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Disposition of Dietary Polyphenols in Breast Cancer Patients' Tumors, and Their Associated Anticancer Activity: The Particular Case of Curcumin

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Scope: Some polyphenol-derived metabolites reach human breast cancer (BC) tissues at concentrations that induce cell senescence. However, this is unknown for isoflavones, curcuminoids, and lignans. Here, their metabolic profiling in normal (NT) and malignant (MT) mammary tissues of newly-diagnosed BC patients and the tissue-occurring metabolites' anticancer activity are evaluated.

Methods and results: Patients ($n = 26$) consumed 3 capsules/day (turmeric, red clover, and flaxseed extracts plus resveratrol; 296.4 mg phenolics/capsule) from biopsy-confirmed diagnosis to surgery (5 ± 2 days) or did not consume capsules ($n = 13$). NT and MT, blood, and urine are analyzed by UPLC-QTOF-MS using targeted metabolomics. Anticancer activity was tested in MCF-7 and MDA-MB-231 BC cells. Mainly phase-II metabolites were detected (108, 84, 49, and 47 in urine, plasma, NT, and MT, respectively). Total metabolite concentrations reached 10.7 ± 11.1 and 2.5 ± 2.4 μmol L^{-1} in NT and MT, respectively. Free curcumin, but not its glucuronide, was detected in the tissues (1.1 ± 1.8 and 0.2 ± 0.2 μmol L^{-1} in NT and MT, respectively). Breast tissue-occurring metabolites' antiproliferation was mainly exerted in p53-wild-type MCF-7 cells by curcuminoids through cell cycle arrest, senescence, and apoptosis induction via p53/p21 induction, while isoflavone-derived metabolites exerted estrogenic-like activity.

Conclusion: Curcuminoids could be coadjuvants that might help fight BC upon regular consumption.

1. Introduction

One in eight women will be diagnosed with breast cancer (BC) in their lifetime, making BC the first cause of cancer-associated death and the most commonly diagnosed malignant tumor worldwide in women.[1] Healthy lifestyles are the first preventive barrier to fight breast cancer.[2] An inverse association between high intake of plant foods and low BC risk has been reported,[3–5] and dietary polyphenol-rich patterns, including Mediterranean diet,[6] and isoflavone-rich Asian diets,[7] have been linked to this protective effect. However, despite these associations and extensive preclinical research dealing with the anticancer activity of polyphenols through a plethora of mechanisms,[8] the human evidence is still limited.[8,9] Nevertheless, resveratrol, quercetin, isoflavones, epigallocatechin gallate (EGCG), lignans, and curcumin, seem to be promising candidates.[8] Flaxseed consumption has been reported to increase the apoptotic cell rate, reduction of Ki-67 and human epidermal growth factor receptor

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type 2 (HER-2) expression,[10] and endogenous estrogen production in BC patients,[11] which could be relevant in the antiestrogen therapy in estrogen-receptor-positive (ER+) tumors.[12] Green tea extracts containing EGCG have also been studied, although no significant effects on anticancer markers were observed after 6 months in ER- and progesterone receptor-negative (PgR-) BC patients.[13,14]

Overall, phenolic compounds are xenobiotics that undergo extensive phase-II and gut microbial metabolism, which drastically limits phenolics' anticancer activity.[15,16] A crucial question is whether (poly)phenols and(or) their derived metabolites can reach malignant mammary tissues (MT) to exert their potential anticancer activity. In this regard, the disposition of phenolic-derived metabolites in human breast cancer tissues has been scarcely approached.[8] For example, silybin and EGCG-derived metabolites were detected in MT from ER+ BC patients after consuming milk thistle and green tea extracts, respectively.[17,18] In the case of isoflavones, Pumford et al.[19] reported the detection of genistein and daidzein in breast tissues from BC patients, but the tissues were enzymatically hydrolyzed, and the real metabolic profiling is unknown so far. Recently, Ávila-Gálvez et al.[20] described the metabolic profiling of phenolic-derived metabolites in MT and normal mammary tissues (NT) from BC patients after consuming 37 different phenolics, including flavanones, ellagic acid, proanthocyanidins, hydroxytyrosol, and resveratrol, and identified a total of 39 and 33 phase II-derived metabolites in NT and MT, respectively. However, metabolites, as detected in breast tissues, did not exert antiproliferative or estrogenic/antiestrogenic activities in BCcells upon short-term treatments. The authors launched two hypotheses: i) the pre-surgery fasting of the patients could have hampered both the detection of some metabolites with fast clearance and the occurrence of higher metabolite concentrations and ii) perhaps, phase-II metabolites could exert long-term tumor-senescent chemopreventive effects. A further pharmacokinetic rat study, with an equivalent dose of the same phenolics assayed in BC patients, described the kinetic disposition in mammary tissues of phase-II, but not free, metabolites derived from resveratrol, hesperetin, urolithins, and hydroxytyrosol at higher concentrations than those detected in the BC long-fasting patients.[21] Besides, a further study showed that phase-II resveratrol metabolites, as found in MT from BC patients, were not deconjugated to release free resveratrol but entered BCcells and exerted long-term tumor-senescent chemoprevention, using clonogenic assays, through p53/p21$^{Cip1/Waf1}$ and p16^{INK4a}/Rb pathways.[22]

To the best of our knowledge, the metabolic profiling of dietary curcuminoids, lignans, and isoflavones-derived metabolites has not been previously reported in MT from BC patients. Besides, the cause-effect relationship between the effects on cancer-related biomarkers and phenolic-derived metabolites has not been fully established. For example, curcumin decreased carcinoembryonic antigen and vascular endothelium growth factor levels in BC patients, but no correlation between these effects and plasma curcumin was found.[23]

Here, we aimed at determining the metabolic profiling of isoflavones, lignans, and curcuminoids for the first time in breast tissues from BC patients. We hypothesized that polyphenols administration a few hours before the surgery could increase metabolites' concentrations in MT from BC patients. We also hypothesized that the physiologically relevant breast tissue-occurring metabolites could exert anticancer activity in BC cell models upon long-term treatments.

2. Experimental Section

2.1. Chemicals and Polyphenol-Rich Supplement

Reagents and standards of phenolic-derived metabolites were obtained as detailed in Supporting Information.

The study's design and formulation of the extract mixture were not planned to evaluate any patients' specific health effect. The formulation of this supplement tried to cover a broad range of phenolics present in the diet and commercial dietary supplements. Each capsule (505 mg of blend) contained 65 mg *trans*-resveratrol, 190 mg turmeric extract, 125 mg flaxseed extract, and 125 mg red clover extract. The components were blended and encapsulated in hard gelatin capsules (Capsugel, Bornem, Belgium) by Laboratorios Admira S.L. (Alcantarilla, Murcia, Spain). The capsules were manufactured, tested, and checked following the European Union's Good Manufacturing Practices requirements. The supplement was analyzed by High Performance Liquid Chromatography-Electrospray Tandem Mass Spectrometry (HPLC-ESI-MS/MS) to evaluate the content of phenolic compounds. The detailed composition is provided in the Supporting Information. Each capsule contained 20 different phenolic compounds with a mean content (±SD) of 296.43 ± 17.52 mg (Table S1, Supporting Information).

2.2. Patients and Study Design

This trial was a dietary intervention that followed the ethical guidelines outlined in the Helsinki Declaration of 1975 and its amendments. The trial was registered at clinicaltrials.gov as NCT03482401 (last update posted on September/10/2020) and was conducted in Murcia (Spain) between October-2018 and June-2019. The safety issues and protocol were addressed and approved (reference 25/09/2018) by the Clinical Ethics Committee at the Reina Sofia University Hospital (Murcia, Spain) and by the Spanish National Research Council's Bioethics Committee (Madrid, Spain).

The primary outcome was to evaluate the disposition and metabolic profiling of curcuminoids, isoflavones, resveratrol derivatives, and lignans in malignant mammary tissues (MT) from BC patients. Secondary outcomes included: i) The characterization of the metabolic profiling in normal mammary tissues (NT), plasma, and urine and ii) the evaluation in BC cell models of the antiproliferative, cytotoxic, estrogenic, antiestrogenic, anticlonogenic, apoptotic, and cellular senescence effects of representative metabolite mixtures that could reach MT.

The patients' flowchart is shown in **Figure 1**. Seventy-five patients with suspected BC were interviewed. Thirty-six patients did not meet the inclusion criteria or declined to participate. Patients with suspected intolerance to any component of fruits or vegetables were excluded. Recruited patients included those over 18 years with newly biopsy-confirmed BC, programmed surgery at least 3 days after the recruitment, and no neoadjuvant treatment.

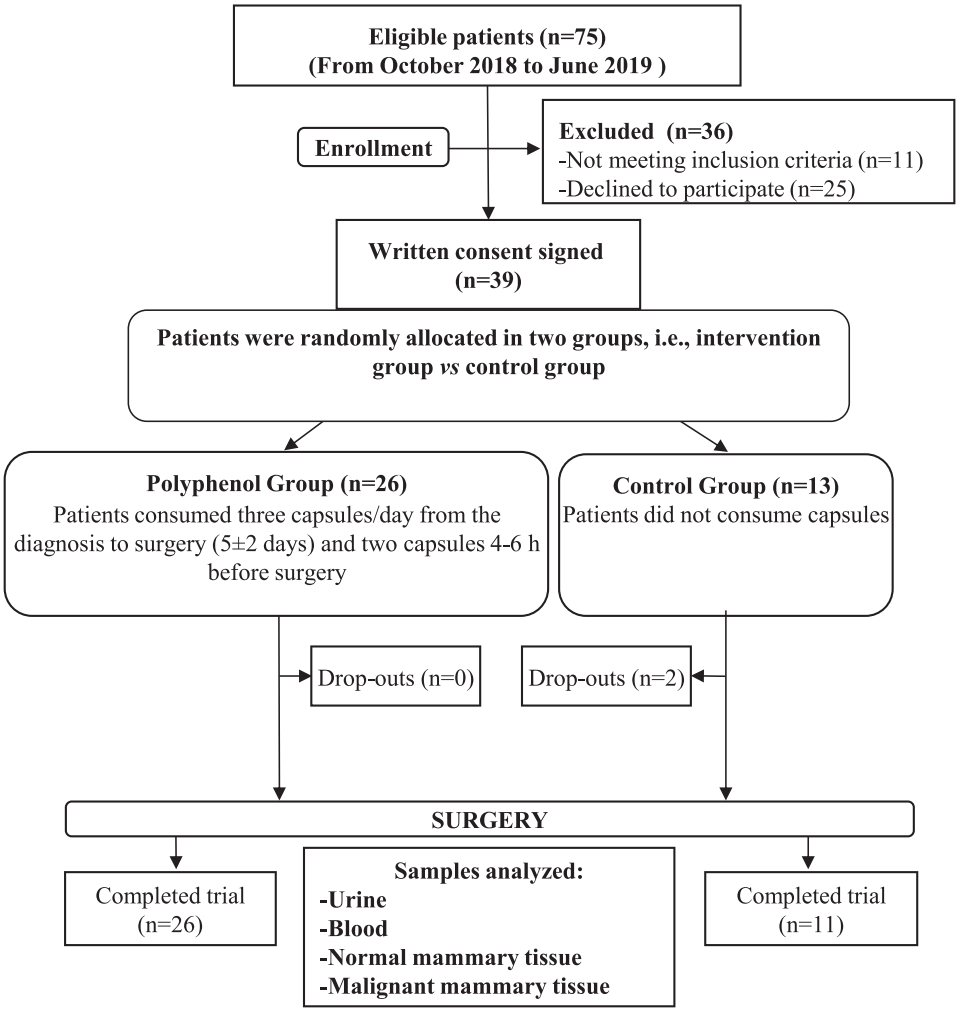

Figure 1. The flow of patients through the trial (CONSORT diagram).

The study was fully explained to the patients, and those that met the inclusion criteria ($n = 39$) gave their written informed consent before participating. The recruited patients were randomized into two groups (polyphenol and control groups) using a randomization method. For every three patients, two were randomly allocated to the polyphenol group and one to the control group to increase the sample size in the polyphenol group (Figure 1). All patients compiled the consumption of all food items for 24 h before surgery.

No placebo was included in the study's design since the trial was not designed to evaluate changes in specific clinical variables. The sample size was based on a previous study,[20] since there is not enough available evidence to accurately power (minimum sample size) this type of study (i.e., tissue disposition of dietary polyphenols).

2.3. Dosage Information

Patients from the polyphenol group consumed three capsules daily from the biopsy-confirmed cancer until the day of the surgery. This daily dose was based on the previous study with other phenolics, also in patients with breast cancer.[20] Besides, the patients also consumed two capsules between 2–6 h before the surgery (the anaesthetists only allowed the intake of two capsules). The control group did not receive any supplementation.

2.4. Sampling Procedure

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 the concentration of urine samples. During surgery (2–6 h after the last consumption of capsules), blood, normal mammary tissue adjacent to the tumor (NT), and malignant mammary tissue (MT) were taken. Venous blood was collected in heparinized vacutainers, and the corresponding plasma was obtained. Urine and plasma samples were kept at −80 °C until analysis. The resected breast tissues were immediately taken to the Anatomical Pathology Service for their examination and classification. Tissue samples were snap-frozen in liquid nitrogen and stored at −80 °C until further analysis.

2.5. Analysis of Phenolics and Derived Metabolites in Urine, Plasma, and Breast Tissues

Plasma samples (200 µL) were extracted with 600 µL acetonitrile:formic acid (98:2, v/v), centrifuged, and the supernatant reduced to dryness using a speed vacuum concentrator. NT and MT samples were weighed and extensively washed (5x) with phosphate buffered saline (PBS) to remove blood contamination, and avoid possible overestimation of metabolites. Samples tissues were homogenized with methanol (MeOH):HCl (99.9:0.1 v/v) in a Bullet Blender Homogenizer (Next Advance, Averill Park, NY, USA) for 5 min. Two internal standards, 0.2 ppm dihydroxycoumarin, and 0.1 ppm chrysin, were added to each sample before and after sample extraction.

The resulting supernatants (plasma and breast tissues) were evaporated in a speed vacuum, resuspended in 100 µL of MeOH, and filtered through a 0.22 µm polyvinylidene fluoride filter before analysis by ultra-high performance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight mass spectrometry (UPLC-ESI-QTOF-MS).

Urine samples were diluted (1:3, v/v) with acidified water (0.1% formic acid), and excretion of creatinine was measured according to the kinetic Jaffé method as reported elsewhere.[24]

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). Data were processed using the Mass Hunter Qualitative Analysis software (version B.06.00, Agilent). A previously validated method for analyzing phenolic compounds in breast tissues was used,[20] which is summarized as follows. A target screening strategy was applied for the qualitative analysis of possible metabolites that could be present after consuming the capsules containing the mixture of extracts. The exact mass of the proposed compound was extracted using an extraction window of 0.01 m/z. The identification of compounds was carried out by taking information about their elution order, molecular weight, fragmentation by MS/MS, and, whenever possible chromatographic comparison with authentic standards.

Phenolic-derived metabolites detected in mammary tissues, urine, or plasma were quantified by peak area integration of their extracted ion chromatograms (EICs) (only when standards were available). Only compounds present in at least 50% of the patients and with apparent differences with respect to the control were considered. The method was validated for linearity, precision intra- and inter-day, limit of detection (LOD), and quantification (LOQ), as well as, for matrix effect (Supporting Information). The analytical parameters for the determination of phenolic compounds and derived metabolites in mammary tissues using UPLC-ESI-QTOF-MS are shown in Table S2, Supporting Information. LODs and LOQs were calculated assuming the criterion of signal-to-noise ratio (S/N) of 3 for the LOD and 10 for the LOQ.[20] The recovery of metabolites from breast tissues, and to check possible chemical deconjugation in the conjugated metabolites due to the processing, control samples were spiked in triplicate with a mixture of available both free and conjugated standards at 0.5 µM. A recovery yield between 43 ± 3% (resveratrol 3-*O*-sulfate) and 98 ± 2% (formononetin) was obtained (Table S2, Supporting Information).

2.6. Enzymatic Treatment of Samples and Standard Conjugates

For the complete deconjugation of metabolites, urine, plasma, MT, and NT samples were treated overnight with glucuronidase/sulfatase from *Helix pomatia* as previously described.[20] After treatment with glucuronidase/sulfatase, special care was taken to subtract the content of other plant components present in commercial *H. pomatia* extracts, including isoflavones.[25,26]

A pilot study regarding the kinetics of metabolites deconjugation using commercial *H. pomatia* and mammary tissues was conducted. Ex vivo deconjugation was assayed as follows: MT or NT (100 mg, $n = 4$) from the control group were homogenized with a bullet blender in 500 µL (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES) buffer (pH = 7.4). After centrifugation, acetate buffer (200 µL, pH = 5) was added, and the samples were spiked with 10 µm L^{-1} curcumin glucuronide, genistein 4'-*O*-glucuronide, DHR 3-*O*-glucuronide or a mix containing the three metabolites. After incubating at 37 °C for 90 min, 4, 8, and 20 h, samples were extracted twice with 2 mL ethyl acetate and evaporated, further re-dissolved in MeOH in the same initial volume that was evaporated, and analyzed by UPLC-ESI-QTOF-MS. In the in vitro deconjugation of standards with the commercial enzymatic extract, the reaction was started by adding 2.500 U of *H. pomatia* extract to the reaction medium containing the same assay conditions and metabolites described for the ex vivo experiment.

2.7. Cell Lines, Cell Culture Conditions, and Treatments

MCF-7 human estrogen receptor (ER)-positive and MDA-MB-231 ER-negative breast adenocarcinoma cells were obtained from the American Type Culture Collection (Rockville, USA) and grown as reported elsewhere.[16]

To evaluate the potential antiproliferative effect (in both BC cells), as well as, the estrogenic/anti-estrogenic activities (in MCF-7 cells), five mixtures of phenolics and derived metabolites were prepared to represent the qualitative and quantitative profile of both total and specific family compounds (curcuminoids, resveratrol, isoflavones, and lignans) that may reach the mammary gland following the consumption of extract. Each representative mixture was assayed at a final concentration in the medium of 2.5 and 10 µmol L^{-1} based on pooled data of concentrations detected in both malignant and normal mammary tissues from breast cancer (BC) patients ($n = 26$). Table S3, Supporting Information, shows the composition of each mixture assayed. All tested compounds were solubilized in DMSO (<0.5% in the culture medium) and filter sterilized (0.2 µm) before addition to the culture media. Control cells were also run in parallel and subjected to the same changes in the medium with sterile DMSO.

2.8. Cell Viability Assay

The effect of each mixture (2.5 and 10 µmol L^{-1}) on cell viability and proliferation were measured by the MTT reduction assay and CV methods according to Giménez-Bastida et al.[22,27] Data are presented as the mean ± standard deviation (SD) of at least three independent experiments ($n = 6$ wells per experiment).

2.9. Estrogenic and Anti-Estrogenic Activity by E-Screen Assay

To evaluate estrogenic-like and anti-estrogenic activity effects of the five mixtures a sensitive E-screen cell proliferation assay was performed, which consisted of using a phenol red-free Eagle's minimal essential medium as described by Payne et al.[28] with some modifications. Briefly, 1500 cells/well in 96 well-plates were seeded during 48 h cells and then washed with PBS and the medium replaced with estrogen-free medium containing the same supplements plus 5% CD-FBS (fetal bovine serum (FBS) treated with dextran coated charcoal) to remove steroids, for 3 days prior either treatment. Afterward, representative mixtures were incubated for 7 days at 2.5 and 10 μmol L^{-1} in the absence or the presence of 17β-estradiol (1 nmol L^{-1}) for the determination of estrogenic or antiestrogenic activities, respectively. The treatments were refreshed after 72 h. Finally, the cell proliferation was measured using the XTT assay as described elsewhere.[16] Data are shown as the mean ± SD of three independent experiments ($n = 6$ wells per experiment).

2.10. Clonogenicity Assay

The MCF-7 and MDA-MB-231 BC cells were seeded at 250 cells/well in 12-well plates, incubated for 48 h, and treated with each mixture (2.5 and 10 μmol L^{-1}) for 14 days. The treatments were replaced every 3–4 days. At the end of the assays, the colonies were fixed with the Carnoy solution, followed by staining with 0.1% CV. The 12-well plate was photographed, analyzed, and quantified using the ImageJ plugin "ColonyArea" to quantify colonies formation as described by Guzmán et al.[29] Data are shown as the mean ± SD of three independent experiments.

2.11. Cell Cycle Analysis

The effect of the mixtures for 3 and 5 days of treatment (10 μmol L^{-1}) on cell cycle distribution in both BC cell lines was carried out as described previously.[22] Data are shown as the mean ± SD of 3 independent experiments (2 wells per treatment) for each time point.

2.12. Assessment of Apoptosis Induction

Apoptosis induction (by identifying both early and late apoptosis) exerted by the mixtures for 3 and 5 days of treatment (10 μmol L^{-1}) was examined using the annexin V/PI detection kit (Molecular Probes, Life Technologies Inc.) as described previously.[30] Briefly, 25 000 cells per sample were analyzed by flow cytometry (Coulter, EPICS XL-MCL, Miami, USA) and staurosporine 5 μmol L^{-1} was used as a positive control. Data were shown as the mean ± SD of 3 independent experiments (2 wells per treatment) for each time point.

2.13. Senescence-Associated β-Galactosidase Assay

The estimation of senescence-associated β-Galactosidase (SA-β-gal) activity was determined using the FDG method and were normalized at the end of the assay measuring cell density by the CV method assay as described elsewhere.[27,31] Data are shown as the mean ± SD of 3 independent experiments (6 wells per treatment) for each time point (3 and 5 days).

2.14. Western-Blot Analysis

Based on the senescence data, BC cells were treated for 5 days at 10 μmol L^{-1}. At the end of assays, the whole-cell protein was extracted and quantified to use equal protein amounts (20 μg protein/lane) as described elsewhere.[27] Primary human antibodies (Cell Signaling, MA, USA) p53 and $p21^{Cip1/Waf1}$, as well as, the anti-mouse horseradish peroxidase-linked secondary antibodies were used. Proteins were quantified by densitometry (ImageJ) using glyceraldehyde-3-phosphate dehydrogenase as the protein loading control. Western-blot analyses were done in triplicate ($n = 3$).

2.15. Statistical Analysis

Data were expressed as the mean ± 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 each metabolite concentration in NT versus MT. In cell cultures, the effects of treatments versus controls were evaluated by the analysis of the variance (ANOVA) followed by *Dunnet posthoc* analysis. Statistical analyses were carried out using the SPSS Software, version 23.0 (SPSS Inc., Chicago, IL, USA). Figures and graphs were performed using Sigma Plot 13.0 (Systat Software, San Jose, CA, USA). Statistical significance was set at $^*p < 0.05$.

3. Results

3.1. Patients' Baseline Characteristics

Seventy-five patients were interviewed after confirmation of BC diagnosis by biopsy. Thirty-nine patients were recruited, and 37 completed the trial; being 26 patients assigned to the polyphenol group and 11 patients to the control group. All patients completed a 24-h recall, as well as, a food frequency questionnaire. After surgery, patients from the polyphenol group confirmed the full compliance of the trial, and no remaining capsules were returned. The dietary supplement was well tolerated, and no adverse events were reported.

The baseline characteristics of BC patients are summarized in **Table 1**. The average participating patient in the polyphenol group was a 54 years old woman, overweight, menopausal, smoker, reporting low physical activity, no family history of cancer, and invasive ductal carcinoma (subtype luminal A, ER+, and progesterone receptor + (PgR+)) (Table 1). The sentinel lymph node biopsy was carried out in all patients, performing conservative surgery in all possible cases, generally tumorectomy (69%).

3.2. Polyphenols Consumed by the Patients

Patients consumed three capsules daily from the diagnosis until the night before the surgery (5 ± 2 days) plus two capsules

Table 1. Baseline characteristics of breast cancer patients and patients' tumors.

	Groups	
	Polyphenol group ($n = 26$)	Control group ($n = 13$)
Mean age ± SD, (range)	54 ± 11, (27–81)	55 ± 14, (35–76)
Mean BMI ± SD, (range) kg m^{-2}	27 ± 5, (20–37)	28 ± 6, (24–36)
Menopause (yes/no)	20/6	10/3
Smoking		
>10 cigarettes/day	12	2
<10 cigarettes/day	3	0
No smoking	11	11
Physical activity[a)]		
Low	16	10
Medium	9	3
High	1	0
Family history of breast cancer[b)] (yes/no)	7/19	2/11
Tumor localization (right/left)	19/7	8/5
Type of surgery		
Tumorectomy	18	8
Mastectomy	5	4
Lumpectomy	3	1
Histological type of tumor		
Ductal	23	11
Lobular	1	0
Mix	2	2
Molecular subtype of tumor		
Luminal A	18	11
Luminal B	6	1
Others	2	1
ER +/−	25/1	12/1
PgR +/−	23/3	12/1
HER-2 +/−	2/24	0/13
TNBC	1	1
Size of tumor[c)]		
Tis	3	3
T0	0	0
T1	16	3
T2	7	7
T3	0	0
Nodal status[c)]		
N0	21	10
N1	5	3
Metastasis[c)]		
MX	19	8
M0	7	5

[a)] According to "Global Recommendations on Physical Activity for Health";[32] [b)] Taking into account only first degree (mother, sister, or daughter); [c)] According to TNM staging; HER-2, human epidermal growth factor receptor type 2; TNBC, triple-negative breast cancer.

between 2 and 6 h before the intervention, depending on the programmed surgery's hour. The composition of the dietary supplement is detailed in Table S1, Supporting Information. In the trial, each patient consumed 20 different phenolics, mainly curcuminoids (180.34 ± 9.46 mg per capsule), resveratrol (64.51 ± 6.08 mg per capsule), lignans (32.44 ± 0.81 mg per capsule), isoflavones (17.80 ± 0.99 mg per capsule), and hydroxycinnamic acid derivatives (1.34 ± 0.18 mg per capsule) (Table S1, Supporting Information).

3.3. Phenolic Compounds and Derived Metabolites in Urine, Plasma, and Breast Tissues

The UPLC-ESI-QTOF-MS analysis led to the tentative detection of 108 compounds in urine, including some native phenolics present in the capsule, their phase-II derived metabolites, and also from microbial origin. From all these compounds, 84 were detected in plasma, and 49 compounds reached NT and 47 MT (Table S4, Supporting Information). All these compounds were derived from isoflavones (38 metabolites), curcuminoids (16 metabolites), lignans (26 metabolites), resveratrol (18 metabolites), and low-molecular-weight phenolic metabolites (10 metabolites) (Table S4, Supporting Information). For all the compounds, molecular formulas were obtained with a high score (<90) and low error (3 ppm) (Table S4, Supporting Information). According to the patients' food questionnaires, their normal diet and the food items consumed 24 h before the surgery by the patients did not present sources of isoflavones, curcuminoids, resveratrol, and lignans. In contrast, the consumption of coffee, bread, cereals, and other plant food sources could explain the low-molecular-weight metabolites' presence. This was confirmed in the control patients, where these low-molecular-weight phenolics were detected but not those metabolites derived from isoflavones, curcuminoids, resveratrol, and lignans.

Table 2 shows the polyphenols and their derived metabolites quantified in urine, plasma, and mammary tissues. Those metabolites with no authentic standards were tentatively identified (Table S4, Supporting Information) but not quantified because the signal in UPLC-ESI-QTOF was significantly different depending on the metabolite and type of conjugation. **Figure 2** shows representative EICs of quantified compounds in mammary tissues (NT and MT), urine, and plasma from a BC patient.

As expected, high interindividual variability was observed in the quantification of phenolics and their derived metabolites in urine, plasma, and mammary tissues. This variability, together with the lack of standards for the correct quantification of many metabolites, makes it challenging to obtain full quantitative conclusions (Table 2). Overall, metabolite concentrations were higher in NT than MT, although no statistically significant difference was observed between MT and NT for any metabolite, including those identified in Table S4, Supporting Information, when comparing their integrated areas (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).

The compounds quantified in urine, plasma, NT, and MT were mainly phase-II metabolites (99.9%, 99%, 84%, and 82%, respectively), although some free forms were also relevant in MT and

Table 2. Phenolic compounds and derived metabolites quantified in urine, plasma, and normal and malignant mammary tissues from breast cancer patients ($n = 26$).

N°	Metabolites	RT	Urine	Plasma	Malignant tissue		Normal tissue	
			(nmol mg creatinine^{-1})	(nmol L^{-1})	pmol g^{-1}	nmol L^{-1}*	pmol g^{-1}	nmol L^{-1}*
2	Daidzein 7-*O*-glucuronide	6.21	11.57 ± 6.69	29.54 ± 36.12	19.54 ± 19.46	33.69 ± 33.55	19.99 ± 16.09	111.06 ± 89.39
5	Daidzein 4'-*O*-glucuronide	7.45	18.76 ± 22.32	118.39 ± 179.69	100.17 ± 76.36	172.71 ± 131.66	356.15 ± 550.76	1979 ± 3060
6	Genistein 7-*O*-glucuronide	8.20	1.47 ± 1.21	10.88 ($n = 1$)	5.32 ($n = 1$)	9.17 ($n = 1$)	ND	ND
8	Genistein 4'-*O*-glucuronide	9.28	1.65 ± 0.99	8.58 ± 4.30	8.13 ($n = 1$)	14.02 ($n = 1$)	17.65 ($n = 1$)	98.06 ($n = 1$)
11	Equol 7-*O*-glucuronide	9.49	6.07 ± 7.32	31.24 ± 22.53	18.72 ± 12.38	32.28 ± 21.34	17.32 ± 21.86	96.22 ± 121.44
12	Daidzein 4'-*O*-sulfate	9.60	3.84 ± 7.15	99.11 ± 69.11	30.21 ± 7.46	52.09 ± 12.86	39.51 ± 33.22	219.50 ± 184.55
20	Daidzein	11.42	0.18 ± 0.13	3.91 ± 2.26	14.10 ± 21.88	24.31 ± 37.72	11.48 ± 21.19	63.78 ± 117.72
22	Dihydrodaidzein	11.65	0.11 ± 0.08	4.48 ± 2.85	6.42 ± 3.00	11.07 ± 5.17	3.52 ± 1.59	19.56 ± 8.83
24	Equol 4'-*O*-sulfate	12.24	0.42 ± 0.47	41.98 ± 46.31	3.69 ± 2.31	6.36 ± 3.98	3.79 ± 1.81	21.06 ± 10.06
31	Genistein	14.01	0.02 ± 0.02	2.03 ± 2.19	2.98 ± 1.77	5.14 ± 3.05	5.18 ± 4.15	28.78 ± 23.06
35	Dihydroformononetin	15.42	0.03 ± 0.01	2.49 ± 1.21	8.18 ± 7.50	14.10 ± 12.93	7.21 ± 7.01	40.06 ± 38.94
36	Formononetin	15.46	0.02 ± 0.01	5.54 ± 4.30	27.62 ± 52.60	47.62 ± 90.69	21.36 ± 18.86	118.67 ± 104.78
38	Biochanin A	17.24	0.02 ± 0.01	3.80 ± 2.84	7.69 ± 10.36	13.26 ± 17.86	4.51 ± 5.13	25.06 ± 28.50
52	Bisdemethoxycurcumin	17.01	0.03 ± 0.03	3.89 ± 5.03	2.73 ± 2.46	4.71 ± 4.24	8.78 ± 10.15	48.78 ± 56.39
53	Demethoxycurcumin	17.24	0.02 ± 0.02	2.45 ± 3.82	13.01 ± 8.93	22.43 ± 15.40	44.50 ± 68.07	247.22 ± 378.17
54	Curcumin	17.46	0.03 ± 0.03	5.26 ± 5.04	108.61 ± 207.15	187.26 ± 357.16	197.86 ± 340.56	1099 ± 1892
77	Enterodiol	11.83	0.01 ± 0.01	1.10 ± 0.84	4.39 ± 4.08	7.57 ± 7.03	0.77 ± 0.48	4.28 ± 2.67
80	Enterolactone	14.31	0.14 ± 0.13	2.69 ± 3.17	7.79 ± 7.24	13.43 ± 12.48	1.75 ± 0.94	9.72 ± 5.22
89	Resveratrol 3-*O*-glucuronide	7.71	69.02 ± 79.85	687.74 ± 632.88	154.91 ± 128.95	267.09 ± 222.33	231.84 ± 135.18	1288 ± 751
90	Dihydroresveratrol 3-*O*-glucuronide	8.37	252.60 ± 163.86	2637 ± 2398	394.24 ± 407.57	679.72 ± 702.71	410.01 ± 258.06	2278 ± 1434
92	Resveratrol 4'-*O*-sulfate	8.77	3.24 ± 2.83	70.20 ± 61.94	13.32 ± 8.95	22.97 ± 15.43	14.34 ± 11.87	79.67 ± 65.94
94	Resveratrol 3-*O*-sulfate	10.40	57.87 ± 69.57	1723 ± 1542	502.97 ± 433.68	867.19 ± 747.72	518.29 ± 505.58	2879 ± 2809
Total conjugated metabolites			427.12 ± 362.74	5495 ± 5026	1455 ± 1424	2508 ± 2455	1936 ± 2013	10754 ± 11182
Total free metabolites:			0.61 ± 0.48	37.64 ± 33.55	203.52 ± 326.97	350.90 ± 563.73	306.92 ± 478.13	1705 ± 2656

Values are shown as mean ± SD. From all the metabolites detected (Table S4, Supporting Information), only quantified metabolites using available standards are shown. N°, peak number in the extracted ion chromatograms (EICs) shown in Figures 2 and 3. *Estimated according to the blood flow and water distribution volume for tumor and normal mammary tissues.[33]

NT (Table 2, Table S4, Supporting Information). This was the case of curcumin, where higher aglycone concentrations were observed in the tissues than in plasma (Table 2). Among non-conjugated metabolites, curcumin was the polyphenol with the highest concentration detected in mammary tissues (Table 2). Table S5, Supporting Information, shows the estimated concentration range (nmol L^{-1}) of metabolites detected in NT and MT. Some metabolites in some patients, under our assay conditions, reached remarkable concentrations, including daidzein 4'-*O*-glucuronide (6.5 µmol L^{-1} in NT vs 0.3 µmol L^{-1} in MT), demethoxycurcumin (1.2 µmol L^{-1} in NT vs 0.06 µmol L^{-1} in MT), curcumin (6.8 µmol L^{-1} in NT vs 1.3 µmol L^{-1} in MT), resveratrol 3-*O*-glucuronide (2.7 µmol L^{-1} in NT vs 0.8 µmol L^{-1} in MT), dihydroresveratrol 3-*O*-glucuronide (5 µmol L^{-1} in NT vs 2.8 µmol L^{-1} in MT), and resveratrol 3-*O*-sulfate (10.6 µmol L^{-1} in NT vs 3 µmol L^{-1} in MT).

The relatively high LOQ values for some metabolites (either free forms or conjugates) such as resveratrol, equol, and genistein glucuronides, among others (Table S2, Supporting Information), could prevent their quantification under our assay conditions. This could also be the case of curcumin glucuronide, which was not detected in the tissues (Table 2). We next treated some samples ($n = 6$, due to the limited availability of breast tissue samples) with glucuronidase/sulfatase (Table S6, Supporting Information) and high concentrations of the isoflavones genistein, formononetin, and biochanin A in their aglycone forms were observed. In contrast, the concentration of other aglycones such as resveratrol and equol did not increase or even were not detected, most likely due to their relatively high LOQ (Table S2, Supporting Information). However, free curcumin concentration, quantified in the tissues (Table 2), did not increase after glucuronidase/sulfatase treatment, suggesting that

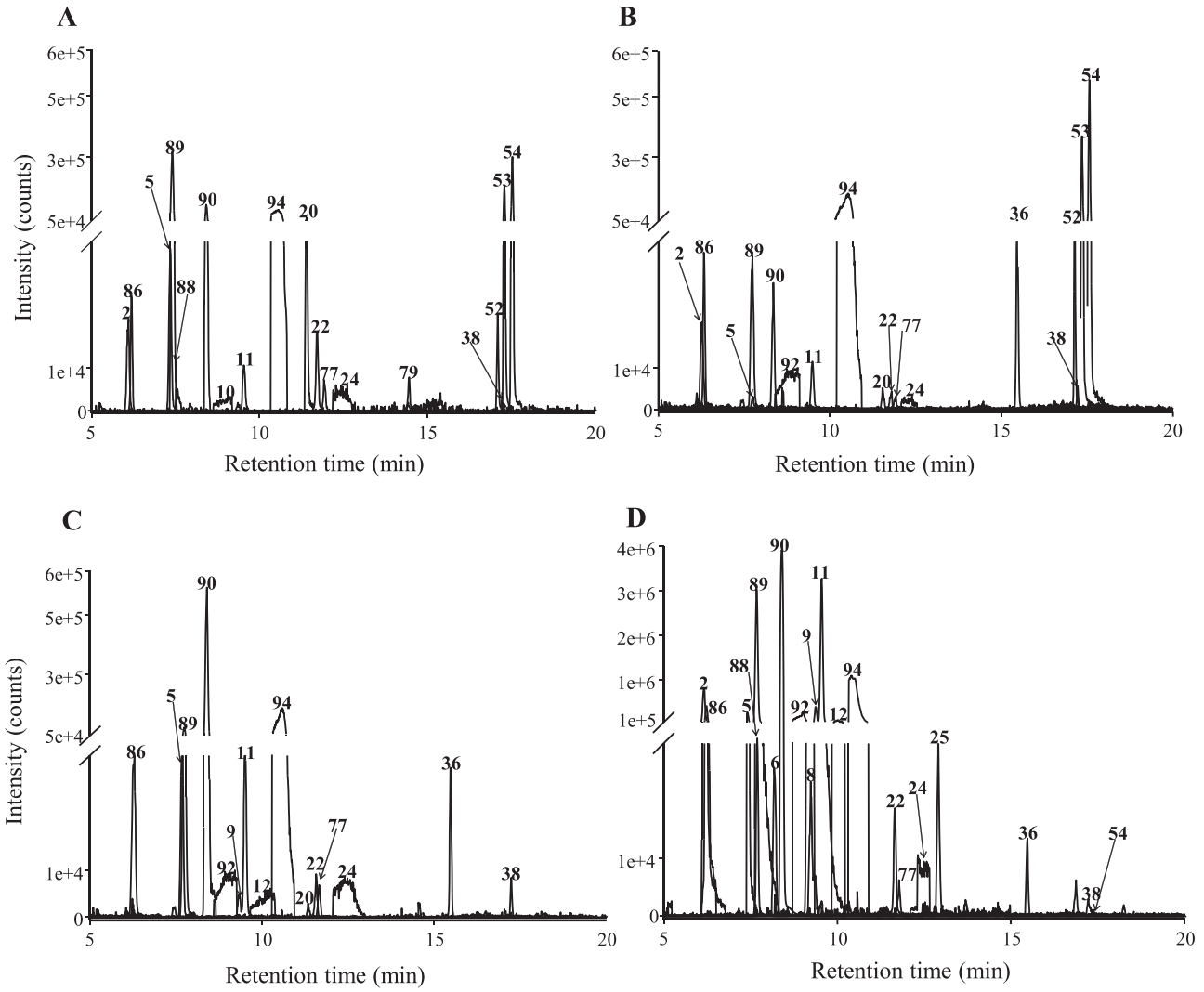

Figure 2. Representative extracted ion chromatograms (EICs) showing phenolic-derived metabolites in (A) malignant (MT), and (B) normal (NT) mammary tissues, (C) plasma, and (D) urine from a breast cancer patient. EICs were obtained using UPLC-ESI-QTOF-MS. Metabolites (peak numbers) can be found in Table S4, Supporting Information.

no curcumin conjugates, especially curcumin glucuronide, were present in non-enzymatically treated tissues. Next, to discard a possible chemical deconjugation upon tissue processing, we supplemented control breast tissues with some available conjugated metabolites, with low or no detection in the tissues (i.e., genistein 7-*O*-glucuronide, equol 7-*O*-glucuronide, and curcumin glucuronide). After processing the tissues, no chemical deconjugation was observed in any case, obtaining good recoveries for the three metabolites. We also observed complete deconjugation and no significant differences in the kinetics (reaction rate or substrate affinity) of genistein 4'-*O*-glucuronide, DHR 3-*O*-glucuronide, and curcumin glucuronide, neither after incubation with MT and NT homogenates from control BC patients nor with the commercial glucuronidase from *H. pomatia* (results not shown). Overall, these results suggest that the relatively high LOQ values of genistein 7-*O*-glucuronide, equol 7-*O*-glucuronide, equol, and free resveratrol prevented their quantification in the tissues. However, in the case of curcumin: i) No chemical deconjugation was observed, ii) no detection of the glucuronide in the tissues was observed, and iii) no increase of the free form was obtained after glucuronidase/sulfatase treatment of the tissues, although both breast tissue homogenates and the commercial enzymatic extract catalyzed the hydrolysis of curcumin glucuronide efficiently. Therefore, these results might suggest that unlike free curcumin, curcumin glucuronide did not reach the mammary tissues.

We did not find any apparent correlation between the concentration of any metabolite detected in the tissues and tumor characteristics (the type of tumor, location, TNM stage, degree of infiltration, etc.). Despite the high interindividual variability, total aglycone values in NT showed an inverse correlation with the patients' BMI values (Spearman coefficient = −0.409; $p = 0.038$). However, this correlation was not observed in MT (Spearman coefficient = −0.189; $p = 0.460$).

3.4. Effect of Representative Metabolite Mixtures on Cell Cytotoxicity, Proliferation, and Clonogenic Growth in Breast Cancer Cell Models

Among the representative mixtures of metabolites, only curcuminoids significantly reduced the cell viability in both BC cell lines at 10 μmol L^{-1} after 3 days and at 2.5 μmol L^{-1} after 5 days (Table S7, Supporting Information). The dose-dependent reduction was higher in MCF-7 than in MDA-MB-231 cells. The growth inhibition effect reached 20% versus control at 2.5 μmol L^{-1} after 5 days in MCF-7, while 28 and 65% inhibition versus control were observed after 3 and 5 days, respectively, at the highest dose (10 μmol L^{-1}). In MDA-MB-231 cells, lower and non-dose dependent effects were observed, and only for the curcuminoids mixture, reaching significant values (around 16% of inhibition; $p < 0.05$) at the highest concentration (10 μmol L^{-1}) after 3 and 5 days (Table S7, Supporting Information).

We also treated both BC cell lines with each mixture at 2.5 and 10 μmol L^{-1} for 2 weeks to test whether longer exposure times affected the clonogenic growth. Notably, the total mixture showed a significant dose-dependent decrease in the colony formation capacity in MCF-7 cells, compared to the control cells, at 2.5 (65%; $p < 0.05$) and 10 μM (80%; $p < 0.05$) (Figure S1A,C, Supporting Information), whereas no effect in colony formation was observed in MDA-MB-231 cells (Figure S1B,D, Supporting Information). The effect of the total mixture was mainly due to the presence of curcuminoids since this mixture exerted a high and dose-dependent reduction ($p < 0.05$) in colony formation (over 90% and 95%, respectively) in the MCF-7 cells (Figure S1A,C, Supporting Information), whereas this effect was lower in MDA-MB-231 cells (around 70% and 85% at 2.5 and 10 μmol L^{-1}, respectively) (Figure S1B,D, Supporting Information). No anti-clonogenic effect was observed for the rest of the mixtures at the doses tested against both BC cell lines (Figure S1, Supporting Information).

We next evaluated whether the observed reduction in the clonogenic effect of both curcuminoids and total metabolites was mediated via cell cycle regulation, apoptosis induction, and(or) cellular senescence induction in both BC cell lines using the highest dose (10 μmol L^{-1}) after 5 days of treatment. Despite the absence of phenotypic effects, we also run the experiments with the rest of the representative mixtures to discard possible early molecular events.

3.5. Effect of Representative Mixtures on Apoptosis Induction

Only the curcuminoids mixture increased the number of early apoptotic cells (twofold; $p < 0.05$) in the MCF-7 cell line, and the late apoptotic cells in both BC cell lines when compared to control cells (around threefold and twofold; in MCF-7 and MDA-MB-231 cells, respectively; $p < 0.05$) (Figure S2, Supporting Information). However, the rest of the treatments did not exert a statistical apoptosis induction (Figure S2, Supporting Information).

3.6. Effect of Representative Mixtures on Cell Cycle Status

In line with the clonogenic assay data, the cell cycle distribution was affected in both BC cell lines, especially in MCF-7 cells, after the treatment with the curcuminoids mixture. In MCF-7 cells, the curcuminoid mixture significantly arrested the cycle at the G_2/M phase ($p < 0.05$), concomitant with a decrease in G_0/G_1 and(or) S phases ($p < 0.05$), compared to the control cells (Figure S3A, Supporting Information). In the case of MDA-MB-231 cells, a lower but significant arrest was also observed in the G_2/M phase ($p < 0.05$) (Figure S3B, Supporting Information). Similarly, the total mixture significantly increased (around 10%) the percentage of cells in the G_2/M phase ($p < 0.05$) in MCF-7 cells (Figure S3A, Supporting Information), but not in MDA-MB-231 cells (Figure S3B, Supporting Information). Finally, no effects on cell cycle distribution were observed for the rest of the treatments at the doses and time tested (Figure S3, Supporting Information).

3.7. Effect on Cellular Senescence Induction and Senescence-Associated Proteins

To assess whether the anti-clonogenic effect, as well as the cell cycle arrest, by both curcuminoids and total phenolic mixture, was associated with cellular senescence induction, we determined the SA-β-Gal activity at 10 μmol L^{-1} after 3 and 5 days of treatment. The treatment of MCF-7 cells with the curcuminoid mixture significantly increased β-Gal activity, compared to the control cells (1.6 ± 0.2-fold and 1.9 ± 0.3-fold; $p < 0.05$, after 3 and 5 days, respectively, Figure S4A, Supporting Information). However, no significant cellular senescence induction was observed at the concentrations investigated with the rest of the treatments (Figure S4A, Supporting Information). On the other hand, as expected, no cellular senescence induction was observed in MDA-MB-231 cells with any mixture treatment at any dose and time tested (Figure S4B, Supporting Information).

Next, we attempted to identify the molecular mechanisms involved in the induction of cellular senescence in MCF-7 cells by analyzing p53 and $p21^{Cip1/Waf1}$, two well-known early senescence-associated molecular markers. Western-blot analyses showed that p53 was only significantly increased in the MCF-7 cells (wild-type p53), but not in the MDA-MB-231 (mutant-type p53) after 5 days of treatment with the curcuminoids mixture (1.7 ± 0.1-fold at 10 μM after 5 days; $p < 0.05$) (**Figure 3**).

Besides, concomitant with the increase of p53, a significant increase of $p21^{Cip1/Waf1}$ was also observed (1.9 ± 0.1-fold) (Figure 3A). The total mixture also significantly increased $p21^{Cip1/Waf1}$ levels, although to a lower extent than the curcuminoids mixture (1.3 ± 0.1-fold, after 5 days; $p < 0.05$) (Figure 3A). On the other hand, none of the mixtures induced significant changes in p53 or $p21^{Cip1/Waf1}$ protein levels in the p53-null MDA-MB-231 cells, except in the case of the curcuminoids mixture that reached a statistically significant increase of $p21^{Cip1/Waf1}$ (1.4 ± 0.2-fold at 10 μmol L^{-1} after 5 days, $p < 0.05$) (Figure 3B).

3.8. Estrogenic and (or) Antiestrogenic Effects

Once we confirmed the concentrations assayed (2.5 and 10 μmol L^{-1}) were not cytotoxic for the representative mixtures tested on the ER+ MCF-7 cells, except for the curcuminoids mixture (Table S7, Supporting Information), we next evaluated the estrogenic/antiestrogenic activities exerted by the five representative

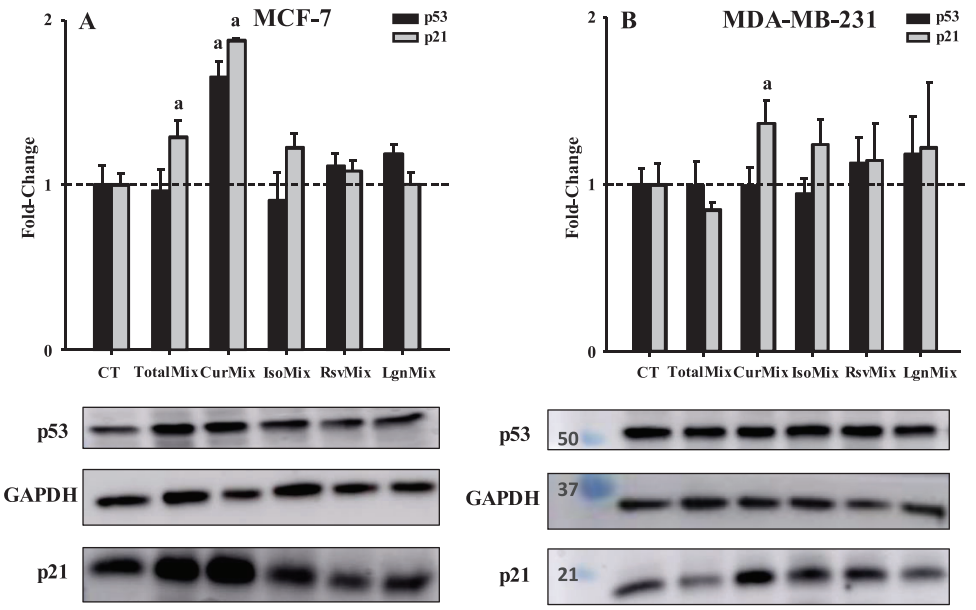

Figure 3. Determination of p53 and $p21^{Cip1/Waf1}$ using protein lysates from A) MCF-7 cells and B) MDA-MB-231 cells after 5 days of treatment at 10 μmol L^{-1} of representative mixtures of total phenolics (TotalMix) and specific family compounds (curcuminoids (CurMix), isoflavones (IsoMix), resveratrol (RsvMix), and lignans (LgnMix)). Glyceraldehyde-3-phosphatedehydrogenase (GAPDH) was used as loading control. Values are expressed as mean ± SD ($n = 3$). [a]Significant difference ($p < 0.05$) compared to control cells.

mixtures (2.5 and 10 μmol L^{-1}) using the E-Screen assay in ER+ MCF-7 cells.

As expected, 17β-estradiol treatment (1 nmol L^{-1}) induced significant cell proliferation (around 2.8-fold change; $p < 0.05$) over control cells (Figure S5, Supporting Information). The treatments with the mixtures for 7 days in the absence of 17β-estradiol in an estrogen-free medium led to determine their estrogenic activities measuring as the induction-fold of cell proliferation compared to the non-treated (control) (Figure S5A, Supporting Information). Estrogenic data showed a dose-dependent significant induction of cell proliferation compared to control cells for both the total mixture (2.2-fold and 2.5-fold change at 2.5 and 10 μmol L^{-1}, respectively; $p < 0.05$) and isoflavones mixture (2.5-fold and 3.7-fold change at 2.5 and 10 μmol L^{-1}, respectively; $p < 0.05$). Besides, a lower but significant increase was also observed for curcuminoids and lignans (1.7-fold in both cases; $p < 0.05$), but only at the highest dose assayed (10 μmol L^{-1}). However, it should be taken into account that this lower effect, mainly for the curcuminoids mixture, could also be explained by the antiproliferative effect observed, mainly at 10 μmol L^{-1}. On the contrary, the resveratrol conjugates mixture at 2.5 or 10 μmol L^{-1} did not increase MCF-7 cells proliferation compared to control cells, suggesting no estrogenic effect at the doses tested (Figure S5A, Supporting Information).

Regarding the antiestrogenic activity of each representative mixture, measured as the prevention of the 17β-estradiol-induced cell proliferation, no effects were observed for most treatments, showing similar increased cell proliferation data to control cells in the presence of 17β-estradiol (Figure S5B, Supporting Information). Only the treatments with the curcuminoids mixture showed a significant dose-dependent decreased in cell proliferation (23 and 41.3% at 2.5 and 10 μmol L^{-1}, respectively; $p < 0.05$), as well as, a significant decrease (21%; $p < 0.05$) for the lignans mixture, but only at the highest concentration assayed (10 μmol L^{-1}) (Figure S5B, Supporting Information). Like the estrogenic effect, the lower cell proliferation observed for the curcuminoid mixture could be partly explained by its antiproliferative effect (Table S7, Supporting Information).

4. Discussion

The evaluation of dietary polyphenols' disposition and metabolic profiling in malignant mammary tissues (MT) from breast cancer (BC) patients could help establish a possible direct causality between the anticancer effects upon dietary polyphenol supplementation and those metabolites that reach MT. However, only a few studies have addressed this issue.[17–20] This approach is challenging due to i) the high interindividual variability in the human polyphenol metabolism, ii) the dissimilar bioavailability, pharmacokinetics, and metabolism of the different polyphenols assayed, including the different turnover and clearance for each metabolite, iii) the different amount of polyphenols consumed in the human diet, iv) the analytical procedures to detect and quantify polyphenols and derived metabolites with high precision and accuracy, and v) the (limited) availability of authentic standards to quantify physiologically relevant metabolites.[34]

In the present study, we describe for the first time the occurrence of metabolites derived from curcuminoids, isoflavones, and lignans in MT from BC patients. Besides, we have answered crucial open questions previously raised by Ávila-Gálvez et al.[20]

dealing with the effect of pre-surgery fasting of patients on the occurrence of metabolites in MT and the long-term anticancer effect of the MT-occurring metabolites on p53-wild and -mutant breast cancer cells. We also assayed here resveratrol for comparative purposes. In this regard, the administration of polyphenols a few hours (2–6 h) before the surgery increased around fourfold the concentration of resveratrol-derived metabolites in MT, NT, and plasma (from 3.2-fold higher in MT for dihydroresveratrol 3-*O*-glucuronide to 4.7-fold higher in MT for resveratrol 3-*O*-sulfate) compared to the concentrations detected after 10–12 h of pre-surgery fasting in the previous study.[20] Therefore, we assume the present trial design also increased the metabolites' concentration derived from the rest of the polyphenols assayed.

The metabolic profile in plasma, urine, MT, and NT revealed mostly phase-II conjugated metabolites, although the percentages varied. In plasma and urine, 99.3% phase-II metabolites versus 0.7% free metabolites and 99.9% versus 0.1% were detected, respectively, while 86.3% versus 13.7%, and 82% versus 18% were observed in NT and MT, respectively. Since chemical hydrolysis associated with the extraction protocol was discarded, the higher presence of free forms in MT and NT could be due to glucuronidase-catalyzed deconjugations.[35–38] It is known that the in situ conjugation/deconjugation might depend on the tissue microenvironment and has been suggested in different contexts and for different metabolites.[39] For example, Patel et al.[40] suggested that resveratrol-sulfates could serve as an intracellular reservoir for the generation of free resveratrol. In our study, the above percentages of conjugated/free metabolites were affected by the relatively high LOQ values for some metabolites. This could be the case, as previously reported, for free resveratrol and their conjugated metabolites[20] or also genistein 7-*O*-glucuronide and curcumin glucuronide. However, while glucuronidase treatments greatly increased free genistein concentration in the tissues, this was not observed in curcumin.

Curcumin is one of the most promising polyphenols to help combat cancer,[41,42] but the metabolism associated with curcumin is complex,[43] which hampers a causal relationship between anticancer effects and curcumin metabolites.[23] Recently, several outstanding studies have revealed that unlike inactive curcumin glucuronide, free bioactive curcumin exerts bone-protective effects upon deconjugation of curcumin glucuronide by glucuronidase-catalyzed reactions in animal and cell models.[38,44,45] Indeed, curcumin glucuronide has been acknowledged as the primary circulating metabolite in rodents and humans.[44] However, although the LOD values in our present study were in the order of those previously published for curcumin and curcumin glucuronide,[38] we did not detect curcumin glucuronide in most patients, neither after glucuronidase treatment, despite we observed curcumin glucuronide deconjugation ex vivo and in vitro. Therefore, our results suggest that curcumin was hardly glucuronidated in the patients, supporting that curcumin glucuronide did not reach MT or NT. Although more research is needed, we hypothesize that the administration of the 20 different polyphenols could have saturated the glucuronidation process as previously suggested.[46,47] Besides, the amount of curcumin ingested by the BC patients (364.5 mg in three capsules; 243 mg in the two capsules taken the morning of the surgery) was significantly lower than that administered to mice (human equivalent dose of 2.5 g)[44] or colorectal cancer patients (2 g) where circulating curcumin glucuronide was clearly identified.[41] The relatively low curcumin dose consumed by the BC patients (low substrate concentration for glucuronyl transferases), together with the possible saturation by the rest of polyphenols' coadministration, could have favored the absorption and disposition of free curcumin in MT and NT. Remarkably, free curcumin was also detected in plasma but at a much lower concentration than in MT and NT. This issue also deserves further research because it could indicate a possible accumulation of free curcumin in mammary tissues, which could be relevant upon long-term consumption of curcumin.

In our previous study, the MT-occurring metabolites did not exert any antiproliferative effect in breast cancer cells after short-term exposure, mainly due to the low activity of phase-II conjugates.[20] However, we further observed that phase-II resveratrol metabolites, mainly resveratrol 3-*O*-sulfate, as found in MT from BC patients, exerted long-term tumor-senescent chemoprevention through p53/$p21^{Cip1/Waf1}$ and $p16^{INK4a}$/Rb pathways in p53-wild but not p53-mutant breast cancer cells.[22] However, this effect was observed only upon incubation of metabolites individually. Remarkably, the co-incubation of various resveratrol metabolites abolished the effect because of competition for cell ABC transporters.

In the present study, we confirmed the same results with the mixture of resveratrol metabolites, which showed neither short-term nor long-term anticancer effects, that is, cytotoxic, apoptotic, senescent, or estrogenic/antiestrogenic effects. Enterolignan and isoflavone mix only exerted some estrogenic/antiestrogenic effects, especially the isoflavone mix, which could be relevant in the anti-estrogen therapy for ER+ tumors.[12] Although phase-II conjugation has been reported to abolish the activity of the antioestrogen tamoxifen,[48,49] however, we observed such effects in an isoflavone mixture with a predominance of conjugates (63%), mainly daidzein 4'-*O*-glucuronide (42.5%), which also supports the findings of a recent study.[50] In contrast to the other mixes, with discrete activities, the curcuminoid mix exerted significant anti-clonogenic, apoptotic, cell cycle arrest, and senescence-induction effects, mainly in p53-wild MCF-7 cells through p53 and $p21^{Cip1/Waf1}$ induction. These effects were likely due to the presence of free curcumin as found in breast tissues since similar mechanisms have been previously reported for curcumin.[42,51,52]

The present pilot trial has some limitations. Our results illustrate the potential anticancer activity exerted by phenolic-derived metabolites that reached MT from BC patients. We assayed two BC cell lines (p53-wild and p53-mutant), but this was just an example to explore the anticancer effects since we are aware of the high heterogeneity of human breast cancers that cannot be mimicked with just two cell models. Besides, we acknowledge that the limited availability of authentic standards, mainly phase-II conjugates, led us to identify several metabolites tentatively and prevented their quantification. Consequently, other possible relevant metabolite mixes that reached MT were not assayed in the present study. We also acknowledge that other BC cell models and mechanisms of action remain to be explored. Besides, we used a previously sensitive and validated UPLC-QTOF-MS method to screen a rich phenolic-derived metabolic profile in a complex matrix such as MT.[20] However, a few metabolites showed relatively

high LOD and LOQ values, most likely due to tissue-matrix effects, which probably hampered their quantification.

Our results show that a complex mixture of phenolic-derived metabolites reached the MT from BC patients and suggest that some metabolites, especially MT-occurring free curcumin, might exert anticancer effects upon long-term exposure. The present study also suggests that the simultaneous and repeated oral administration of polyphenols might disturb or even disrupt their own metabolism, as suggested elsewhere.[24,47,53] As commented above, epidemiological studies failed to correlate clearly the binomial disease-dietary polyphenols. Indeed, the specific dose of either the combination or specific (poly)phenols required to exert the effects remains elusive and prevents the precise link between polyphenols and the effects observed.[34] Our previous results, combined with the present study, suggest that taking a significant amount of a complex mixture of polyphenols could be translated into a somewhat paradoxical situation. On the one hand, the presence of relevant concentrations of many phase-II metabolites could result in competition and lower activity instead of synergy through competition for transporters and(or) receptors. We confirm here our previous study in which phase-II resveratrol metabolites exerted senescence in p53-wild cells individually, but not when the breast-tissues occurring resveratrol mix was assayed.[22] On the other hand, our results also suggest the consumption of high amounts of a wide variety of polyphenols could saturate glucuronidation processes, which could allow some dietary phenolics to reach systemic tissues in their free, much more bioactive forms. This could be the particular case of curcumin.

Overall, this is a puzzle for the recommendations on the consumption of polyphenols in the diet and contributes to the great interindividual variability that might prevent conclusive evidence on cancer prevention (including BC) by dietary polyphenols.

Supporting Information

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

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

J.C.E. conceived and designed the study; M.A.A.-G., B.A., and A.J.F.-L. recruited the patients; B.A., and A.J.F.-L. provided the resected tissues; F.M.-D. and A.J.M.-T. performed the clinical pathological analysis of the mammary tissues; M.A.A.-G., J.A.G.-B., and A.G.-S., performed the experiments and analyzed the data; J.C.E. wrote the manuscript; All the authors critically reviewed the manuscript.

Keywords

breast cancer, clinical trial, curcumin, isoflavone, lignan, resveratrol

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