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Comparison of 24-h volume and creatinine-corrected total urinary polyphenol as a biomarker of total dietary polyphenols in the Invecchiare InCHIANTI study

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Abstract

Polyphenols have beneficial effects on several chronic diseases but assessing polyphenols intake from self-reported dietary questionnaires tends to be inaccurate and not very reliable. A promising alternative is to use urinary excretion of polyphenols as a proxy measure of intake. The best method to assess urinary excretion is to collect 24-h urine. However, since collecting 24-h urine method is expensive, time consuming and may be difficult to implement in large population-based studies, measures obtained from spot urine normalized by creatinine are commonly used. The purpose of the study was to evaluate the correlation between polyphenols dietary intake and total urinary polyphenol excretion (TPE), expressed by both 24-h volume and urinary creatinine normalization in 928 participants from the InCHIANTI study. Dietary intake data were collected using a validated food frequency questionnaire. Urinary TPE was analyzed by Folin–Ciocalteau assay. Both urinary TPE expression models were statistically correlated ($r = 0.580$), and the partial correlation coefficient improved (pr = 0.722) after adjusting for the variables that modify the urinary creatinine excretion (i.e. gender, age, BMI, physical activity and renal function). In crude

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Conflict of interest

The authors are not aware of any conflict of interest.

models, polyphenol intake was associated with TPE corrected by 24-h volume ($r = 0.211$; $P < 0.001$), but not with creatinine normalization ($r = 0.014$; $P = 0.692$). However, urinary TPE expressed by creatinine correction was significantly correlated with dietary polyphenols after adjusting for covariates (pr = 0.113; $P = 0.002$). We conclude that urinary TPE expressed by 24-h volume is a better biomarker of polyphenol dietary intake than by urinary creatinine normalization. After covariate adjustment, both can be used for studying the relationships between polyphenol intake and health in large-scale epidemiological studies.

Keywords

Polyphenols; Urine 24-h; Creatinine normalization; Biomarker; InCHIANTI study

1. Introduction

Polyphenols are widespread, naturally occurring, secondary plant compounds, with more than 8000 different phenolic structures currently identified [1]. Both clinical and epidemiological studies provide evidence that polyphenol-rich foods and polyphenol-rich diet have protective effects against chronic diseases such as cancer, obesity cardiovascular and neurodegenerative diseases. These beneficial effects are due to their antioxidant, antiviral, anti-inflammatory and chemopreventive activities [2,3].

There are, however, a number of limitations related to studies concerning the beneficial effects of polyphenols. First, it is very difficult to assess dietary exposure to polyphenols because dietary data are usually obtained by means of food frequency questionnaires (FFQs), which provide inaccurate information [4]. Moreover, information on total polyphenol content is extremely limited. There are some food composition data using the global Folin–Ciocalteau (F–C) assay, although a lower amount of data is available using more specific and accurate techniques (such as HPLC and GC coupled with DAD or MS detectors) [5–7]. Finally, nutritional status also depends on the absorption and metabolism of polyphenols, the bioavailability of which differs enormously from one molecule to another [8] and among individuals [9]. These problems could be solved using a biomarker as a reliable proxy measure of polyphenol consumption [10].

Specific polyphenols and flavonoids that can be quantified by LC–MS/MS are promising biomarkers for the intake of fruits and vegetables [11,12], polyphenol-rich beverages [13] and polyphenol-rich foods consumption [14]. Currently, F–C assay in urine samples is considered the best biomarker for total polyphenol intake [15–18]. The F–C method, with a previous solid-phase extraction, is a rapid and global measure of total polyphenol content. This methodology has been optimized to apply to large-scale epidemiological studies because it is quick and economic [15].

The urine sample when expressed by volume (24-h) is considered to be the most reliable and non-invasive biological specimen for assessing polyphenol biomarkers [10]. However, its collection is tedious for both patients and researchers. When total volume in 24 h is not available, the normalization of urine samples can be performed in different ways, such as by urinary creatinine (creatinuria), specific gravity or osmolarity, although creatinine is the

most usual correction method [15,19] and provides results that are better than, or at least as good as, the others [20,21]. To date, the F–C assay has only been used in large studies using urine normalized by creatinine [15,22], although there are some small nutritional studies on polyphenol excretion that show relevant between estimates obtained by urinary creatinine or 24-h urine volume normalization [23]. Using data from a large, population-based survey, the aim of this study was to assess the correlation between measures of polyphenolic content by the F–C assay expressed by 24-h volume or by urinary creatinine correction. In addition, we also studied the correlation of both measures with self-reported measures of dietary intake.

2. Methods

2.1. Population

The InCHIANTI (Invecchiare in Chianti) study (www.inchiantistudy.net) is a prospective cohort investigation aimed at assessing factors affecting loss of mobility in later life. The study design and data collection have been described elsewhere [24]. Briefly, 1453 predominantly elderly subjects were randomly selected from the populations of Greve in Chianti and Bagno a Ripoli, two small towns located in the Chianti countryside of Tuscany, Italy. Of these, 928 participants, who had both dietary assessment data and biological samples, were available and were included in the present study. The participants were all European and of *Caucasian* race. The Italian National Institute of Research and Care on Aging Ethical Committee approved the study protocol, and written informed consent was obtained from each participant.

2.2. Dietary and physical activity assessment

At baseline, usual food and energy intake were estimated through personal interviews using the Italian version of the FFQ developed and validated in the European Prospective Study into Cancer and Nutrition (EPIC) study [25]. Total dietary polyphenols were calculated according to Ovaskainen et al. [7]. These total polyphenol data (expressed as mg/100 g) were aggregated from phenolic acids, flavonoids (anthocyanidins, flavan-3-ols, proanthocyanidins, flavanones, flavones, flavonols and isoflavones), lignans and ellagitannins, which were analyzed by HPLC [7]. For several food items, values were completed using data from US Department of Agricultural databases [6,26] and Phenol-Explorer [5].

The level of physical activity in the year prior to the interview was classified on an ordinal scale, based on responses to a modified standard questionnaire [27], into: (1) hardly any physical activity; (2) mostly sitting/some walking; (3) light exercise 2–4 h/week; (4) *r* light >4 h/week or moderate 1–2 h exercise; (5) moderate exercise >3 h/week; (6) intense exercise many times/week; and (7) walks >5 km day^{-1}, >5 days/week, >5 years.

2.3. Standards and reagents

All samples and standards were handled with no exposure to light. F–C reagent, boric acid 99.5% and gallic acid were obtained from Sigma®, and sodium carbonate from Panreac. Anhydrous sodium acetate, concentrated hydrochloric acid, methanol, acetic acid 99.8% and

formic acid 98–100% were purchased from Scharlau, and ultrapure water (Milli-Q) from Millipore (Bedford, MA).

2.4. Analytical methods

Twenty-four hour urine samples were collected from all participants. Urine samples were coded, boric acid was added as a preservative, and the samples were then stored at −80 °C until analysis. The clinical investigators and laboratory technicians were not provided with clinical data. Total urinary polyphenol was analyzed by F–C assay, after a solid-phase clean-up as described elsewhere [15]. Urine samples were centrifuged for 10 min at 4 °C, and 800 μL of supernatants were diluted with 800 μL of Milli-Q water and acidified with 34 μL of concentrated hydrochloric acid. After equilibrating the cartridges (Oasis® MAX 60 mg, Waters, Milford, MA, USA), 800 μL of urine was loaded. The cartridges were washed with 1 mL of sodium acetate 50 mM (pH 7) in 5% of methanol. Then, polyphenols were eluted with 1800 μL of 2% formic acid in methanol. 15 μL of the eluted fractions were mixed with 170 μL of Milli-Q water in the thermo microtiter 96-well plate (nunc™, Roskilde, Denmark), and 12 μL of F–C reagent and 30 μL of sodium carbonate (200 g L^{-1}) were added. The mixtures were incubated for 1 h at room temperature in the dark. After the reaction period, 73 μL of Milli-Q water were added, and finally absorbance was measured at 765 nm in a UV–vis Thermo Multiskan Spectrum spectrophotometer (Vantaa, Finland). Total polyphenol equivalents (TPEs) were expressed as mg gallic acid equivalent (GAE)/24-h urine or mg GAE/g of creatinine.

Urinary creatinine was measured using a compensated modified Jaffe kinetic method for a Roche/Hitachi analyzer (Roche Diagnostics GmbH, Mannheim, Germany) [28].

Renal function was calculated by Cockcroft–Gault equation: (140 − age) × weight × 0.85 (if female)/(serum creatinine × 72). All participants were classified as having normal renal function (equal or higher than 60 mL min^{-1}), impaired renal function (≥30 to < 60 mL min^{-1}), profoundly impaired renal function or renal failure (lower than 30 mL min^{-1}).

2.5. Statistical analysis

All analyses were performed using SPSS software version 15.0 (SPSS Inc., Chicago, IL, USA). Baseline characteristics of the participants were expressed as means (SD) for normal distribution or percentages. Skewed variables (Kolmogorov with the Lilliefors Significance correction and Levene tests) were described as median (interquartile range). Total dietary polyphenol was normalized by natural logarithm for further analysis. Variables such as urine TPE, which were not normalized by the logarithmic transformation, were Box–Cox transformed with $\alpha = 0.00001$ and $\lambda = 0.15$. Pearson correlation and the partial correlation coefficient in a multiple linear regression model with transformed urine TPE (expressed as mg GAE/24-h urine) as a dependent variable, and transformed urine TPE (expressed as mg GAE/g of urinary creatinine) as an independent variable, as well as age, sex, BMI, physical activity, energy intake (expressed as 1000 kcal day^{-1}) and renal function (normal, impaired, and profoundly impaired or failure) as covariates, were calculated to estimate the association between both expression types of transformed urine TPE. The association between transformed total dietary polyphenols and crude urine TPE (expressed as 100 mg GAE/24-h

urine or as 100 mg GAE/g of urinary creatinine) was assessed by multiple linear regressions (partial correlation coefficient), adjusting for gender, age, BMI, physical activity, energy intake (expressed as 1000 kcal day^{-1}), and renal function. In the last models, urinary TPEs were expressed as 100 mg GAE because the B-coefficients were very low. The difference between both correlation coefficients of transformed total dietary polyphenols and crude urine TPE models was assessed using the Hotelling's *t*-test for dependent correlations within a population. All the regression models were tested for the detection of outliers, multicollinearity, homoscedasticity, and the normality and independence of errors. All statistical tests were two-tailed, and the significance level was $P < 0.05$.

3. Results

The participant characteristics stratified by urinary creatinine quartiles are summarized in Table 1. The mean age was about 70 years, 55.3% or the participants were women and 56.4% had impaired renal function.

Urinary TPE expressed by 24-h volume and by urinary creatinine correction were highly correlated ($r = 0.580$; $P < 0.001$). A partial correlation coefficient in the multiple linear regression model confirmed this association (pr = 0.722; $P < 0.001$), showing, in addition, that gender, age, physical activity, energy intake and renal function were also significantly associated, but BMI was not (Table 2).

The estimated dietary polyphenol intake from validated FFQs was correlated directly in a raw model with urinary TPE expressed by 24-h volume ($r = 0.211$; $P < 0.001$), but it was not correlated with urinary creatinine-corrected TPE ($r = 0.014$; $P = 0.692$). However, in multiple linear models the association between dietary polyphenol and both transformed TPE expressions was statistically significant (partial correlation coefficient (pr) = 0.164; $P < 0.001$ for24-h volume; and pr = 0.113; $P = 0.002$ for urinary creatinine correction, respectively), after adjusting for gender, age, BMI, physical activity, energy intake and renal function (Table 3). In both models the significant covariates were gender, age, physical activity and energy intake, but not BMI and renal function. The Hotelling's *t*-test showed statistically significant differences between correlation coefficients of both models ($P < 0.001$).

4. Discussion

In the current study, we observed a significant correlation between urinary TPE expressed as 24-h volume and as normalized by urinary creatinine, in an elderly free-living population. The correlation was considerably higher after adjusting for age, gender, physical activity, energy intake and renal function. We also show that both urinary TPE measures were statistically associated with total polyphenol intake.

As expected, urinary TPE 24-h volume is the best method to quantify polyphenol biomarkers urinary excretion as it was best correlate with intake. However, 24-h urine collection it is not practical in large-scale epidemiological studies [4,10]. Our findings suggest that normalizing TPE with urinary creatinine provides a good measurement alternative to 24-h urine collection [15], as long as such values are used in analyses that

account for covariates that can modify creatinine excretion. Renal function is obviously important because creatinine is the most commonly used indicator of renal function [29]; in this study, subjects with renal failure or renal impairment were classified in lower urinary creatinine quartiles than normal subjects (P of trend < 0.001). Gender, age and race are covariates that affect creatinine excretion and clearance rate [30,31]; men produced more creatinine than women – mean (SD) 89.7 (39.8) and 61.7 (29.2) mg dL^{-1}, respectively – which may be due, in part, to their higher muscular mass. Moreover, there was also a negative significant correlation between age and creatinine excretion ($r = -0.234$, $P < 0.001$). Although ethnic group is an important covariate, this was not included in the regression model because all subjects in the cohort were *Caucasian*, and the effect of race should be evaluated in future studies. Diet, assessed as energy intake ($r = 0.283$; $P < 0.001$), physical activity ($r = 0.268$; $P < 0.001$) and BMI ($r = 0.081$; $P = 0.021$) were also statistically correlated with urinary creatinine. That is because creatinine is a by-product of muscle catabolism, so its production is expected to depend on body composition, exercise and food patterns [21,32]. Indeed, the importance of all these variables, with the exception of BMI, was also shown when they were included in the regression model between both urinary TPE, as the partial correlation coefficient improved from 0.580 to 0.722.

The secondary aim of the current study was to assess both urinary TPE measures as biomarkers of total polyphenol intake. We found a significant crude correlation between urinary TPE expressed as mg GAE/24-h volume and total polyphenol intake ($r = 0.211$; $P <$ 0.001. However, TPE expressed as mg GAE/g creatinine and total polyphenol intake were not significantly correlated. In part, these differences could be due to the high variability of creatinine excretion, depending on gender, age, physical activity, renal function and diet. Our Pearson correlation coefficient was lower than that published by our group in other studies using urine creatinine normalization [15,22]. However, these differences could be explained in part because the InCHIANTI population is older and has more renal dysfunction than the populations recruited in the other studies. Moreover, these differences could also be due to dietary polyphenol quantification, because in the current study, food composition data came from HPLC analysis, while in the other studies food data were analyzed by F–C assay. Composition data on total polyphenol using F–C could be overestimated if one does not take into account or eliminate the interfering substances that can react with the F–C reagent. In our study, this is solved by using a cleanup before the F–C assay to eliminate interfering substances, such as sulfur dioxide, ascorbic acid, sugar, aromatic amines, organic acid, and Fe(II) [16].

Covariate adjustments in urinary TPE expressed by creatinine correction model increased the partial regression coefficient with dietary intake, which became statistically significant (pr = 0.116; $P = 0.002$). The fit of the model in which urinary TPE was expressed as 24-h volume remained significantly better than the fit for TPE urinary creatinine normalized model.

Interestingly, renal function is not a significant covariate in models including urinary TPE expressed by 24-h volume because renal function can vary the total urinary volume excreted, but it would not alter the ratio between 24-h TPE and urinary 24-h volume. However, renal

function would be a significant variable in models in which TPE was corrected by urinary creatinine, because creatinine excretion varies with renal function [29].

Polyphenol biomarkers measured in urine normalized by 24-h volume usually show higher regression coefficients than when creatinine correction is applied; for instance, 24-h urinary levels of alkylresorcinols and isoflavones as biomarkers of cereal fiber intake ($r = 0.40$) and soy ($r = 0.52$) intakes, respectively, versus urinary creatinine normalized levels of 4-O-methylgallic acid and isoferulic acid as biomarkers of tea ($r = 0.50$) and coffee ($r = 0.26$) intakes, respectively [33–35]. For other urinary biomarkers, using creatinine correction, for example for resveratrol metabolites as a biomarker of wine consumption, provides high regression coefficients ($r = 0.89$) [19,36]. The main limitation of the study may be the use of an FFQ, because it is not the best dietary assessment technique [37], although this FFQ has been validated for this population and the EPIC Italian population [38]. Moreover, in EPIC – Europe, this FFQ was also used to positively correlate plasma carotenoids and fruit and vegetable consumption [39], and fruits and vegetables are the second main polyphenol source, after coffee, in the Finnish population [7]. For this reason, we consider this FFQ suitable for assessing polyphenol intake. Another drawback may be that the participants of the study were older subjects and there was a high prevalence of renal impairment, thus the results cannot probably be extrapolated to the general population. In this case, when we compare the same analytical measure (TPE) expressed in two different ways (by 24-h volume and by urinary creatinine correction) renal function is statistically significant. However, when we have an analytical variable (urinary TPE) and a dietary variable (total dietary polyphenols) which was assessed with more error than analytical ones [40], renal function is not significant, although in a homogenous population, as with the men in the current study, renal function was of borderline significance

In conclusion, we showed that urinary TPE expressed in both 24-h volume and by urinary creatinine correction are highly correlated, especially after adjusting for covariates. Furthermore, our data support that urinary TPE expressed by 24-h volume is a better biomarker of polyphenol dietary intake than by urinary creatinine normalization, although creatinine-corrected urinary TPE adjusted for covariates may also be a suitable biomarker of total polyphenol in a free-living population. For these reasons, the biomarker, expressed by 24-h volume and by urinary creatinine correction, would be an additional and more reliable tool for studying the relationships between polyphenol intake and health effects in large epidemiological studies.

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Abbreviations

TPE total polyphenol excretion

F–C	Folin–Ciocalteau
GAE	gallic acid equivalents
EPIC	European Prospective Investigation into Cancer and Nutrition
FFQ	food frequency questionnaire

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Table 1

Characteristics of participants at baseline stratified by urinary creatinine quartiles.

Characteristic[a]	All (*n* = 928)	Q1 (*n* = 232)	Q2 (*n* = 232)	Q3 (*n* = 232)	Q4 (*n* = 232)	*P*-test
Gender (% female)	55.3	75.3	59.7	41.4	28.4	<0.001
Age (year)	69.8 (13.6)	73.8 (9.8)	70.3 (12.6)	69.9 (12.5)	64.6 (16.4)	<0.001
BMI (kg m^{-2})	27.3 (4.0)	27.0 (3.8)	27.3 (4.5)	27.3 (3.8)	27.8 (4.0)	0.169
Physical activity (%)						<0.001
Hardly any physical activity	5.7	54.5	18.2	13.6	13.6	
Mostly sitting/some walking	14.4	53.7	22.8	11.8	11.8	
Light exercise 2–4 h/week	39.7	39.9	27.7	13.5	19	
Moderate 1–2 h or light >4 h/week	32.6	28.3	23.6	18	30.1	
Moderate exercise >3 h/week	5.5	14	16	12	58	
Intense exercise many times/week	1.4	25	0	8.3	66.7	
Walks >5 km day^{-1}, >5 days/week, >5 years	0.7	28.6	42.9	14.3	14.3	
Energy intake (kcal day^{-1})	1965.9 (581.5)	1794.4 (496.9)	19312.0 (515.5)	2049.1 (665.7)	2205.2 (628.7)	<0.001
Renal function (%)						<0.001
Normal	43.6	26	23.9	15.5	34.6	
Impaired	54.7	43.5	25.2	14.3	16.9	
Profoundly impaired or failure	1.7	76.9	23.1	0	0	
Total dietary polyphenol (mg day^{-1})	436.6 (344.9–549.1)	412.7 (336.1–535.1)	425.1 (352.6–523.8)	455.9 (322.0–569.9)	474.1 (367.4–598.3)	<0.001
Urinary TPE (mg GAE/24-h volume)	151.0 (113.3–199.8)	159.5 (120.3–211.7)	165.7 (123.3–210.3)	151.3 (111.0–184.8)	129.2 (102.9–172.4)	<0.001
Urinary TPE (mg GAE/g creatinine)	156.6 (117.8–213.2)	216.0 (178.4–269.0)	166.9 (137.4–207.2)	128.7 (109.0–162.8)	106.2 (86.9–133.9)	<0.001
Urinary creatinine (mg dL^{-1})	74.2 (37.1)	41.0 (8.6)	65.0 (5.0)	84.5 (5.0)	127.3 (29.7)	<0.001

[a]Baseline characteristics of the participants were expressed as means (SD) for normal distribution or percentages. Skewed variables (Kolmogorov with the Lilliefors Significance correction and Levene tests) were described as median (interquartile range).

Table 2

Multiple linear regression model explaining the association between transformed urine TPE expressed as mg GAE/24-h urine (as dependent variable) and transformed urine TPE expressed as mg GAE/g of urinary creatinine (as independent variable), after adjusting for covariates.

	B-coefficients (IC 95%)	***P*-value**
Constant of the model	3.628 (2.978 to 4.279)	<0.001
Transformed urinary TPE (mg GAE/g of creatinine)	0.693 (0.646 to 0.740)	<0.001
Gender (female vs. male)	−0.452 (−0.552 to −0.352)	<0.001
Age (per year)	−0.015 (−0.019 to −0.010)	<0.001
BMI (kg m^{-2})	−0.001 (−0.012 to 0.009)	0.786
Physical activity	0.085 (0.034 to 0.136)	0.001
Energy intake (1000 kcal day^{-1})	0.084 (0.002 to 0.166)	0.045
Renal function		
Impaired vs. normal	−0.207 (−0.307 to −0.106)	<0.001
Profoundly impaired or failure vs. normal	−0.612 (−0.971 to −0.254)	0.001

Table 3

Results from multiple linear regression analysis which assessed the association between transformed total dietary polyphenols and transformed urine TPE expressed as 100 mg GAE/24-h urine (model 1) and expressed as 100 mg GAE/g urinary creatinine (model 2), after adjusting for covariates.

	Model 1 (expressed by 24-h volume)		**Model 2 (expressed by creatinine)**	
	B-coefficients (IC 95%)	***P*-value**	**B-coefficients (IC 95%)**	***P*-value**
Constant of the model	5.170 (4.896 to 5.445)	<0.001	5.297 (5.028 to 5.565)	<0.001
Crude urine TPE	0.072 (0.042 to 0.103)	<0.001	0.030 (0.012 to 0.049)	0.002
Gender (female vs. male)	0.085 (−0.131 to −0.039)	<0.001	−0.103 (−0.149 to −0.056)	<0.001
Age (per year)	0.003 (0.001 to 0.005)	0.002	0.002 (0.001 to 0.004)	0.013
BMI (kg m^{-2})	−0.002 (−0.007 to 0.003)	0.483	−0.002 (−0.007 to 0.003)	0.431
Physical activity	0.027 (0.003 to 0.051)	0.025	0.031 (0.007 to 0.055)	0.012
Energy intake (1000 kcal day^{-1})	0.344 (0.305 to 0.382)	<0.001	0.346 (0.308 to 0.385)	<0.001
Renal function				
Impaired vs. normal	−0.023 (−0.070 to 0.025)	0.347	−0.034 (−0.081 to 0.014)	0.161
Profoundly impaired or failure vs. normal	−0.014 (−0.182 to 0.154)	0.868	−0.045 (−0.214 to 0.124)	0.600