RESEARCH ARTICLE

Breast Cancer

Molecular Nutrition & Food Research

www.mnf-journal.com

Metabolic Profiling of Dietary Polyphenols and Methylxanthines in Normal and Malignant Mammary Tissues from Breast Cancer Patients

*María Ángeles Ávila-Gálvez, Rocío García-Villalba, Francisco Martínez-Díaz, Beatriz Ocaña-Castillo, Tamara Monedero-Saiz, Adela Torrecillas-Sánchez, Beatriz Abellán, Antonio González-Sarrías, and Juan Carlos Espín**

Scope: Dietary polyphenols may protect against breast cancer. However, it is unknown whether polyphenols reach human malignant breast tumors in molecular forms and(or) at concentrations likely to act against cancer.

Methods and Results: Ninteen breast cancer patients consumed three capsules daily from biopsy-confirmed diagnosis to surgery (6 ± 2 days). The capsules contained pomegranate, orange, lemon, olive, cocoa, and grapeseed extracts plus resveratrol, providing 37 different phenolics (473.7 mg), theobromine and caffeine (19.7 mg). A total of 101 metabolites are identified in urine, 69 in plasma, 39 in normal (NT), and 33 in malignant (MT) tissues by UPLC-ESI-QTOF-MS. Eight control patients did not consume extracts. Phenolic-derived metabolites in MT and NT are mainly glucuronidated and(or) sulfated. Some representative metabolites detected in MT (median and range, pmol g^{-1}) are urolithin-A-3-*O*-glucuronide (26.2; 3.2—66.5), 2,5-dihydroxybenzoic acid (40.2; 27.7—52.2), resveratrol-3-*O*-sulfate (86.4; 7.8—224.4), dihydroresveratrol-3-*O*-glucuronide (109.9; 10.3—229.4), and theobromine (715.0; 153.9—3,216). Metabolites, as detected in breast tissues, do not exert antiproliferative or estrogenic/antiestrogenic activities in MCF-7 breast cancer cells.

Conclusion: This is the first study that describes the metabolic profiling of dietary phenolics and methylxanthines in MT and NT comprehensively. Although phase-II conjugation might hamper a direct anticancer activity, long-term tumor-senescent chemoprevention cannot be discarded.

1. Introduction

Breast cancer is the most commonly diagnosed cancer in women worldwide and the second leading cause of cancer death. Overall, one in eight women will be diagnosed with breast cancer in their lifetime and prevention through the improvement of lifestyle, including dietary habits, remains as the first-choice strategy.[1,2]

Fruits, vegetables, and derivatives such as tea, olive oil, coffee, and cocoa contain phytochemicals (phenolics, carotenoids, glucosinolates, and others) with potential cancer chemopreventive activity.[3,4] Among them, (poly)phenolic compounds show a broad spectrum of activities (antioxidant, antiinflammatory, cardioprotective, anticancer, etc.).[4,5] There is abundant research in animal models that highlights a significant role of polyphenols and polyphenol-rich plant extracts in the prevention of breast cancer, including resveratrol,[6] ellagic acid,[7] berries,[8,9] pomegranate,[10] and citrus flavanones.[11] However, this evidence remains elusive in humans. The recent Continuous Update Project (CUP) reported the only limited association between breast cancer risk and the intake of fruits, vegetables, tea and coffee, and some phytochemicals such as isoflavones and carotenoids.[12] To date, observational studies provide results suggestive of protection only for vegetable consumption against estrogen receptor-negative (ER-) breast cancer risk, a subtype of cancer more abundant in premenopausal women.[13–15] The explanation could lie in an inaccurate estimation of individuals' exposure to the polyphenol metabolome.[16]

M. Á. Ávila-Gálvez, Dr. R. García-Villalba, T. Monedero-Saiz, Dr. A. González-Sarrías, Prof. J. C. Espín
Laboratory of Food & Health
Research Group on Quality, Safety and Bioactivity of Plant Foods, CEBAS-CSIC
Campus de Espinardo
30100 Murcia, Spain
E-mail: jcespin@cebas.csic.es

Dr. F. Martínez-Díaz, B. Ocaña-Castillo
Reina Sofía University Hospital
Service of Anatomical Pathology
Avda. Intendente Jorge Palacios s/n, 30003 Murcia, Spain

A. Torrecillas-Sánchez
Reina Sofía University Hospital
Service of Breast Unit
Avda. Intendente Jorge Palacios s/n, 30003 Murcia, Spain

Dr. B. Abellán
Reina Sofía University Hospital
Service of Surgery
Avda. Intendente Jorge Palacios s/n, 30003 Murcia, Spain

DOI: 10.1002/mnfr.201801239

Methylxanthines (MX), mainly caffeine, theophylline, and theobromine are alkaloids consumed worldwide through the intake of coffee, cocoa, tea, cola drinks, and yerba mate. Years ago there was concern about coffee consumption (also for isoflavones) and the risk of breast cancer. However, no consistent observations currently support this risk.[12,17] Nowadays, the role of MX as psychostimulants is well-known,[18] although other, somewhat indirect, health benefits have also been proposed such as enhancers of the effects of cocoa on cardiovascular function through the increase of flavanols' bioavailability.[19]

Overall, it is not clear yet whether dietary phytochemicals, including polyphenols and MX, can exert a direct effect on malignant breast tissues (MT). To date, a comprehensive disposition and metabolic profiling of a wide range of dietary polyphenols and MX in MT from breast cancer patients have not yet been described. To the best of our knowledge, the evidence is limited to soy isoflavones[20] and hop prenylflavonoids[21] in normal breast tissues (NT) and epigallocatechin gallate (EGCG) in breast cancer patients.[22] Therefore, we assessed here the disposition and metabolic profiling of 37 different dietary phenolics, theobromine, and caffeine in NT and MT from newly diagnosed breast cancer patients. We also explored whether the molecular forms and concentrations of metabolites that could reach tumor tissues exert antiproliferative, estrogenic, and(or) antiestrogenic activities in the MCF-7 cells model.

2. Experimental Section

2.1. Chemicals and Polyphenol-Rich Supplement

Standards of phenolic-derived metabolites (PM) and MX were obtained as detailed in Supporting Information.

The polyphenol-rich supplement used in this study was conceived by the principal investigator of this research and consisted of a blend of resveratrol and commercially available dietary plant extracts (orange, lemon, pomegranate, cocoa, olive, and grape seed). The formulation of this supplement tried to cover a broad range of phenolics present in the diet as well as in commercial dietary supplements. Both the design of the study and formulation were not planned to evaluate specific health effects on the patients but to increase the current knowledge about the metabolism and disposition of phenolic compounds and MX in breast cancer tissues. The components were blended and encapsulated in hard gelatin capsules by Laboratorios Admira S.L. (Alcantarilla, Murcia, Spain). Each capsule (700 mg of blend) contained 53.85 mg trans-resveratrol, 161.5 mg pomegranate extract (rich in punicalagin and ellagic acid), 53.85 mg orange extract (very rich in hesperidin), 53.85 mg lemon extract (rich in eriocitrin), 161.5 mg olive extract (rich in hydroxytyrosol), 161.5 mg cocoa extract (containing procyanidins and rich in theobromine), and 53.85 mg grape seed extract (very rich in procyanidins). The capsules were manufactured, tested, and checked following the standards of the European Union's good manufacturing practices requirements. As determined by HPLC-ESI-IT-MS/MS analyses, each capsule contained 37 different phenolic compounds with a mean (±SD) of 158.0 ± 13.5 mg plus theobromine (6.41 ± 0.19 mg) and caffeine (0.16 ± 0.01 mg) (Table S1, Supporting Information).

2.2. Patients and Study Design

This study was a randomized dietary intervention that followed the ethical guidelines outlined in the Helsinki Declaration of 1975 and its amendments. The safety issues and protocol were addressed and approved (reference 29/03/2017) by the Clinical Ethics Committee at Reina Sofia University Hospital (Murcia, Spain) and by the Spanish National Research Council's Bioethics Committee (Madrid, Spain). The trial was registered at clinicaltrials.gov as NCT03482401 and was conducted in Murcia (Spain) between June and December 2017.

The study was fully explained to the patients who gave their written informed consent before participating. Eligible patients were those over 18 years with newly biopsy-confirmed breast cancer, programmed surgery at least 3 days after the recruitment, and no neoadjuvant treatment. Patients with suspected intolerance to any component of fruits or vegetables were excluded.

The design of the study and the flow of participants are shown in **Figure 1**. All the participants were asked to record their diet for 3 days before the surgery, especially when consuming fruits, vegetables, cocoa, coffee, tea, and olive oil. From the eligible patients interviewed ($n = 45$), 28 patients (27 women and one man) signed the written consent. Patients were informed that this intervention was not intended to evaluate cancer markers and thus, they were told that some of them would consume capsules and others would participate as a control group following a random sequence. Patients that signed the written consent were sequentially cited by the surgeons who randomly allocated the patients in the polyphenol group that consumed the capsules from diagnosis to surgery and in the control group who did not consume any capsules. No placebo was included in the design of the study since the trial was not designed to evaluate changes in specific clinical variables. Besides, there was not enough available evidence to accurately power (minimum sample size) this type of studies (i.e., tissue disposition of dietary polyphenols). To increase the sample size in the polyphenol group, every three patients, two of them were randomly allocated to the polyphenol group and one to the control group. The primary outcome was to evaluate the disposition and metabolic profiling of a broad spectrum of dietary phenolic compounds and MX in malignant mammary tissues. Secondary outcomes were to characterize the metabolic profiling in normal breast tissues, plasma, and urine of breast cancer patients as well as to evaluate in vitro, in the MCF-7 cells model, the antiproliferative, cytotoxic, estrogenic, and antiestrogenic effects of a mixture of those metabolites that could reach the malignant breast tissues.

Urine and blood samples were obtained in the morning, just before anesthesia on the day of the surgery. As the pooling of 24 h-volume urine was not possible, creatinine was measured for normalizing concentration of urine samples. Venous blood was collected in heparinized vacutainers and the corresponding plasma was obtained. Urine and plasma samples were kept at -80 °C until analysis. At resection surgery, a sample of malignant tissue as well as normal tissue adjacent to the tumor was taken in each patient. Rapidly, breast tissues were taken to the Anatomical Pathology Service for their examination and classification. A portion of glandular tissue (malignant and normal) as well as normal adipose tissue, whenever possible, were provided for their

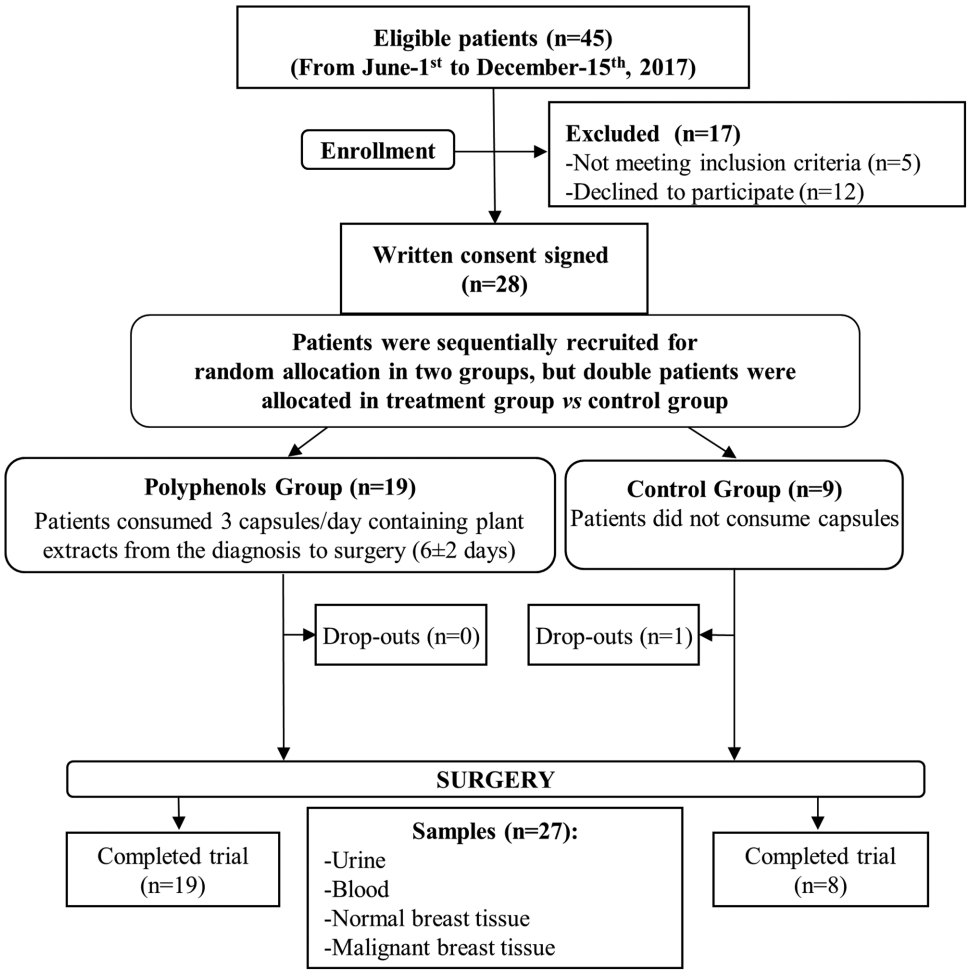

Figure 1. The flow of patients through the trial (CONSORT diagram).

analysis in this study. Tissue samples were snap frozen in liquid nitrogen and stored at −80 °C until further analysis.

2.3. Dosage Information

Patients were randomly allocated to consume (polyphenol group, $n = 19$) three capsules daily from biopsy-confirmed diagnosis to surgery (6 ± 2 days) or not consume any capsule (control group, $n = 8$). The capsules contained pomegranate, orange, lemon, olive, cocoa, and grape seed extracts plus resveratrol, providing daily 37 different phenolics (473.7 mg) plus theobromine and caffeine (19.7 mg).

The patients were instructed to consume the capsules daily from the confirmed cancer diagnosis to the day of the surgery (i.e., presurgery period). In this period, capsules could be taken at the same time or distributed throughout the day. The only specific instruction was given for the day before the surgery where the patients should consume the three capsules at night, before bedtime. In this trial, it was not allowed to provide the capsules in the morning, before surgery (no enough previous evidence to demonstrate the lack of potential interactions with anesthesia). Therefore, patients followed a mandatory fast before surgery, which resulted in a median interval of 12 h between the last intake of capsules and surgical resection of the tumor.

2.4. Analytic Methods

2.4.1. Phenolics and Methylxanthines in the Dietary Supplement

The supplement was analyzed by HPLC-ESI-IT-MS/MS to evaluate the content of phenolic compounds and MX. Detailed information is provided in Supporting Information.

2.4.2. Phenolics and Methylxanthines, and Their Derivatives, in Urine, Plasma, and Breast Tissues

Urine samples were centrifuged, filtered through a 0.22 µm polyvinylidene fluoride (PVDF) filter and diluted with acidified water (0.1% formic acid) before analysis by UPLC-ESI-QTOF-MS. The urinary excretion of creatinine was measured to allow standardization of diuresis as reported elsewhere.[20]

Plasma samples (200 µL) were extracted with 600 µL acetonitrile:formic acid (98:2, v/v), centrifuged, and the supernatant reduced to dryness in a speed vacuum concentrator. The evaporated samples were re-suspended in 100 µL of methanol (MeOH), filtered through a 0.22 µm PVDF filter, and injected in the UPLC-ESI-QTOF-MS equipment.

Normal (NT) and malignant (MT) glandular breast tissue samples were cut and 250 mg were weighed. Samples were extensively washed with PBS to remove traces of blood and other possible contaminants. Samples tissues were homogenized with 1.25 mL of MeOH:HCl (99.9:0.1 v/v) in a Bullet Blender Homogenizer (Next Advance, Averill Park, NY, USA) for 5 min. Some MT samples needed a second cycle of 5 min for complete disaggregation. Stainless steel beads (3 and 2 tablespoons containing 0.9–2 and 3.2 mm beads, respectively) were used for breast tissue homogenization. Then, samples were sonicated in an ultrasonic bath for 10 min and centrifuged at 10 000 × *g* for 5 min at 4 °C. Each supernatant was evaporated in a speed vacuum, resuspended in 200 µL of MeOH, and filtered through a 0.22 µm PVDF filter before analysis by UPLC-ESI-QTOF-MS. Adipose tissues were available only in three patients from the polyphenol group due to the majority of conservative surgeries that were approached. Adipose tissues were processed as described for glandular tissues except for a first homogenization with 0.4 mL hexane. After centrifugation, the supernatant was discarded and the pellet was then homogenized in the MeOH:HCl solution as described above. Two internal standards, 0.2 ppm dihydroxycoumarin and 0.1 ppm chrysin were added to each sample before and after sample extraction. Glandular NT and MT samples were also hydrolyzed using β-glucuronidase and sulfatase as detailed in Supporting Information.

2.5. UPLC-ESI-QTOF-MS Analyses of Plasma, Urine, and Breast Tissues

Analyses were performed on an Agilent 1290 Infinity UPLC system coupled to a 6550 Accurate-Mass quadrupole-time-of-flight (QTOF) mass spectrometer (Agilent Technologies, Waldbronn, Germany) using an electrospray interface (Jet Stream Technology). A previously validated method for the analysis of PM in colon tissues was used.[23] The injection volume was 3 µL for plasma and urine samples and 5 µL for breast tissue samples. Spectra were acquired in the m/z range from 100 to 1100, in negative and positive polarity mode and an acquisition rate of 1.5 spectra per second. Data were processed using the Mass Hunter Qualitative Analysis software (version B.06.00, Agilent). A target screening strategy was applied for the qualitative analysis of possible metabolites that could be present after consumption of the capsules containing the mixture of extracts. More than 180 possible compounds were browsed in the different samples. These compounds included parent phenolic compounds present in the extract as well as derived metabolites both unconjugated and conjugated (glucuronides, sulfates, sulfoglucuronides, etc.). The exact mass of the proposed compound was extracted using an extraction window of 0.01 m/z. Only compounds present in at least 50% of the patients and with clear differences respect to the control were considered. Besides, targeted MS/MS analysis provided additional information to achieve a reliable compound identification. MS/MS product ion spectra were collected at m/z 50–800 range using retention time window of 1 min, collision energy of 20 V, and acquisition rate of 4 spectra per second.

From all the compounds identified in breast tissues, those with available standards were quantified in all samples (plasma, urine, and breast tissues) by peak area integration of their extracted ion chromatograms (EICs). PM and MX were quantified in negative and positive mode, respectively. The method was validated for linearity, precision, accuracy, limit of detection (LOD) and limit of quantification (LOQ) as well as for matrix effects and the values for the breast tissue are shown in Tables S2 and S3, Supporting Information. Calibration curves of PM were linear in the range LOQ-5 µM and for MX in the range LOQ-20 µM with significant correlation coefficient $r^2 \geq 0.997$. Matrix effect in the tissues was calculated by comparing the peak area of each compound (0.5 µmol L^{-1}) in MeOH and each matrix. In general, no or low (<20%) matrix effect was observed. Quantification was done by interpolation in the calibration curve prepared in the corresponding matrix. Recovery of metabolites was calculated in triplicate using control samples spiked with a mixture of standards at 0.5 µmol L^{-1}. A recovery yield between 43 ± 3% and 85 ± 4% was obtained for PM (Table S2, Supporting Information) and between 81 ± 8% and 108 ± 26% for MX (Table S3, Supporting Information). LODs and LOQs were obtained by injecting successively diluted standard solutions. LOD and LOQ were calculated by following the criterion of signal-to-noise ratio (S/N) of 3 for the LOD and of 10 for the LOQ. LODs ranged from 0.7 to 17 nmol L^{-1} for PM and from 5 to 10 nmol L^{-1} for MX. LOQs ranged from 2 to 59 nmol L^{-1} for PM and from 17 to 35 nmol L^{-1} for MX. Precision was evaluated from measurements of each sample three times on the same day (intra-day) and in three different days (inter-day). The precision expressed as the relative standard deviation (% RSD) of peak areas was in all cases <3% for intra-day assay and <15% for inter-day assay (Tables S2 and S3, Supporting Information). Hydrolyzed breast tissues were also analysed as described above. LODs and LOQs for unconjugated metabolites are detailed in Supporting Information.

2.6. Cell Culture Conditions and Treatments

MCF-7 human ER-positive (ER+) breast adenocarcinoma cells, expressing both α- and β-ERs, were obtained from the American Type Culture Collection (Rockville, USA). Cells were maintained at 37 °C in a humidified atmosphere (95%) with 5% v/v CO_2 and using Eagle's minimal essential medium (EMEM) containing 10% fetal bovine serum (FBS), 1.5 g L^{-1} sodium bicarbonate, and 2 mmol L^{-1} L-glutamine, and supplemented with 0.1 mmol L^{-1} nonessential amino acids, 1 mmol L^{-1} sodium pyruvate, and 0.01 mg mL^{-1} bovine insulin (Gibco, Invitrogen S.A., Barcelona, Spain). Test samples were solubilized in DMSO (<0.5% in the culture medium) and filtered (0.22 µm) before addition to the culture media. Control cells were also run in parallel and subjected to the same changes in medium with DMSO. For the determination of estrogenic and antiestrogenic activities, the medium consisted of a phenol red-free EMEM with the same supplements and containing 5% dextran coated charcoal-FBS

treated to remove steroids. The antiproliferative and estrogenic/antiestrogenic activities of representative mixtures of metabolites that reached the breast tissues were evaluated. Mixtures were assayed at a final concentration similar to that detected in breast tissues, and also at 10 and 50 μmol L^{-1}. Cell proliferation was measured by the XTT method.[24] Cells were seeded in 96-well plates at a density of 5000 cells per well and were maintained at appropriate culture conditions for 48 h before each treatment. Final results in treated cells were expressed as the percentage of those values obtained for control cells. The analyses of cell proliferation were performed at least in triplicate ($n = 12$ wells per experiment).

2.7. Determination of Estrogenic and Antiestrogenic Activities by the E-Screen Assay

Estrogenic-like and antiestrogenic-like effects of metabolite mixtures were performed using the E-screen assay as described by Payne et al.[25] with some modifications. Briefly, cells were seeded at 1500 cells per well in 96 well-plates. Cells were washed with PBS after 48 h, replaced with the estrogen-free medium, and preincubated for 3 days before either treatment. Afterward, representative mixtures were incubated at 10 and 50 μmol L^{-1} in the absence or presence of 17β-estradiol (1 nmol L^{-1}) for 7 days. Treatments were refreshed after 72 h and cell proliferation was measured using the XTT assay. Estrogenic activity was measured as the induction-fold of proliferation in metabolites-treated cells versus the nontreated (control) cells, whereas antiestrogenic activity was measured as the induction-fold of proliferation in metabolites-treated cells versus the nontreated (control) cells, in the presence of 17β-estradiol (1 nmol L^{-1}). Data were presented as the mean ± standard deviation (SD) of three separate experiments ($n = 12$ wells per experiment).

2.8. Statistical Analysis

Statistical analyses were carried out using the SPSS Software, version 23.0 (SPSS Inc., Chicago, IL, USA). Data were expressed as means ± SD. The empirical distribution of data with the normality assumption was tested with the Shapiro–Wilk test. Intrasubject comparisons were evaluated using the Wilcoxon Signed Rank Test to detect significant differences in the concentration for each metabolite in NT versus MT. In the case of cell cultures, the effects of treatments versus controls were analyzed by the analysis of variance (ANOVA). Plots of data were performed using Sigma Plot 13.0 (Systat Software, San Jose, CA, USA). Statistical significance was set at $^{*}p < 0.05$. A trend toward significance was acknowledged when $0.1 > {}^{\#}p > 0.05$.

3. Results

3.1. Study Population

Eligible patients ($n = 45$) were contacted when the breast cancer was biopsy-confirmed, and the surgery programmed, which explains the short interval time between diagnosis and surgery (6 ± 2 days). Twenty-eight breast cancer patients were recruited, 27 completed the dietary intervention trial (26 women and one man) and one patient from the control group revoked her written consent (Figure 1). The polyphenol-rich supplement was well tolerated, and no adverse effects such as intolerance, dyspepsia, or allergic reactions were reported. There were also no effects on serobiochemical variables such as renal and hepatic enzymes, etc. (results not shown). After surgery, patients from the polyphenol group confirmed the full compliance of the trial and no remaining capsules were returned. The study was stopped in December because enough samples had been obtained to reach the primary objective.

The average participating patient was a 56 years old menopausal woman, nonsmoker, with overweight and family history of cancer (69%) (**Table 1**). All patients (from both groups) were prescribed a benzodiazepine drug the night before the surgery, and just before the intervention they also consumed another benzodiazepine. The most frequently associated chronic pathologies in the population study were anxiety (41% under chronic treatment with benzodiazepine-derived drugs) and high arterial blood pressure (33% under treatment with antihypertensive drugs).

Sentinel lymph node biopsy was carried out in all the patients who were treated with the most conservative type of intervention, generally tumorectomy (59%). Most of the tumors (69%) were infiltrating ductal carcinomas, 96% of tumors were ER+, 77% were PgR+ (progesterone-receptor-positive), and only two patients showed HER2+ tumors (human epidermal receptor type 2-positive) who showed ER+ and PgR+. No patient showed HER2+, ER- and PgR-tumors (Table 1). Mean percentage of the proliferation marker Ki-67 in the study population was 18%, ranging from 1% to 80%.

3.2. Polyphenols and Methylxanthines Intake

Patients from the polyphenol group consumed three capsules daily from the diagnosis until the night before the surgery, with a median presurgery fasting period of 12 h. Overall, each patient consumed daily 37 different phenolic compounds (473.7 ± 40.5 mg day^{-1}) as well as theobromine (19.2 ± 0.6 mg day^{-1}) and caffeine (0.48 ± 0.03 mg day^{-1}) with a total intake during the trial (mean of 6 days) of 2.84 ± 0.24 g phenolics plus 115.2 ± 3.6 mg theobromine and 2.9 ± 0.2 mg caffeine. The detailed qualitative and quantitative composition (phenolic compounds + MX) of the dietary supplement can be found in Table S1, Supporting Information.

3.3. Disposition and Metabolic Profiling of Dietary Polyphenols, Methylxanthines, and Their Derived Metabolites in Urine, Plasma, and Breast Tissues

Through a target screening strategy, 187 potential metabolites derived from phenolics (PM) and MX were screened. A total of 101 metabolites were identified in urine (90 PM and 11 MX). Of the 101 total metabolites present in urine, 69 also occurred in plasma.

Table 1. Characteristics of breast cancer patients and patients' tumors.

	Groups	
	Polyphenol group [$n = 19$]	Control group [$n = 8$]
Mean age ± SD, (range)	56 ± 10, (41–76)	56 ± 10, (45–76)
Mean BMI ± SD, (range) kg m^{-2}	28 ± 6, (22–45)	29 ± 8, (21–45)
Females/Males	18/1	8/0
Menopause (yes/no)	12/6	6/2
Smoking		
>10 cigarettes per day	4	2
<10 cigarettes per day	2	0
No smoking	13	6
Tumor localization (right/left)	12/7	4/4
Type of surgery		
Tumorectomy	10	6
Mastectomy	5	1
Lumpectomy	3	1
Quadrantectomy	1	0
Type of tumor		
Ductal	15	6
Lobular	1	0
Mix	1	1
Others	2	1
Size of tumor[a)]		
Tis	1	1
T0	0	0
T1	11	2
T2	6	5
T3	1	0
Nodal status[a)]		
N0	17	5
N1	2	3
Metastasis[a)]		
MX	6	3
M0	13	5
HER2 +/−	2/17	0/8
ER +/−	19/0	7/1
PgR +/−	13/6	7/1

[a)] According to TNM staging; HER2, human epidermal growth factor receptor type 2; ER, estrogen receptor; PgR, progesterone receptor.

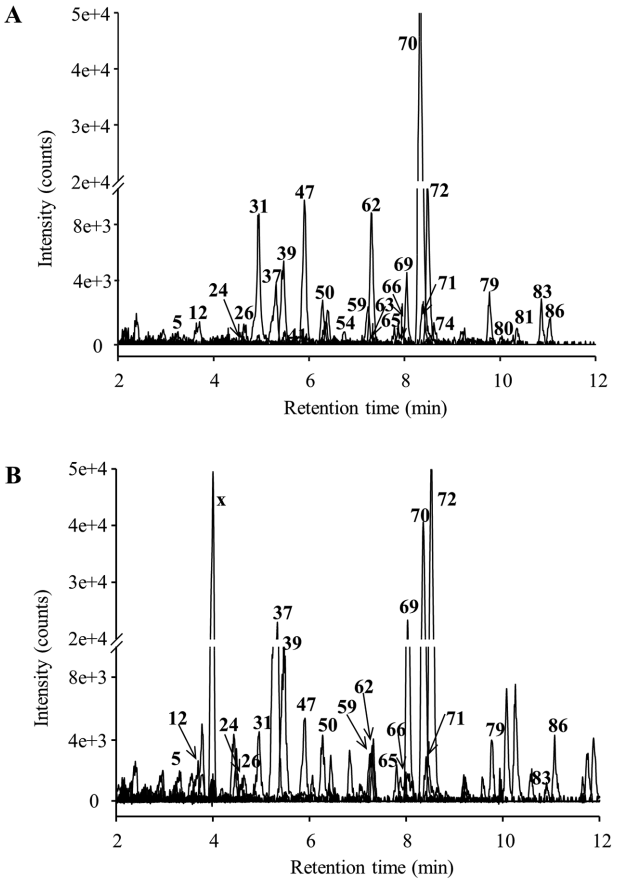

Figure 2. Extracted ion chromatograms (EICs) showing PM in A) normal and B) malignant breast tissues. EICs were obtained using UPLC-ESI-QTOF-MS. Peaks: **5**, Hydroxyhippuric acid (isomer-1); **12**, Hydroxyhippuric acid (isomer-2); **24**, Urolithin A-sulfoglucuronide; **26**, 2,5-Dihydroxybenzoic acid; **31**, Resveratrol sulfoglucuronide (isomer-1); **37**, 5-(3′,4′-Dihydroxyphenyl-γ-valerolactone 3′-sulfate; **39**, 2,6-Dihydroxybenzoic acid; **41**, Resveratrol sulfoglucuronide (isomer-2); **47**, Resveratrol 4′-*O*-glucuronide; **50**, Urolithin-A 3-*O*-glucuronide; **54**, Isourolithin-A 3-*O*-glucuronide; **59**, Resveratrol 4′-*O*-sulfate; **62**, Resveratrol 3-*O*-glucuronide; **63**, Dihydroresveratrol 4′-*O*-glucuronide; **65**, Dihydroresveratrol sulfate (isomer-1); **66**, Urolithin-A sulfate; **69**, Dihydroresveratrol 3-*O*-glucuronide; **70**, Resveratrol 3-*O*-sulfate; **71**, Tyrosol sulfate (isomer-1); **72**, Dihydroresveratrol sulfate (isomer-2); **74**, Tyrosol sulfate (isomer-2); **79**, Urolithin-B 3-*O*-glucuronide; **80**, Hesperetin 7-*O*-glucuronide; **81**, Hesperetin 3′-*O*-glucuronide; **83**, Hesperetin 7-*O*-sulfate; **86**, Urolithin-B 3-*O*-sulfate; and **x**, unidentified. The list of PM can be found in Table S4, Supporting Information.

In the mammary tissues, a total of 39 and 33 metabolites were detected in NT and MT, respectively. For all the compounds, molecular formulae were obtained with a high score (<90) and low error (5 ppm). Chromatographic characteristics, the occurrence of metabolites (urine, plasma, MT, and NT), and other details are shown in Table S4, Supporting Information, for PM and in Table S5, Supporting Information, for MX. **Figures 2** and **3** show representative extracted ion chromatograms (EICs) of identified PM and MX, respectively, in both NT and MT. EICs of urine and plasma are shown in Figures S1 and S2, Supporting Information, for PM and MX, respectively. **Tables 2** and **3** show the comparative metabolic profiling of those quantified PM and MX, respectively, in MT, NT, urine, and plasma.

Only metabolites with available standards were quantified, that is, 15 PM (Table 2) and 4 MX (Table 3). Those metabolites with no authentic standards were tentatively identified but not quantified as the signal in UPLC-ESI-QTOF-MS was dramatically different depending on the metabolite and type of conjugation. Quantitative analysis after hydrolysis was only possible for 2,5- and 2,6-dihydroxybenzoic acids, hesperetin, urolithin A, isourolithin A, and urolithin B (Supporting Information). However, and paradoxically, although resveratrol derivatives were the most abundant metabolites in breast tissues, no unconjugated resveratrol

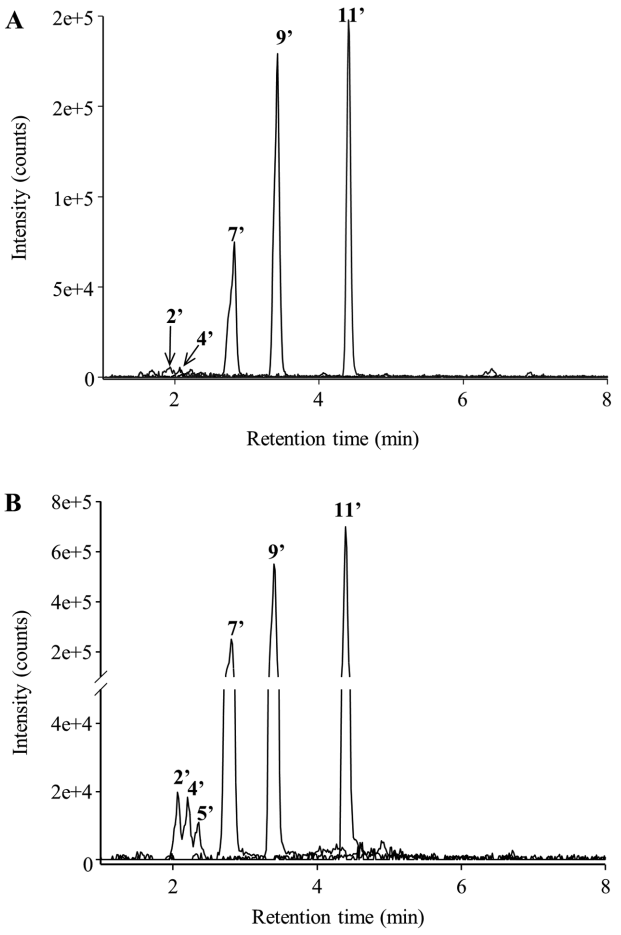

Figure 3. Extracted ion chromatograms (EICs) showing MX in A) normal and B) malignant breast tissues. EICs were obtained using UPLC-ESI-QTOF-MS. **2′**, 7-Methylxanthine; **4′**, 3-Methylxanthine; **5′**, 1-Methylxanthine; **7′**, Theobromine; **9′**, Theophylline; **11′**, Caffeine. The list of all MX can be found in Table S5, Supporting Information.

was quantified after enzymatic hydrolysis. The latter was due to its high LOQ (130 nmol L^{-1}) compared to those of its phase II metabolites detected in the tissues (from 2 nmol L^{-1} for resveratrol 4′-*O*-sulfate to 12 nmol L^{-1} for resveratrol 3-*O*-glucuronide, Table S2, Supporting Information). For the same reason, the unconjugated metabolite DHPV could not be quantified either.

As the patients did not follow a restricted diet between diagnosis and surgery, some PM were also detected in breast tissues from control patients. This was the case for the isomers of 2,5- and 2,6-dihydroxybenzoic acids, which were found in all the control patients, and some flavanone-derived conjugated metabolites (hesperetin 7- and 3-glucuronides and hesperetin 7-sulfate) detected in one control patient, which was consistent with the intake of one glass of orange juice the night before the surgery according to the food record provided by the patient. The metabolite DHPV-3′-sulfate (peak no. 37, Table 2, Table S4, Supporting Information), which is a metabolite derived from procyanidins-containing dietary sources, was also found in three control patients. The concentration found for these metabolites in breast tissues from control patients was very low, close to their corresponding LOQs (Table S2, Supporting Information). These metabolites were also found in plasma and urine from these patients. All the MX identified in urine, plasma, NT, and MT of patients that consumed the dietary supplement were also identified in the control group, and at similar concentrations, despite not all the patients consumed cocoa-derived products, coffee, or tea the day before the surgery according to their food records.

In the ongoing trial, we randomly selected one patient (not included in the main trial) to consume only cocoa extract (2.1 g day^{-1}), which provided a dose of around 82.5 mg day^{-1} theobromine and 2 mg day^{-1} caffeine. The purpose was to check whether a fourfold higher intake of theobromine and caffeine could increase the concentrations of MX in breast tissues. However, approximately the same profiling and concentrations of MX were found in MT, NT, urine, and plasma from this patient (results not shown).

A 63-year-old male with morbid obesity participated in this trial. This patient presented an infiltrating ductal breast cancer, classified as T1N0MX, ER+, PgR+, and HER2-, and underwent a mastectomy. Resveratrol was the most abundant polyphenol present in the supplement, and its derived metabolites were the most abundant compounds detected in women patients. However, no resveratrol metabolites were detected in MT or NT from this male patient. The rest of the metabolic profiling was similar to that of the women.

The washing of the breast tissue samples to eliminate the remaining blood traces was essential to avoid an overestimation of metabolite concentrations since around twofold higher concentrations were detected in unwashed samples due to the blood that soaked the external surface of the tissues (results not shown). We did not find an association between patients' BMI and tissue disposition of metabolites as well as between type or amount of metabolites and tumor characteristics (the type of tumor, infiltration degree, TNM stage, location, etc.).

In general, the concentration of PM tended to be higher in MT than in NT. When intra-subject differences were explored for all identified metabolites in tissues, trend differences were observed for resveratrol 3-*O*-sulfate (1.5-fold higher in MT, $p = 0.07$), reaching statistical significance in the case of DHPV-3′-sulfate (2.5-fold higher in MT, $p = 0.02$). In the case of MX, the concentration of caffeine tended to be higher in MT (1.8-fold higher in MT, $p = 0.06$), reaching statistical significance in the rest of MX, that is, 3-methylxanthine (twofold higher in MT, $p = 0.02$), theobromine (twofold higher in MT, $p < 0.001$), theophylline (1.5-fold higher in MT, $p = 0.04$), and 7-methylxanthine (fivefold higher in MT, $p = 0.03$) (although 7-methylxanthine was not quantified with its corresponding standard, the comparison between MT and NT was possible using the relative peak areas for this metabolite. This approach is not correct for quantification purposes, but it is a valid strategy for comparing the abundance of the same specific metabolite in different groups or conditions).

No significantly different conjugation profiling was found in MT versus NT. All MX were detected unconjugated in both MT and NT. Overall, PM were mostly glucuronidated and(or) sulfated in MT and NT (85.5% and 86.6%, respectively). Among these conjugates, the percentage of sulfated PM was slightly higher in MT (42%) than in NT (31%), while sulfated PM in plasma and urine accounted for 29% and 32%, respectively, which was close to the percentage of sulfation observed in normal tissues. The

Table 2. Phenolic compounds and derived metabolites quantified in normal and malignant breast tissues, urine, and plasma from breast cancer patients.

No.[a]	Metabolites	Mean concentration ± SD; median and (range)			
		Malignant tissue [pmol g^{-1}]	Normal tissue [pmol g^{-1}]	Urine [nmol mg^{-1} creatinine]	Plasma [nmol L^{-1}]
26	2,5-Dihydroxybenzoic acid	40.2 ± 17.5; 40.2 (27.7–52.2)	31.1 ± 25.2; 20.1 (14.9–83.5)	2.6 ± 1.3; 1.9 (0.6–5.8)	36.6 ± 19.1; 28.1 (13.5–69.5)
37	5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone 3′-sulfate	19.8 ± 19.4; 14.6 (3.8–61.5)	6.9 ± 8.6; 10.6 (0.8–75.0)	10.1 ± 12.2; 5.6 (0.1–40.6)	56.3 ± 67.1; 31.7 (1.7–227.7)
39	2,6-Dihydroxybenzoic acid	12.3 ± 5.8; 13.6 (3.9–22.7)	7.7 ± 3.8; 8.3 (2.5–15.0)	0.6 ± 0.6; 0.6 (0.1–2.4)	56.9 ± 38.2; 42.3 (14.2–152.3)
50	Urolithin-A 3-*O*-glucuronide	29.4 ± 23.9; 26.2 (3.2–66.5)	27.4 ± 21.2; 27.4 (1.9–65.3)	16.6 ± 16.1; 14.3 (0.07–56.2)	140.6 ± 142.2; 99.5 (21.0–534.8)
54	Isourolithin-A 3-*O*-glucuronide	ND	9.6 ± 5.4; 8.4 (5.4–16.9)	17.6 ± 14.6; 13.9 (5.7–37.2)	100.4 ± 91.4; 91.6 (12.3–206.1)
59	Resveratrol 4′-*O*-sulfate	8.1 ± 3.9; 8.1 (3.5–13.3)	3.9 ± 3.6; 3.2 (1.3–13.3)	4.5 ± 3.8; 2.9 (0.3–12.2)	30.4 ± 19.1; 28.3 (4.0–71.2)
62	Resveratrol 3-*O*-glucuronide	29.2 ± 8.7; 28.7 (20.0–41.1)	29.2 ± 31.4; 20.3 (1.7–104.9)	95.3 ± 81.4; 77.9 (7.9–275.5)	142.2 ± 105.3; 153.4(11.4–336.5)
66	Urolithin-A 3-*O*-sulfate	4.8 ± 3.5; 4.8 (2.2–7.4)	5.4 ± 4.8; 2.5 (1.8–11.9)	0.9 ± 1.2; 1.3 (0.01–11.0)	18.0 ± 15.0; 14 (1.9–42.7)
69	Dihydroresveratrol 3-*O*-glucuronide	100.1 ± 85.5; 109.9 (10.3–229.4)	82.4 ± 71.3; 73.8 (1.7–234.6)	73.3 ± 131.3; 33.9 (0.07–586.4)	665.6 ± 660.0; 263.1(6.8–1,811)
70	Resveratrol 3-*O*-sulfate	89.3 ± 71.1; 86.4 (7.8–224.4)	56.5 ± 75.0; 25.3 (3.9–280.1)	94.8 ± 82.1; 74.7 (1.7–270.8)	359.7 ± 260.1; 309.5 (3.0–918.7)
79	Urolithin-B 3-*O*-glucuronide	11.3 ± 1.8; 11.3 (10.0–12.3)	14.4 ± 5.1; 13.9 (8.2–213.0)	15.9 ± 11.5; 17.4 (3.1–28.7)	137.8 ± 76.3; 178.4 (13.2–195.9)
80	Hesperetin 7-*O*-glucuronide	5.0[b]	5.0 ± 3.3; 5.8 (1.2–7.9)	0.8 ± 1.0; 0.2 (0.04–3.4)	16.0 ± 11.3; 12.1 (5.0–38.5)
81	Hesperetin 3′-*O*-glucuronide	10.0 ± 6.2; 12.9 (2.7–14.1)	3.7 ± 2.7; 4.1 (1.0–10.0)	2.3 ± 3.5; 0.6 (0.2–11.8)	27.0 ± 23.5; 18.7 (5.9–79.9)
83	Hesperetin 7-*O*-sulfate	6.0 ± 4.4; 6.0 (1.3–10.7)	2.3 ± 0.2; 2.5 (1.3–3.8)	0.5 ± 0.5; 0.2 (0.02–1.8)	20.0 ± 20.9; 10.8 (4.2–77.9)
86	Urolithin-B 3-*O*-sulfate	1.0 ± 0.6; 1.0 (0.6–1.2)	1.3 ± 0.6; 1.3 (0.6–1.8)	0.03 ± 0.03; 0.02 (0.006–0.06)	9.7 ± 5.2; 8.8 (4.9–16.2)

[a] Quantification with UPLC-ESI-QTOF-MS/MS and using authentic standards; ND, not detected; [b] metabolites quantified only in one patient; No., peak number in the extracted ion chromatograms, EICs, (Figure 2; Figure S1, Supporting Information) and listed in Table S4, Supporting Information; SD, standard deviation.

Table 3. Methylxanthines quantified in normal and malignant breast tissues, urine, and plasma from breast cancer patients.

No.[a]	Metabolites	Mean concentration ± SD; median and (range)			
		Malignant tissue [pmol g^{-1}]	Normal tissue [pmol g^{-1}]	Urine [nmol mg^{-1} creatinine]	Plasma [nmol L^{-1}]
4′	3-Methylxanthine	523.5 ± 201.2 468.1 (303.0–875.9)	229.5 ± 172.9 149.4 (56.0–571.1)	57.8 ± 45.8 40.0 (10.8–148.2)	388.9 ± 287.7 270.0 (101.1–1,208)
7′	Theobromine	919.4 ± 827.2 715.0 (153.9–3216)	620.0 ± 898.9 246.1 (20.6–3,903)	32.8 ± 30.0 24.4 (2.8–111.1)	2,345 ± 2,072 1,963 (279.6–7,941)
9′	Theophylline	570.0 ± 547.8 314.4 (22.8–1,881)	402.2 ± 561.7 179.4 (3.9–2,134)	31.1 ± 41.1 23.9 (0.2–177.2)	1,389 ± 1,595 495.4 (23.0–4,898)
11′	Caffeine	496.4 ± 692.8 181.4 (27.3–2,197)	409.3 ± 561.3 89.2 (5.7–2,011)	3.6 ± 3.6 2.6 (0.2–11.9)	1,469 ± 1,793 353.8 (26.0–6,348)

[a] Quantification using UPLC-ESI-QTOF-MS/MS in the positive mode, and only when authentic standards were available; No., peak number in the extracted ion chromatograms, EICs, (Figure 3; Figure S2, Supporting Information) and listed in Table S5, Supporting Information; SD, standard deviation.

only quantified unconjugated PM in both MT and NT were 2,5- and 2,6-dihydroxybenzoic acids.

Regarding normal adipose tissue, the concentrations of PM and MX were much lower than those found in glandular tissues (both MT and NT). The metabolites hesperetin 3′-*O*-glucuronide, isourolithin-A 3-*O*-glucuronide, urolithin-B 3-*O*-glucuronide, resveratrol 3-*O*-glucuronide, resveratrol 4′-*O*-sulfate, urolithin-B 3-*O*-sulfate, and urolithin-A 3-*O*-sulfate were not detected in adipose tissues. The isomers 2,5- and 2,6-dihydroxybenzoic acids, hesperetin 7-*O*-glucuronide, and hesperetin 7-*O*-sulfate were detected below their LOQs. The NT/adipose tissue biodistribution ratio of PM was very low, that is, 99.97/0.03 for urolithin-A 3-*O*-glucuronide, 99.99/0.01 for DHPV-3′-sulfate, 99.98/0.02 for dihydroresveratrol 3-*O*-glucuronide, and 99.99/0.01 for resveratrol 3-*O*-sulfate. These ratios were similar to those obtained for MX, that is, 99.96/0.04 for 3-methylxanthine, 99.95/0.05 for theobromine, 99.98/0.02 for theophylline, and 99.93/0.07 for caffeine.

3.4. The Pool of Bioavailable Metabolites That Reach Malignant Tissues Do Not Exert In Vitro Antiproliferative, Cytotoxic, Estrogenic, or Antiestrogenic Activities

Representative mixtures (Table S6, Supporting Information) of the bioavailable metabolites (both PM and MX) that reached the malignant mammary tissues, even at much higher concentrations (10 and 50 μmol L^{-1}) than those found in the tissues did not exert significant antiproliferative or cytotoxic effects after 72 h (data not shown).

As expected, the E-Screen assay data showed that the positive estrogenic control (1 nmol L^{-1} 17β-estradiol treatment for 7 days) increased threefold cell proliferation compared to untreated (control) cells ($p < 0.05$). However, in the absence of 17β-estradiol the mixtures of metabolites assayed (10 and 50 μmol L^{-1}), that is, PM, MX, or the combination PM+MX did not induce cell proliferation compared to control cells, which suggested the lack of estrogenic-like effect for these metabolites (Figure S3A, Supporting Information). Neither antiestrogenic effects were observed as the mixtures could not prevent the induction of cell proliferation when 17β-estradiol was already present in the cell medium (Figure S3B, Supporting Information).

4. Discussion

The current evidence on the chemopreventive activity of polyphenols in breast cancer patients is still limited. Two critical issues need to be addressed: i) Can dietary polyphenols and(or) their derived metabolites reach malignant human mammary tissues? and ii) Do the molecular forms and concentrations of the metabolites that reach the malignant tissue exert potential anticancer activity? We provide here the first evidence on the relative disposition and metabolic profiling of a wide range of dietary phenolics and methylxanthines in malignant versus normal mammary tissues from breast cancer patients.

Recently, Lazzeroni et al.[22] described the occurrence of EGCG in mammary tissues from eight breast cancer patients who consumed a lecithin formulation of green tea extract. Unfortunately, the metabolic profiling of EGCG was not provided, and no control groups were included, i.e., patients consuming tea extract without lecithin formulation, and patients that did not consume extract. Bolca et al. reported for the first time the occurrence of soy isoflavones[20] and hop prenylflavonoids[21] as well as their derived metabolites in NT from healthy women. These polyphenols had been previously acknowledged with estrogenic/antiestrogenic activities and could exert beneficial effects mainly against ER+ breast cancers.[26,27] These authors also hypothesized on the different possible disposition of isoflavones in breast tissues from men.[20] In our trial, the metabolic profiling in the participating obese man mainly differed from that of women in the lack of resveratrol metabolites in both MT and NT. However, we acknowledge this is a single case report, and future studies are needed to confirm these differences as well as the potential role of obesity in the tissue distribution of dietary polyphenols.

The previously reported conjugation of isoflavones[20] and prenylflavonoids[21] in NT (98% and >90%, respectively), mostly as glucuronides, was in agreement with the extensive conjugation observed for the phenolic-derived metabolites in our study (Table 2; Table S4, Supporting Information). Bolca et al. also speculated with the potential role of conjugated metabolites as a source of bioactive aglycones in tissues[20,21] since glucuronidation usually hinders the activity of the compound.[28,29] This theory could be especially relevant in breast tumor tissues where the glucuronyltransferase/glucuronidase ratio is lower than in normal tissues.[30] However, these authors could not contrast their hypothesis as no malignant tissues were available to compare with.[20,21] The in situ conjugation/deconjugation can depend on the tissue microenvironment and has been suggested in different contexts and for different compounds.[31,32] However, there is still controversy. For example, the increase of unconjugated luteolin in plasma was described as a consequence of the deglucuronidation of the flavonoid upon induction of β-glucuronidase in lipopolysaccharide (LPS)-treated rats.[31] However, the increase of unconjugated resveratrol in plasma was further attributed to an abnormal absorption of the orally administered resveratrol due to the interaction between LPS and ABC transporters.[33] Patel et al.[34] reported the absorption of an orally administered resveratrol-sulfate in humans and suggested that resveratrol-sulfates could serve as an intracellular reservoir for the generation of resveratrol. The deconjugation of resveratrol-sulfate to resveratrol was assayed in vitro and depended on the cell line.[34]

In our study, the qualitative metabolic profiling of a wide range of dietary phenolics was quite similar in MT and NT (Tables 2 and 3; Table S4, Supporting Information). The higher blood flow could partially explain the higher amount of some metabolites in MT versus NT.[35] However, this is not a general rule, and tissue type could also be critical in the disposition of metabolites since higher concentrations of urolithins were found in normal colorectal tissues versus malignant ones.[23]

We also found slight higher sulfation of metabolites in MT, in agreement with the increased sulfation activity in breast tumor cells.[36,37] However, despite the low glucuronyltransferase/glucuronidase ratio in breast tumors, we did not detect higher concentrations of deconjugated metabolites in MT to unequivocally support the in situ conjugation/deconjugation theory in tumor breast tissues, which might depend on many variables

such as metabolite type, physiological context, tissue type, precise sampling time, etc. Nevertheless, we cannot exclude in our study the presence of a higher concentration of unconjugated metabolites such as resveratrol in nonhydrolyzed MT, which might not be detected in breast tissues due to its high LOD and LOQ (Supporting Information). Consequently, the in vitro assay of conjugated PM pool that were quantified in MT did not exert antiproliferative or cytotoxic activity even at much higher concentrations than those reached in breast tissues, which was in agreement with the limited antiproliferative activity previously reported for conjugated PM.[29,38] Unlike the unconjugated metabolites resveratrol, genistein, daidzein, urolithin A, and urolithin B, which have been reported to exert in vitro estrogenic and antiestrogenic activities in MCF-7 cells,[37] our results suggest that their corresponding conjugated metabolites lacked these activities in the same model even at supraphysiological concentrations (Figure S4, Supporting Information). Therefore, the potential antiproliferative/cytotoxic as well as estrogenic/antiestrogenic activities in breast tissues could be mainly governed by the molecular form of each metabolite attained in the tissue. Although phase-II conjugation might hamper a direct antiproliferative activity in the MCF7 cell model, however, long-term tumor-senescent chemoprevention cannot be discarded. In this regard, Patel et al.[34] described the antiproliferative effect of resveratrol-sulfate in the colorectal cancer cell line HT-29, which was not mediated by a cell growth arrest, apoptosis, or necrosis, but by autophagy-induced senescence.[34] Therefore, the findings of Patel et al. open new opportunities to investigate the potential role of (conjugated) phenolic-derived metabolites against cancer.

We also report here for the first time the disposition of methylxanthines in breast tissues, and thus we cannot compare our results with previous studies. It is of note the relatively high concentration of MX detected in MT and NT despite the fast absorption and clearance of these molecules[39] as well as the presurgical fasting of the patients (10–12 h). The lack of dose-response after fourfold supplementation of MX in one patient requires further research, but it was probably due to the fasting (10–12 h) before the surgery. Control patients also showed MX in their tissues, and we acknowledge that the amount of MX provided by the supplement was scarce in comparison with a simple cup of coffee, tea, or a glass of cola beverage. In general, despite the rapid absorption and effect of MX acting as enhancers of attention and alertness as in the case of caffeine,[40] our results suggest that MX might persist in the body much longer than previously thought. In our study, the combination of MX plus PM as detected in malignant tissues did not exert anticancer activity in MCF-7 cells under our specific assay conditions. However, cancer chemopreventive effects of MX cannot be excluded. For example, MX have been reported i) to inhibit the nuclear enzyme poly(ADP-ribose)polymerase-1 at physiological concentrations,[41] ii) reverse multidrug resistance through the downregulation of the breast cancer resistance protein (BCRP/ABCG2), which could improve the efficacy of anticancer drugs that are ABCG2 substrates,[42] and iii) also may sensitize tumor cells to chemotherapeutic drugs such as tamoxifen and reduce breast cancer growth.[43]

The present trial has some limitations. The number of patients is still low to draw unequivocal conclusions on the differences in the disposition of PM and MX in MT versus NT, especially taking into account the high inter-individual variability for polyphenols metabolism. Tissue sampling was carried out at 10–12 h after the last supplement intake, which most likely prevented a higher disposition of PM and MX in breast tissues. Besides, the high LOD and LOQ for some unconjugated phenolics, mainly resveratrol, probably prevented its quantification in breast tissues. We also acknowledge that the daily dose of phenolics provided to the patients could be dietary achievable but perhaps not representative of a regular diet. Finally, in addition to the logical limitations of in vitro studies, the lack of in vitro activity of PM and MX in our specific test conditions does not unequivocally exclude other possible biological activities[34] after long-term exposure to dietary polyphenols and MX. This could be especially relevant if the conjugated metabolites exert a tumor-senescent activity, and(or) the in situ conjugation/deconjugation process is, at least, partially assumed.

Taking into account the results and limitations of this study, we report here the most comprehensive study carried out so far on the disposition and metabolic profiling of dietary polyphenols and methylxanthines in mammary tissues from breast cancer patients. Overall, this study provides a step forward for future clinical trials to unravel the role of polyphenols against breast cancer.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

The research reported in this article was supported by the Project 201770E081 from the Spanish National Research Council (CSIC, Spain). The products used in this study were kindly provided by Laboratorios Admira S.L. (Alcantarilla, Murcia, Spain). The authors are grateful to Dr. M. Madrigal and other surgeons from the Reina Sofía Hospital (Murcia, Spain) for their contribution to the recruitment of patients and the revision of the manuscript. The authors also thank J.C. Espín-Aguilar (IES Alcántara, Alcantarilla, Murcia, Spain) for his technical support in some parts of the study.

J.C.E. designed the study; T.M.S., A.T.S., M.A.A.G., and B.A. recruited the patients, supervised the protocol implementation, and collected urine and blood samples; B.A. collected breast tissue samples at surgery; F.M.D. and B.O.C. performed histological analysis of breast tissues; M.A.A.G. and R.G.V. processed the samples and analyzed the metabolites; A.G.S. and M.A.A.G. performed cell assays; A.G.S. and R.G.V. critically reviewed the manuscript; J.C.E. wrote the manuscript. All authors have read and approved the final manuscript for publication.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

breast cancer, clinical trials, methylxanthine, polyphenols, tissue disposition

Received: November 13, 2018
Revised: January 9, 2019
Published online: February 4, 2019

[1] A. Jemal, F. Bray, M. M. Center, J. Ferlay, E. Ward, D. Forman, *Ca-Cancer J. Clin.* **2011**, *61*, 69.
[2] C. Catsburg, R. S. Kim, V. A. Kirsh, C. L. Soskolne, N. Kreiger, T. E. Rohan, *Am. J. Clin. Nutr.* **2015**, *101*, 817.
[3] C. A. Thomson, E. Ho, M. B. Strom, *Nutr. Rev.* **2016**, *74*, 432.
[4] J. A. Rothwell, V. Knaze, R. Zamora-Ros, *Curr. Opin. Clin. Nutr. Metab. Care.* **2017**, *20*, 512.
[5] J. C. Espín, A. González-Sarrías, F. A. Tomás-Barberán, *Biochem. Pharmacol.* **2017**, *139*, 82.
[6] D. Sinha, N. Sarkar, J. Biswas, A. Bishayee, *Semin. Cancer Biol.* **2016**, *40–41*, 209.
[7] N. Wang, Z. Y. Wang, S. L. Mo, T. Y. Loo, D. M. Wang, H. B. Luo, D. P. Yang, Y. L. Chen, J. G. Shen, J. P. Chen, *Breast Cancer Res. Treat.* **2012**, *134*, 943.
[8] F. Aqil, J. Jeyabalan, R. Munagala, I. P. Singh, R. C. Gupta, *Mol. Nutr. Food Res.* **2016**, *60*, 1470.
[9] S. Amatori, L. Mazzoni, J. M. Alvarez-Suarez, F. Giampieri, M. Gasparrini, T. Y. Forbes-Hernandez, S. Afrin, A. Errico Provenzano, G. Persico, B. Mezzetti, A. Amici, M. Fanelli, M. Battino, *Sci. Rep.* **2016**, *6*, 30917.
[10] A. Bishayee, A. Mandal, P. Bhattacharyya, D. Bhatia, *Nutr. Cancer* **2016**, *68*, 120.
[11] J. Singhal, L. Nagaprashantha, S. Chikara, S. Awasthi, D. Horne, S. S. Singhal, *Oncotarget* **2017**, *8*, 75025.
[12] World Cancer Research Fund International/American Institute for Cancer Research. *Diet, Nutrition, Physical Activity and Breast Cancer. WCRF/AICR*; Continuous Update Project Report, WCRF International, London **2017**, 1.
[13] S. Jung, D. Spiegelman, L. Baglietto, L. Bernstein, D. A. Boggs, P. A. van den Brandt, J. E. Buring, J. R. Cerhan, M. M. Gaudet, G. G. Giles, G. Goodman, N. Hakansson, S. E. Hankinson, K. Heizisouer, P. L. Horn-Ross, M. Inoue, V. Krogh, M. Lof, M. L. McCullough, A. B. Miller, M. L. Neuhouser, J. R. Palmer, Y. Park, K. Robien, T. E. Rohan, S. Scarmo, C. Schairer, L. J. Schouten, J. M. Shikany, S. Sieri, et al., *JNCI, J. Natl. Cancer Inst.* **2013**, *105*, 219.
[14] K. E. Bradbury, P. N. Appleby, T. J. Key, *Am. J. Clin. Nutr.* **2014**, *100*, 394S.
[15] M. J. Emaus, P. H. Peeters, M. F. Bakker, K. Overvad, A. Tjønneland, A. Olsen, I. Romieu, P. Ferrari, L. Dossus, M. C. Boutron-Ruault, L. Baglietto, R. T. Fortner, R. Kaaks, H. Boeing, A. Trichopoulou, P. Lagiou, D. Trichopoulos, G. Masala, V. Pala, S. Panico, R. Tumino, S. Polidoro, G. Skeie, E. Lund, E. Weiderpass, J. R. Quirós, N. Travier, M. J. Sánchez, M. D. Chirlaque, E. Ardanaz, et al., *Am. J. Clin. Nutr.* **2016**, *103*, 168.
[16] R. Zamora-Ros, M. Touillaud, J. A. Rothwell, I. Romieu, A. Scalbert, *Am. J. Clin. Nutr.* **2014**, *100*, 11.
[17] A. Tavani, A. Pregnolato, C. La Vecchia, A. Favero, S. Franceschi, *Eur. J. Cancer Prev.* **1998**, *7*, 77.
[18] A. Oñatibia-Astibia, R. Franco, E. Martínez-Pinilla, *Mol. Nutr. Food Res.* **2017**, *61*, 1600670.
[19] R. Sansone, J. I. Ottaviani, A. Rodriguez-Mateos, Y. Heinen, D. Noske, J. P. Spencer, A. Crozier, M. W. Merx, M. Kelm, H. Schroeter, C. Heiss, *Am. J. Clin. Nutr.* **2017**, *105*, 352.
[20] S. Bolca, M. Urpi-Sarda, P. Blondeel, N. Roche, L. Vanhaecke, S. Possemiers, N. Al-Maharik, N. Botting, D. De Keukeleire, M. Bracke, A. Heyerick, C. Manach, H. Depypere, *Am. J. Clin. Nutr.* **2010**, *91*, 976.
[21] S. Bolca, J. Li, D. Nikolic, N. Roche, P. Blondeel, S. Possemiers, D. De Keukeleire, M. Bracke, A. Heyerick, R. B. van Breemen, H. Depypere, *Mol. Nutr. Food Res.* **2010**, *54*, S284.
[22] M. Lazzeroni, A. Guerrieri-Gonzaga, S. Gandini, H. Johansson, D. Serrano, M. Cazzaniga, V. Aristarco, D. Macis, S. Mora, P. Caldarella, G. Pagani, G. Pruneri, A. Riva, G. Petrangolini, P. Morazzon, A. DeCensi, B. Bonanni, *Cancer Prev. Res.* **2017**, *10*, 363.
[23] M. A. Nuñez-Sánchez, R. García-Villalba, T. Monedero-Saiz, N. V. García-Talavera, M. B. Gómez-Sánchez, C. Sánchez-Álvarez, A. M. García-Albert, F. J. Rodríguez-Gil, M. Ruiz-Marín, F. A. Pastor-Quirante, F. Martínez-Díaz, M. J. Yáñez-Gascón, A. González-Sarrías, F. A. Tomás-Barberán, J. C. Espín, *Mol. Nutr. Food Res.* **2014**, *58*, 1199.
[24] N. W. Roehm, G. H. Rodgers, S. M. Hatfield, A. L. Glasebrook, *J. Immunol. Methods* **1991**, *142*, 257.
[25] J. Payne, C. Jones, S. Lakhani, A. Kortenkamp, *Sci. Total Environ.* **2000**, *248*, 51.
[26] C. A. Lamartiniere, *Am. J. Clin. Nutr.* **2000**, *71*, 1705S.
[27] B. M. Dietz, A. Hajirahimkhan, T. L. Dunlap, J. L. Bolton, *Pharmacol. Rev.* **2016**, *68*, 1026.
[28] L. E. Pritchett, K. M. Atherton, E. Mutch, D. Ford, *J. Nutr. Biochem.* **2008**, *19*, 739.
[29] A. González-Sarrías, J. A. Giménez-Bastida, M. Á. Núñez-Sánchez, M. Larrosa, M. T. García-Conesa, F. A. Tomás-Barberán, J. C. Espín, *Eur. J. Nutr.* **2014**, *53*, 853.
[30] N. Albin, L. Massaad, C. Toussaint, M. C. Mathieu, J. Morizet, O. Parise, A. Gouyette, G. G. Chabot, *Cancer Res.* **1993**, *53*, 3541.
[31] K. Shimoi, N. Saka, R. Nozawa, M. Sato, I. Amano, T. Nakayama, N. Kinae, *Drug Metab. Dispos.* **2001**, *29*, 1521.
[32] C. Menendez, M. Dueñas, P. Galindo, S. González-Manzano, R. Jimenez, L. Moreno, M. J. Zarzuelo, I. Rodríguez-Gómez, J. Duarte, C. Santos-Buelga, F. Perez-Vizcaino, *Mol. Nutr. Food Res.* **2011**, *55*, 1780.
[33] M. Larrosa, M. Azorín-Ortuño, M. J. Yañez-Gascón, M. T. García-Conesa, F. A. Tomás-Barberán, J. C. Espín, *Eur. J. Nutr.* **2011**, *50*, 673.
[34] K. R. Patel, C. Andreadi, R. G. Britton, E. Horner-Glister, A. Karmokar, S. Sale, V. A. Brown, D. E. Brenner, R. Singh, W. P. Steward, A. J. Gescher, K. Brown, *Sci. Transl. Med.* **2013**, *5*, 205ra133.
[35] R. Choe, M. E. Putt, P. M. Carlile, T. Durduran, J. M. Giammarco, D. R. Busch, K. W. Jung, B. J. Czerniecki, J. Tchou, M. D. Feldman, C. Mies, M. A. Rosen, M. D. Schnall, A. DeMichele, A. G. Yodh, *PLoS One* **2014**, *9*, e99683.
[36] J. P. Harrelson, M. W. Lee, *Pharmacol. Ther.* **2016**, *167*, 60.
[37] M. Larrosa, A. González-Sarrías, M. T. García-Conesa, F. A. Tomás-Barberán, J. C. Espín, *J. Agric. Food Chem.* **2006**, *54*, 1611.
[38] M. Á. Ávila-Gálvez, J. C. Espín, A. González-Sarrías, *J. Agric. Food Chem.* **2018**, *66*, 8547.
[39] D. Wikoff, B. T. Welsh, R. Henderson, G. P. Brorby, J. Britt, E. Myers, J. Goldberger, H. R. Lieberman, C. O'Brien, J. Peck, M. Tenenbein, C. Weaver, S. Harvey, J. Urban, C. Doepker, *Food Chem. Toxicol.* **2017**, *109*, 585.
[40] EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA), *EFSA J.* **2011**, *9*, 2054. https://doi.org/10.2903/j.efsa.2011.2054
[41] L. Geraets, H. J. Moonen, E. F. Wouters, A. Bast, G. J. Hageman, *Biochem. Pharmacol.* **2006**, *72*, 902.
[42] R. Ding, J. Shi, K. Pabon, K. W. Scotto, *Mol. Pharmacol.* **2012**, *81*, 328.
[43] A. H. Rosendahl, C. M. Perks, L. Zeng, A. Markkula, M. Simonsson, C. Rose, C. Ingvar, J. M. Holly, H. Jernström, *Clin. Cancer Res.* **2015**, *21*, 1877.