Hippuric Acid in 24-Hour Urine Is a Potential Biomarker for Fruit and Vegetable Consumption in Healthy Children and Adolescents¹,²

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Abstract

An objective noninvasive biomarker for fruit and vegetable (FV) consumption would help to more reliably characterize the relationship between FV intake and health status in observational studies. Because increases in urinary hippuric acid (HA) were observed after consumption of several FV varieties, we aimed to investigate whether 24-h urinary HA may represent a potential biomarker for FV consumption in children and adolescents. The association of FV and juice (FVJ) intake calculated from 3-d weighed dietary records with 24-h urinary HA was analyzed in 240 healthy children and adolescents and compared with associations of the established biomarkers urinary nitrogen (uN) and potassium (uK) with protein and potassium intake, respectively. Spearman correlation coefficients (r) and cross-classifications were calculated for all diet-biomarker associations. Potential confounders for the HA-FVJ association were examined in linear regression models. In children, correlations of HA with FVJ (r = 0.62), uN with protein (r = 0.64), and potassium intake with uK (r = 0.69) were comparable. In adolescents, the HA-FVJ association was weaker (r = 0.41) compared with the biomarkers uN (r = 0.60) and uK (r = 0.58) (all P < 0.0001). Cross-classification into the same/adjacent quartile by dietary and urinary data were >85% for all analyzed comparisons except for a 75% classification agreement between HA and FVJ in adolescents. Unadjusted and adjusted linear regression models indicated significant (P < 0.0001) HA-FVJ associations in both age groups. FVJ explained more of the variability in HA excretion in children (R² = 0.38) than in adolescents (R² = 0.22). Our findings in children showing HA-FVJ associations comparable to those for well-established biomarkers with their respective dietary intakes suggest that HA may represent a useful biomarker for FVJ. J. Nutr. doi: 10.3945/jn.112.159319.

Introduction

Most observational studies report a favorable association between higher fruit and vegetable (FV)³ consumption and lower risk of several chronic diseases including coronary heart disease (1) and stroke (2). Interventional studies that show reductions in blood pressure (3) as well as the inflammatory marker C-reactive protein (4) after higher FV intake support these associations. Even in adolescents, a higher FV consumption has been related to lower markers of inflammation and oxidative stress (5). Although an inverse FV–coronary heart disease association is discernible in most studies, weak and partly controversial relations are also reported (6). This may be due, among other factors, to differing and sometimes inaccurate dietary assessment methods in observational studies that do not adequately capture habitual FV consumption (7). To further characterize the relationship between this food group and chronic diseases in epidemiologic studies, an objective and accurate nutritional biomarker for FV intake is of interest. So far, however, no consistent and noninvasive biomarker has been established for dietary intake of FV (8).

Urinary hippuric acid (HA), the glycine conjugate of benzoic acid, was traditionally used as a biomarker of toluene exposure in industrial workers (9). More recent human and animal studies [as well as older studies (10)] indicated increases in HA after the ingestion of several FV varieties, including blueberries (11), cranberries (10,12), apple products (13), cherries (14), black currants (15), and a mixed FV meal (16). The increases in HA seem to be mainly attributed to the metabolism of different dietary polyphenols (16) for which colored FV are a major food source. After passing into the large intestine, these dietary phenolic compounds and flavonoids are cleaved by the colonic microbiota into smaller aromatic compounds such as phenolic acids and hydroxycinnamates, which can be absorbed from the intestine. Further metabolism in the liver leads to phase II metabolites, including HA, that are excreted in urine in substantial quantities (17).
A metabolome-wide association study identified higher HA excretion in individuals residing in south compared with north China. The higher HA levels appeared concurrently with a lower prevalence of heart diseases and stroke, lower blood pressure, and a more favorable diet including higher intakes of vitamin C and potassium in south China (18). Further metabolomic analyses supported these findings, showing a positive association between HA excretion and mixed-fruit intake (19) or a vegetarian-type diet (20) as well as an inverse association between HA excretion and blood pressure (21).

The above findings indicate that HA may represent a biomarker for FV consumption in epidemiologic studies on diet-disease relationships. To investigate whether HA may actually represent an appropriate biomarker for FV consumption in healthy children and adolescents, we examined the association between dietary intake of FV and 24-h urinary HA excretion in 9–10- and 12–15-y-olds. We also compared the dietary intake–potential biomarker relationship for HA with that for the established urinary biomarkers urinary nitrogen (uN) and urinary potassium (uK), representing protein and potassium intake, respectively.

**Participants and Methods**

**Participants.** For the present investigation, participants were included from the Dortmund Nutritional and Anthropometric Longitudinally Designed (DONALD) Study, an ongoing open-cohort study that was started in 1985. The DONALD Study gathers information on diet, development, and metabolism in healthy volunteers between infancy and young adulthood (22,23). The regular examinations include 24-h urine collections, 3-d weighed dietary records, anthropometric measurements, and medical assessments and interviews on lifestyle. The study protocol was approved by the Ethics Committee of the University of Bonn (Germany). All examinations were conducted with parental, and later on with children’s, written consent.

For the current analysis, we choose 120 children (aged 9–10 y; 60 boys, 60 girls) and 120 adolescents (60 girls aged 12–14 y and 60 boys aged 13–15 y) who had provided a plausible 3-d weighed dietary record including a 24-h urine collection. For all urine samples, uN and uK measurements had to be available as major selection criteria. The different age ranges for female and male adolescents were chosen to account for the earlier puberty onset in girls (24). Because of possibly incomplete collection, urine samples with a 24-h creatinine excretion rate <0.1 mmol/ (kg body weight - d) (22) were excluded. The plausibility of dietary recording was estimated by using the ratio between reported total energy intake and predicted basal metabolic rate [calculated according to Schofield (25)]. Ratios below age- and sex-specific cutoff values (26) were considered implausible and excluded from the analysis.

**Dietary recording.** Diet was assessed on 3 consecutive days, and all consumed foods and beverages during the 3-d dietary record were weighed with electronic food scales to the nearest 1 g. If weighing was not possible, semiquantitative recording (e.g., number of cups) was allowed. The mean 3-d intakes of foods and nutrients used in the present analysis were calculated by using our continuously updated in-house nutrient database LEBTAB (27). The food group used for evaluation of the biomarker HA in the current study consisted of FV (including fresh, frozen, and canned products) as well as FV juices (FVJ). All of these items were calculated as the sum of (native) separately ingested fruit, vegetables, or juices and ingredients of processed or prepared foods. For beverages such as diluted juices, only the part that consisted of 100% juice contributed to the amount of juice intake in our calculation. We also calculated FV intake without juices to examine the association of this food group with HA excretion.

**Urinary measurements.** Annual 24-h urine collections for children ≥3 y old are routinely performed in the DONALD Study on the third day of the 3-d weighed dietary record under standardized conditions (22). All urine samples collected during the 24-h collection period are immediately stored frozen at –7–12°C in Extran (Extran, MA03; Merck)-cleaned, preservative-free 1-L plastic containers before being transported to the Research Institute of Child Nutrition where they are stored at –20°C until analysis. Creatinine excretion was determined by the kinetic Jaffé procedure on a creatinine analyzer (Beckman-2; Beckman Instruments). uN, the biomarker for protein intake, was measured by the method of Kjeldahl (Büchi 430 Digestor and Büchi Distillation unit B-324; Büchi Labortechnik) and uK, the biomarker for K intake, was analyzed by flame atomic absorption spectrometry (Perkin Elmer 1100 Spectrometer; Perkin Elmer). Urinary HA was measured by direct colorimetry according to the method reported by Tomokuni and Ogata (28), with minor modifications as follows: methanol instead of ethanol was used to dilute the colored reaction product of HA and benzzenesulfonyl chloride (azlactone) in pyridine after 60 min reaction time, and the color intensity was photometrically detected at 436 nm (Lambda 11 UV/VIS Spectrometer; Perkin Elmer). HA measurements were performed in triplicate, and the arithmetic means of the 3 measurements were used in the analysis. Intra- and interassay precision, expressed as CV, were 3.8% and 5.8%, respectively, at a HA concentration of 1.84 mmol/L.

**Anthropometric measurements.** Anthropometric measurements were performed at the time of dietary recording and urine collection following standard procedures. Participants’ standing height was measured to the nearest 0.1 cm with a digital stadiometer (Harpenden; Holtain Ltd). Body weight was assessed to the nearest 0.1 kg by using an electronic scale (Sega 753 E; Seca Weighing and Measuring Systems). Body weight and height were used to calculate participants’ BMI. Individual age- and sex-independent BMI SD scores (SDS) were calculated according to German reference curves (29). Body surface area (BSA) was calculated according to the formula by Du Bois and Du Bois (30) as follows:

$$\text{BSA}(m^2) = 0.007184 \times \text{height(cm)}^{0.725} \times \text{weight(kg)}^{0.425}.$$ 

**Statistical analysis.** All calculations were carried out by using SAS procedures (version 9.1.3; SAS Institute). Because some of the dietary and urinary variables were not normally distributed, Spearman correlation coefficients were calculated to investigate crude associations between the urinary measurements and the respective nutritional variables. By means of cross-classification we computed for both age groups the percentage of persons classified into either the same or an adjacent quartile of the nutrient or food groups by dietary recording and urinary data or who were misclassified into the opposite quartile. In preliminary linear regression models with In-transformed HA as the dependent variable and FVJ as the independent variable, potential confounders, including participants’ age and sex, urine volume, energy and protein intake, and individual BSA, were checked for their relevance. Because urine volume and BSA showed significant associations with HA excretion in children and adolescents, these variables were included in the multivariable regression model (adjusted model 1). Other potential confounders were either not associated with the outcome or they did not substantially modify the association between HA and FVJ. Because increases in urinary HA have been observed after the intake of coffee (31), green and black tea (32), and cocoa products (33), we controlled for the potential influence of these polyphenol-rich dietary components in an additional regression model (adjusted model 2). In this model, in addition to adjustment for urine volume and BSA, tea and coffee consumption were included as categorical variables (consumption during the 3-d dietary record: yes/no) and cocoa consumption was included as a continuous variable. Coffee and green or black tea consumption was defined as any recorded intake of the dry (instant) powder or brewed beverage including intake from composite beverages such as ice tea. Cocoa intake was calculated as the sum of cocoa powder and cocoa butter consumed separately or as ingredients of, e.g., chocolate.

Tests for sex-by-FVJ interactions for HA as the dependent variable were nonsignificant. Hence, linear regression models were combined for both sexes. An ANCOVA was used to compare the mean ln HA excretion

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adjusted for urine volume and BSA (model 1) between quartiles of FVJ consumption. P values <0.05 were considered significant.

Results
The BMI of the group studied (Table 1) was comparable to the German reference population (29) as indicated by a BMI-SDS close to zero for both age groups. Intakes of potassium, protein, and vegetables were higher (P < 0.05) in adolescents than in children with regard to absolute intakes but not in terms of energy-adjusted intakes, whereas energy-adjusted intakes of fruit were higher (P = 0.004) in children. BSA-adjusted urinary excretion rates in the older age group were lower for uN (P = 0.001) and uK (P = 0.006) but did not differ for HA.

Spearman correlations (Table 2) indicated a comparable strength in the association of uN with protein (r = 0.64, P < 0.0001), potassium intake with uK (r = 0.65, P < 0.0001), and HA with FVJ (r = 0.62, P < 0.0001) in children. In adolescents, the correlation between HA and FVJ was weaker (r = 0.41, P < 0.0001) in comparison with the diet-biomarker association for uN (r = 0.62, P < 0.0001) and uK (r = 0.58, P < 0.0001) in the same population and when compared with the HA-FVJ association in the younger age group. Concerning cross-classification (Table 2), 85–90% of the children were classified into the same or an adjacent category for all 3 biomarker–dietary intake associations, whereas misclassification into the opposite quartile occurred in ≤3.3% of children. For uN and uK, ≥85% of the adolescents were classified into the same or an adjacent quartile by urinary data and dietary records, whereas the agreement in classification between HA and FVJ was slightly lower (75%). Less than 5% of the adolescents were misclassified in all biomarker-intake associations.

In unadjusted linear regression models, FVJ and ln-transformed HA showed a highly significant (P < 0.0001) association in both age groups (Table 3), but FVJ intake explained a higher proportion of the variability in HA excretion in children (38%) than in adolescents (22%).

When FV intake was calculated without consideration of FV juices, β values for the FV-HA association were comparable to the FVJ-HA association in children (β = 0.0012, P < 0.0001) and somewhat higher in adolescents (β = 0.0012, P < 0.0001) in the unadjusted models. However, the correlation coefficients decreased in both age groups (r = 0.33 and r = 0.35, respectively; P < 0.0001). A total of 78% of the children and 71% of the adolescents were classified correctly/adjacently by HA excretion and FV intake (data not shown).

Regarding FVJ, the adjustments for urine volume and BSA (adjusted model 1) slightly attenuated the regression coefficients, but the highly significant associations with HA excretion remained in both age groups. According to the multivariable-adjusted regression equation (model 1), a 10-g increase in FVJ consumption predicted an increase in ln-HA excretion by 0.009 in children and 0.0063 in adolescents, which corresponds to a 0.9% and 0.63% change in absolute HA excretion, respectively.

### TABLE 1 General characteristics of 240 DONALD participants

<table>
<thead>
<tr>
<th></th>
<th>Children (aged 9–10 y)</th>
<th>Adolescents (F: aged 12–14 y; M: aged 13–15 y)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>120 (60 M)</td>
<td>120 (60 M)</td>
</tr>
<tr>
<td><strong>Anthropometry</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>16.5 (15.3, 18.3)</td>
<td>18.7 (17.0, 20.7)</td>
</tr>
<tr>
<td>BMI-SDS</td>
<td>0.00 ± 0.89</td>
<td>−0.06 ± 0.95</td>
</tr>
<tr>
<td>BSA, m²</td>
<td>1.16 ± 0.12</td>
<td>1.49 ± 0.20</td>
</tr>
<tr>
<td><strong>Dietary variables</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Energy intake, MJ/d</td>
<td>7.26 ± 1.14</td>
<td>8.54 ± 1.81</td>
</tr>
<tr>
<td>Protein intake, g/d</td>
<td>56.8 ± 12.7</td>
<td>66.4 ± 17.3</td>
</tr>
<tr>
<td>Protein intake, g/MJ</td>
<td>7.8 ± 1.2</td>
<td>7.8 ± 1.1</td>
</tr>
<tr>
<td>FVJ, g</td>
<td>411 (281, 556)</td>
<td>430 (316, 576)</td>
</tr>
<tr>
<td>FVJ, g/MJ</td>
<td>56 (39, 81)</td>
<td>51 (38, 66)</td>
</tr>
<tr>
<td>Fruit, g</td>
<td>138 (75, 201)</td>
<td>119 (52, 192)</td>
</tr>
<tr>
<td>Fruit, g/MJ</td>
<td>19 (10, 29)</td>
<td>13 (6, 22)</td>
</tr>
<tr>
<td>Vegetables, g/MJ</td>
<td>87 (65, 133)</td>
<td>109 (63, 151)</td>
</tr>
<tr>
<td>Juice, g</td>
<td>163 (65, 274)</td>
<td>190 (84, 299)</td>
</tr>
<tr>
<td>Juice, g/MJ</td>
<td>22 (8, 41)</td>
<td>24 (9, 33)</td>
</tr>
<tr>
<td>Potassium intake, g/d</td>
<td>2.41 ± 0.61</td>
<td>2.68 ± 0.74</td>
</tr>
<tr>
<td>Potassium intake, mg/MJ</td>
<td>332 ± 67</td>
<td>316 ± 64</td>
</tr>
<tr>
<td><strong>Urinary variables</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24-h urine volume, mL</td>
<td>742 (681, 978)</td>
<td>871 (680, 1084)</td>
</tr>
<tr>
<td>Creatinine excretion, mmol/d</td>
<td>5.54 ± 1.29</td>
<td>8.74 ± 2.50</td>
</tr>
<tr>
<td>Nitrogen excretion, mmol/d</td>
<td>538 ± 140</td>
<td>628 ± 170</td>
</tr>
<tr>
<td>Nitrogen excretion, mmol/m²</td>
<td>465 ± 107</td>
<td>420 ± 100</td>
</tr>
<tr>
<td>HA excretion, mmol/d</td>
<td>2.10 [1.57, 2.63]</td>
<td>2.82 [2.17, 3.50]</td>
</tr>
<tr>
<td>HA excretion, mmol/m²</td>
<td>1.78 ± 0.46</td>
<td>1.89 ± 0.47</td>
</tr>
<tr>
<td>Potassium excretion, mmol/d</td>
<td>47.7 ± 16.4</td>
<td>53.9 ± 18.4</td>
</tr>
<tr>
<td>Potassium excretion, mmol/m²</td>
<td>41.1 ± 13.1</td>
<td>36.5 ± 12.6</td>
</tr>
</tbody>
</table>

1 Values are means ± SD or medians (25th, 75th percentiles). BSA, body surface area; FVJ, fruit and vegetables including juices; HA, hippuric acid; SDS, SD score.

2 Values are daily urinary excretion rates divided by individual BSA.
The additional inclusion of the dietary variables coffee, tea, and cocoa (adjusted model 2) did not influence HA excretion or the HA-FVJ association in children. In adolescents, however, these polyphenol-rich dietary components additionally explained 4% of the variance in HA excretion and strengthened the observed HA-FVJ association (13% higher $b$ value in model 2 compared with model 1). Whole grains and potatoes are additional dietary sources of polyphenols that might influence HA excretion. In our analysis, adjustment for whole-grain intake in model 1 improved the model $R^2$ in children by 3%, but neither the inclusion of whole grains nor of potatoes in model 1 changed the HA-FVJ association ($b$ value) in any age group (data not shown).

The ANCOVA comparing mean adjusted (urine volume, BSA) HA excretion between quartiles of FVJ was significant ($P < 0.01$) in both age groups. The difference in HA-excretion between the lowest and the highest FVJ quartile was comparable in children and adolescents (1.02 and 0.95 mmol/d, respectively), but adolescents presented a higher mean HA excretion level and a wider 95% CI in each FVJ intake quartile (Fig. 1).

**Discussion**

To our knowledge, the present study is the first to directly investigate HA excretion as a biomarker for FV consumption in a healthy population. Our results indicate that 24-h urinary HA may represent a useful, noninvasive biomarker for FV intake in healthy children and adolescents. Both multiple linear regression models and the ANCOVA indicated strong associations between HA and FVJ in both age groups. Moreover, <5% of the studied children and adolescents were misclassified into the opposite quartile comparing classifications by 3-d dietary record FVJ intake and HA excretion levels. Juices produced from FV can have lower polyphenol concentrations compared with native FV, although this may not always be the case (34). When we calculated FV intake without juices, the correlation with HA excretion was attenuated in our cohort. Because marked increases in HA have also been reported after exclusive ingestion of fruit juices (15,35) and juice intake accounted for 40% of the consumed amount of FVJ (in g/d) in our cohort, juices might substantially contribute to FV-related polyphenol intake and thus HA excretion at least in children and adolescents. So far, few studies have investigated biomarkers for FV intake in children and adolescents. In Australian prepubertal children,

### TABLE 2

<table>
<thead>
<tr>
<th>Predictor: FVJ</th>
<th>In Values of hippuric acid excretion</th>
<th>$eta^2$</th>
<th>$P$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Children</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unadjusted model</td>
<td>0.0012</td>
<td>&lt;0.0001</td>
<td>0.38</td>
<td></td>
</tr>
<tr>
<td>Adjusted model 1$^3$</td>
<td>0.0009</td>
<td>&lt;0.0001</td>
<td>0.46</td>
<td></td>
</tr>
<tr>
<td>Adjusted model 2$^4$</td>
<td>0.0009</td>
<td>&lt;0.0001</td>
<td>0.46</td>
<td></td>
</tr>
<tr>
<td><strong>Adolescents</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unadjusted model</td>
<td>0.00077</td>
<td>&lt;0.0001</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td>Adjusted model 1$^3$</td>
<td>0.00063</td>
<td>&lt;0.0001</td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td>Adjusted model 2$^4$</td>
<td>0.00071</td>
<td>&lt;0.0001</td>
<td>0.36</td>
<td></td>
</tr>
</tbody>
</table>

1 DONALD, Dortmund Nutritional and Anthropometric Longitudinally Designed Study; FVJ, fruit and vegetables including juices. A $P < 0.0001$. FVJ, fruit and vegetables including juices; HA, hippuric acid; uK, urinary potassium; uN, urinary nitrogen.

![FIGURE 1](image-url) Geometric means and 95% CI of 24-h hippuric acid excretion (in mmol/d) adjusted for urine volume and body surface area in quartiles of FVJ consumption calculated from 3-d weighed dietary records for 120 children ($n = 30$/quartile) (A) and 120 adolescents ($n = 30$/quartile) (B). ANCOVA was significant ($P < 0.01$) in both age groups. FVJ, fruit and vegetables including juices.
uK was significantly correlated (r = 0.13) with FV consumption assessed with a semiquantitative FFQ (36). In Japanese children and adolescents (aged 10–14 y), correlations between intake of fruit, green-yellow vegetables, or other vegetables assessed with a dietary history questionnaire and the sum of serum carotenoids ranged from r < 0.1 to r = 0.3 (37). Possible biomarkers of general FV intake include carotenoids and vitamin C in plasma as well as urinary flavonoids (8). Reported correlations with different dietary assessment methods in healthy adults are mainly in the range of r = 0.3–0.4 for total urinary flavonoids (38–40) and 0.2–0.4 for total plasma or serum carotenoids (39,41–43). For the correlation between plasma vitamin C and FV intake, a wide range (r = 0.3–0.6) is reported in the literature (43–45). Most of these correlation coefficients seem to be slightly lower than those observed for urinary HA in the present study, especially with respect to our 9–10- y-old age group.

For use in epidemiologic studies, a biomarker that can also be determined in spot urines would be more feasible. A circadian rhythm in HA excretion was reported in one older study in healthy volunteers (46). Therefore, it cannot be determined at the moment whether HA in spot urines might also be useful as a biomarker for FV.

In our analysis, Spearman correlation and agreement in cross-classification suggested that the strength in the HA-FVJ association was comparable to the well-established biomarkers uN and uK (47,48) in 9–10-y-old children. In adolescents, however, correlation coefficients as well as the agreement in classification into the same or an adjacent quartile by dietary recording and urinary excretion were higher for the biomarkers uN and uK and their respective nutrient intake than for the HA-FVJ association. A theoretical reason for the weaker association observed between HA excretion and FVJ consumption in the older compared with the younger age group could be a lower accuracy of self-reported dietary intake in adolescents, as has been reported in the literature (49,50). However, the difference in validity for the biomarker HA between children (r = 0.61) and adolescents (r = 0.42) observed in the present study seems not to be mainly attributable to a lower general accuracy in dietary recording in the older age group, because correlation coefficients for the biomarkers uN and uK were comparable between both age groups. Another possible explanation for the lower agreement between HA excretion and FVJ consumption in adolescents may be the higher intake of polyphenol-rich dietary components other than FV in this age group when compared with younger children. Although intake of coffee, tea, and cocoa had no influence on HA excretion in children in the present study, adjustment for these dietary variables improved the HA-FVJ association in adolescents. In addition, in adjusted model 2, HA-excretion in adolescents significantly (P = 0.04) increased with increasing cocoa consumption, and there was a trend (P = 0.09) for lower HA values in adolescents without reported tea consumption compared with those who drank tea during the 3-d dietary record (data not shown). A possible influence of these polyphenol-rich beverages may be taken into account as a confounding factor in studies of HA as a biomarker for FVJ in adults, because coffee and tea can be a major source of phenolic acid intake in this age group. The increasing contribution of coffee, tea, and cocoa to HA generation in older adolescents and adults could weaken the relevance of HA as a biomarker for FVJ in observational studies. However, in large-population studies, the intake of coffee and tea can be more reliably estimated from FFQ than FV consumption (51). Thus, by controlling for the intake of tea and coffee using dietary data, urinary HA may still represent an appropriate biomarker for FV intake in adults.

In both age groups studied in the present analysis, urine volume and BSA were significantly and independently associated with HA excretion, whereas age, sex, and intakes of energy and protein did not influence urinary HA levels. Our findings of a significant association between urinary HA and urine volume is in line with previous studies reporting that the excretion of other nutritional biomarkers, including excretion of iodine (52) and vitamin B-12 (53), is affected by urine volume.

HA contributes to the organic acid fraction excreted in human urine (54). This organic acid fraction is closely associated with BSA in children, adolescents, and adults (55), probably because BSA is highly correlated with body lean mass, which itself is an important determinant of glomerular filtration rate (56). A similar mechanism could explain the HA-BSA association observed in the present study. Moreover, HA excretion seems to differ between normal-weight and obese individuals (57). Also in our analysis, BMI-SDS was associated with urinary HA. However, because BSA and BMI-SDS were closely correlated (r > 0.7 in our study), and BSA was the stronger predictor of HA in both age groups, we decided to include only BSA in the adjusted regression models.

The colorimetric method used for HA determination in the present study may not yield completely identical results in comparison to chromatographic (11,13,16) and nuclear magnetic resonance spectroscopic (18,58) methods to identify urinary HA. However, nuclear magnetic resonance spectroscopic analysis of urinary HA in healthy children and adolescents (aged 7–18 y) (58) yielded mean HA values (311 mmol/mol creatinine) that were not substantially different from our data (393 and 319 mmol HA/mol creatinine for children and adolescents, respectively). One recent interventional study (14) also used the colorimetric method proposed by Tomokuni and Ogata (28) for HA measurements. In this study, the mean normal HA values [–1 g (5.58 mmol)/d] reported for 10 healthy, middle-aged adults exceeded the HA values found in our adolescent age group by >2-fold. A higher BSA in the adults may partly explain the observed difference to our findings.

The colorimetric method has been shown to give higher HA values when compared with chromatographic methods (59), which can be explained by the fact that the colorimetric detection covers additional glycine conjugates of benzoic acids such as hydroxyhippuric acids and methylhippuric acid (59). Urinary hydroxyhippuric acids, however, have also been shown to increase after the consumption of black currants (15,60) or a mixed-FV meal (16) and may thus contribute to the HA-FVJ association in the present study.

One limitation of our analysis is the use of only one 24-h urine and one 3-d dietary record, which does not allow for intra-individual variation in dietary intake and urinary excretion. Several 24-h urine collections and dietary records could more accurately compare individual dietary intake with urinary excretion (48). A recent study (19) also showed relatively high interindividual variation in HA excretion. These different HA excretion levels between individuals might be partly due to differences in intestinal microbiota, because altered gut microbiota has been associated with urinary HA excretion levels in animals (61) and humans (62). The possible variation in HA attributable to the intestinal microbiota could not be accounted for in our study. In addition, we were not able to control for the influence of added benzoic acid as a food preservative. A recent Austrian study (63) estimated mean daily intake of benzoic acid in children to be 1.6 mg/(kg body weight · d) [0.013 mmol/(kg body weight · d)]. On the basis of mean body weights in our study populations (34 kg in children and 49 kg in adults, because coffee and tea can be a major source of phenolic acid intake in this age group. The increasing contribution of coffee, tea, and cocoa to HA generation in older adolescents and adults could weaken the relevance of HA as a biomarker for FVJ in observational studies. However, in large-population studies, the intake of coffee and tea can be more reliably estimated from FFQ than FV consumption (51). Thus, by controlling for the intake of tea and coffee using dietary data, urinary HA may still represent an appropriate biomarker for FV intake in adults.

In both age groups studied in the present analysis, urine volume and BSA were significantly and independently associated with HA excretion, whereas age, sex, and intakes of energy and protein did not influence urinary HA levels. Our findings of a significant association between urinary HA and urine volume is in line with previous studies reporting that the excretion of other nutritional biomarkers, including excretion of iodine (52) and vitamin B-12 (53), is affected by urine volume.

HA contributes to the organic acid fraction excreted in human urine (54). This organic acid fraction is closely associated with BSA in children, adolescents, and adults (55), probably because BSA is highly correlated with body lean mass, which itself is an important determinant of glomerular filtration rate (56). A similar mechanism could explain the HA-BSA association observed in the present study. Moreover, HA excretion seems to differ between normal-weight and obese individuals (57). Also in our analysis, BMI-SDS was associated with urinary HA. However, because BSA and BMI-SDS were closely correlated (r > 0.7 in our study), and BSA was the stronger predictor of HA in both age groups, we decided to include only BSA in the adjusted regression models.

The colorimetric method used for HA determination in the present study may not yield completely identical results in comparison to chromatographic (11,13,16) and nuclear magnetic resonance spectroscopic (18,58) methods to identify urinary HA. However, nuclear magnetic resonance spectroscopic analysis of urinary HA in healthy children and adolescents (aged 7–18 y) (58) yielded mean HA values (311 mmol/mol creatinine) that were not substantially different from our data (393 and 319 mmol HA/mol creatinine for children and adolescents, respectively). One recent interventional study (14) also used the colorimetric method proposed by Tomokuni and Ogata (28) for HA measurements. In this study, the mean normal HA values [–1 g (5.58 mmol)/d] reported for 10 healthy, middle-aged adults exceeded the HA values found in our adolescent age group by >2-fold. A higher BSA in the adults may partly explain the observed difference to our findings.

The colorimetric method has been shown to give higher HA values when compared with chromatographic methods (59), which can be explained by the fact that the colorimetric detection covers additional glycine conjugates of benzoic acids such as hydroxyhippuric acids and methylhippuric acid (59). Urinary hydroxyhippuric acids, however, have also been shown to increase after the consumption of black currants (15,60) or a mixed-FV meal (16) and may thus contribute to the HA-FVJ association in the present study.

One limitation of our analysis is the use of only one 24-h urine and one 3-d dietary record, which does not allow for intra-individual variation in dietary intake and urinary excretion. Several 24-h urine collections and dietary records could more accurately compare individual dietary intake with urinary excretion (48). A recent study (19) also showed relatively high interindividual variation in HA excretion. These different HA excretion levels between individuals might be partly due to differences in intestinal microbiota, because altered gut microbial metabolism has been associated with urinary HA excretion levels in animals (61) and humans (62). The possible variation in HA attributable to the intestinal microbiota could not be accounted for in our study. In addition, we were not able to control for the influence of added benzoic acid as a food preservative. A recent Austrian study (63) estimated mean daily intake of benzoic acid in children to be 1.6 mg/(kg body weight · d) [0.013 mmol/(kg body weight · d)]. On the basis of mean body weights in our study populations (34 kg in children and 49 kg in adults, because coffee and tea can be a major source of phenolic acid intake in this age group. The increasing contribution of coffee, tea, and cocoa to HA generation in older adolescents and adults could weaken the relevance of HA as a biomarker for FVJ in observational studies. However, in large-population studies, the intake of coffee and tea can be more reliably estimated from FFQ than FV consumption (51). Thus, by controlling for the intake of tea and coffee using dietary data, urinary HA may still represent an appropriate biomarker for FV intake in adults.
adolescents) and assuming comparable benzoic acid intakes per kilogram body weight in each age group, HA excretion attributable to benzoic acid intake of 0.45 mmol/d in children and 0.64 mmol/d in adolescents can be estimated to correspond to ~20% of the HA excretion observed in our study populations.

Using 3-d weighed dietary records as the reference tool for biomarker validation in our study is a further limitation because even a very accurate recording method can provide only an estimate of true dietary intake (64). Weighed dietary records are, however, considered the most exact dietary assessment instruments in epidemiologic studies (65), and we excluded dietary records with implausible energy intake from our present analysis to improve the quality of the investigated dietary records. In addition, the use of the well-established urinary biomarkers uN and uK for comparison with the HA-FVJ association in the same study populations further substantiates our findings in healthy children and adolescents. Although uN and uK might be less reliable for estimation of absolute protein and potassium intake in children and adolescents due to a positive N and K balance during growth (50), the individual differences in protein and potassium retention in the narrow age ranges of our 9–10- and 12–15-y-old study populations should be small enough to allow a reasonable comparison of the respective biomarker associations with the HA-FVJ association.

In conclusion, we found a reasonably strong association between the intake of FVJ and the urinary excretion of the aromatic metabolite HA in healthy children and adolescents. The comparison with the established biomarkers uN and uK suggested that, especially in children, HA may represent a valid biomarker for FVJ intake. The investigation of HA as a biomarker for FVJ intake in adults would further extend the present findings.

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T.R. designed the research; N.D. performed part of the laboratory analyses; N.D. and D.K. performed the statistical analysis; D.K. wrote the manuscript; and L.S. and T.R. provided critical input on the data analyses and on early versions of the manuscript. All authors read and approved the final manuscript and reviewed the manuscript for important intellectual content.

Literature Cited
Hippuric acid as a fruit and vegetable biomarker