

Sugar profiling in participants of the KarMeN study

Sugar profile of human urine and biological variability

With the analytical method described here, we provide a straightforward and reliable tool to obtain sugar profiles and

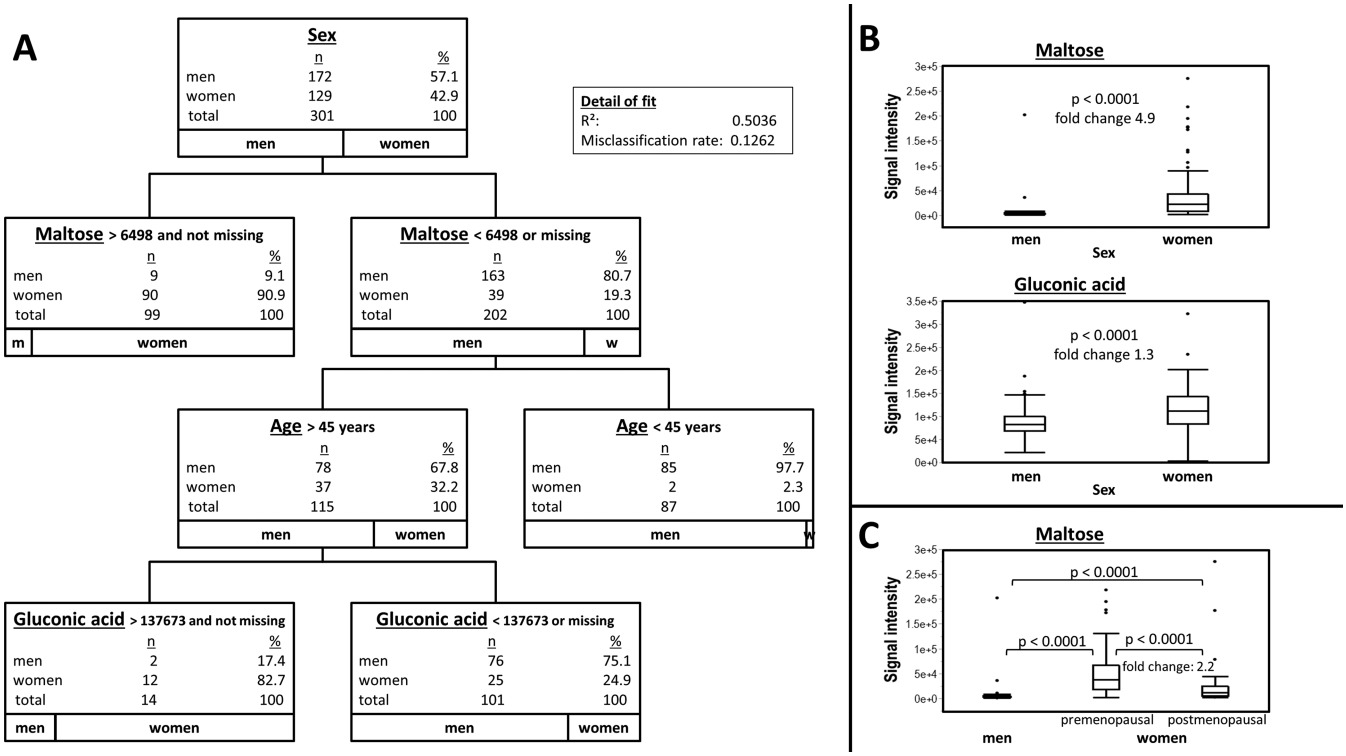

FIGURE 3 Identification of urinary markers discriminating sex via the CART approach. (A) Decision tree with splitting rules, the number of men or women, and the ratio between men and women for each branch; (B) box plots for the 2 top sugar compound candidates to differentiate sex; (C) association of age with maltose excretion in women (pre- and postmenopausal). Significance was established with the use of the Wilcoxon test, with participants excluded where the sugar compound was not detected. CART, classification and regression tree.

TABLE 3
Results of building a decision tree (CART) for the identification of possible markers to differentiate sex. Possible candidate sugar compounds for splitting are listed only if significant *P* values were achieved after Bonferroni correction[1]

Sugar compound	Candidate G^2	$-\log_{10}$ (*P* value)	Cut point[2]
First node: split candidates			
Maltose	152.6	55.8316	6.50E + 03
Gluconic acid	56.6	17.0557	1.05E + 05
Unknown U05	37.8	9.1285	6.66E + 05
Fructose[3]	27.1	5.0777	2.25E + 05
Second node: split candidates			
Age	34.7	8.6471	45.363
Gluconic acid	27.1	5.8762	1.38E + 05
Sedoheptulose[3]	25.3	5.2497	1.32E + 05
Unknown U05	19.8	3.4428	7.07E + 05
Third node: split candidates			
Gluconic acid	20.0	4.0476	1.38E + 05
First leaf: split candidates[4]			
Second leaf: split candidates[4]			
Third leaf: split candidates			
Mannonic acid	21.6	4.6179	5.62E + 04
Unknown U03	17.5	3.3893	4.73E + 04
Fourth leaf: split candidates[4]			

[1] G^2, likelihood ratio chi-square; highest values indicate best split.
[2] Best value for splitting the variables (cut point).
[3] For reasons of readability, only the higher-ranking derivative was listed.
[4] Candidate *P* values were below the significance level.

semiquantitative data in biofluids like urine. We observed a considerably wider range of sugar compounds than commonly known or expected in human urine (see Figure 2, Table 1, and Supplemental Table 1). There are many less known urinary sugar compounds with unclear origin such as psicose, perseitol, or mannoheptulose; their origin could be exogenous or endogenous. However, urinary sugar profiles partly showed a remarkable variability (see Figure 2), and thus, we believe that many individual sugars are dependent on sex, health status, or are a surrogate of a dietary pattern or the consumption of distinct food items.

Association of the urinary sugar profile with sex

The most important metabolites to differentiate between male and female sugar profiles were maltose and gluconic acid. Maltose has been reported to be present in very low concentrations in human urine, but no differences with respect to sex have been described so far (10, 12, 23, 36–39). We hypothesize that the maltose excretion seen in women may be associated with the vaginal microbiota (dominated by lactic acid–producing *Lactobacillus* species). Spear et al. (40) demonstrated that vaginal fluid possesses α-amylase activity, and thus is able to degrade free glycogen to maltose, maltotriose, and maltotetraose, which can then be utilized by *Lactobacillus* species (41, 42). This degradation pathway of free glycogen released from the vaginal epithelium might be responsible for the higher excretion rate of maltose in female urine.

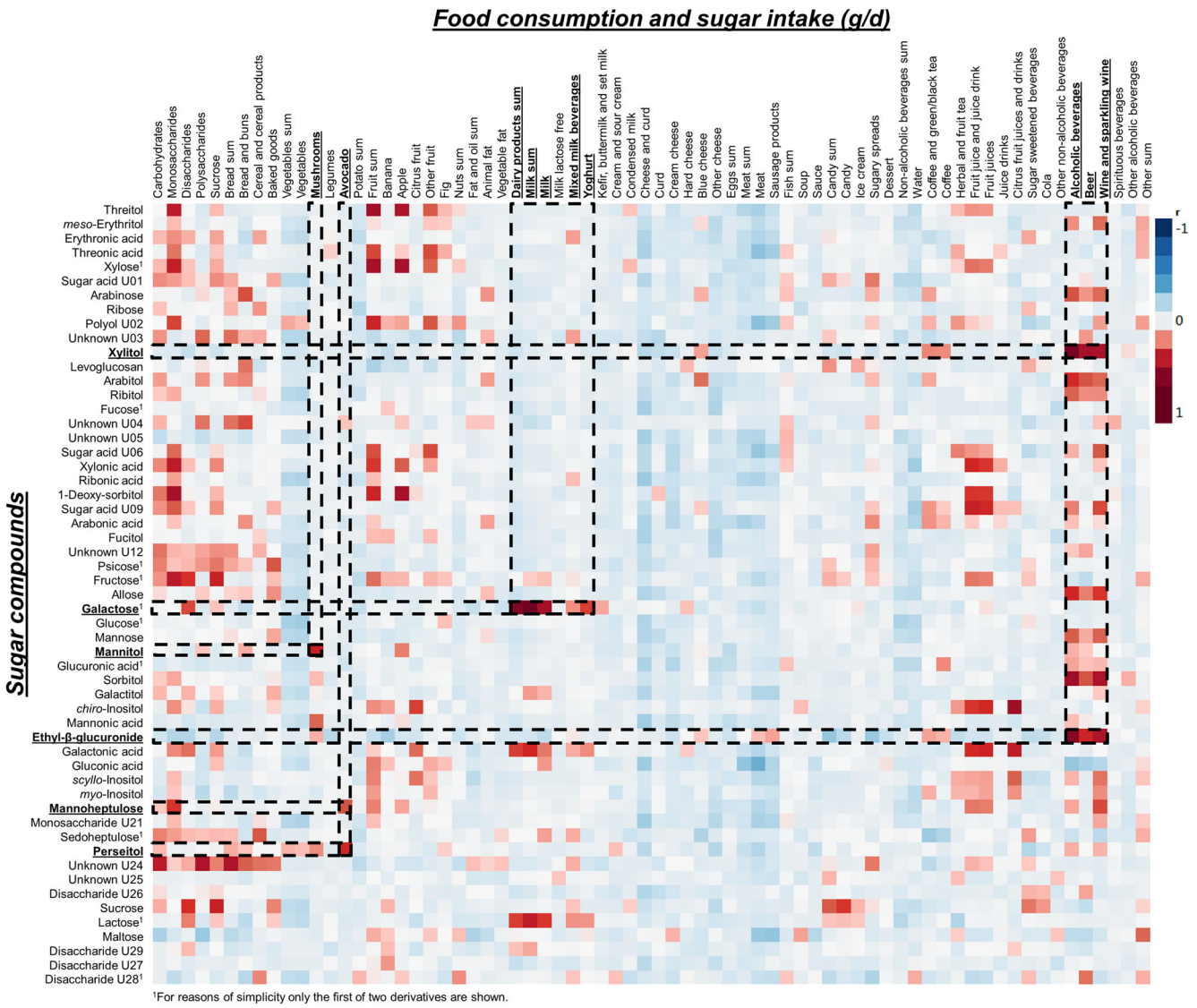

FIGURE 4 Heat map of Spearman rank correlation coefficients for the correlations of sugar compound excretion with food consumption and sugar intake (for an explanation of variables, see Supplemental Table 2) during the 24-h collection of urine. Correlations were estimated by a pairwise method. Boxes indicate the highest (most significant) correlations. The amount of consumed food is always given in g/d.

We observed a significantly lower maltose content in the urine of postmenopausal women in comparison with premenopausal women (see Figure 3). Postmenopausal women have significantly lower amounts of glycogen and *Lactobacilli* counts in the vaginal fluid as a result of reduced estrogen concentrations (41, 43, 44). Collectively, these observations concur with our finding of reduced maltose concentrations in postmenopausal women and add plausibility to a link between maltose excretion and the vaginal microbiota.

For gluconic acid and fructose we could not find a plausible biological explanation for the observed sex-dependent differences in urine.

Association of the urinary sugar profile with diet

Based on the correlation analysis, potential dietary markers for the consumption of various food items as well as food groups were identified (Figure 5 and Table 4). This analysis suggests the following sugar compounds to serve as specific dietary markers: mannoheptulose and perseitol for avocado and galactose and lactose for dairy products. These sugars are known constituents of these respective foods (45–50). Although an increase of mannoheptulose and perseitol excretion in urine after avocado consumption (25) has been described in an intervention with 3 volunteers, it was not specifically defined as a dietary marker. An increase of galactose and lactose after pure lactose ingestion has been observed (49, 51–53). Moreover, in a recent intervention study with milk, both galactose and lactose were suggested as specific dietary markers for milk consumption (54).

Although mannitol appears to be a plausible dietary marker for mushroom consumption (55), its specificity is questioned because there are many other sources of mannitol in the human diet (48) (see also Table 2) — a fact that confounds the aforementioned identified association (see Figure 5).

TABLE 4
Correlations between analytes and the consumed amounts of certain foods, food groups, or nutrients as determined by a 24-h recall (see Supplemental Table 2)[1]

Sugar compounds	Dietary intake	Participants		Spearman[2]
		$n_{excr.}$	$n_{ing.}$	ρ
Perseitol	Avocado	219	9	0.3388
Galactose[3]	Milk sum	301	234	0.6644
Galactose[3]	Dairy products sum	301	279	0.6082
Galactose[3]	Milk	301	174	0.4779
Galactose[3]	Yoghurt	301	84	0.3017
Lactose[3]	Milk sum	300	234	0.4204
Lactose[3]	Dairy products sum	300	279	0.3364
Lactose[3]	Milk	300	174	0.3180
Galactonic acid	Milk sum	296	234	0.3403
Galactonic acid	Dairy products sum	296	279	0.3005
Xylitol	Alcoholic beverages	301	96	0.6379
Xylitol	Beer	301	47	0.4667
Xylitol	Wine and sparkling wine	301	55	0.4531
Ethyl-β-glucuronide	Alcoholic beverages	144	96	0.5885
Ethyl-β-glucuronide	Wine and sparkling wine	144	55	0.4446
Ethyl-β-glucuronide	Beer	144	47	0.3457
Sorbitol	Alcoholic beverages	299	96	0.4948
Sorbitol	Wine and sparkling wine	299	55	0.4496
Allose	Alcoholic beverages	301	96	0.3425
Allose	Wine and sparkling wine	301	55	0.3128
Arabitol	Alcoholic beverages	301	96	0.3340
Mannitol	Mushrooms	301	35	0.3633
Mannitol	Button mushroom	301	32	0.3489
chiro-Inositol	Citrus fruit juices and drinks	260	65	0.4941
chiro-Inositol	Citrus fruit	260	37	0.3000
Galactonic acid	Citrus fruit juices and drinks	296	65	0.3825
Threitol	Fruit sum	301	228	0.4904
Threitol	Apple	301	106	0.4359
Xylose[3]	Fruit sum	301	228	0.4768
Xylose[3]	Apple	301	106	0.4736
1-Deoxy-sorbitol	Apple	299	106	0.4653
1-Deoxy-sorbitol	Fruit sum	299	228	0.3504
1-Deoxy-sorbitol	Fruit juice and juice drink	299	131	0.3130
1-Deoxy-sorbitol	Fruit juices	299	123	0.3097
Sugar acid U09	Fruit juice and juice drink	301	131	0.3649
Sugar acid U09	Fruit juices	301	123	0.3395
Polyol U02	Fruit sum	301	228	0.3516
Galactonic acid	Fruit juices	296	123	0.3498
Galactonic acid	Fruit juice and juice drink	296	131	0.3320
Xylonic acid	Fruit juice and juice drink	301	131	0.3408
Xylonic acid	Fruit juices	301	123	0.3107
Xylonic acid	Fruit sum	301	228	0.3091
chiro-Inositol	Fruit juices	260	123	0.3350
chiro-Inositol	Fruit juice and juice drink	260	131	0.3119
1-Deoxy-sorbitol	Monosaccharides	299	301	0.4676
Unknown U24	Polysaccharides	301	301	0.4477
Unknown U24	Bread sum	301	293	0.4463
Unknown U24	Carbohydrates	301	301	0.3949
Fructose[3]	Monosaccharides	301	301	0.3966
Fructose[3]	Sucrose	301	301	0.3581
Fructose[3]	Disaccharides	301	301	0.3192
Threitol	Monosaccharides	301	301	0.3955
Xylonic acid	Monosaccharides	301	301	0.3919
Xylose[3]	Monosaccharides	301	301	0.3785
Sucrose	Sucrose	301	301	0.3620
Sucrose	Disaccharides	301	301	0.3243
Sucrose	Candy	301	104	0.3132
Mannoheptulose	Monosaccharides	300	301	0.3135

[1] $n_{excr.}$, number of participants who excreted a certain sugar compound; $n_{ing.}$, number of participants who ingested individual foods or food groups.
[2] Spearman rank correlation coefficients < -0.30 or > 0.30. All listed correlations had significant P values < 0.0001.
[3] For reasons of readability, only the higher-ranking derivative was listed.

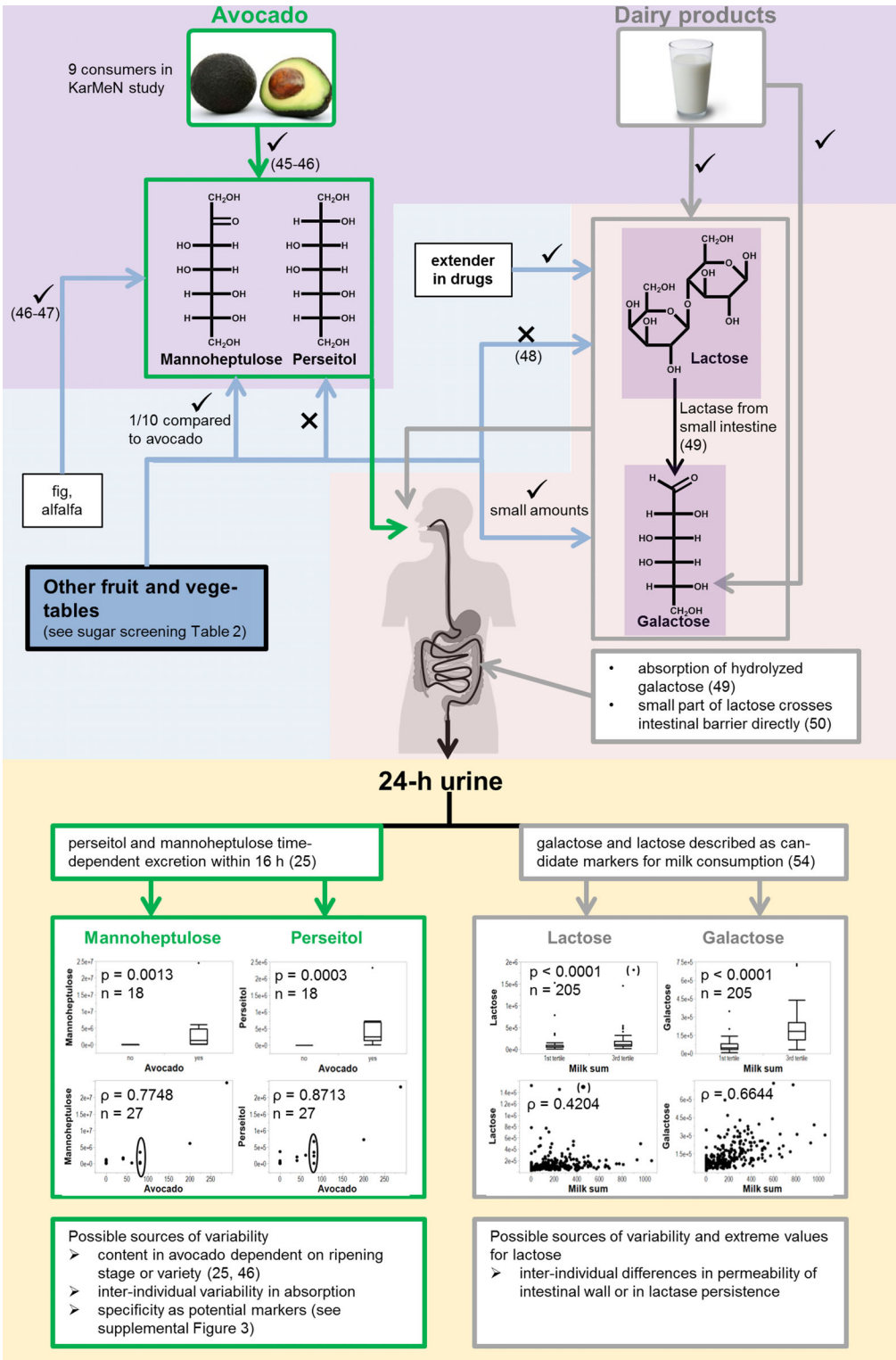

FIGURE 5 (*Continued*)

We also identified xylitol as a potential dietary marker for alcoholic beverage consumption. An increase of xylitol in urine after administration of ethanol (56) has been described before, but the causality underlying the relation between alcohol consumption and urinary xylitol output (56, 57) warrants further research. Ethyl-β-glucuronide has already been described as a dietary marker for alcoholic beverage consumption (58–60); we observed a moderate association (see Figure 5 and Table 4). In

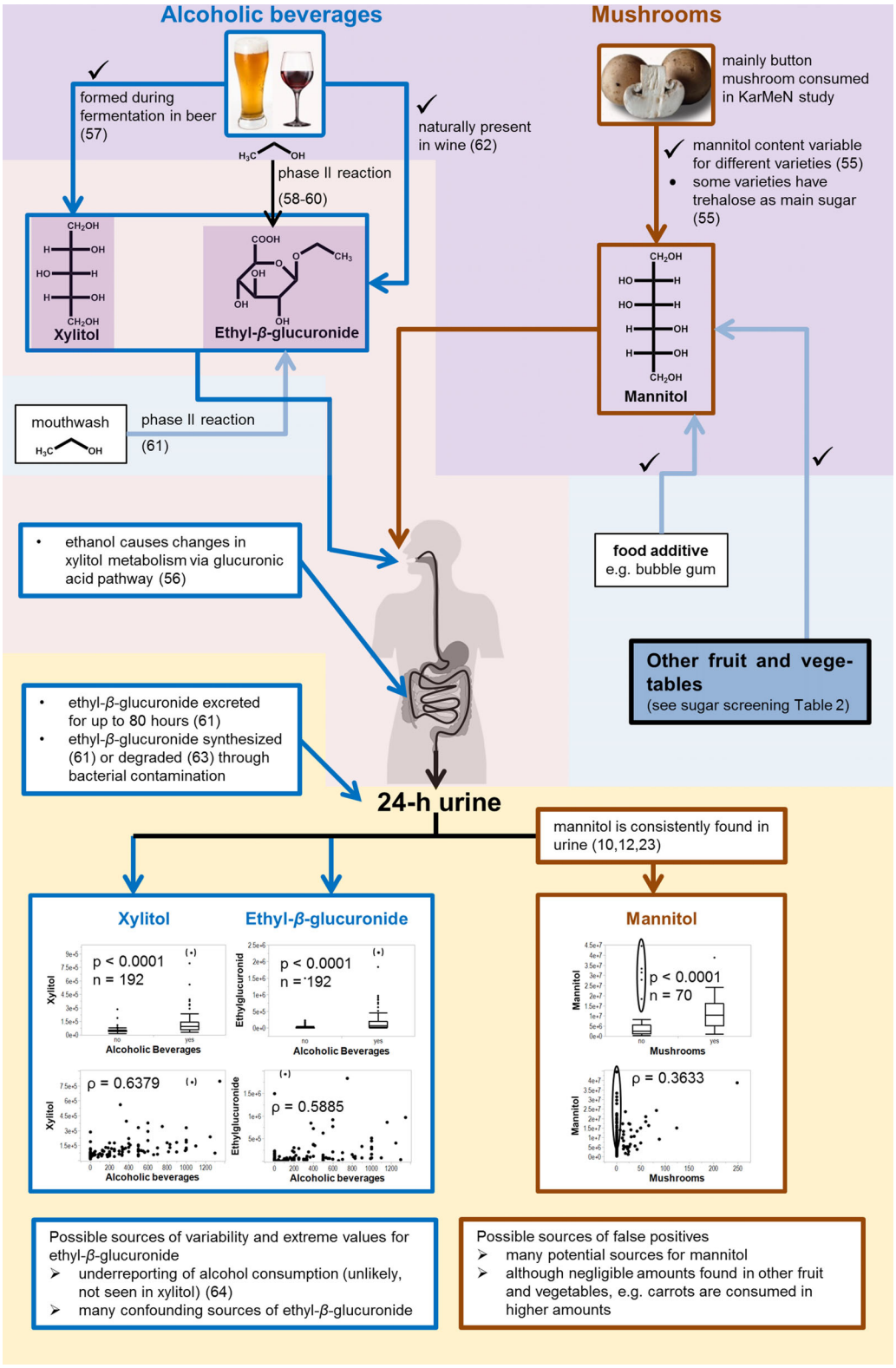

FIGURE 5 Overview of potential dietary markers (includes results of the KarMeN study and sugar screening of fruit and vegetables as well as literature data). Potential dietary markers of food consumption with the strongest associations in the correlation analysis, their plausibility in terms of origin, and their specificity in terms of potential confounders or other interferences. Shaded violet: food/nutrient level; shaded light blue: interfering sources for potential dietary markers (foods, drugs, results from sugar screening of fruit and vegetables); shaded red: metabolization in human; shaded yellow: results in 24-h urine samples. Colors of boxes and arrows as follows: green (part 1): consumption of avocado; grey (part 1): consumption of dairy products; dark blue (part 2): consumption of alcoholic beverages; brown (part 2): consumption of mushrooms; light blue: interfering sources for potential dietary markers (foods, drugs, results from sugar screening of fruit and vegetables). Check: compound occurs in specific food; cross: compound does not occur in specific food. The amount of consumed food is given in g per day. Significance was established by use of the Wilcoxon test. Spearman rank correlation coefficients were calculated using all 301 participants, except for avocado, where the 9 avocado consumers plus 18 randomly selected participants were used.

light of the many other potential confounders and interferences for ethyl-β-glucuronide detection (61–64) (see Figure 5), we recommend to use measurements of additional metabolites such as ethylsulfate (65) or in combination with xylitol.

It would be highly desirable to use some of these dietary markers in future as an objective measure of food consumption in comparison with self-reported consumption, where biases such as under- or over-reporting in cases of perceived unhealthy or healthy foods often occur (64). Objective dietary markers would allow more reliable insights into health aspects, and thus, relations between diet and health could be more accurately ascertained.

In more general terms, a dietary marker should fulfil a number of criteria such as its specificity, the dose-response relation, plausibility of origin, and suitability in free-living populations, and, importantly, analytical robustness (66). Questions around the quality of dietary markers also cover issues on whether a metabolite is a short-term marker of intake over a 24–36 h period or whether it can also serve as a long-term reporter molecule especially in epidemiologic studies (67). Moreover, whether there are saturation effects and whether the dietary marker can quantitatively assess consumption are also important issues (66).

Specificity and dose-response effect, plausibility, and suitability in a free-living population as in our KarMeN population on an unrestricted diet as well as methodological validity were all addressed in the present study. The main limitations in our approach were *1)* the low number of participants consuming some specific food items such as avocado, *2)* the potential bias through the use of self-reported food consumption data for the correlation analysis, and *3)* owing to our study design so far only a conclusion about metabolites' usefulness as short-term markers can be drawn. Other limitations might be that only a single urine collection was measured potentially leading to exaggerated interindividual variation and that only fruit and vegetables, but not processed food and beverages, were screened during the sugar profiling of food. However, our aim is that the developed analytical method and the approaches used to identify some crucial sugar compound determinants will be taken into larger and more diverse cohorts as the next step to deriving quantitative dietary markers and to shedding light on the diet-health relation for one of the most important food substrates in the human diet and metabolism, namely the sugars.

In conclusion, we have demonstrated that the human urinary sugar profile is complex and comprises many more compounds than previously anticipated. With the large number of sugar compounds detected, we identified also a huge gap in knowledge regarding the metabolism of most of these sugar compounds, in particular along the diet-health-disease trajectory. We therefore suggest that future research should not only encompass analyzing common and well-known sugar compounds, but rather strive for a more comprehensive view on sugar compounds. However, the data from our study can be used as a reference for normal sugar profiles of healthy humans with respect to the occurrence of individual sugar compounds along with variances in excretion. For some sugars, we identified crucial determinants such as sex and pre- compared with postmenopausal women. However, these determinants need further study. We also identified a considerable number of sugar compounds as potential dietary markers for individual food items and groups (see Figures 4 and 5), for which confirmation and assessment of their quantitative dimension and their usability as long-term markers in epidemiologic studies are required in future studies. Although our newly developed semitargeted GC-MS method is only semiquantitative, it clearly offers a rapid and cost-effective strategy to obtain comprehensive insights into the sugar profile by detecting not only numerous known, but also some unknown sugar-like compounds that also deserve identification. Our analytical method may also be useful in identifying the underlying physiologic processes that allow assessing determinants for absorption/permeation from the intestine into blood circulation as well as for renal secretion/reabsorption. Ultimately, this analytical method may not only help to identify dietary markers, but also to identify disease biomarkers in the future.

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The authors' responsibilities were as follows—SEK and HD: designed the research project; AB, BW, and IH: developed the KarMeN concept and design; CM: conducted the analytical experiments; CM, BE, PGF, and CHW: analyzed data and performed statistical analysis; CM: wrote the initial draft of the manuscript; CHW, SEK, BW, IH, and HD: critically reviewed and contributed to the manuscript; CM and SEK: had primary responsibility for final content; and all authors: read and approved the final version of the manuscript. None of the authors has a conflict of interest to declare.

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