RESEARCH ARTICLE

Occurrence of urolithins, gut microbiota ellagic acid metabolites and proliferation markers expression response in the human prostate gland upon consumption of walnuts and pomegranate juice

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Epidemiology supports the important role of nutrition in prostate cancer (PCa) prevention. Pomegranate juice (PJ) exerts protective effects against PCa, mainly attributed to PJ ellagitannins (ETs). Our aim was to assess whether ETs or their metabolites ellagic acid and urolithins reach the human prostate upon consumption of ET-rich foods and to evaluate the effect on the expression of three proliferation biomarkers. Sixty-three patients with BPH or PCa were divided into controls and consumers of walnuts (35 g walnuts/day) or pomegranate (200 mL PJ/day) for 3 days before surgery. Independently of the ETs source, the main metabolite detected was urolithin A glucuronide, (3,8-dihydroxy-6H-dibenzo[b,d]pyran-6-one glucuronide) (up to 2 ng/g) together with the traces of urolithin B glucuronide, (3-hydroxy-6H-dibenzo[*b*,*d*]pyran-6-one glucuronide) and dimethyl ellagic acid. The small number of prostates containing metabolites was likely caused by clearance of the compounds during the fasting. This was corroborated in a parallel rat study and thus the presence of higher quantities of metabolites at earlier time points cannot be discarded. No apparent changes in the expression of *CDKN1A*, *MKi-67* or *c-Myc* were found after consumption of the walnuts or PJ. Our results suggest that urolithin glucuronides and dimethyl ellagic acid may be the molecules responsible for the beneficial effects of PJ against PCa.

Keywords:
Bioavailability / Cancer / Ellagitannins / Gene expression / Polyphenol

1 Introduction

Prostate cancer (PCa) is the most common non-cutaneous malignancy and the second leading cause of cancer mortality in men living in the developed countries [1, 2]. Benign prostatic hyperplasia (BPH) is also the most common benign tumor in adult men. Both BPH and localized PCa can be eliminated by surgery or radiotherapy, although chemoprevention through the administration of synthetic or naturally occurring compounds to delay or even suppress carcinogenesis is being increasingly

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Abbreviations: BPH, benign prostate hyperplasia; **CG,** control group; **DMEA,** dimethyl ellagic acid; **EA,** ellagic acid; **EIC,** extracted ion chromatogram; **ETs,** ellagitannins; **HED,** human equivalent dose; **PCa,** prostate cancer; **PE,** pomegranate extract; **PJ,** pomegranate juice; **PJG,** pomegranate juice group; **Uro-A,** urolithin A, (3,8-dihydroxy-6H-dibenzo[*b*,*d*]pyran-6-one); **Uro-B,** urolithin B, (3-hydroxy-6H-dibenzo[*b*,*d*]pyran-6-one); **WG,** walnuts group

studied [3, 4]. Epidemiology supports the important role of nutrition in PCa prevention [5]. High meat intake and dietary saturated fats are associated with a higher PCa risk. A number of protective compounds have also been identified in the diet including carotenoids, sulforafane from cruciferous, selenium, omega-3 fatty acids and polyphenols. All these constituents may prevent and/or inhibit prostate disorders by acting synergistically within a diet rich in fruits and vegetables [6–8].

Ellagitannins (ETs) are polyphenols formed by ellagic acid (EA) subunits [9] and are abundant in walnuts, pomegranates (fruit and juice), persimmon, oak-aged wines and berries [9, 10]. ETs and EA exhibit anti-cancer properties *in vitro* and *in vivo* [11, 12]. In particular, pomegranate exerts protective effects against a number of cancers [4] including PCa [13]. Pomegranate juice (PJ) supplementation has been shown to prolong the doubling time of the PCa biomarker PSA (prostate serum antigen) after surgery or radiotherapy [14]. These results suggest that some components of the pomegranate may be involved in the observed PCa inhibitory effects. ETs have been suggested to be the most active fraction in the PJ [15, 16]. However, ETs are hydrolyzed *in vivo* to yield EA which is further metabolized by the gut microbiota to produce mostly the derivatives urolithins [17, 18]. Although the occurrence of ETs and EA in the bloodstream is almost negligible, urolithins can reach concentrations at the micromolar level [19]. In addition, urolithins have been identified as bioactive molecules that can bind estrogenic receptors and display estrogenic and antiestrogenic activity in human breast cancer MCF-7 cells [20]. Recently, urolithins have also been shown to inhibit colon cancer cells proliferation and alter the expression of genes and proteins ultimately modulating cell cycle and signalling events associated with cancer development [21].

Some of the molecular alterations associated with development and progression of BPH and PCa may be similar but are not yet fully understood [22]. BPH is accompanied by a significant increase in the proliferation rate of epithelial cells in the hyperplasic acini [23] and deregulation of the expression of genes involved in cell proliferation or cell cycle is associated to PCa [24]. Among those genes, *CDKN1A*, cyclin-dependent kinase inhibitor 1A (p21, Cip1) is a key regulator of cell cycle progression at the G_1 phase and is hypothesized to have cancer-promoting functions [25]. The v-myc myelocytomatosis viral oncogene homolog (avian) (*c-Myc*) is a strong positive regulator of cell proliferation and has been found to be amplified in up to 72% of androgen-independent PCa [26]. *MKi-67* or antigen identified by monoclonal antibody Ki-67 is a marker of cell growth and proliferation whose expression is increased in PCa and BPH relative to normal tissue [27, 28]. Since the abnormal expression of these molecules is involved in cancer development and progression, the characterization of the proliferation patterns of BPH and PCa by analyzing the expression of genes involved in the regulation of cell cycle and proliferation may yield important information on the cells response to new therapeutic drugs and treatments.

This study was undertaken to determine whether ETs, EA, urolithins or any other derived conjugate can be found and quantified in the human prostate gland upon consumption of PJ or walnuts. The results will allow us to establish which compounds may enter the prostatic tissue and may be the actual molecules responsible for a putative chemopreventive effect in this organ. This information will be very helpful in the design of better *in vitro* studies using realistic concentrations of the metabolites detected *in vivo*. In an effort to gain a deeper insight into the molecular changes that may occur in the prostate tissues as a consequence of the exposure to the pomegranate or walnuts, we have also used RT-PCR to measure the expression differences of three proliferation biomarkers, *CDKN1A, MKi-67* and *c-Myc* in human samples of BPH and PCa between two groups of patients, control group (CG) and patients supplemented with PJ or walnuts.

2 Materials and methods

2.1 Chemicals

Urolithins A (Uro-A; 3,8-dihydroxy-6H-dibenzo[*b*,*d*]pyran-6-one; 95% purity) and B (Uro-B; 3-hydroxy-6H-dibenzo[*b*,*d*]-pyran-6-one, 98% purity) were synthesized by Kylolab S.L. (Murcia). EA, β-glucuronidase (EC 3.2.1.31; 100 000 units) from bovine liver and sulfatase (EC 3.1.6.1; 10 000 units) from *Helix pomatia* were purchased from Sigma (St Louis, MO, USA). Punicalagin was isolated and purified from pomegranate husks as described previously [29]. Organic solvents such as methanol (MeOH), acetone, ACN, *etc*., were obtained from Merck (Darmstadt, Germany). Milli-Q system (Millipore, Bedford, MA, USA) ultrapure water was used throughout this experiment.

2.2 Walnuts and pomegranate samples

Peeled walnuts were purchased in a local supermarket, processed and analyzed by LC-MS/MS to characterize and quantify the polyphenols content [19]. PJ was obtained from fresh pomegranates (*Punica granatum*, cv Mollar de Albatera) using a laboratory pilot press and polyphenols were also determined by LC-MS/MS [29]. PJ was bottled and stored frozen at −20°C. Pomegranate extract (PE) was kindly supplied by Nutracitrus S.L. (Elche, Spain). Ten milligrams of PE were dissolved in 1 mL water:formic acid (99:1). The solution was filtered through 0.45 μm membrane filter Millex-HV_{13} (Millipore) and a 10 μL aliquot was analyzed by LC-MS/MS to determine polyphenols according to Cerdá *et al*. [29]. PJ and walnuts polyphenols were fully stable along the study period.

2.3 Subjects and clinical study design

This study conforms to the principles outlined in the Declaration of Helsinki and its amendments. The protocol

was approved by the Committee of Clinical Investigation of the ''Hospital Reina Sofia'' (reference October 3, 2007) and was fully explained to the volunteers who gave their written informed consent prior to participation. The inclusion criteria for participating in this trial were clinically diagnosed BPH or PCa. Exclusion criteria included vegetarian diet, consumption of vitamin supplements or nutraceuticals, renal insufficiency, previous gastrointestinal surgery, gastrointestinal disease (hepatic insufficiency, intestinal inflammation) or removal of gall bladder, as well as intake of antibiotics (at least 2-wk before the trial). The patients that fulfilled the criteria were interviewed 1 wk before surgery and were included consecutively in the study. From all patients interviewed, 63 men (mean BMI, $27.7 \pm 3.6\,kg/m^2$) were finally enrolled for the study. Patients' age ranged from 56 to 90 with an average age of 68.9 ± 7.6 years. Fourteen subjects (BMI $27.9 \pm 3.8\,kg/m^2$) were randomly selected to consume walnuts and 19 patients (BMI $28 \pm 3.1\,kg/m^2$) to drink PJ. The other 30 patients did not consume PJ or walnuts but also gave their permission to analyze a piece of their removed prostate tissue (CG). All the participants were asked to avoid the consumption of ETs-containing foodstuffs at least 5 days before the trial including pomegranates, hazelnuts, pistachios, walnuts, strawberries, raspberries, oak-aged wines and spirits, blackberries, persimmons, peaches, plums, as well as their derivatives such as juices, jams and jellies. In addition, each patient provided the name of medications consumed. Patients belonging to the pomegranate juice group (PJG) ingested one small bottle (200 mL) *per* day, whereas the volunteers enrolled in the walnut group (WG) consumed individual packages of 35 g peeled walnuts. The walnuts package contained an oxygen-free atmosphere (100% N_2) to avoid rancidity. In both groups, the patients ingested the foodstuffs daily in the morning for 3 days before surgery. The last intake was approximately 18–24 h before surgery. All the patients provided urine and blood samples before the intake of PJ or walnuts (baselines) as well as before the surgery. Patients from the CG group provided urine and blood samples before surgery. Rapidly after surgery, the entire prostate or a piece of tissue (depending on the type of surgery applied, *i.e.* prostatectomy, transurethral resection or adenomectomy) was taken to the Anatomical Pathology Service where it was examined and classified by two expert pathologists. Thirteen prostate samples (six from CG, three from WG and four from PJG) were diagnosed as PCa (only specimens with a high carcinoma component were included in the analysis). The other 50 samples were diagnosed as BPH and were verified not to contain any cancer cells. Small appropriate adjacent sections of the specimens were cut separately: (i) some were frozen in liquid nitrogen (for the analysis of the presence of EA and metabolites), (ii) other sections were included in paraffin and stained with eosin-haematoxylin for microscopy inspection and detection of cancerous cells and (iii) some small portions were finely cut and immersed in RNAlater Stabilization Reagent (Qiagen) in order to preserve and protect against risk of RNA degradation, and stored at −80°C until use.

2.4 Animal study

Experiments followed a protocol approved by the local animal Ethics Committee (reference 300305440012) and the Local Government in accordance with the recommendations of the European Union regarding animal experimentation (Directive of the European Council 86/609/EC). Male Sprague–Dawley rats ($n = 16$, 200–250 g) were provided by the Animal Centre of the University of Murcia (Murcia, Spain). Rats were quarantined for 1 wk, housed in eight cages (two rats *per* cage), kept at 22 ± 2°C with $55 \pm 10\%$ relative humidity and controlled lighting 12 h light:dark cycles. Rats were distributed in four groups ($n = 4$ *per* group) and fed with standard chow (Panlab, Barcelona, Spain) supplemented with PE or Uro-A for three consecutive days. Two groups received the diet supplemented with PE at two concentrations: (i) high PE (HPE), 885 mg/kg/day corresponding to a human equivalent dose (HED) of 10 g PE/day for a 70 kg-person and (ii) low PE, 175 mg/kg/day (HED of 2 g PE/day). The other two groups were fed with Uro-A-supplemented diet at two concentrations: (i) 22.5 mg/kg/day (HED of 1 g Uro-A/day) and (ii) 4.5 mg/kg/day (HED of 0.2 g Uro-A/day). At the end of the experiment, two rats from each group were fasted for 18 h before sacrifice whereas the other two animals had free access to their corresponding diets until sacrifice. Rats were anesthetised *via* intramuscular injection (1 mL/kg body weight) with a mixture of ketamine (Imalgéne 1000) (Merial, Barcelona, Spain) and xylazine (1:1) (Xilagesic 2%) (Calier, Barcelona, Spain) and sacrificed by exanguination *via* cardiac punction. Prostates were quickly collected, extensively rinsed out in saline phosphate buffer (PBS) and immediately frozen at −80°C until analysis.

2.5 Processing of prostate, plasma and urine samples

Prostate samples (0.15 g) were thawed and homogenised with 1.35 mL of cold methanol:HCl:H_2O (79.9:0.1:20, v:v:v) using an IKA T10 Ultra-Turrax equipment (Janke and Kunkel, Ika-Labortechnick, Germany) at 24 000 rpm for 1 min at 4°C. The mixture was centrifuged at 14 000 *g* for 5 min in a Sigma 1–13 microcentrifuge (Braun Biotech International, Germany) and the supernatant kept at 4°C. The pellet was further extracted with 0.5 mL of the same methanolic solution and centrifuged at 24 000 rpm for 1 min at 4°C. Both supernatants were pooled and evaporated to dryness with N_2 at 40°C. The dry residue was dissolved with 100 μL of MeOH in the ultrasound bath for 5 min and centrifuged at $14\,000 \times g$ for 5 min. The resulting supernatant was evaporated under N_2 to 20 μL. Finally, this

volume was diluted by adding 20 μL of Milli-Q water and a 4 μL aliquot was injected into the LC-MS/MS equipment. The same protocol was ued for the rat prostates. Prostate samples that were going to be treated with sulfatase and glucuronidase were processed in a similar way but, 100 μL of sodium acetate buffer (pH 5) instead of MeOH were used to dissolve the dry residue after the final evaporation step. Then, the protocol described by Cerdá *et al.* [29] was followed. The extraction recovery was checked three times (with and without enzymatic treatment), using EA, Uro-A and Uro-B (2 μM each) with a mean recovery of 90 ± 5% for the three compounds. No EA or urolithins glucuronides were used as there were no available standards.

Plasma samples were obtained by centrifugation at 14 000 × *g* for 15 min at 4°C in a Sigma 1–13 microcentrifuge and immediately frozen at −80°C for further analyses. For extraction, plasma samples (1 mL) were vigorously homogenised with ACN (× 3, 3 mL each). The organic phases (~9 mL) were pooled and evaporated to dryness and the residue was re-dissolved in MeOH (100 μL), diluted (1:1) with Milli-Q water, filtered through a 0.45 μm filter Millex-HV_{13} (Waters, Millipore, MA, USA) and then analyzed (4 μL) by LC-MS/MS. Thawed urine samples were centrifuged at 14 000 × *g* for 5 min in the microcentrifuge at 4°C. The supernatant was then filtered through a 0.45 μm filter Millex-HV_{13} and analyzed (4 μL) by LC-MS/MS.

2.6 HPLC-DAD-MS/MS analyses

Prostate, urine and plasma samples were analyzed using a HPLC-DAD system (1200 series, Agilent) equipped with a HTC Ultra mass detector in series (Bruker Daltonics, Bremen, Germany). The mass detector was an ion-trap mass spectrometer equipped with an ESI system (capillary voltage, 4 kV; dry temperature, 350°C). Mass scan and MS/MS daughter spectra were measured from *m/z* 100 to 800 using the Ultra scan mode (*m/z* 26 000/s). Collision-induced fragmentation experiments were also performed using helium as collision gas, and the collision energy was set at 50%. MS data were acquired in the negative ionization mode. Chromatographic separations of prostate, urine and plasma samples were carried out on a 150 × 0.5 mm id, 5 μm, reverse-phase SB C18 Zorbax column (Agilent) using water/formic acid (99:1, v/v) (A) and ACN (B) as the mobile phases at a flow rate of 10 μL/min. The gradient started with 1% B in A to reach 60% B at 30 min, 90% B at 30 min for 5 min and returning to the initial conditions (1% B). UV chromatograms were recorded at 280, 305, 360 and 510 nm.

2.7 Identification and quantification of ETs and urolithins

ETs from walnuts and PJ were identified according to their UV spectra, ion mass (MS) and daughter fragments (MS/MS) using ion trap [19, 29]. Identification of EA and punicalagin was carried out by chromatographic comparison (UV and MS) with pure commercial standards. ETs and EA derivatives in PJ and PE were quantified as punicalagin at 360 nm and as free EA at 360 nm, respectively. ETs in walnuts were quantified as free EA at 360 nm after hydrolysis as described previously [19]. Anthocyanins were quantified as cyanidin-3-glucoside at 510 nm [29]. Urolithins and EA-derived metabolites were identified according to their UV and MS spectra as well as MS/MS fragments as described previously [18, 19]. Urine and bile samples from acorn-fed Iberian pigs were used to help in the identification of urolithins and EA-derived metabolites [18].

2.8 RNA isolation and characterization from human prostate biopsies

For maximum efficiency of extraction, ~40–50 mg subsamples of each prostate biopsy section were weighed out of the RNAlater solution and processed according to the RNeasy mini kit (Quiagen) protocol. RNA concentration and purity were checked using the Nanodrop spectrophotometer system (ND-1000 3.3 Nanodrop Technologies). Only samples with a ratio Abs_{260}/Abs_{280} between 1.8 and 2.1 were used for RT-PCR experiments. The integrity of the ribosomal RNA was further checked using agarose gel electrophoresis (1%). RNA was extracted from two to four subsamples *per* specimen.

2.9 RT-PCR analysis

Changes in the expression of the selected markers were assessed by one-step quantitative RT-PCR (Taqman system, Applied Biosystems, ABI, Madrid, Spain). Primers and probes were purchased from Assays-on-demand (ABI, Madrid, Spain): Hs00355782_m1, cyclin-dependent kinase inhibitor 1A (p21, Cip1) (*CDKN1A*); Hs01032443_m1, antigen identified by monoclonal antibody Ki-67 (MKi-67); Hs00153408_m1, v-myc myelocytomatosis viral oncogene homolog (avian) (*c-Myc*). The one-step real-time RT-PCRs were run on the ABI 7500 system following manufacturer's conditions, using a total reaction volume of 25 μL in a MicroAmp Optical 96-well plate covered by optical adhesive covers and using Taqman Universal Master Mix (ABI, Madrid, Spain). All assays for a particular gene were undertaken at the same time under identical conditions. The expression levels of target genes were normalized to the levels of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; Hs99999905_m1) utilizing a standard curve method for quantification.

RT-PCR reactions were performed in duplicate aliquots of the RNA extracted from two to four sub-samples of each specimen and mean values were calculated. The intra-sample gene expression variability was studied for each

target and for the housekeeping gene *GAPDH* by determining the CV for replicates of the same specimen ($n = 3$–4). It was observed that expression variation for *GAPDH* was $<10\%$ in most cases and that it was lower than the expression variation for the three target genes (~20%). The usefulness of the housekeeping gene *GAPDH* was also evaluated by comparing the levels of this gene (normalized to the amount of total RNA) between BPH and PCa samples as well as between samples from CG and the PJG and WG. *GAPDH* was not differentially expressed between the groups of samples examined and therefore it was used for the normalization of gene expression.

2.10 Statistical analysis

Differences in the mRNA levels of the studied genes were assessed between the two patient groups, control and supplemented, as well as between BPH and PCa groups using an unpaired Student's *t*-test. Results with a two-sided *p*-value <0.05 were considered statistically significant.

3 Results

3.1 Phenolic composition of PJ, walnuts and PE

Each PJ bottle (200 mL) contained a total of 279 mg of EA-type phenolics. Among ETs, three isomers of punicalagin were the most abundant compounds (265 mg) although other ETs such as punicalin were also detected. In addition, 14 mg of EA derivatives including EA glucoside and free EA were quantified. Other minor polyphenols detected were the anthocyanins delphinidin, cyanidin and pelargonidin 3-glucosides and 3,5-diglucosides. Each walnut package (35 g net weight) contained 210 mg of EA-related phenolic compounds in total, 202 mg as ETs and 8 mg as free EA. The ETs identified by LC-MS/MS analysis were pedunculagin, casuarictin, tellimagrandin I and II, glansrin A, B and C, stenophyllanin A, 2,3-hexahydroxydiphenoyl-β-D-glucopyranoside and rugosin. The patients belonging to PJG and WG consumed a total of 837 and 630 mg, respectively, of EA-related polyphenols for the 3 days of the supplementation period. The PE used in the animal study contained 35% punicalagins, 13% punicalin, 4.5% EA glucoside and 8.9% free EA. The rats fed with the HPE dose ingested a HED of 6.14 g/day of EA-related compounds, and the rats fed with the low dose ingested a HED of 1.23 g/day of these compounds.

3.2 Urine and plasma analyses

The subjects enrolled in this trial were classified into three groups based on the type and amount of urolithins excreted in the urine, *i.e.* high, low and very low urolithins excreters. The high urolithin excreters grouped those volunteers that were characterised by urine concentrations of Uro-A glucuronide (the main metabolite excreted) superior to 5 μM. In these individuals, more than two other urolithins or EA derivatives were easily detected in the UV chromatogram (Fig. 1A). The low-urolithin excreters produced urine with a concentration of urolithin-A glucuronide below 5 μM and the identification of other urolithins or EA derivatives was possible only by ion mass analysis (Fig. 1B). Finally, the very-low-urolithin excreters were characterised by the absence of identifiable urolithins or EA derivatives in the UV chromatograms of urine samples and the identification of the main metabolite urolithin-A glucuronide was possible only by MS/MS analysis. Following this criterion, from the 33 patients who consumed either PJ or walnuts, 48.5% (16 subjects, 9 from WG and 7 from PJG) were identified as high urolithin excreters, 32% (10 subjects, 4 from WG and 6 from PJG) as low excreters and 21% (7 subjects, 1 from WG and 6 from PJG) as very low excreters.

Uro-A glucuronide was found in the plasma of four subjects from the WG and three subjects from the PJG (21% of total supplemented participants) all included in the category of high-urolithin excreters. The mean concentration of Uro-A-glucuronide measured was 0.11 ± 0.05 μM (range 0.05–0.2 μM). Other metabolites such as urolithin-C glucuronide (*m/z* 419 and MS-MS at *m/z* 243 and 175), urolithin-C methyl ether glucuronide (*m/z* 433, and MS-MS

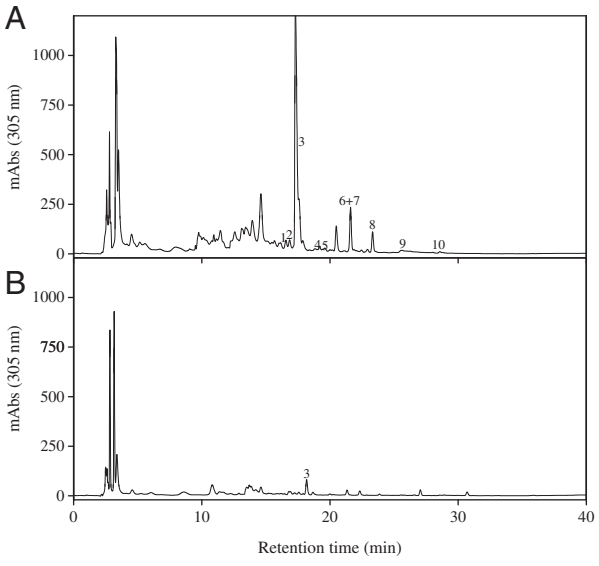

Figure 1. HPLC profile of urine from two patients after consumption of PJ (200 mL/day) for 3 days. (A) High-urolithin excreter. (1) Uro-A sulfoglucuronide (*m/z* at 483, MS-MS at *m/z* 403, 307 and 227), (2) urolithin C (*m/z* at 243), (3) Uro-A glucuronide (*m/z* at 403, MS-MS at *m/z* 227 and 175), (4 and 5) isomers of urolithin-D methyl ether glucuronide (*m/z* at 449, MS-MS at *m/z* 273, 258 and 175), (6+7) co-elution of Uro-B glucuronide (*m/z* at 387, MS-MS at *m/z* 211 and 175) and Uro-A sulfate (*m/z* at 307, MS-MS at *m/z* 227), (8) Uro-A (*m/z* at 227), (9) DMEA glucuronide (*m/z* at 505, MS-MS at *m/z* 329 and 175), (10) Uro-B (*m/z* at 211). (B) Low urolithin excreter, (3) Uro-A glucuronide.

at *m/z* 257, 243 and 175) and dimethyl ellagic acid (DMEA) glucuronide (*m/z* 505 and MS-MS at *m/z* at 329, 315 and 175) were only detected by extracting their corresponding ions and were not quantified.

3.3 Human prostates analyses

A very high inter-individual variability was observed between these samples and urolithin and/or EA derivatives were only detected and identified in eight prostate samples (24% of the supplemented patients), mostly belonging to the high excreters group (Table 1). Despite consuming less EA-related compounds, more metabolites were detected in the prostate samples from patients who consumed the walnuts than from those who consumed the PJ. Uro-A glucuronide was identified by its ion mass (*m/z* 403, MS-MS at *m/z* 227 and 175) (Fig. 2) as well as in the UV chromatograms (Fig. 3A) of six prostate samples (four samples from WG and two from PJG). The content of Uro-A glucuronide was in the range between 0.5 and 2 ng/g of tissue. Uro-B glucuronide was identified only in two samples by its ion mass (*m/z* 387, MS-MS at *m/z* 175) (Fig. 2) and it was not quantified. No other urolithin derivatives or aglycone urolithins were detected. After enzymatic treatment of the prostate samples with glucuronidase/sulfatase, we were not able to detect the aglycone urolithins although different enzymes concentration and incubation times (from 30 min to 8 h) were assayed. Since we were not able to recover the original glucuronides either and, given the small quantities of compounds handled, it is likely that degradation and (or) losses occurring during the incubation and processing of the samples may be the cause of these results. DMEA was clearly identified in four prostate samples from the high excreters (both consumers of walnuts or PJ; Table 1) but only by its ion MS (*m/z* 329, MS-MS at *m/z* 315 and 301) (Fig 2) and therefore was not quantified. In two prostate samples from the group of low excreters (one sample from the WG and another one from the PJG), only DMEA was identified (Fig. 2 and Table 1). DMEA was absent in all the 29 prostate control samples. In contrast to the urolithins, DMEA was detected as aglycone instead of the glucuronide derivative in plasma and urine. No metabolites were found in the prostates from the low excreters group (Table 1). No clear correlation was found between metabolite occurrence and histological type of prostate tissue (glandular parenchyma or stroma) or pathological status (PCa or BPH).

3.4 Animal study

In order to assess the effect of the fasting period before surgery on the detection of metabolites in the prostate, a parallel study was conducted in which the levels of metabolites detected in the prostate of rats fasted for 18 h were compared with those found in rats that had access to the diet until sacrifice. Uro-A glucuronide was the only metabolite detected in the prostates from rats that had been fed with the HPE (HED of 10 g/day) or with the high dose of Uro-A (HED of 1 g/day) and had not fasted before sacrifice (levels detected in tissue, 6–8.5 and 14–17 ng/g, respectively) (Figs. 3B and C). However, no metabolites were found in the prostate tissues from rats fed with the high doses of PE or Uro-A but that had been fasting for 18 h before sacrifice. Moreover, no metabolites were found in the prostates from rats fed with low doses of PE or Uro-A.

3.5 Gene expression analysis

The expression levels of *CDKN1A*, *MKi-67* and *c-Myc* were measured in prostate tissue specimens from all the patients included in the study. Besides the high inter-sample variability observed, the three genes were found to be expressed in all the tissues examined with *CDKN1A* being the most highly expressed gene, followed by *MKi-67* and *c-Myc* (Fig. 4). When the expression levels of each of the three genes were compared between the BPH and PCa samples, no statistically significant differences were observed in the transcript levels of *MKi-67*. However, *CDKN1A* was more expressed in the BPH samples than in the PCa specimens ($p<0.003$) (Fig. 5). The mean relative *CDKN1A* expression value was 1.51 (median 1.21) in the BPH group and 0.88 (median 0.91) in the PCa group. On the other hand, *c-Myc* showed a stronger signal ($p<0.003$) in the carcinoma samples (mean value: 3.06; median: 2.64) than

Table 1. Distribution of WG and PJG and occurrence of metabolites in the prostate

	High excreters			Low excreters			Very low excreters			Total
	Uro-A gluc	Uro-B gluc	DMEA	Uro-A gluc	Uro-B gluc	DMEA	Uro-A gluc	Uro-B gluc	DMEA	
WG	a,b,c,d	a	a,b,d	nd	nd	nd	nd	nd	g	5[a] (15%)[b]
PJG	e,f	e	e	nd	nd	nd	nd	nd	h	3[a] (9%)[b]
Total	6[a] (37.5%)[c]						–	2[a] (28.5%)[d]		8[a] (24%)[b]

nd, not detected.
a) Number of samples with the presence of metabolites. The letters indicate different prostate samples.
b) Percentage over the total of patients that consumed either walnuts or PJ.
c) Percentage over the number of high excreters.
d) Percentage over the very-low-excreters.

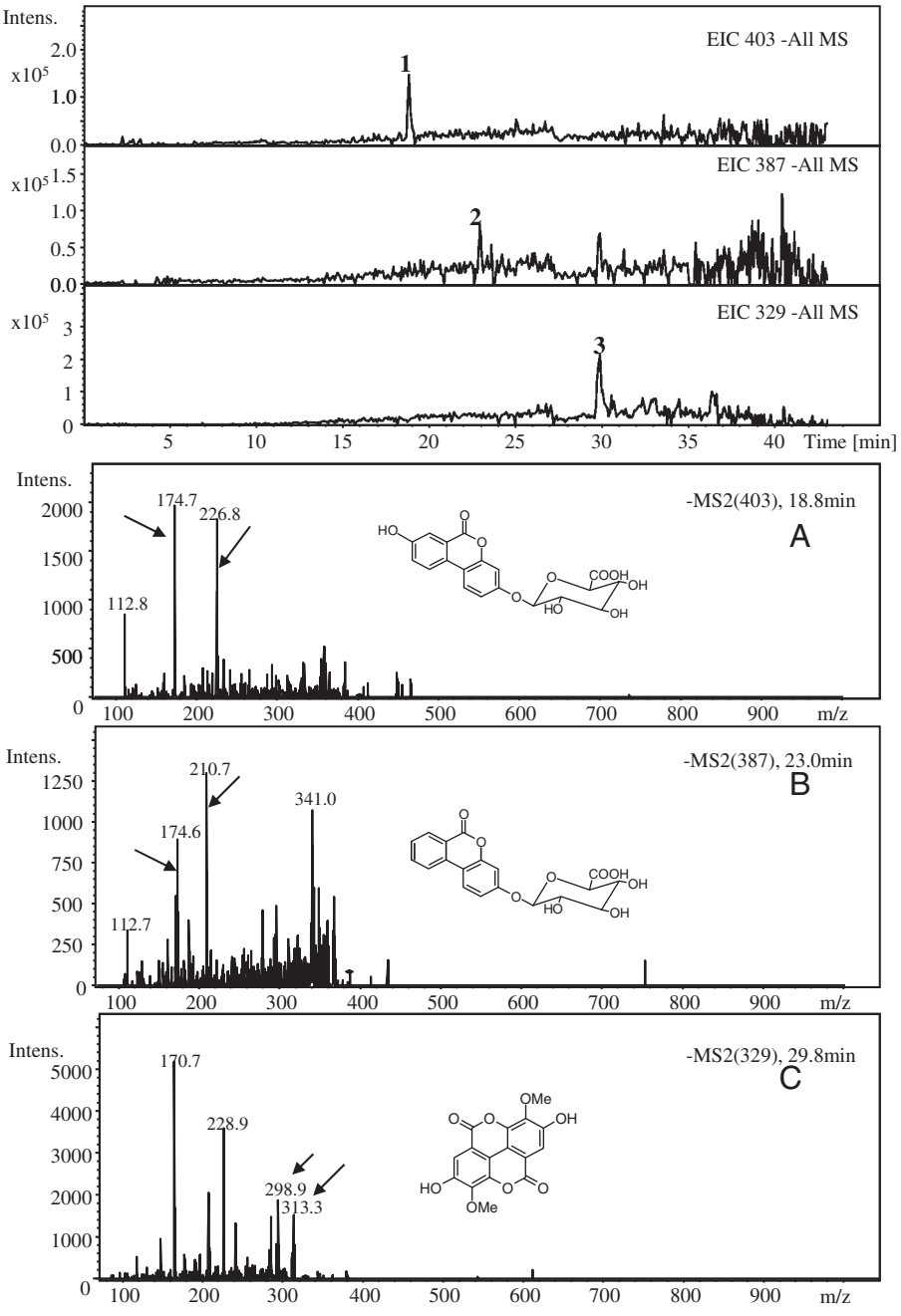

Figure 2. LC-MS/MS analysis of human prostate from a volunteer after consuming PJ (200 mL/day) for 3 days. (1) extracted ion chromatogram (EIC) at *m/z* 403 showing the metabolite Uro-A glucuronide, (2) EIC at *m/z* 387 showing the metabolite Uro-B glucuronide, (3) EIC at *m/z* 329 showing the metabolite DMEA. (A) MS/MS daughter spectra from the glucuronide *m/z* 403 showing the glucuronyl residue (*m/z* at 175) and the aglycone part (Uro-A) (*m/z* at 227). (B) MS/MS daughter spectra from the glucuronide *m/z* 387 showing the glucuronyl residue (*m/z* at 175) and the aglycone part (Uro-B) (*m/z* at 211). (C) MS/MS daughter spectra from dimethyl EA *m/z* 329 showing the fragments *m/z* at 313 and 299.

in the BPH specimens (mean value: 1.20; median: 1.01). Whether there were any significant differences in the transcript levels of the above genes between the two groups of patients, CG and PJG or WG, was subsequently investigated. Results (Fig. 6 and Table 2) indicated that there were not significant differences between these two groups for any of the genes studied. Since we found differences in the metabolic capacity of the volunteers to produce and eliminate the urolithins, we further compared the expression levels of these genes between the control biopsies and the samples obtained from volunteers classified as high excreters. Again, no significant differences were found between the two groups. We specifically looked at the expression levels of the three genes in those samples from patients in which metabolites had been clearly detected in the prostate tissue but no significant differences against controls were found.

4 Discussion

There is a large body of evidence that supports an important role of nutrition on the risk of developing PCa [5]. In the

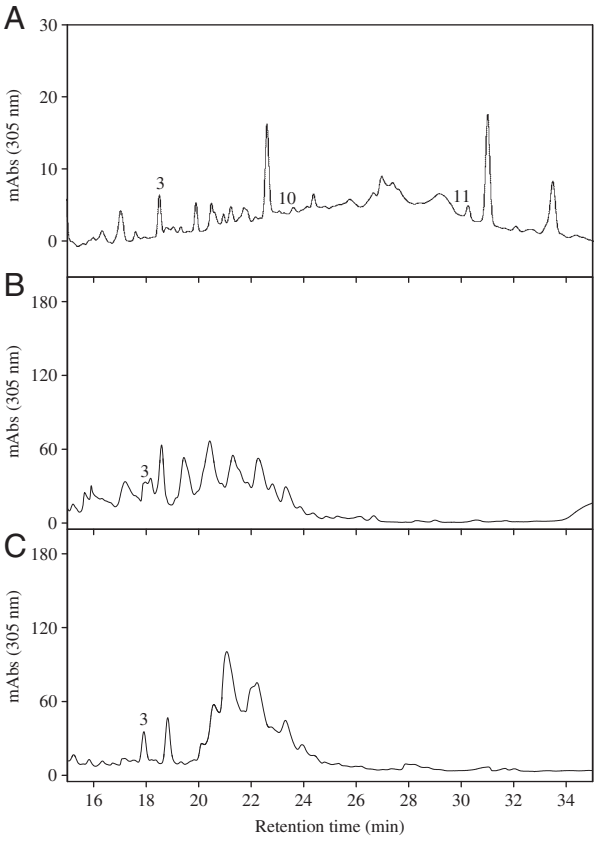

Figure 3. HPLC profiles of human (A) and rat (B, C) prostates. (A) Prostate from a patient after consuming walnuts (35 g/day) for 3 days. (3) Uro-A glucuronide, (10) Uro-B glucuronide, (11) DMEA. (B) Rat prostate from a rat fed with PE-supplemented diet (HPE of 10 g/day) for 3 days. The rat had free access to food until sacrifice. (3) Uro-A glucuronide. (C) Rat prostate from a rat fed with synthetic Uro-A-supplemented diet (high dose of 1 g/d) for 3 days. The rat had free access to food until sacrifice.

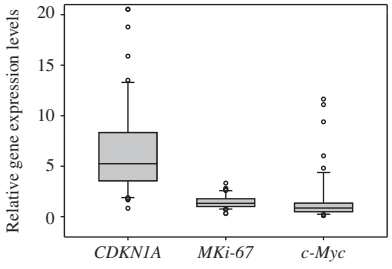

Figure 4. Box and whisker plots displaying the distribution of the relative expression levels for the proliferation-related markers: *CDKN1A*, *MKi-67* and *c-Myc*, in all the prostate tissue samples analyzed by real-time RT-PCR. The boxes indicate the area of 50% of the samples, the horizontal line in the boxes indicate the median. The whiskers display the range.

search for new dietary products for cancer chemoprevention, pomegranate and its derivatives are promising candidates [4]. PEs have been reported to suppress the

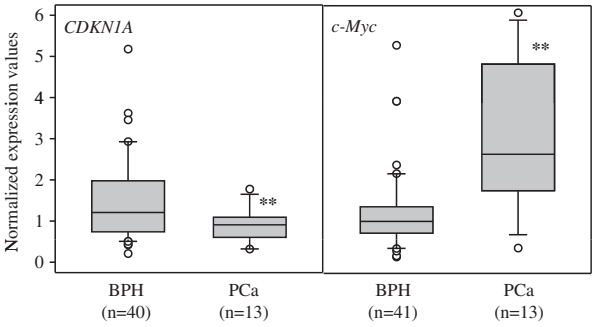

Figure 5. *CDKN1A* and *c-Myc* expression in BPH *versus* PCa samples analyzed by real-time RT-PCR. Values were normalized to *GAPDH* to yield relative units. Statistical differences were calculated by an unpaired *t*-test ($p < 0.01$, **).

proliferation, xenograft growth and invasion of human PCa cells [30]. The most striking evidence supporting the potential effect of pomegranate derivatives on PCa was provided by Pantuck *et al.* [14] who reported a prolongation of the PSA doubling time in patients with PCa upon consumption of PJ. Unfortunately, in this study, the authors did not try to establish a correlation between the observed effects and any of the components of the PJ or any metabolites determined in the urine of the volunteers. Despite the increasing evidence of the beneficial effects of pomegranate derivatives on PCa, no conclusive data have been reported regarding the compounds and (or) derived metabolites that may be the actual molecules responsible for these effects.

The most abundant polyphenols in PJ, ETs, have been suggested to be the main active fraction with effects against PCa [16]. If so, it is plausible that other ETs-rich foods should also be beneficial against PCa. However, Simon *et al.* [31] reported a lack of effect on serum PSA levels upon daily walnut intake (35 g) in healthy men for 6 months and Spaccarotella *et al.* [32] have also recently shown that the ratio of free PSA:total PSA was mildly increased, but not significantly, upon consumption of walnuts (75 g) for 2 months. The differences observed in the PSA outcome between consumption of PJ or walnuts may be caused by more than one reason, *i.e.*, (i) other non-ETs compounds present in the PJ, and not in the walnuts, are responsible for the observed effect on the PSA levels; (ii) the walnut studies were too short to produce a significant change and (iii) the walnut studies were carried out in healthy men whereas the pomegranate study was done with PCa patients. The aim of this study was to contribute to clarify whether the ETs are able to reach the human prostate gland, or if it is their hydrolysis product, EA, and (or) the gut microbiota EA-derived urolithins the actual molecules that can reach the prostate upon consumption of PJ. We also included in our study a second group of patients consuming walnuts to determine whether there were any differences against pomegranate consumers that may support the different reported effects on PSA levels. The detection of metabolites

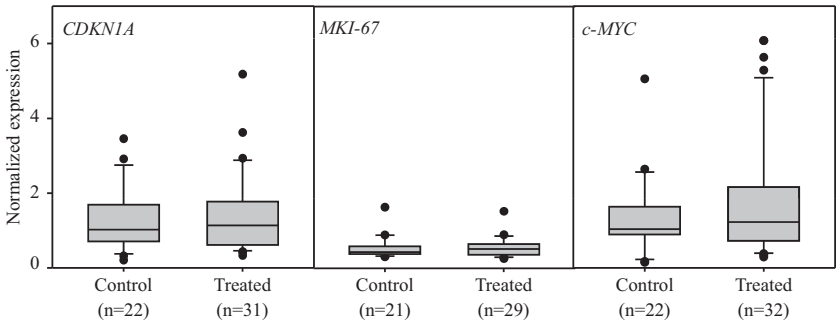

Figure 6. *CDKN1A, MKi-67* and *c-Myc* expression in prostate samples from control patients (CG) *versus* samples from volunteers supplemented with PJ or walnuts (PJG+WG) analyzed by real-time RT-PCR. Values were normalized to *GAPDH* to yield relative units.

Table 2. RT-PCR analysis of *CDKN1A, MKi-67* and *c-MYC* mRNA expression in prostate samples from control and patients that consumed PJ and walnuts

	CDKN1A	*MKi-67*	*c-MYC*
Control	1.28[a] ± 0.84 (0.21–3.46)[b]	0.55 ± 0.30 (0.31–1.64)	1.38 ± 1.03 (0.14–5.06)
PJG+WG	1.41 ± 1.07 (0.33–5.18)	0.55 ± 0.26 (0.26–1.52)	1.87 ± 1.63 (0.29–6.08)

a) The values represent the mean expression units of each gene normalized to GAPDH ± SD.
b) Range.

in the human prostate gland would also provide valuable information to design more relevant *in vitro* mechanistic studies.

Our study reports for the first time the occurrence of EA-derived metabolites in the human prostate gland upon consumption of ETs rich foods. The presence and quantity of metabolites in the prostate were independent of the intake of PJ or walnuts (Table 1) and the major metabolite detected was Uro-A glucuronide (Fig. 3). Besides the differences in the type and content of ETs, consumption of PJ and walnuts yielded mostly Uro-A metabolites as shown by the metabolites found in urine and plasma in agreement with the previous reports [19, 29]. Some of the detected metabolites in urine (Fig. 1) matched with those previously reported by us in different biological fluids and organs of pigs fed with acorn [18]. No ETs were detected in the prostate, plasma or urine samples from any of the volunteers which may be partially explained by the *in vivo* hydrolysis of ETs to EA and its further transformation by the gut microbiota to yield urolithins [17–19, 29]. Although the urolithins are bioavailable and can reach micromolar concentration in the bloodstream [29], the levels of these compounds found in the human prostates after consumption of pomegranate or walnuts were very low (in the range of ng/g). In a previous study, Seeram *et al.* [15] reported the presence of Uro-A derivatives in the mouse prostate upon a moderate oral administration of synthetic Uro-A (equivalent to a HED of 57 mg for a 70 kg-person). They found a peak of concentration after 4 h with a maximum total concentration of Uro-A conjugates of ~1000 ng/g but no metabolites were detected after 24 h. In addition, no EA or urolithin derivatives were detected in the prostate upon oral administration of PE at any of the time points examined. These results and ours are in agreement with an active enterohepatic circulation of these metabolites [18] which implies that urolithins are present in the systemic bloodstream for a short period of time and that they are excreted in urine for many hours [19, 29]. In this study, the analysis of metabolites in prostate was carried out at one-time point only, approximately 18–24 h after the last PJ or walnut intake, due to the required fasting period before surgery. This may explain, in part, that EA-derived metabolites, including the main metabolite urolithin-A glucuronide, were only detected in eight prostate samples (Table 1) and at very low concentrations. These prostate samples were all from volunteers in which EA-derived metabolites had also been detected in plasma, which suggests that these metabolites are not accumulated in the prostate but rather circulate with the bloodstream that irrigates the prostate (despite the poor irrigation of this gland). The results of the rat study confirmed that the absence or low levels of metabolites in most of the human prostates examined may be in part due to the 18 to 24 h fasting period. The occurrence of higher content of metabolites in the human prostate cannot be ruled out.

In an effort to investigate a potential effect in the prostate gland at the gene expression level derived from the intake of ETs-rich food, we measured and compared the mRNA quantities of *CDKN1A (p21), MKi-67* and *c-Myc* in prostate tissues from control and supplemented patients. The pre-selection of these cancer-related markers was based on their key role on cell cycle and cell proliferation regulation [25], the reported differential expression of these genes in BPH and in PCa [27, 28, 33–36] and the modulation of their expression in PCa cells after exposure to PE [13, 30]. In agreement with the previous reports [26, 35], our results show a statistically significant increase in the expression of *c-Myc* in the PCa samples over the BPH group. In contrast, we did not find differences in the levels of *MKi-67* between PCa and BPH specimens although *MKi-67* has been repeatedly shown to be more expressed in PCa [27, 28]. It has also been reported that immunoexpression of p21 was higher in BPH specimens than in normal prostates although not as elevated as in PCa

[36]. The role of p21 in PCa has not been yet clarified. *CDKN1A* increased expression has been associated with poor prognosis after prostatectomy and radiotherapy [37]. In contrast, the downmodulation of the tumor suppressors p21 and p27 produces a more aggressive PCa phenotype [38]. Our results show that p21 is significantly repressed in the PCa samples in comparison to the BPH samples. p21 induces cell cycle arrest and inhibits proliferation and a number of oncogenes such as *c-Myc* repress p21 to promote cell growth and tumorigenesis [39]. In this study, *c-Myc* is highly expressed in the PCa samples which may be associated with the lower expression of *CDKN1A* in the tumor tissues.

Our results also indicate that consumption of ETs-rich pomegranate or walnuts had no apparent effect on the expression of *p21*, *c-Myc* or *MKi-67* in the prostate gland, even if the patients were producing significant quantities of some metabolites and these were entering the prostate tissue. The absence of significant differences between the two groups may be influenced by the high variability between individuals. We fully recognize the difficulty to circumvent possible causes of bias, such as the heterogeneity of the prostate tissues obtained by the different procedures (transurethral resection or adenomectomy) or the fact that small specimens may not be representative of neighboring tissues. Where this study was implemented more accurate methods to obtain more homogenous cell populations, such as laser-microdissection, were not available. Other previous studies have shown the feasibility of RT-PCR transcript detection in prostatic tissues even without previous laser microdissection [40]. In an attempt to overcome some of the tissues heterogeneity, we carried out duplicate RT-PCR analysis in RNA extracted from two to four sub-samples of the same specimen and mean values were calculated. The intra-sample gene expression variability was found to be lower than 20% for most specimens and for the three genes studied. We believe our results are valid to the extent that mean values of several sub-samples from the same specimen may be able to detect twofold expression changes. Our results did not confirm previous *in vitro* results in which PEs have been shown to suppress the proliferation of several human PCa cell lines [13, 16, 30] and to cause molecular changes such as a twofold induction of *WAF1/p21* [13, 30] or the 0.6 downregulation of *c-Myc* [30]. However, these studies were all carried out with whole PEs at high concentrations and for long periods of continuous exposure (between 4 and 48 h) which are not representative of the physiological situation in which only very small quantities of certain metabolites have been detected in the prostate gland. In a recent work, the proliferation inhibiting properties of urolithin and its methyl derivatives were tested in several PCa cells [15]. Although this is a step in the right direction in the study of the bioactive properties of metabolites, the reported IC_{50}s are rather high (micromolar concentrations) compared with the expected *in vivo* tissue levels and the methyl derivatives of the urolithins have not so far been detected in the prostate gland.

Our results show that some conjugates of urolithins (glucuronides) and DMEA can reach and enter the human prostate gland upon consumption of ETs-rich sources such as PJ and walnuts. The low amounts of these metabolites detected (ng/g) in only some of the patients may have been in part caused by the required 18 h fasting period before surgery which allows for almost complete clearance of the compounds. It is, however, plausible that higher levels may be found short after consumption. No changes in the expression levels of several proliferation markers were associated to the consumption of the ETs-containing foods or to the presence of high quantities of derived metabolites in the urine. The results presented here corroborate the need to design better *in vitro* studies that should focus on the bioactivity of the actual *in vivo* metabolites formed upon consumption of ETs, Uro-A glucuronide and DMEA, at the very low concentrations (ppb) detected. Another important issue that needs to be clarified is the exposure time. These studies will help to determine the molecular mechanisms and gene targets involved in the cells response that will have to be then confirmed *in vivo*.

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