Gene expression changes in colon tissues from colorectal cancer patients following the intake of an ellagitannin-containing pomegranate extract: a randomized clinical trial

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Abstract

The clinical evidence of dietary polyphenols as colorectal cancer (CRC) chemopreventive compounds is very weak. Verification in humans of tissue-specific molecular regulation by the intake of polyphenols requires complex clinical trials that allow for the procurement of sufficient pre- and postsupplementation tissue samples. Ellagitannins (ETs), ellagic acid (EA) and their gut microbiota-derived metabolites, the urolithins, modify gene expression in colon normal and cancer cultured cells. We conducted here the first clinical trial with 35 CRC patients daily supplemented with 900 mg of an ET-containing pomegranate extract (PE) and evaluated the expression of various CRC-related genes in normal and cancerous colon tissues before (biopsies) and after (surgical specimens) 5–35 days of supplementation. Tissues were also obtained from 10 control patients (no supplementation) that confirmed a large, gene- and tissue-specific interindividual variability and impact of the experimental protocol on gene expression, with some genes induced (*MYC, CD44, CDKN1A, CTNNB1*), some repressed (*CASP3*) and others not affected (*KRAS*). Despite these issues, the consumption of the PE was significantly associated with a counterbalance effect in the expression of *CD44, CTNNB1, CDKN1A, EGFR* and *TYMs*, suggesting that the intake of this PE modulated the impact of the protocol on gene expression in a gene- and tissue-specific manner. These effects were not associated with the individuals' capacity to produce specific urolithins (*i.e.*, metabotypes) or the levels of urolithins and EA in the colon tissues and did not reproduce *in vitro* effects evidencing the difficulty of demonstrating *in vivo* the *in vitro* results.
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1. Introduction

Numerous preclinical *in vitro* and animal studies have given evidence of the regulation of cell processes by polyphenols *via* (epi)genetic changes as a general mechanism of action underlying the health benefits attributed to these compounds [1]. Demonstration in humans of these changes remains essential to better comprehend the link between polyphenols intake and health effects, but, in this

area, clinical trials are limited and show weak evidence of the effects of these compounds on molecular expression in human tissues. Various reasons are responsible for this: (a) the high interindividual variability in the human capacity to metabolize and absorb the compounds as well as in the ability to respond to these compounds and (or) their metabolites [2] and (b) difficulties in obtaining sufficient number of participants and high-quality samples.

Ellagitannins (ETs) and ellagic acid (EA) are phenolic compounds abundant in foods such as pomegranate, walnuts or berries. The metabolic conversion of these compounds by the gut microbiota to form some intermediate urolithins (Uro) (Uro-M5, Uro-M6,Uro-M7, Uro-D, Uro-C) and the main final metabolites Uro-A, IsoUro-A and Uro-B in animals and humans has been thoroughly reported [3–6]. Considerably high concentrations of these urolithins can be found in the feces (mM range) and in the colon tissues (µM range) of volunteers that consumed pomegranate extracts (PEs) and/or walnuts [7,8]. It is also known now that the capacity of producing urolithins varies between individuals depending on their microbiota composition, and thus, humans can be classified into different metabolizing phenotypes (metabotypes): (a) metabotype A that produces Uro-A, (b) metabotype B that produces IsoUro-A and/or Uro-B in addition to Uro-A and (c) metabotype 0 or nonproducers [7,9]. A recent and interesting appreciation is that the distribution of these metabotypes appears to be related to the health status. Along these lines, the urolithin metabotype B has been reported to be more abundant in persons with gut dysbiosis such as obese subjects, individuals with metabolic syndrome or colorectal cancer (CRC) patients [8,9].

Urolithins, the main colon microbial metabolites derived from the polyphenols ETs, have gained a lot of interest for their potential health benefits. Among these, the potentiality of these molecules to reduce or prevent CRC development has been repeatedly evidenced in cultured colon cancer cells [10]. Each of the main urolithins, EA and mixtures of these metabolites (reproducing the concentrations found in the colon tissues from individuals with metabotypes A or B) is able to inhibit the growth of these cells and interfere with cell cycle and apoptotic death mechanisms. These phenotypic responses are accompanied by substantial molecular changes in several key cancer deregulated genes (e.g., CDKN1A, MYC, TP53) and microRNAs (miRs; miR-224, miR-215) [11,12]. Also, there were marked differences in the response between normal and cancerous cells, changes commonly observed in all the cells investigated (e.g., induction of CDKN1A) with independence of the metabolite tested, and metabolite- and cell-specific changes [12]. These in vitro results need to be verified in humans, and thus, clinical studies with appropriate designs to investigate tissue molecular changes must be implemented [13]. We conducted the first clinical trial in CRC patients where we provided evidence of the in vivo availability and concentrations of urolithins in the colon following a short-term supplementation with ET-containing PEs [8] and also reported some specific and significant changes in various miRs in the human colon normal and malignant tissues after the intake of the PE [14].

Using the same colon samples obtained from those CRC patients, the aim of the present research was to test whether it was also possible to detect significant differential expression for various specific genes in colon human tissues in response to the supplementation with a PE. The selection of these genes was based on: (a) genes that are key hallmarks for cancer development (e.g., APC, CTNNB1, KRAS, EGFR, TP53, MYC, BIRC5) [15] and (b) previous in vitro studies have shown changes in these genes in colon normal and cancer cultured cells exposed to the urolithins and EA (e.g., CDKN1A, ABCG2, CASP3) [12]. The partial objectives of this research were (a) to examine the interindividual variability in the expression levels of the selected molecular markers in the human normal and cancerous colon tissues, (b) to evaluate the impact of the experimental protocol on the expression levels of these markers and (c) to try to discriminate specific changes in gene expression in the human colon tissues that

might be attributed to the consumption of the PE and (or) the presence of urolithins in the colon tissues.

2. Materials and methods

2.1. Pomegranate extracts

PEs (PE-1 and PE-2) were kindly supplied by Laboratorios Admira S.L. (Alcantarilla, Murcia, Spain). The composition of PE-1 and PE-2 has been previously reported in detail [8] and differs primarily in their ET (punicalagin)/EA ratio: (a) PE-1 (low ET/EA ratio) contains 72 mg/g punicalagin, 2 mg/g punicalin and 294 mg/g EA derivatives, and (b) PE-2 (high ET/EA ratio) contains 155 mg/g punicalagin, 5.4 mg/g punicalin and 28 mg/g EA derivatives.

2.2. Study design and intervention

This randomized controlled trial (Clinicaltrials.gov, NCT01916239, 30 July 2013) was designed to investigate the potential effects of a supplementation with ET-containing PE in the expression of several genes in the colon tissues obtained from CRC patients. The study was approved by the Spanish National Research Council's Bioethics Committee (Madrid, Spain) and by the Clinical Ethics Committee at Reina Sofia University Hospital (Murcia, Spain) (reference 03/2011) and conducted in accordance with the guidelines in the Declaration of Helsinki and its amendments. Details of the trial can be found in previous reports [8,14]. Briefly, eligible patients with programmed colonoscopy (n=2501) gave their written informed consent prior to participating. Inclusion criteria were as follows: age over 18 years and confirmed CRC diagnosis with resectable tumor by programmed surgery, World Health Organization performance status between 0 and 3, alanine aminotransferase >2.5-fold, hemoglobin >10 g/dl, creatinine <140 mM and serum bilirubin >1.5-fold above the normal values range. Exclusion criteria were as follows: patients that did not satisfy the inclusion criteria and (or), treatment with chemotherapy or radiotherapy 1 month prior to recruitment, alcoholism, active peptic ulcer, pregnancy/breastfeeding, habitual intake of food supplements and treatment with steroids or other anti-inflammatory drugs 1 week prior to recruitment.

Fifty-seven patients were recruited and several initial (baseline) endoscopic colon biopsies taken: two samples from normal tissue (normal biopsy, Nb) and four samples from malignant tissues (malignant biopsy, Mb). The samples were rapidly stabilized in RNAlater reagent (Qiagen, Madrid, Spain) and stored at −80°C until use. Patients were then randomly allocated into three groups: a control group (n=14) that did not consume any PE and two groups that consumed two capsules (2×450=900 mg) daily of the PE-1 (n=22) or the PE-2 (n=21). The supplementation period lasted from the initial colonoscopy until the programmed surgery and was on average 13.6±7.5 days (range: 5–35 days). The last dose was consumed approximately 10–12 h before surgery. It is important to note that, during the supplementation period, none of the participants was taking any anticancer drug or treatment. At surgery, normal (Ns, ~10 cm adjacent to the tumor) and malignant (Ms) tissues were taken approximately 2 h after initiating the surgical resection. These colon tissue specimens were rapidly taken to the Anatomical Pathology Service. Portions of both types of tissue were cut out, immersed in RNAlater stabilization reagent and stored at −80°C until use. A total of 45 patients completed the trial: control group (n=10), PE-1 group (n=19) and PE-2 (n=16).

2.3. RNA extraction and cDNA preparation

All colon tissue samples (0.02 g obtained from a pool of different pieces of each sample) were homogenized with lysis buffer (Qiagen) using an IKA T10 Ultra-Turrax equipment (Janke and Kunkel, Ika-Labortechnick, Germany) at 24,000 rpm at 4 C for 1 minute. Total RNA extraction and quality assessment were performed as reported elsewhere [14]. Only samples with a ratio Abs_{260}/Abs_{280} between 1.8 and 2.1 and RNA Integrity Number values above 6.0 that indicate acceptable RNA integrity for reverse transcriptase polymerase chain reaction (RT-PCR) assays [16] were used for gene expression analysis. cDNA was obtained from RNA following reverse transcription according to the manufacturer's instructions (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, CA, USA) and quantified using the Nanodrop spectrophotometer system (ND-1000 3.3 Nanodrop Technologies, USA).

2.4. Gene expression analyses: RT-PCR arrays

Gene expression levels were evaluated using TaqMan Array 96-Well Plates (Applied Biosystems, ABI, CA, USA) designed to analyze 12 genes. A total quantity of 100 ng of cDNA obtained from each treatment was added to each well of the TaqMan Array Plate together with the standard master mix (Applied Biosystems, CA, USA) to a final volume of 20 µl, and the quantitative PCRs were run on the 7500 RT-PCR System (Applied Biosystems) following manufacturer's conditions. These plates contained gene-specific primers (ABI) for four generally accepted reference genes: 18S (Eukaryotic 18S rRNA, Hs99999901_s1), GAPDH (Glyceraldehyde 3-phosphate dehydrogenase, Hs99999905_m1), HPRT1 (Hypoxanthine phosphoribosyltransferase 1, Hs99999909_m1) and GUSB (Glucuronidase, beta, Hs99999908_m1). HPRT1, GUSB and GAPDH were considered suitable reference genes for gene expression normalization in tumor and normal tissue from colon cancer patients based on geNorm and/or

Table 1
Demographic, clinical and tumor features of the CRC patients included in the study [a]

Patients		PE group ($n=35$)		Control group ($n=10$)
		PE-1 [b] group ($n=19$)	PE-2 [c] group ($n=16$)	
Mean age and range		75.2±10.8 (51–89)	75.2±9.3 (53–88)	71.3±12.9 (51–92)
Mean BMI [d] (kg/m^2) and range		28±5 (21–40)	30±4 (24–42)	31±8 (23–45)
Females		9	7	7
WHO status (0/1/2/3)		7/7/3/2	6/6/4/0	2/3/3/2
Hypertension		10	8	7
Dyslipidemia		7	8	6
Diabetics		5	6	4
Metabotype [e] (A/B/0)		8/10/1	8/4/4	–
Tumor				
Location	Cecum	2	1	1
	Ascending	6	1	1
	Hepatic flexure/transverse colon	1	2	1
	Sigmoid colon	5	8	2
	Rectum/sigmoid	5	4	5
Histology	Differentiation, high/moderate/poor	1/16/2	1/12/3	1/9/0
Classification (TNM)	T1/T2/T3/T4	0/4/10/5	3/5/7/1	0/2/8/0
	N0/N1/N2/N3	11/6/2/0	12/2/2/0	7/2/1/0
	M0/M1	19/0	15/1	10/0

[a] Adapted from Núñez-Sánchez et al. [14].
[b] Low ET/EA ratio.
[c] High ET/EA ratio.
[d] Obesity: BMI ≥30 kg/m^2; overweight: 25 kg/m^2 < BMI <30 kg/m^2; normoweight: BMI ≤25 kg/m^2.
[e] Adapted from Núñez-Sánchez et al. [8].

NormFinder stability analyses [17–19]. We tested the stability (M) in our colon tissue samples using the reference gene stability analysis software NormFinder [20]. *GAPDH* exhibited the lowest M value (<0.15) and was selected for normalization of gene expression data. The 12 genes included in this study and some of their main biological functions in relation with cancer are listed in Supplementary Table S1. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method, and values were multiplied by $\times 10^4$ [21]. Fold-change (FC) values ≥1.2 and ≤−1.2 were considered indicative of up-regulation and down-regulation, respectively. A percentage value ≥50% of individuals in the sample population exhibiting up- or down-regulation was considered indicative of a tendency.

2.5. MYC immunohistochemical analyzes

Formalin-fixed, paraffin-embedded samples of Mb, Ns and Ms tissues were supplied by the Anatomical Pathology Service of the Hospital Reina Sofia (Murcia, Spain). Paraffin sections (5 μm) were deparaffinized in xylene substitute and rehydrated in ethanol. MYC protein expression was determined immunohistochemically using a Cell and Tissue Staining Kit (R&D Systems, Abingdon, UK) following manufacturer's recommendations. This system ensures high detection sensitivity by using high-quality anti-mouse secondary biotinylated antibodies and high-sensitivity streptavidin conjugated to horseradish peroxidase. Two sections of each sample were processed following the protocol, and whereas one section was incubated with 7.5 μg/ml of anti-human c-MYC antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), the other section was incubated with buffer only and used as a null control to discard nonspecific staining of the secondary reagents. The samples were next hematoxylin-stained and dehydrated in ethanol and cleared in xylene substitute prior to mounting the samples. Mounted samples were stored and protected from light until image analysis. Pictures were taken using a Leica DM6000B microscope (optical magnification 20×) connected to a Leica DFC280 camera and using the Leica Application Suite V2.5.0 acquisition software (Leica Microsystems Ltd., Barcelona, Spain). Representative photographs of MYC protein expression levels in immunohistochemically stained colon Ns and Ms tissues from control patients and from those that consumed the PE are shown in Supplementary Fig. S1A–D. Reactivity was measured by image analysis using a Leica QWin image analysis (Leica Microsystems Ltd., Barcelona, Spain). MYC protein levels were calculated using stained area and staining intensity values averaged from 30 fields per sample covering different representative tissue areas (crypts, muscle and connective tissue).

2.6. Statistical analysis

Plots of the gene expression data were performed using Sigma Plot 12.5 (Systat Software, San Jose, CA, USA), and statistical analyses were performed using the SPSS Software, version 21.0 (SPSS Inc., Chicago, IL, USA). All the data were included in the analysis. The variability of gene expression was measured by the coefficient of variation (CV) expressed as %. Chi-square test was used for baseline comparison of categorical variables. The results of the Shapiro–Wilk test indicated that the gene expression values did not follow a normal distribution, and thus, the FC values between groups are indicated by the median. Analyses for paired and unpaired data comparisons were performed using the nonparametric Wilcoxon signed rank and the Mann–Whitney U methods, respectively. To adjust for within-patient variation and interpatient differences, age, gender, urolithin metabotype, body mass index (BMI) and tumor location as well as TNM and differentiation degree of tumors were included as covariates in the statistical analysis for each gene analyzed. Potential associations between gene expression and metabotypes or metabolites levels in the same colon tissues were examined using the Spearman's rank correlation coefficient. The accepted level of statistical significance was $P<.05$. Other P values are also indicated for comparison.

3. Results

3.1. Characteristics of CRC patients and tumor features

The main demographic and clinical characteristics of the CRC patients included in this study are shown in Table 1. Most patients ($n=30$) were obese–overweight ($n=17$ obese with BMI ≥30 kg/m^2 and $n=13$ overweight with 25 kg/m^2 < BMI<30 kg/m^2), whereas 15 patients were normoweight (BMI ≤25 kg/m^2). Patients' ages ranged from 51 to 92 years old. Most of them were either asymptomatic ($n=15$) or only limited for heavy physical activity ($n=16$) according to WHO status that scores the general well-being and capability of patients for making normal activities of daily life. Individuals were categorized into metabotype A, B or 0 based on the production of urolithins [8].

Patients' tumors mainly presented moderate differentiation, *i.e.*, from 50% to 95% tumor was gland forming. The predominant tumor stage was IIA (T3N0M0) according to TNM classification (equivalent to Dukes' stage B; *i.e., muscularis propria* is invaded by the tumor, but there is no regional lymph node or distant metastasis). No patient reported side effects (dyspepsia, allergic reactions, *etc.*) upon consumption of either PE-1 or PE-2. In addition, no clinically relevant effects were detected in the serobiochemical variables related to kidney and liver functions (results not shown). The duration time of the surgical interventions including anesthesia ranged from 60 to 360 min (mean value: 189±59 min) and depended on tumor localization, type of surgery, previous surgery in the patient and BMI (longer in obese patients). Under the conditions of our study, we did not find significant correlation between colon tissue gene expression data and

any of the following variables: tumor differentiation, tumor location or TMN classification, PE intervention time, sex, age or BMI.

3.2. Gene expression variability in the human colon tissue samples

We first examined the interindividual variability of the expression levels for each gene in the four types of colon tissue samples collected: normal biopsies (Nb), malignant biopsies (Mb), normal surgical tissues (Ns) and malignant surgical tissues (Ms) (results are shown as CV% in Supplementary Table S2). In the Nb, the average CV was ~50%. The least variable genes were *CASP3* and *CDKN1A* (CV ~31%), whereas *MYC* and *ABCG2* exhibited the highest variability (~60% and 70%, respectively). In general, surgery was associated with an increase in the variability of gene expression (Ns, CV: ~75%; Ms, CV: ~118%), which was also higher in the malignant tissues (Mb, CV: ~96% and Ms, CV: ~120%) than in the normal ones. Overall, the variability of gene expression in these samples is high but markedly differs from one gene to another. As an example, the interindividual variability in the

expression levels of *ABGC2* and *CASP3* is shown in Supplementary Figs. S2 and S3, respectively.

We additionally compared gene variability and protein variability in these tissues for one of these markers, the oncoprotein MYC. Generally, the variability of the immunohistochemically determined MYC levels was lower than that of the gene expression levels (Ns, CV: 43.4% *vs.* 84.8%; Ms, CV: 39.9% *vs.* 62.8%; Mb, CV: 43.5% *vs.* 48.9%, protein *vs.* gene expression, respectively). For this molecule, protein variability was not so much increased in the malignant tissues as compared with the normal ones (CV: 39.9% *vs.* 43.4% for Ns and Ms samples, respectively) or by the experimental protocol (CV: 43.5% *vs.* 39.9% for Mb and Ms samples, respectively).

3.3. Gene expression data presentation and validation of the study

The results of the analysis of gene expression differences between the various tissues examined are listed in Supplementary Tables S3–S5. Gene expression data are presented as the % of individuals

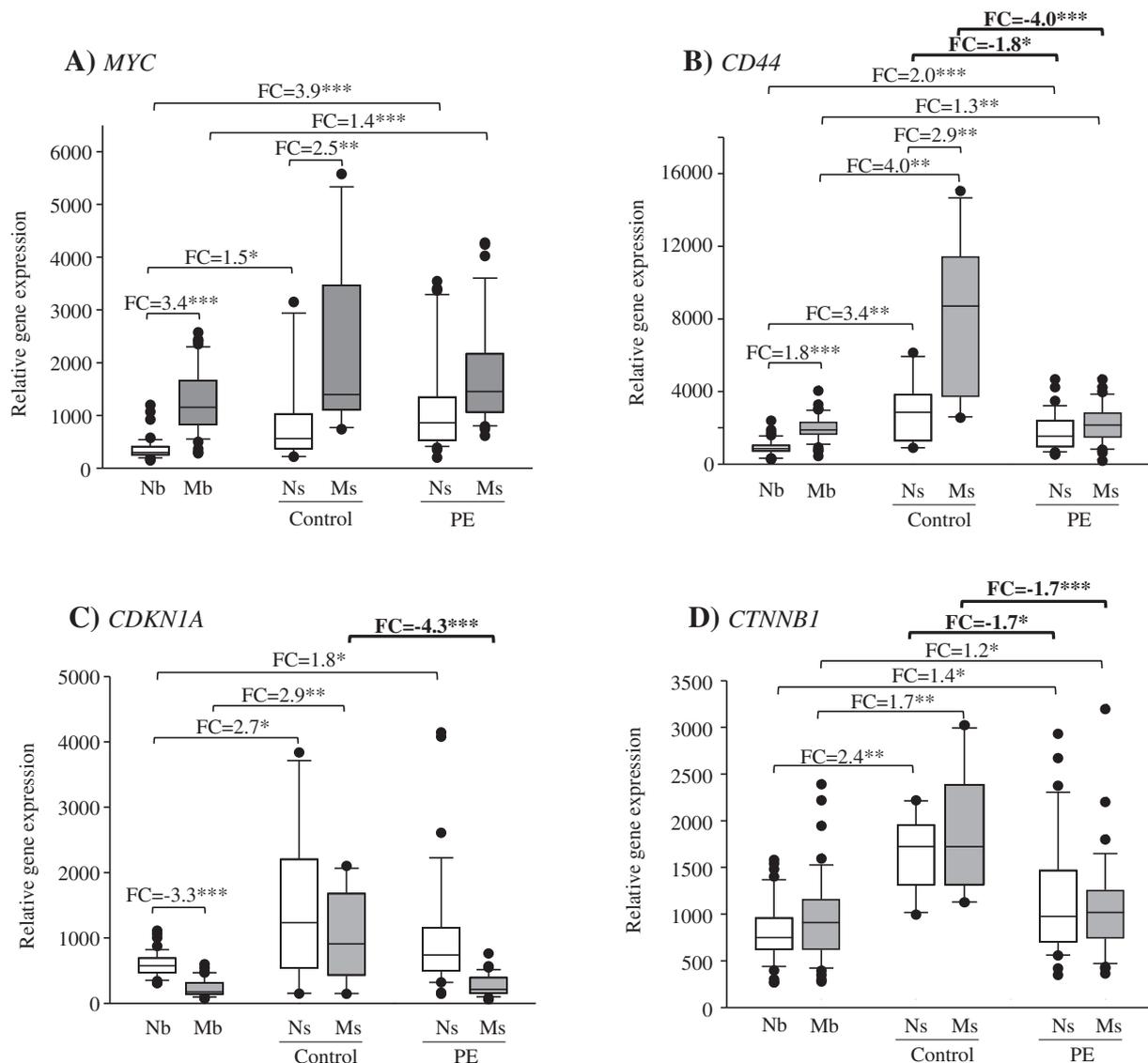Fig. 1. Relative gene expression levels for (A) *MYC*, (B) *CD44*, (C) *CDKN1A* and (D) *CTNNB1* in Nb and Mb and in Ns and Ms specimens. Box plots indicate the median, the 25th/75th percentiles and the SD. Only significant FC differences between tissue samples and between CRC patients that consumed the PE and the CRC control group that did not consume the PE are indicated. *P<.05, **P<.01, ***P<.001.
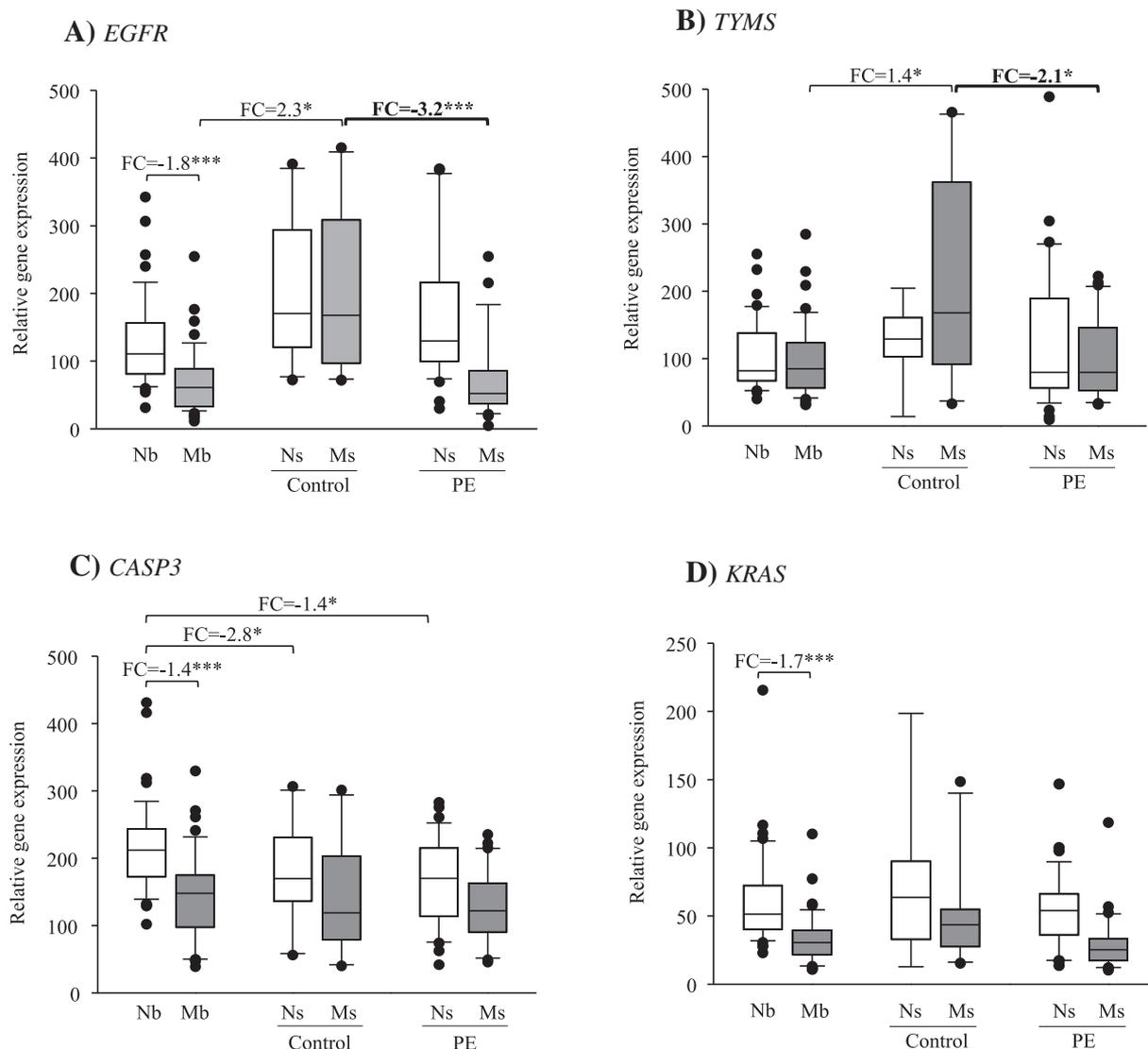
Fig. 2. Relative gene expression levels for (A) *EGFR*, (B) *TYMS*, (C) *CASP3* and (D) *KRAS* in Nb and Mb and in Ns and Ms specimens. Box plots indicate the median, the 25th/75th percentiles and the SD. Only significant FC differences between tissue samples and between CRC patients that consumed the PE and the CRC control group that did not consume the PE are indicated. *P<.05, **P<.01, ***P<.001.

exhibiting a change, the magnitude of the change (FC median and range) and the significance of these changes (P values) [14]. To illustrate and compare the most relevant and representative responses in the different tissues examined, Fig. 1A–D displays the expression changes for *MYC*, *CD44*, *CDKN1A* and *CTNNB1* and Fig. 2A–D for *EGFR*, *TYMS*, *CASP3* and *KRAS*.

Because of the high variability of the gene expression data and as a means to validate our study, we evaluated gene expression differences between malignant and normal tissues for several genes that have been confirmed to be overexpressed or down-regulated in CRC [22–27]. The results are included in Supplementary Table S3. Our data supported that *MYC* (Fig. 1A), *CD44* (Fig. 1B) and *BIRC5* were mostly overexpressed in Mb vs. Nb (>85% of the subjects exhibited higher levels of these transcripts in the malignant tissues) and that *CDKN1A* (Fig. 1C), *ABCG2* and *APC* were predominantly repressed in Mb vs. Nb (>70% of the participants exhibited down-regulation) with very significant (P<.001) FC values (3.9, 1.8, 2.1 and −22.3, −3.3, −1.8, respectively). These differences were also shown when we compared Ms vs. Ns from the control patients, although they were only significant in the case of *MYC* (Fig. 1A), *CD44* (Fig. 1B) and *ABCG2*.

Interindividual variation comparing gene and protein differential expression (FC values) between Ms and Ns samples is additionally shown in Supplementary Fig. S4. Of the total individuals examined, 40% exhibited a concordant overexpression of the gene and protein in the malignant tissue. Overall, there was a good agreement between the MYC protein and gene differences between Ms and Ns specimens, confirming the overexpression of this molecule in the malignant colon tissues (Supplementary Table S6).

3.4. Gene expression changes attributable to the experimental procedure

We next investigated those changes in gene expression that might be a consequence of the experimental protocol (surgery and tissue handling procedures). For this purpose, we compared gene levels between surgical specimens (postsurgery) and initial biopsies (presurgery) in both the malignant and normal tissues obtained from control patients that did not consume any of the PEs (n=10) (Supplementary Table S4). The genes most largely and significantly up-regulated by the protocol in both the normal and malignant tissues were *CD44*, *CDKN1A* and *CTNNB1* (80%–90% patients exhibited up-

regulation; FC: 2.0–4.0; Fig. 1B–D). *MYC* was also moderately up-regulated following surgery and sample handling (60%–70% individuals; FC: 1.5 in the normal tissues and 1.3 in the malignant tissues; Fig. 1A). MYC up-regulation was also confirmed at the protein level in the malignant tissues (FC=1.5; Supplementary Table S4).

Other genes such as *EGFR* (Fig. 2A), *TYMS* (Fig. 2B) and *TP53* resulted significantly up-regulated after surgery only in the malignant tissues (>60% individuals; FC: 1.4 to 2.3, *P*<.05), whereas *CASP3* (Fig. 2D) was significantly down-regulated only in the normal tissue (60% individuals; FC: −2.8, *P*<.05). The rest of the examined genes exhibited consistent but no significant changes or were not apparently altered by the experimental protocol as in the case of *KRAS* (Fig. 2D).

3.5. Gene expression changes in human colon samples following the consumption of PE

A preliminary analysis (data not shown) revealed that there were no significant differences between the patients that consumed the PE-1 extract and those that consumed the PE-2 extract, and thus, the effects of the intake of one or another PE (*n*=35) are presented jointly. Results of the comparison between the surgical tissue (post-PE intake) and the biopsy (previous to PE intake) for each gene are included in Supplementary Table S5. To discriminate changes that might be attributed to the consumption of the PE, we focused on those genes that responded substantially different in the group that consumed the PE in comparison with the control group (no supplementation). The consumption of the PE for several days appeared to counterbalance the changes observed in the control group for some of the investigated genes. This effect could be clearly and significantly distinguished in both the malignant and normal tissues for *CD44* (Fig. 1B) and *CTNNB1* (Fig. 1D) or only in the malignant tissue for *CDKN1A* (Fig. 1C), *EGFR* (Fig. 2A) and *TYMs* (Fig. 2A). Other genes like *MYC* (Fig. 1A), *CASP3* (Fig. 2C) and *KRAS* (Fig. 2D) displayed similar results in the PE group to those in the control group, indicating that these genes were not seemingly modified following the intake of PE. We additionally explored potential associations between the expression changes of those genes exhibiting a change and the individuals' metabotypes (A, B or 0) or the quantity of each of the urolithins detected in the colon tissues. However, we did not find any significant correlation.

4. Discussion

In vitro and animal studies investigating the effects of plant bioactive compounds and, in particular, of polyphenols on gene expression are numerous and have been used to identify potential molecular targets and mechanisms underlying the health benefits of these compounds. The translation of these preclinical results to human evidences requires the implementation of complex clinical trials that are usually hampered by the safety and ethical restrictions that limit tissue sample accessibility [13]. To date, very few trials have investigated and reported significant associations between the dietary intake of bioactive compounds and specific gene expression changes in human tissues and even less in human colon tissues. Early studies looking at the expression of *MYC* and *GJA1* (*Connexin 43*) in colonic tissues following the consumption of β-carotene showed that expression changes in these tissues were variable and could also be detected in the placebo group, evidencing the difficulty in detecting significant changes associated with the intake of the compound and (or) the appearance of metabolites in the serum or tissues, especially when comparing groups of small sample size (*n*=7–8 placebo, *n*= 5–10 β-carotene) [28,29]. Nevertheless, a number of genes have been reported to be significantly regulated in normal colonic mucosa following the intake of folic acid in comparison with a placebo group even though the group sizes were very small (*n*=8 placebo, *n*=6 folic acid) [30]. Importantly, in a pilot clinical trial looking at the effects of

the intake of resveratrol or of a grape extract on colon mucosa gene expression, it was shown that the effects on the expression of specific genes could be different in normal and in cancer tissues [31]. Taking into consideration all these issues, the present randomized controlled clinical trial was conceived to try to demonstrate for the first time expression changes in specific CRC genes attributable to the intake of an ET-containing PE extract or to the presence of the derived ET metabolites in the colon tissues. For this purpose, we measured and compared the expression levels of several cancer-related genes in both normal and cancerous colon biopsies (baseline) and surgical samples (postsupplementation) in CRC patients from a control group (*n*=10) and a PE-supplemented group (*n*=35).

Our study corroborated a large interindividual variability and impact of the experimental protocol on the expression of the genes investigated. Gene expression variation is inherent to genes and is influenced by many factors. Often, the most highly variable genes are associated with diseases and cellular responses. Variability is additionally increased by tissue heterogeneity and by the sampling protocols [32]. Regarding sample procurement, it has been shown that surgery (clamping-associated ischemia, anesthetics) and postsurgery (temperature, handling time) factors can induce or repress the expression of oncogenes, transcription factors and signaling molecules involved in cancer processes in normal and CRC tissues [33,34]. Although biopsies (usually obtained by less drastic procedures than surgical specimens) should be the preferred tissue samples in clinical trials looking at gene expression, it is not always possible to obtain a sufficient number of biopsies from pre- and postintervention time points. This is even more complicated when working with patients diagnosed with CRC due to ethical restrictions and little margin for an intervention with a food or dietary product in the absence of other treatments. The gap between the initial diagnostic colonoscopy and the scheduled surgery, as implemented in our study, represented a unique opportunity to investigate the potential effects of the consumption of the PE extract on gene expression in colon tissues before any cancer treatment is applied. To obtain more reliable gene expression data, we made an effort to standardize the procedure and to shorten the handling time of the tissue samples [34] to less than 30 min. Further, we specifically examined the gene expression variability and its alteration by the experimental protocol in our sample population. Analysis of the tissues obtained from the control patients confirmed that the experimental procedure affected the variability and the expression of some of the investigated genes in the normal and (or) in the malignant tissues. As an example, the variability and expression of *MYC* were enhanced in the normal (Ns) and malignant (Ms) surgical tissues as compared to the initial biopsies. However, the variability and (or) expression levels of other genes (*CASP3*, *KRAS*) were not modified, indicating that the experimental protocol does not affect gene expression globally and equally but that the effect is gene specific. We additionally show that the effects of the experimental protocol can be extended to the protein levels since the oncoprotein MYC was also significantly induced in the control patients.

As a means to show the validity of our experimental design, we tried to confirm in our sample population the differential expression in malignant *vs.* normal colon tissues for some specific molecules that have been previously documented, *i.e.*, the down-regulation of *CDKN1A* [22], *ABCG2* [23] and *APC* [24] or the induction of *MYC* [25], *CD44* [26] and *BIRC5* [27]. Comparison between Mb and Nb and between Ms and Ns specimens in our sample population confirmed tissue differences for these markers, suggesting that our study was sufficiently adequate despite the effects of the experimental procedure, the interindividual variability and the sample size.

We then investigated whether we were able to discriminate significant changes in specific genes occurring in the patients that consumed the PE for several days before their programmed surgery. We found that the intake of the PE extract could be associated with a

significant counteracting effect to that caused by the protocol in the expression of some genes in the normal and malignant tissues (*CD44, CTNNB1*) (Fig. 1B and D) or in the malignant tissues (*CDKN1A, EGFR, TYMs*) (Figs. 1C and 2A and B). These results suggested a potential gene- and tissue-specific influence of the PE that was evidenced by a down-regulating or preventing effect promoted by the PE intake over the change caused by the experimental protocol. One of the purposes of this clinical study was to see whether it was possible to reproduce some of the gene expression changes caused by the urolithins *in vitro*. Our results did not reflect any of the changes reported in colon cultured cells exposed to either mixed or single ET metabolites. For example, it appears that colon cancer cultured cells commonly up-regulate the expression of *CDKN1A* in response to the presence of urolithins and EA, either mixed or individually [11,12,35], as opposed to the down-regulating or counteracting effects associated to the PE intake in this clinical study. The lack of concordance between *in vitro* and *in vivo* results has been broadly debated and may be attributed both to the problems intrinsic to the human studies as well as to the limitations of the cultured cell models [13]. One additional consideration may be that the CRC cell lines generally used in *in vitro* studies derive from different individual cancer patients, and thus, the results obtained with each one cannot represent the results obtained *in vivo* from a heterogeneous sample population. Indeed, different cultured cell models also exhibit different responses [12]. We were not able to find any significant association between the expression changes observed in the human tissues in *CD44*, *CTNNB1*, *CDKN1A* or *TYMS* and the corresponding individuals metabotypes (A, B or 0) or the quantity of urolithins detected in the tissues, confirming the difficulty in linking *in vivo* gene expression effects with the presence of specific metabolites. We may hypothesize that the *in vivo* observed effects can be caused by other components present in the PE, or synergistic effects between the urolithins and other compounds or *via* secondary changes associated with modulatory effects on the microbiota composition [36]. Further, the presence in the human colon of a thick mucus gel layer may also represent a critical difference with the common cancer cultured cells models. This layer protects the epithelial cells from toxic insults in the luminal environment and plays an essential role in the interaction–communication between the microbiota and the host. Comparative studies between cell models with and without the mucus layer show critical differences in the response of the gut epithelial cells to the presence of bacteria or xenobiotic compounds. Like so, it has been evidenced that *Salmonella* exhibits higher adhesion and invasion capacity against HT29-MTX-E12 mucus-producing cells than against the classic HT29 or Caco-2 cell models [37]. Also, D'Agostino et al. [38] demonstrated that tea epigallocatechin gallate was more toxic to normal HT29 cells than to HT29-MTX-E12 mucus-producing cells. Future *in vitro* research on colon gene expression regulation by dietary compounds should be implemented with models that reproduce this essential feature of the intestinal barrier. Our results support the need to further pursue the research to understand and validate gene expression responses to dietary compounds both *in vitro* and *in vivo*.

This is the first study to report *in vivo* colon tissue gene expression changes attributable the intake of an ET-containing PE. Despite our efforts to increment the sample size (a total of 45 CRC patients involved), we acknowledge that the number of volunteers is still limited to overcome the variability of gene expression and to draw more conclusive results. Nevertheless, we have evaluated and reported the interindividual variability in the expression levels of each of the investigated genes, a type of data not commonly reported but essential to understand the complexity of gene expression responses and to establish a not-yet-defined adequate "*n*" size for this type of trials. Gene expression studies have long been and continue to be a means of investigating the molecular mechanisms of action underlying the health effects of bioactive compounds, although

mostly in *in vitro* studies. Because of the variability inherent to gene expression, the rapidity of the transcription process and the modest correlation between mRNA and protein levels [39], it may be advisable to move the focus of these studies to the protein level, even more in the case of *in vivo* studies. Indeed, we have shown here that the variability of the MYC protein is lower and less affected by the protocol than that of the transcript, but more studies looking at the variability of gene and protein tissue expression should be pursued.

5. Concluding remarks

In this clinical study, we report for the first time colon tissue gene expression changes influenced by the oral intake of an ET-containing PE. We also confirm that interindividual variability and experimental procedures can largely and specifically influence gene expression in the human tissues, and thus, the inclusion of a control group is crucial to discern between changes caused by the intervention and those attributable to the protocol. Further, the observed *in vivo* gene expression changes did not reflect previously reported *in vitro* results and were not significantly associated with the individuals' metabotypes or with the presence of specific metabolites in the colon tissues. We postulate that increasing the sample size might provide further and more powerful evidence of the effects of the intake of this product on colon tissue gene expression changes and will potentiate the research linking *in vivo* and *in vitro* results. Nevertheless, we should develop further the study of protein(s) as targets that respond to the intake of bioactive compounds.

Chemical compounds

Urolithin A (PubChem CID: 5488186); Urolithin B (PubChem CID: 5380406); Urolithin C (PubChem CID: 60198001); Urolithin D (PubChem CID: 5482042); Urolithin M6 (PubChem CID: 101461104); Ellagic acid (PubChem CID: 5281855); Punicalagin (PubChem CID: 44584733); Punicalin (PubChem CID: 5388496).

Authors' contribution

J.C.E. designed the study; M.A.N.S. and A.G.S. performed the gene expression analyses; M.A.N.S. and M.T.G.C. analyzed the data; T.M.S., N.V.G.T., M.B.G.S. and C.S.A. recruited and coordinated CRC patients; A.M.G.A. and F.J.R.G. collected biopsy samples; M.R.M. collected surgical samples; F.A.P.Q. and F.M.D. carried out the histopathological analyses; F.A.T.B., R.G.V., A.G.S., J.C.E. and M.T.G.C. contributed to the discussion of the manuscript; J.C.E. and M.T.G.C. wrote the manuscript. All authors have read and approved the final manuscript.

Conflicts of interests

None of the authors have any competing interests in the manuscript.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jnutbio.2017.01.014.

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