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Ellagic Acid Metabolism by Human Gut Microbiota: Consistent Observation of Three Urolithin Phenotypes in Intervention Trials, Independent of Food Source, Age, and Health Status

ABSTRACT: Three phenotypes for urolithin production after ellagitannin and ellagic acid intake are consistently observed in different human intervention trials. Subjects can be stratified into three urolithin-producing groups. "Phenotype A" produced only urolithin A conjugates, which included between 25 and 80% of the volunteers in the different trials. "Phenotype B" produced isourolithin A and/or urolithin B in addition to urolithin A, this being the second relevant group (10−50%). "Phenotype 0" (5−25%) was that in which these urolithins were not detected. The three phenotypes were observed independently of the volunteers' health status and demographic characteristics (age, gender, body mass index (BMI)) and of the amount or type of ellagitannin food source ingested (walnuts and other nuts, strawberries, raspberries, and other berries or pomegranates). Interestingly, a higher percentage of phenotype B was observed in those volunteers with chronic illness (metabolic syndrome or colorectal cancer) associated with gut microbial imbalance (dysbiosis). These urolithin phenotypes could show differences in the human gut microbiota and should be considered in intervention trials dealing with health benefits of ellagitannins or ellagic acid. Whether this phenotypic variation could be a biomarker related to differential health benefits or illness predisposition deserves further research.

KEYWORDS: *polyphenol, meta-analysis, metabolite, microbiota, interindividual variability*

INTRODUCTION

Ellagitannins and ellagic acid are polyphenols often underestimated in our diet. However, they can play an important role in the health effects observed after the intake of different food products such as pomegranates, nuts, some berries (raspberry, blackberry, strawberry, cloudberry, etc.), tea, muscadine grapes, many tropical fruits, oak-aged wines, and medicinal plants. Ellagitannins and ellagic acid are transformed by the gut microbiota to produce urolithins, bioavailable metabolites that can exert anti-inflammatory and anticarcinogenic effects,[1−3] and can reach high concentrations in both normal and tumor human colonic tissues.[4] There is, however, a large interindividual variability in these health effects. The gut microbial composition in healthy humans and also the gut microbioma imbalance (dysbiosis), which is associated with different chronic illnesses, such as inflammatory bowel diseases (colitis, Crohn's disease, etc.), colon cancer, metabolic syndrome, obesity, and cirrhosis could be behind the different polyphenol metabolic profiles.[4]

Differences in urolithin production, both quantity and chemical type, could explain, at least partly, the large variability in the health effects observed in vivo. Urolithin A (**1**) is the main metabolite observed in human plasma and urine, where it mainly occurs as conjugate forms with glucuronic acid (**4**) and/or sulfate. However, isourolithin A (**2**) and urolithin B (**3**) (Figure 1) conjugates are also observed in some volunteers, whereas in others urolithins are not produced or are present below the limit of detection.[4] Therefore, it is important to evaluate whether these metabolic behaviors are consistent in different human populations (gender, age, health status, racial background, and geographic location) and if they are produced after consumption of different ellagitannin-containing foods.

Our aim was to evaluate the ellagitannin/ellagic acid bioconversion capacity of humans to produce urolithins. For this purpose, we evaluated the metabolic behavior of three groups of healthy volunteers by analyzing their urinary urolithin profiles after the intake of walnuts and pomegranate extracts. In addition, we performed a small meta-analysis by re-examining the metabolic profiles observed in previous studies with volunteers of different ages, gender, and health status including some characterized by a gut dysbiosis.

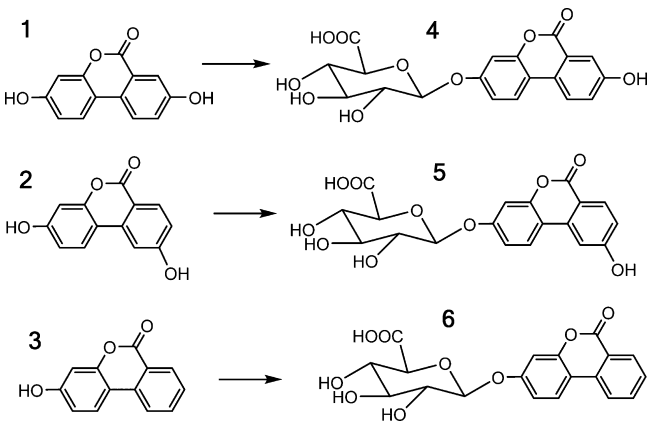

Figure 1. Urolithin metabolites observed after ellagitannin intake: urolithin A (**1**); isourolithin A (**2**); urolithin B (**3**); urolithin A glucuronide (**4**); isourolithin A glucuronide (**5**); urolithin B glucuronide (**6**).

MATERIALS AND METHODS

Chemicals. Urolithins were chemically synthesized (Villapharma, Murcia, Spain), as described elsewhere.[4] Purity was >95% in all tested compounds.

Intervention Studies and Study Products. Three intervention studies were performed and were in accordance with the Helsinki

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Declaration and approved by the Ethics Committee from CSIC (Madrid, Spain) (Table 1). All volunteers gave their written informed consent prior to participation, and they had not taken antibiotics during 4 weeks before the trials. In the three intervention trials, volunteers consumed their usual diet but avoided ellagitannin-containing food products during the study and 1 week before starting.

In the first intervention study, healthy volunteers (n = 20, 11 men and 9 women) aged between 21 and 55 years consumed 30 g of walnuts (edible part)/day for 3 days. Walnuts were purchased in a local supermarket. After the last intake, 24 h urine samples were collected. Urinary volume was measured, and the samples were immediately stored at −20 °C.

In the second intervention study, healthy volunteers (n = 20, 10 men and 10 women) aged between 18 and 23 years ingested four capsules of pomegranate extract in a single intake (total dose = 1.8 g of extract). Urine samples were also collected for 24 h after the intake and were immediately stored at −20 °C.

In the third trial, overweight healthy subjects (n = 49, 32 men, 17 women; body mass index (BMI) > 27 kg/m^2) aged between 40 and 65 years ingested two daily capsules of pomegranate extract (0.9 g/day) for 3 days. Urine samples were collected for 24 h after the last intake, and the urine was stored at −20 °C until further analysis. In both interventions 2 and 3 capsules containing pomegranate extract were kindly supplied by Laboratorios Admira S.L. (Alcantarilla, Murcia, Spain).

Extraction Protocol of Urolithin Metabolites. Urine and feces samples were processed as previously reported.[4,5]

LC-MS Analysis. Samples were analyzed by HPLC-DAD-ESI-MS/MS or UPLC-ESI-QTOF-MS as previously reported.[4]

RESULTS AND DISCUSSION

We report here that human subjects can be consistently clustered within three phenotypes, that is, "phenotype A", "phenotype B", or "phenotype 0" according to their different capacities for excreting urolithins, independent of their demographic characteristics, health status, or ellagitannin-containing food product (Table 1). This has been observed in three dietary interventions in healthy volunteers (present study) as well as after re-examining available data for urinary profiles from previous studies.

Urolithins are mainly detected either as aglycones in feces or as phase II metabolites in systemic organs or biological fluids (glucuronide or sulfate derivatives). Overall, urolithin A (**1**) is the main metabolite produced in humans and previously identified as a urinary biomarker after consumption of ellagitannins/ellagic acid from different food products.[6] In the present study (Table 1), interventions 1−3 with healthy volunteers, some volunteers excreted only urolithin A metabolites (phenotype A), whereas other subjects, in addition to urolithin A (**1**), also excreted urolithin B (**3**) and isourolithin A (**2**) showing a more complex urinary metabolite profile (phenotype B) (Figure 2). Furthermore, a third group that did not excrete urolithins in urine was also identified (phenotype 0). The same three phenotypes were observed after intake of walnuts (intervention 1) or pomegranate extracts (interventions 2 and 3).

This observation prompted us to re-examine the excretion profiles observed in previous studies that we had previously completed in our laboratory or in collaboration with other research groups, as well as data available from other published studies. These three phenotypes were also found in intervention studies either with healthy volunteers or with those volunteers having some disease situations, confirming that these three phenotypes are quite robust and consistent (Table 1). In accordance with the three interventions described here, the three phenotypes were also observed no matter the

Table 1. Urolithin Urinary Phenotype Distribution in Different Human Trials

trial	food (quantity ingested, total ellagic acid content after hydrolysis/day)	analytical technique	N	age	BMI (kg/m^2)	health status	phenotype		
							A	B	0
intervention 1 (this study)	walnuts (30 g/day, 162.8 mg of total ellagic acid)	HPLC-DAD-ESI-IT-MS/MS	20 (10 men, 10 women)	21−55	23.8 ± 2.3	healthy	13 (65%)	4 (20%)	3 (15%)
intervention 2 (this study)	pomegranate extract (1.8 g, 220 mg of total ellagic acid)	UPLC-ESI-QTOF-MS	20 (10 men, 10 women)	18−23	20.5 ± 2.1	healthy	16 (80%)	2 (10%)	2 (10%)
intervention 3 (this study)	pomegranate extract (0.9 g/day, 110 mg of total ellagic acid)	UPLC-ESI-QTOF-MS	49 (32 men, 17 women)	40−65	30.4 ± 3.4	healthy-overweight	30 (60%)	15 (30%)	4 (10%)
González-Sarrías et al.[7]	walnuts (35 g/day, 190 mg of total EA), pomegranate juice (200 mL/day, 165.7 mg of total ellagic acid)	HPLC-DAD-ESI-IT-MS/MS	28 (men)	56−90	27.7 ± 3.6	prostate cancer or benign prostate hyperplasia	17 (60%)	4 (15%)	7 (25%)
Truchado et al.[8]	strawberry (200 g of fresh strawberries or the equivalent in puree, 150 mg of total ellagic acid)	HPLC-DAD-ESI-IT-MS/MS	20 (8 men, 12 women)	25−30	not available	healthy	16 (80%)	3 (15%)	1 (5%)
Puupponen-Pimia et al.[9]	strawberry (100 g/day, 75 mg total EA) + raspberry (100 g/day, 147 mg of total ellagic acid) + cloudberry (100 g/day)	HPLC-DAD-ESI-IT-MS/MS	20 (10 men, 10 women)	50−65	31.8 ± 4.4	metabolic syndrome	5 (25%)	10 (50%)	5 (25%)
Tulipani et al.[11]	nuts (unpeeled), (30 g/day, 81.4 mg of total ellagic acid) (15 g of walnuts + 7.5 g of almonds + 7.5 g of hazelnuts)	HPLC-DAD/ESI-QqQ-MS/MS and HPLC-ESI-IT-MS	22 (13 men, 9 women)	31−63	31.0 ± 2.9	metabolic syndrome	11 (50%)	9 (41%)	2 (9%)
Núñez-Sánchez et al.[4]	pomegranate extract (0.9 g/day, 110 mg of total ellagic acid)	UPLC-ESI-QTOF-MS	26 (14 men, 12 women)	52−89	28.9 ± 3.9	colorectal cancer	11 (42.3%)	11 (42.3%)	4 (15.4%)

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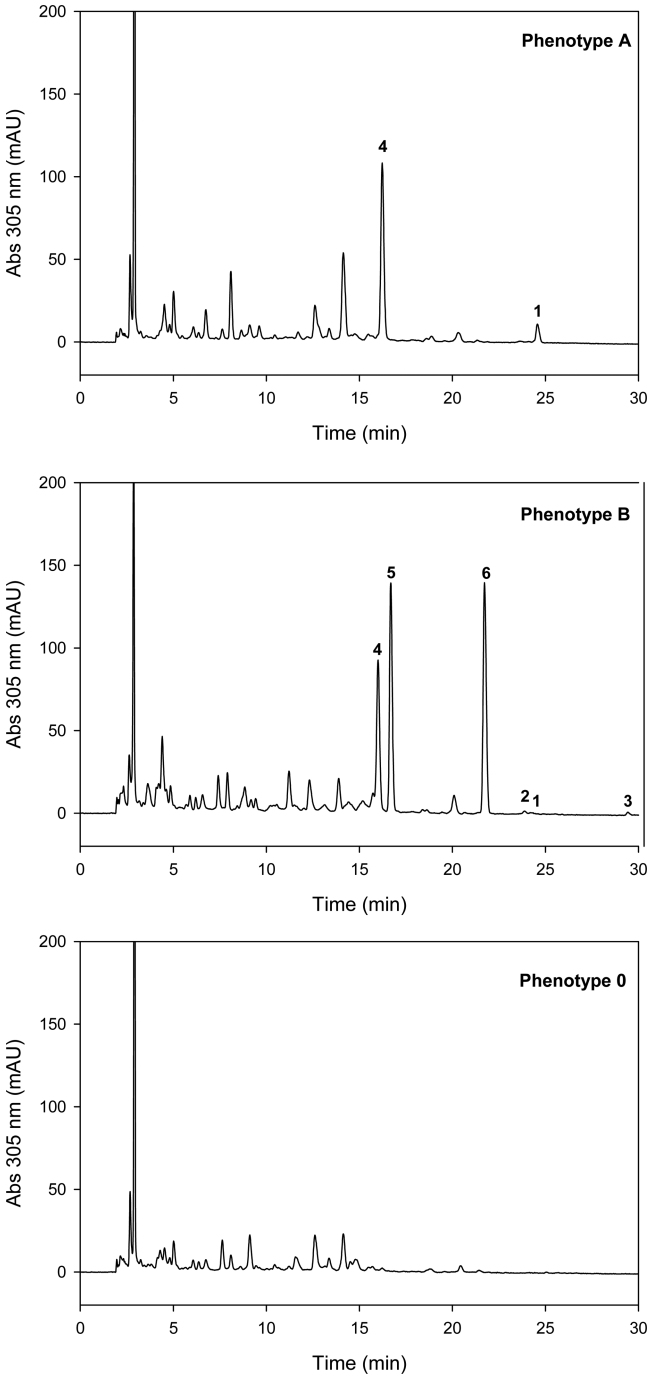

Figure 2. Characteristic HPLC-UV chromatograms showing the urolithin metabolite profiles corresponding to the three urinary phenotypes observed after the intake of walnuts.

source of ellagitannins and the amount of ellagic acid ingested. They were identified after the intake of walnuts, pomegranate juice,[7] pomegranate extracts,[4] strawberries,[8,9] raspberries,[9,10] cloudberries,[9] and unpeeled nuts.[11]

In healthy volunteers as well as in those patients with illnesses that are not characterized by gut dysbiosis (prostate cancer or benign hyperplasia), phenotype A was the main urinary profile (Table 1; Figure 3). In this regard, phenotype A was also the most abundant profile in patients with chronic obstructive pulmonary disease (age 60 ± 11 years) that consumed pomegranate juice for 5 weeks,[12] although data were not available for quantitative re-examination of the urinary

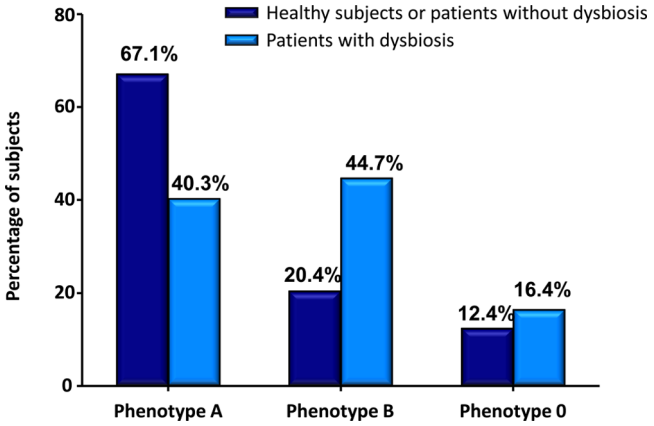

Figure 3. Mean distribution of the three phenotypes present in healthy subjects and patients without dysbiosis (n = 137) as well as in those patients with illnesses associated with gut dysbiosis (metabolic syndrome, n = 42; and colorectal cancer, n = 26).

profiles (results not shown). In contrast, the ratio between phenotypes A and B changed in patients with illness associated with gut dysbiosis such as metabolic syndrome or colorectal cancer, where phenotype B gained increasing relevance (Table 1; Figure 3). Age, gender, BMI, and geographical location did not affect urolithin urinary profiles (Table 1). We hypothesize that gut dysbiosis is the cause of the increase in the abundance of isourolithin A and urolithin B (phenotype B) in the patients with colon cancer and metabolic syndrome (Table 1). Therefore, a urolithin production test could be a useful biomarker to monitor certain gut microbial imbalances or to assess treatment efficacy.

Previous studies from other groups also showed the presence of these three phenotypes, which were within the ranges shown here. For example, a previous study of the urinary urolithins produced after the intake of raspberries by healthy volunteers (n= 10) showed that 70% were only urolithin A excreters (phenotype A), 20% excreted in addition isourolithin A and urolithin B (phenotype B), and 10% were nonexcreters (phenotype 0).[10] Interestingly, despite the relatively low number of volunteers, the distribution of the three phenotypes was similar to that observed in the three trials with healthy subjects shown here. A recent study[13] has reported the presence of three urinary urolithin excretion patterns after the intake of tropical highland blackberry juice by healthy volunteers that is also consistent with our results. However, demographic characteristics (age, gender, BMI) of volunteers were not described, and the ratio between phenotypes A and B was closer to that found in patients with gut dysbiosis than that observed in healthy subjects. In addition, in this previous study, the correlation of the production of urolithin B with that of isourolithin A was not described, as isourolithin A was not analyzed.

Our results show that stratification of subjects according to their urolithin phenotype should be considered in ellagitannin and ellagic acid intervention trials. Three consistent enterotypes, with different gut microbiota communities, have been described in humans.[14] In line with the trend highlighted by a previous study,[15] the challenge now is to investigate the correlation between these enterotypes, the three urolithin phenotypes, and the possible impact on human health.

Urolithins seem to be responsible for some health effects after ellagitannin intake;[2] therefore, volunteers in phenotype 0

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will probably not benefit from them, and this should be demonstrated in further studies. Ellagic acid either remains unmetabolized in these volunteers or could be metabolized in a different way, leading to compounds different from urolithins that need to be further investigated.

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Notes

The authors declare no competing financial interest.

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