Phenolic Secoiridoids in Extra Virgin Olive Oil Impede Fibrogenic and Oncogenic Epithelial-to-Mesenchymal Transition: Extra Virgin Olive Oil As a Source of Novel Antiaging Phytochemicals

Alejandro Vazquez-Martin,^{1,2,*} Salvador Fernández-Arroyo,^{3,*} Sílvia Cufí,^{1,2,*}
Cristina Oliveras-Ferraro,^{1,2,*} Jesús Lozano-Sánchez,^{3,*} Luciano Vellón,⁴ Vicente Micol,⁵
Jorge Joven,⁶ Antonio Segura-Carretero,^{3,†} and Javier A. Menéndez^{1,2,†}

Abstract

The epithelial-to-mesenchymal transition (EMT) genetic program is a molecular convergence point in the life-threatening progression of organ fibrosis and cancer toward organ failure and metastasis, respectively. Here, we employed the EMT process as a functional screen for testing crude natural extracts for accelerated drug development in fibrosis and cancer. Because extra virgin olive oil (EVOO) (*i.e.*, the juice derived from the first cold pressing of the olives without any further refining process) naturally contains high levels of phenolic compounds associated with the health benefits derived from consuming an EVOO-rich Mediterranean diet, we have tested the ability of an EVOO-derived crude phenolic extract to regulate fibrogenic and oncogenic EMT *in vitro*. High-performance liquid chromatography (HPLC) coupled to time-of-flight (TOF) mass spectrometry assays revealed that the EVOO phenolic extract was mainly composed (~70%) of two members of the secoiridoid family of complex polyphenols, namely oleuropein aglycone—the bitter principle of olives—and its derivative decarboxymethyl oleuropein aglycone. EVOO secoiridoids efficiently prevented loss of proteins associated with polarized epithelial phenotype (*i.e.*, E-cadherin) as well as *de novo* synthesis of proteins associated with mesenchymal migratory morphology of transitioning cells (*i.e.*, vimentin). The ability of EVOO to impede transforming growth factor- β (TGF- β)-induced disintegration of E-cadherin-mediated cell-cell contacts apparently occurred as a consequence of the ability of EVOO phenolics to prevent the upregulation of SMAD4—a critical mediator of TGF- β signaling—and of the SMAD transcriptional cofactor SNAIL2 (Slug)—a well-recognized epithelial repressor. Indeed, EVOO phenolics efficiently prevented crucial TGF- β -induced EMT transcriptional events, including upregulation of *SNAIL2*, *TCF4*, *VIM* (Vimentin), *FN* (fibronectin), and *SERPINE1* genes. While awaiting a better mechanistic understanding of how EVOO phenolics molecularly shut down the EMT differentiation process, it seems reasonable to suggest that nontoxic *Oleaceae* secoiridoids certainly merit to be considered for aging studies and, perhaps, for ulterior design of more pharmacologically active second-generation anti-EMT molecules.

¹Catalan Institute of Oncology, Girona, Catalonia, Spain.

²Girona Biomedical Research Institute, Girona, Catalonia, Spain.

³Department of Analytical Chemistry, Faculty of Sciences, University of Granada, Granada, Spain.

⁴Fundación INBIOMED, Cell Reprogramming Unit, San Sebastián. Basque Country, Spain.

⁵Molecular and Cellular Biology Institute (IBMC), Miguel Hernández University, Elche, Alicante, Spain.

⁶Centre de Recerca Biomèdica, Hospital Universitari Sant Joan de Reus, Institut d'Investigació Sanitària Pere Virgili, Universitat Rovira i Virgili, Reus, Catalonia, Spain.

*These authors have contributed equally to this research and are listed in random order.

†Co-senior authors.

Introduction

A DEREGULATED EPITHELIAL-TO-MESENCHYMAL (EMT) trans-differentiation process (*i.e.*, the generation of motile mesenchymal cells from epithelial sheets)^{1–3} is an ideal molecular scenario to test the notion that “an anti-aging drug must delay age-related diseases in order to extend lifespan (*i.e.*, unless a drug delays age-related diseases, it will not extend lifespan and vice versa, if a drug prevents age-related diseases, it must extend lifespan).”⁴ On the one hand, EMT overactivation is a critical phenomenon in age-related human diseases exhibiting end-state organ fibrosis (*e.g.*, kidney fibrosis in chronic renal disease, liver fibrosis in nonalcoholic steatohepatitis, or myocardial fibrosis in heart failure).^{5–8} The pleiotropic cytokine transforming growth factor- β (TGF- β) is a major regulator of these types of pathophysiological EMT.

Indeed, chronic diseases characterized by excessive fibrosis can be explained in terms of repeated and sustained infliction of TGF- β -induced EMT, which significantly increases synthesis and accumulation of collagen and extracellular matrix (ECM) in the affected organ.^{7,9–11} In this scenario, highly differentiated epithelial cells in the specific organs (*e.g.*, tubular epithelial cells in the kidney) will respond to noxious stimuli by undergoing TGF- β -driven EMT. Then, the transdifferentiated epithelial cells migrate into the interstitial space and some of them will become activated (*i.e.*, myofibroblasts *de novo* expressing mesenchymal markers such as α -smooth muscle cell actin [SMA]) to become the main source of excessive ECM. TGF- β -induced EMT not only constitutes a pivotal mechanism contributing to the supply of activated myofibroblasts in renal fibrosis but it also similarly turns adult hepatocytes into activated fibroblasts, contributing to liver fibrosis in nonalcoholic steatohepatitis.⁸ Spontaneous activation of TGF- β -driven EMT has also been shown to profibrotic responses during myocardial fibrosis in aged mice hearts.¹²

While end-stage renal disease will need lifelong renal replacement therapy with maintenance dialysis or kidney transplantation, it is also noteworthy that renal transplant recipients frequently start or restart dialysis because of the unusual propensity of these allografts to develop interstitial fibrosis and tubular atrophy as tubular epithelial cells react to certain fibrogenic stimuli to (re)engage in the process of EMT.^{13–16} Fibrosis, ranging from mild inflammation to severe sclerosing peritonitis and encapsulating sclerosing peritonitis, is also responsible for negative changes in the peritoneal membrane; this preservation is crucial for long-term survival in peritoneal dialysis. Glucose and glucose degradation products stimulate the profibrotic factor TGF- β by mesothelial cells and induce EMT, the pivotal triggering mechanism of peritoneal membrane fibrosis in peritoneal dialysis patients.^{17–19} Accordingly, the quality of life on renal replacement therapy is much impaired, and the life expectancy of these patients is substantially shorter when compared to the general population.²⁰

On the other hand, the EMT developmental process has gained much attention in oncology because some aspects of embryonic EMT are instrumentally employed by human cancer cells to facilitate metastasis. Tumor cells undergoing the EMT acquire the capacity to migrate and invade the surrounding stroma; they subsequently spread via blood and lymphatic vessels to repopulate distant sites as metas-

tases.^{21–25} More importantly, it has recently been discovered that tumor cells undergoing EMT acquire stem cell-like characteristics, thus showing a link between EMT and pathways involved in promoting cellular stemness.^{26–31} Induction of nontumorigenic, immortalized human mammary epithelial cells to the EMT phenotype likewise results in the loss of the epithelial phenotype (*e.g.*, downregulation and relocation of the epithelial marker E-cadherin) and the acquisition of the mesenchymal phenotype (*e.g.*, gain of the mesenchymal marker vimentin) concomitant with the acquisition of the CD44^{high}/CD24^{low} immunophenotype, a molecular signature associated with stem cells and increased tumor-initiating capacity in breast cancer disease.^{26,31} Because EMT-phenotypic tumor cells acquire stem-like cell signatures characterized by increased metastatic capacity and self-renewal ability, the stem-like cells or cancer stem cells (CSCs) generated from (*e.g.*, TGF- β driven) EMT induction provide a resource for cancer to recur because these cells are well known to be highly drug resistant.^{32–35}

A definitive understanding of the cellular and molecular events driving excessive fibrosis causally underlying end-state organ failures is crucial to designing effective and specifically targeted therapeutic interventions aimed to impede “fibrogenic EMT.” Similarly, a better molecular understanding and biological characterization of EMT phenotypic cells, cancer stem-like cells, and CSCs should allow us to screen for potential drugs that could cause selective killing of these cells that are the “root cause” of tumor development, maintenance, recurrence, and metastasis.^{36–38} Indeed, it is reasonable to suggest that molecular characterization of agents able to impede the generation of EMT-phenotypic tumor cells (*i.e.*, “oncogenic EMT”) will allow not only the development of newer therapies for complete eradication of tumors, which will certainly improve the overall survival of patients diagnosed with cancers, but also to ameliorate progressive tissue fibrosis in various age-related chronic diseases.^{2,39}

Supporting the hypothesis that EMT-focused studies can promise the generation of new anticancer and antifibrosis drugs, it should be noted that the gerosuppressant drug rapamycin (*i.e.*, a blocker of the nutrient-sensing mammalian target of rapamycin [mTOR] pathway that, by slowing down organismal aging, efficiently delays cancer)^{40–42} has been found to significantly enhance the expression of a protective gene for EMT (*i.e.*, E-cadherin) to impede TGF- β -induced EMT in cultured human peritoneal mesothelial cells.^{43,44} Similarly, the gerosuppressant agent metformin appears to molecularly behave as a *bona fide* antiaging modality owing its ability to prevent TGF- β -driven EMT in cultured Madin-Darby canine kidney (MDCK) cells (an *in vitro* mouse system model to study the critical involvement of the EMT phenomenon in renal fibrosis) and in cultured MCF-7 breast cancer epithelial cells (an *in vitro* human system model to study the loss of E-cadherin expression/function as the central molecular process required for EMT-driven acquisition of malignant and stem cell traits because post-EMT MCF-7/TGF- β mesenchymal phenotypic cells display increased tumorsphere-initiating stem cell-like features compared to their pre-EMT parental MCF-7 epithelial phenotypic cells).^{45,46} Accordingly, metformin treatment decreases both the self-renewal and the proliferation of breast CSC populations and efficiently prevents EMT-promoted ontogenesis

of the breast CSC molecular signature because it ablates the ability of TGF- β to increase the population of breast cancer cells that can form mammospheres in suspension—a feature endowed by CSCs.^{47–50}

Because crude phenolic extracts directly obtained from extra virgin olive oil (EVOO) and naturally enriched in secoiridoids (*i.e.*, a family of complex polyphenols characteristics of *Oleaceae* plants)^{51–54} can efficiently attack drug-resistant EMT-type breast cancer cells intrinsically enriched with CSC-like phenotypes,^{55,56} we recently envisioned that EVOO phenolics can negatively impact TGF- β -triggered fibrogenic and oncogenic EMT. Here we reveal for the first time that an EVOO-derived crude phenolic fraction is sufficient to efficiently impede fibrogenic and oncogenic EMT in cultured MDCK and MCF-7 cells, respectively. Our current study strongly suggests that *Oleaceae* secoiridoids might constitute a novel valuable phytochemical platform for the discovery of previously unrecognized antiaging biomolecules.

Materials and Methods

Olive oil

The EVOO employed in this study was from the mono-variety Picual obtained from Córdoba (Andalusia, Spain) in 2008. Picual olives were processed by continuous industrial plants equipped with a hammer crusher, a horizontal malaxator, and a two-phase decanter. Samples were stored in bottles without headspace at room temperature and in darkness before analysis.

Isolation of EVOO phenolic fraction

To isolate the phenolic fraction of Picual EVOO, we used solid-phase extraction (SPE) with Diol-cartridges. In all, 60 grams of Picual EVOO was dissolved in methanol and loaded onto the column. The cartridge was washed with 15 mL of hexane, which was then discarded to remove the nonpolar fraction of the EVOO. Finally, the sample was recovered by passing it through 40 mL of methanol; the solvent was evaporated under vacuum. The residue was dissolved with 2 mL of methanol and filtered through a 0.25-mm filter before characterization of the phenolic profile.

Qualitative and quantitative characterization of phenolic compounds

To characterize the phenolic profile in the Picual EVOO phenolic extract, rapid resolution liquid chromatography (RRLC) coupled to electrospray interface time-of-flight mass spectrometry (ESI-TOF-MS) was performed in an Agilent 1200-RRLC system (Agilent Technologies, Waldbronn, Germany) of the Series Rapid Resolution equipped with a vacuum degasser, an autosampler, a binary pump, and a UV-Vis detector. The chromatographic separation was carried out on a Zorbax Eclipse Plus C18 analytical column (4.6 mm $\times$ 150 mm, 1.8- μ m particle size). The flow rate was 0.80 mL/min, and the temperature of the column was maintained at 25°C. The mobile phases used were water with 0.25% acetic acid as eluant A and methanol as eluant B. The total run time was 27 min.⁵³ Compounds were monitored in sequence first with diode array detection (DAD; 240 and 280 nm) and then with a mass analyzer (MA) detector. MS was performed using the microTOF (Bruker Daltonik, Bre-

men, Germany), which was coupled to the RRLC system. At this stage, the use of a splitter was required to the coupling with the MS detector because the flow that arrived to the TOF detector had to be 0.2 mL/min to obtain reproducible results and stable spray. The TOF mass spectrometer was equipped with an ESI (model G1607A from Agilent Technologies, Palo Alto, CA) operating in negative ion mode. External mass spectrometer calibration was performed with sodium formate clusters (5 mM sodium hydroxide in water / 2-propanol 1/1 [vol/vol], with 0.2% of formic) in quadratic high-precision calibration (HPC) regression mode.

The calibration solution was injected at the beginning of the run, and all of the spectra were calibrated prior to identification of EVOO polyphenols. The optimum values of the source and transfer parameters were get for a good sensitivity and reasonable resolution of the mass range for compounds of interest (50–1000 *m/z*) to improve ionization performance.⁵³ The accurate mass data for the molecular ions were processed using the software Data Analysis 3.4 (Bruker Daltonik), which provided with a list of possible elemental formulas by using the Generate Molecular Formula Editor. The latter employs a CHNO algorithm that provides standard functionalities such as minimum/maximum elemental range, electron configuration, and ring-plus double bonds equivalent, as well as a sophisticated comparison of the theoretical with the measured isotopic pattern (sigma value) for increased confidence in the suggested molecular formula. The widely accepted accuracy threshold for confirmation of elemental compositions has been established at 5 ppm for most of the compounds.

The individual quantification of the identified phenolic compounds was carried out by RRLC-ESI-TOF using the validated method described above.⁵³ Ten standard calibration graphs for the quantification of the principal compounds found in the samples were prepared using the following commercial available standards. Hydroxytyrosol, tyrosol, vanillin, luteolin, apigenin, *p*-coumaric acid, ferulic acid, and vanillic acid were purchased from Sigma-Aldrich (St. Louis, MO), and (+)-pinosresinol was acquired from Arbo Nova (Turku, Finland). Oleuropein (Ole) was purchased from Extrasynthèse (Lyon, France).

As complementary information, the total phenolic content of the crude EVOO phenolic extract was determined by a spectrophotometric method based on the Folin–Ciocalteu technique.⁵⁷ The absorbance of the solution was measured at a wavelength of 725 nm in a Spectronic Genesys™ 5 spectrophotometer (Spectronic Instruments Inc. Rochester, NY). The extracts were diluted 1:10 with methanol. After this, a 50- μ L aliquot of the diluted methanolic extract of EVOO was used in this determination. Total polyphenols were expressed as caffeic acid equivalents. A calibration curve of freshly prepared caffeic acid solution was carried out. Three replicates of each analysis and for each calibration point were performed to obtain reproducible results.

Cell lines and culture conditions

MDCK cells were obtained from Dr. Manel Esteller (Cancer Epigenetics and Biology Program-PEBC, Bellvitge Biomedical Research Institute-IDIBELL, L'Hospitalet, Barcelona, Spain) and cultured in Dulbecco modified Eagle medium (DMEM; BioSource International; Invitrogen S.A., Barcelona, Spain) supplemented with 10% fetal bovine

TABLE 1. MAIN PHENOLIC COMPOUNDS IDENTIFIED IN EXTRA VIRGIN OLIVE OIL PHENOLIC EXTRACT BY HPLC-DAD-ESI-TOF

Number	Proposed compound	Retention time (min)	[M-H]-	Molecular formula	Structure
1	Hydroxytyrosol	8	153.0557	C ₈ H ₁₀ O ₃
2	Tyrosol	9,9	137.0608	C ₈ H ₁₀ O ₂
3	Vanillin	11,7	151.0401	C ₈ H ₈ O ₃
4	<i>p</i> -Coumaric acid	13,5	163.0401	C ₉ H ₈ O ₃
5	Hydroxytyrosol acetate	14	195.0663	C ₁₀ H ₁₂ O ₄
6	Elenolic acid	15	241.0718	C ₁₁ H ₁₄ O ₆
7	Hydroxy elenolic acid	15,4	257.0667	C ₁₁ H ₁₄ O ₇
8	Decarboxymethyl oleuropein aglycon	16,3	319.1187	C ₁₇ H ₂₀ O ₆

(continued)

TABLE 1. (CONTINUED)

Number	Proposed compound	Retention time (min)	[M-H]-	Molecular formula	Structure
9	Hydroxy D-oleuropein aglycon	16,6	335.1136	C ₁₇ H ₂₀ O ₇
10	Syringaresinol	18,2	417.1555	C ₂₂ H ₂₆ O ₈
11	Pinoresinol	18,8	357.1344	C ₂₀ H ₂₂ O ₆
12	Decarboxymethyl ligstroside aglycon	19,2	303.1229	C ₁₇ H ₂₀ O ₅

(continued)

TABLE 1. (CONTINUED)

Number	Proposed compound	Retention time (min)	[M-H] ⁻	Molecular formula	Structure
13	Hydroxy D-ligstroside aglycon	19,9	319.1187	C ₁₇ H ₂₀ O ₆	
14	10-Hydroxy oleuropein aglycon	23	393.1191	C ₁₉ H ₂₂ O ₉	
15	Oleuropein aglycon	23,2	377.1242	C ₁₉ H ₂₂ O ₈	
16	Luteolin	23,7	285.0405	C ₁₅ H ₁₀ O ₆	
17	Methyl D-oleuropein aglycon	25,4	333.1344	C ₁₈ H ₂₂ O ₆	

+ Me

(continued)

TABLE 1. (CONTINUED)

Number	Proposed compound	Retention time (min)	[M-H] ⁻	Molecular formula	Structure
18	Ligstroside aglycon	25,6	361.1293	C ₁₉ H ₂₂ O ₇	
19	Apigenin	25,8	269.0451	C ₁₅ H ₁₀ O ₅	
20	Methyl oleuropein aglycon	26,2	391.1398	C ₂₀ H ₂₄ O ₈	

serum (FBS), L-glutamine (2 mmol/L), and antibiotics (all from Biowhittaker, Madrid, Spain). MCF-7 human breast cancer cells were obtained from the American Type Culture Collection (ATCC) and they were routinely grown in improved MEM (IMEM) supplemented with 5% FBS and L-glutamine (2 mmol/L). Cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Cells were screened periodically for *Mycoplasma* contamination.

TGF-β1

Recombinant human (carrier-free) TGF-β1 was purchased from R&D Systems Inc. (Minneapolis, MA).

Immunofluorescence staining and high-content confocal imaging

Cells were seeded at approximately 5,000 cells/well in 96-well, clear-bottomed imaging tissue culture plates (Becton Dickinson Biosciences; San Jose, CA) optimized for automated imaging applications. Triton X-100 permeabilization and blocking, primary antibody staining (1:50 dilution), secondary antibody staining using Alexa Fluor 488/594 goat anti-rabbit/mouse immunoglobulin Gs (IgGs) (Invitrogen, Molecular Probes, Eugene, OR), and counterstaining (using

Hoechst 33258; Invitrogen) were performed by following BD Biosciences protocols. Images were captured in different channels for Alexa Fluor 488 (pseudocolored green) and Hoechst 33258 (pseudocolored blue) on a BD Pathway™ 855 Bioimager System (Becton Dickinson Biosciences, San Jose, CA) with 20× or 40× objectives (NA 075 Olympus). Merged images were obtained according to the recommended assay procedure using BD Attovision™ software.

Quantitative, real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from MCF-7 cell cultures supplemented with TGF-β (100 ng/mL), EVOO phenolics, or TGF-β plus EVOO phenolics as specified using a Qiagen RNeasy kit and QIAshredder columns according to the manufacturer's instructions (Qiagen, Valencia, CA). One microgram of total RNA was reverse-transcribed to cDNA using the Reaction Ready™ First Strand cDNA Synthesis Kit (SABiosciences, Frederick, MD) and applied to EMT PCR Arrays (cat. no. PAHS-090; 96-well format) following SABiosciences RT-PCR manual. Plates were processed in an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA), using automated baseline

and threshold cycle detection. Data were interpreted by using SABiosciences' web-based PCR array analysis tool.

Results

Qualitative and quantitative characterization of phenolic compounds in EVOO phenolic extract was performed in a previously reported analytical approach.⁵³ Identification of phenolic compounds was performed by comparing both retention times and MS spectral data from EVOO methanolic extract and standards, as detailed in Materials and Methods. The remaining compounds, for which no commercial standards were available, were characterized by interpretation of their ultraviolet (UV) absorbance maxima, mass spectra provided by the TOF-MS, and other analytical information reported elsewhere because most of these compounds have been previously described in OO samples. Table 1 summarizes the main compounds identified in the Picual EVOO variety, including their structure and the information gen-

erated by TOF analyzer—retention time, calculated m/z , and molecular formula. Twenty compounds from different families (*i.e.*, simple phenols, flavonoids, lignans, and secoiridoids) were identified. Figure 1A shows the resulting chromatogram of EVOO phenolic extract. Several isomers of oleuropein aglycon were tentatively characterized in this sample. This compound and its isomers, for which there are no available commercial standards, have been previously described in OO,⁵⁸ and the accuracy of MS data provided enough confidence during their identification. The analysis of the true isotopic pattern by ESI-TOF-MS in combination with excellent mass resolution and mass accuracy is the perfect choice for molecular formula determination using the Generate Molecular Formula Editor as detailed in Materials and Methods.

Concentrations of individual phenolics were determined using the areas of each individual compound (three replicates) obtained by peak integration in their extracted ion chromatogram (EIC) and by interpolation in the corresponding

FIG. 1. (A) (Left) Base peak chromatogram (BPC) of extra virgin olive oil (EVOO) phenolic extract obtained by high-performance liquid chromatograph–electrospray ionization–time-of-flight mass spectrometry (HPLC-ESI-TOF) with the extracted ion chromatogram (EIC) of the main phenolic compounds. (Right) Percent distribution of main phenolic families identified in Picual EVOO phenolic extract. (B) Percent distribution of identified phenolic compounds in Picual EVOO phenolic extract. The figure shows chemical structures of oleuropein aglycone and its derivative decarboxymethyl oleuropein aglycone, the major components of the Picual EVOO phenolic extract. (Color image available online at www.liebertpub.com/rej)

calibration curve. EIC results of the main phenolic compounds are also shown in Fig. 1A. The phenolic compounds hydroxytyrosol, tyrosol, luteolin, and apigenin were quantified by their respective commercial standards prepared as previously described.⁵³ Other phenolic compounds, which had no commercial standards, were tentatively quantified on the basis of other compounds having similar structures. Secoiridoid and lignan groups were quantified with oleuropein and (+)-pinoresinol standards, respectively. Elenolic acid derivatives, which cannot be considered as true phenolic compounds, were expressed as oleuropein. It should be considered that response of the standards can be different from each one of the EVOO analytes included in the Picual variety, and consequently the quantification of these compounds was only an estimation of their actual concentrations.

Table 2 summarizes the amount of each phenolic from Picual EVOO, with the main components belonging to the secoiridoids group. Among these complex polyphenols, two derivatives of hydroxytyrosol (3,4-DHEPA) linked to the elenolic acid and its decarboxymethylated form, namely oleuropein aglycon (3,4-DHEPA-EA) and decarboxymethyl oleuropein aglycon (3,4-DHEPA-DEA), were the most abundant compounds as they constituted up to 23% and 46% of total phenolics, respectively (Fig. 1B). Regarding other phenolics belonging to the secoiridoid family, the relative contents of the decarboxymethylated form of ligstroside aglycon as well as the hydroxylated and methylated forms of oleuropein aglycon were significantly higher than other phenolic com-

pounds. Simple phenols (*e.g.*, tyrosol, hydroxytyrosol), lignans (*e.g.*, siringaresinol, [+-]pinoresinol), and flavones (*e.g.*, luteolin and apigenin) were notably underrepresented in the phenolic mixture. The average total polyphenols content was 683.579 ± 4.169 expressed as milligrams of analyte/kg of EVOO, as tentatively calculated as the sum of the individual phenolic compound concentrations.

Because an inherent problem with using crude natural extracts in cell-based screens is that many of them are toxic to cells at the dilutions that might generate optimal hit rates because of the high concentrations of salts and other materials they contain, the EVOO phenolic extract was not cytotoxic at tested concentration to avoid unacceptable false-positive results. On the basis of earlier studies using crude EVOO phenolic extracts in human breast cancer cell lines,^{53,54,56} we first confirmed that EVOO phenolics did not significantly alter viability at 200 ng/mL ($\sim 0.01\%$ vol/vol), the concentration employed during 72 hr to test modulation of the EMT process in MDCK and MCF-7 cells.

EVOO phenolics impede "fibrogenic EMT"

We first employed MDCK cells to evaluate the impact of the EVOO-derived crude phenolic extract on several TGF- β -altered EMT parameters including cell morphology. Thus, we established TGF- β -induced EMT and then we measured the response to EVOO phenolics (Fig. 2). Untreated MDCK control cells displayed a typical epithelial-like morphology with respect to their light-microscopic appearance. Upon treatment with TGF- β (50 ng/mL), the cells acquired a spindle-type morphology and a reduced number of cell-cell contacts. These alterations started after 24 hr and they were maximal after 72 hr of treatment, when TGF- β supplementation fully induced a complete conversion of parental MDCK epithelial cells to a fibroblast-like, spindle-shaped, elongated mesenchymal morphology. The changes of morphology were partially reversible when TGF- β treatment was carried out in the presence of the EVOO-derived crude phenolic extract. Whereas EVOO phenolics as sole treatment had no significant effects on EMT-related morphological changes, TGF- β -treated MDCK cells changed from scattered and fibroblast-like shapes in the absence of EVOO phenolics to a more packed cobblestone morphology in their presence. Although MDCK cells co-treated with TGF- β and the EVOO-derived crude phenolic extract failed to maintain entirely the compact cuboidal appearance of parental MDCK control cells, EVOO phenolics notably prevented TGF- β -induced cell scattering and reduced the number of TGF- β -induced spindle-shaped cells and pseudopodia.

Because the above-mentioned findings suggested that exposure to EVOO phenolics triggered molecular changes that were consistent with a partial reversion of the EMT phenomenon, we carried out immunostaining studies to assess the expression status of marker proteins of either the epithelial (*i.e.*, E-cadherin) or the mesenchymal (*i.e.*, vimentin) phenotype in MDCK cell populations (Fig. 2). Compared with untreated MDCK control cells, immunostaining studies showed the reduced expression of E-cadherin in TGF- β 1-treated MDCK cells, especially in the adherens junctions in which the expression of E-cadherin was not detected by immunofluorescence microscopy. The intermediate filament vimentin accumulated profusely in the cytoplasm of TGF- β 1-treated MDCK cells. The

TABLE 2. QUANTITATIVE RESULTS EXPRESSED IN MG ANALYTE/ KG OF PICUAL EXTRA VIRGIN OLIVE OIL PHENOLIC EXTRACT

Phenolic compounds	Concentration	Percent
Simple phenols	14.467 $\pm$ 0.285	2.116
Hydroxytyrosol	9.625 $\pm$ 0.208	1.408
Tyrosol	4.162 $\pm$ 0.109	0.609
Hydroxytyrosol acetate	0.680 $\pm$ 0.003	0.099
Secoiridoids	662.673 $\pm$ 4.782	96.942
Elenolic acid	13.123 $\pm$ 0.125	1.920
Hydroxy elenolic acid	0.097 $\pm$ 0.002	0.014
Oleuropein aglycon	157.917 $\pm$ 1.257	23.102
Decarboxymethyl oleuropein aglycon	314.429 $\pm$ 2.189	45.998
Hydroxy D-oleuropein aglycon	32.373 $\pm$ 1.584	4.736
10-Hydroxy oleuropein aglycon	39.479 $\pm$ 0.806	5.775
Methyl oleuropein aglycon	24.349 $\pm$ 0.516	3.562
Methyl D-oleuropein aglycon	2.903 $\pm$ 0.079	0.425
Ligstroside aglycon	16.261 $\pm$ 0.709	2.379
Decarboxymethyl ligstroside aglycon	53.652 $\pm$ 0.354	7.849
Hydroxy D-ligstroside aglycon	8.087 $\pm$ 0.353	1.183
Lignans	1.587 $\pm$ 0.061	0.232
Pinoresinol	0.815 $\pm$ 0.032	0.119
Syringaresinol	0.772 $\pm$ 0.029	0.113
Flavones	4.852 $\pm$ 0.084	0.710
Luteolin	4.041 $\pm$ 0.065	0.591
Apigenin	0.811 $\pm$ 0.040	0.119
Total phenolic contents	683.579 $\pm$ 4.169	100.000

Value = $\bar{X} \pm$ standard deviation (SD). Percent distribution of identified phenolic compounds.

FIG. 2. Effects of extra virgin olive oil (EVOO) phenolics on transforming growth factor- β (TGF- β)-induced changes in the morphology and expression of epithelial/mesenchymal markers of Madin-Darby canine kidney (MDCK) cells. MDCK cells were grown until 80% confluence, serum-starved for 24 hr, and then treated for 3 days with a minimal amount (0.1%) of fetal bovine serum (FBS) supplemented with vehicle (control [untreated]), TGF- β 1 alone (10 ng/mL), extra virgin olive oil phenolic extract (EVOO-PE) alone (200 ng/mL), or TGF- β 1 plus EVOO-PE. Phase-contrast and immunofluorescence images were obtained and merged on a BD Pathway™ 855 Bioimager System according to the recommended assay procedure using BD Attovision™ software. Images show representative portions of MDCK cultures showing subcellular distribution of vimentin, E-cadherin, and SNAI2 from two to three independent experiments.

EVOO-derived crude phenolic extract significantly prevented TGF- β 1-induced disintegration of E-cadherin at cell-cell contacts. Moreover, EVOO phenolics largely prevented the strong activation of the post-EMT marker vimentin that occurred upon treatment with TGF- β 1. We finally evaluated whether the EVOO-derived crude phenolic extract could impact the expression of EMT-promoting transcription factors such as SNAI2 (Slug), which not only functions as a bona fide epithelial repressor that inhibits *E-cadherin* gene expression through its direct binding to E-boxes within the *E-cadherin* promoter but also interacts with mesenchymal activators such as β -catenin to induce vimentin expression.³⁴ Likewise, SNAI2 protein was significantly upregulated during TGF- β 1-driven mesenchymal conversion of MDCK cells. Of note, the EVOO-derived crude phenolic extract negatively regulated nuclear accumulation of SNAI2 in response to TGF- β 1.

EVOO phenolics impede “oncogenic EMT”

We first confirmed that cellular changes (morphology and size) observed in MCF-7 breast cancer epithelial cells supplemented exogenously with TGF- β 1 resembled those of tumor cells undergoing EMT. Immunoreactivity of the epithelial cell marker E-cadherin was likewise found at sites of cell-cell contact in untreated MCF-7 control cells. This classical expression of E-cadherin in the basolateral membrane of epitheloid cells was significantly altered upon exposure to the

EMT promoter TGF- β 1, which promoted a drastic distribution of E-cadherin from a conspicuous, highly membranous staining to a more diffuse, barely detectable staining in the cytoplasm (Fig. 3). The EVOO-derived crude phenolic extract as sole treatment appeared to significantly accumulate E-cadherin protein expression at sites of cell-cell contact, thus promoting a more cuboidal appearance of MCF-7 cell cultures, which grew in more compact, homogeneously structured monolayers. Of note, TGF- β 1 failed to downregulate membranous E-cadherin when MCF-7 epithelial breast cancer cells were concurrently co-exposed to EVOO phenolics.

To make a preliminary evaluation of whether EVOO phenolics negatively impacted formation and activation of EMT-promoting SMAD complexes (*i.e.*, SMAD proteins [intracellular transcription factors and transducers of TGF- β signaling] have low affinity for DNA and need to interact and form a complex with EMT-promoting transcription factors, including SNAI1 and ZEB, to target the gene promoter of E-cadherin during TGF- β -driven EMT in breast epithelial cells), we employed immunofluorescence microscopy to monitor the expression status of SMAD4, SNAI2, and E-cadherin proteins of MCF-7 epithelial cells cultured with TGF- β 1 in the absence or presence of EVOO phenolics (Fig. 4). TGF- β 1 treatment of MCF-7 cells for 72 hr resulted in enhanced expression of SMAD4 and SNAI2, which paralleled loss of E-cadherin at intercellular junctions. The EVOO-derived crude phenolic extract significantly prevented the ability of TGF- β to upregulate SMAD4 and SNAI2.

EVOO phenolics prevent TGF- β 1-induced activation of the EMT transcriptional program

Finally, we decided to explore whether EVOO phenolics actively regulated a programmed series of genes events controlling TGF- β 1-induced EMT, as suggested by the immunofluorescence studies. Total RNA from untreated MCF-7 control cells and MCF-7 cells treated with TGF- β 1, EVOO phenolics, or TGF- β 1 plus EVOO phenolics were evaluated using qRT-PCR of genes specifically associated with EMT and the reciprocal mesenchymal-to-epithelial transition (MET) (Fig. 5). When a three-fold or greater difference in mRNA expression levels was used as the cutoff to determine significant regulatory effects on genes involved in the EMT genetic program, TGF- β 1 treatment was found to significantly upregulate a total of 11 of the 84 EMT gene regulators assessed in the experiment. MCF-7 cells treated with TGF- β 1 for 72 hr transcriptionally enhanced the expression of developmental EMT genes that have been repeatedly demonstrated to play pivotal roles in malignant progression, including *SNAI2* and *TCF4* (approximately four-fold increases).^{59,60} Accordingly, MCF-7/TGF- β 1 cells notably increased the expression of plasminogen activator inhibitor type-1 (*PAI-1*; *SERPINE1*), a prominent member of the subset of TGF- β -initiated EMT-related signaling events,^{61,62} and the mesenchymal markers vimentin (*VIM*; ~4-fold) and fibronectin (*FN*; ~4-fold). Supporting the notion that EVOO phenolics appear to negatively regulate oncogenic EMT, co-treatment of MCF-7 cells with the EVOO-derived crude phenolic extract efficiently prevented the occurrence of most of TGF- β 1-induced EMT transcriptional events, including up-regulation of *SNAI2*, *TCF4*, *VIM*, and *FN*. TGF- β 1-induced accumulation of *SERPINE1* decreased from ~15 times in the absence of EVOO phenolics to ~4 times in

FIG. 3. Effect of extra virgin olive oil (EVOO) phenolics on distribution of the epithelial marker E-cadherin in MCF-7 cells. Subcellular distribution of E-cadherin was evaluated by indirect immunofluorescence in MCF-7 cells grown until 80% confluence, serum-starved for 24 hr, and then treated for 3 days with a minimal amount (0.1%) of fetal bovine serum (FBS) supplemented with vehicle (control [untreated]), transforming growth factor- β 1 (TGF- β 1) alone (10 ng/mL), EVOO phenolic extract (EVOO-PE) alone (200 ng/mL), or TGF- β 1 plus EVOO-PE. Images show representative portions ($n=3$) of MCF-7 cell cultures treated as specified for 3 days and captured in different channels for E-cadherin (green) and DNA (blue) on a BD Pathway™ 855 Bioimager System. The figure also shows a schematic summarizing EVOO phenolics' preventative activity against TGF- β -promoted downregulation or complete loss of the metastasis suppressor protein E-cadherin. Secoiridoids-targeted E-cadherin expression could efficiently prevent TGF- β -induced conversion of epithelial into migratory mesenchymal cells and may thus be a useful strategy to impede formation of migratory cancer stem cells (CSCs).

their presence. Regardless the presence of the EMT inducer TGF- β 1, the EVOO-derived crude phenolic extract significantly decreased by ~4-fold the baseline expression of the mesenchymal marker osteopontin (*SPP1*, secreted phosphoprotein 1)^{63–65} independently of the presence of TGF- β 1.

Discussion

EVOO, the juice of the olive obtained solely by pressing and consumed without any further refining process, is unique among other vegetable oils because of its high level of naturally occurring phenolic compounds. Here, we have tested the hypothesis that prevention of TGF- β -induced EMT may represent a previously unrecognized mechanism through which phenolic molecules naturally occurring in EVOO might actively regulate the pathophysiology of many age-related human diseases, including end-state organ failures, due to excessive EMT-related fibrosis and cancer metastasis, given that the EMT genetic program is sufficient to

generate migrating CSCs by directly linking the acquisition of cellular motility with the maintenance and enhancement of tumor-initiating capacity.

Using canine MDCK kidney cells and human MCF-7 breast cancer cells, we directly evaluated the impact of a crude EVOO phenolic extract on several TGF- β -altered EMT parameters, including cell morphology and expression levels of epithelial and mesenchymal markers. EVOO phenolics significantly reduced TGF- β 1-induced cell scattering and TGF- β 1-induced intercellular spaces in MDCK cell cultures. The inhibitory effects of EVOO phenolics on TGF- β 1-induced conversion of MDCK epithelial cells to the spindle-like mesenchymal morphology was accompanied by a significant prevention of the cytoplasmic accumulation of the mesenchymal marker vimentin occurring in the TGF- β 1-induced mesenchymal state of MDCK cells. Perhaps more importantly, treatment with EVOO phenolics notably prevented the occurrence of hallmarks of TGF- β -induced acquisition of the mesenchymal phenotype, including the ability of TGF- β to

FIG. 4. Effects of extra virgin olive oil (EVOO) phenolics in transforming growth factor- β -regulated epithelial-to-mesenchymal transition (EMT) signaling cascade. Subcellular distribution of SMAD4, SNAI2, and E-cadherin was evaluated strictly in parallel by indirect immunofluorescence in MCF-7 cells grown until 80% confluence, serum-starved for 24 hr, and then treated for 3 days with a minimal amount (0.1%) of fetal bovine serum (FBS) supplemented with vehicle (control [untreated]), transforming growth factor- β 1 (TGF- β 1) alone (10 ng/mL), EVOO-phenolic extract (EVOO-PE) alone (200 ng/mL), or TGF- β 1 plus EVOO-PE. Images show representative portions ($n = 3$) of MCF-7 cell cultures treated as specified for 3 days and captured in different channels for E-cadherin (green) and DNA (blue) on a BD Pathway™ 855 Bioimager System. This figure also shows a schematic summarizing EVOO phenolics' preventative activity against TGF- β -driven EMT (1: Input layer [ligand/receptor/adaptors]; 2: Signal-processing layer [signaling cascade/transcription factors]; 3: Output layer [epithelial/mesenchymal markers]). TGF- β binding to its receptor (TGFBR) results in activation and nuclear translocation of SMAD transcription factors (e.g., SMAD4), which achieve target gene (e.g., E-cadherin) specificity through interaction with transcriptional cofactors (e.g., SNAI2). Epithelial-to-mesenchymal transition (EMT)-promoting transcription factors, including epithelial repressors such as SNAILs, ZEB, or TWIST, interact with SMADs, thus resulting in the formation of EMT-promoting SMAD complexes that can drive EMT by repressing epithelial genes such as E-cadherin. E, E-boxes; SBE, SMAD-binding elements.

cause loss of plasma membrane staining for E-cadherin and its ability to enhance cytoplasmic punctate and diffuse vesicle E-cadherin staining. Disintegration of cell-cell contacts (tight junctions, adherens junctions, and desmosomes) is a key feature of EMT.

More importantly, several lines of evidence have also substantiated that cell-cell contacts are not passive targets but

they are active regulators of EMT. Loss of the adherens junctions' component E-cadherin is well established to promote EMT in developmental biology and tumor models, whereas forced expression of E-cadherin can restore (noninvasive) epithelial phenotypes in (invasive) mesenchymal tumor cells. In terms of "fibrotic EMT," E-cadherin has been shown to play a central role in the EMT of renal tubular cells. In terms of

FIG. 5. Epithelial-to-mesenchymal transition (EMT) expression profiling of MCF-7 cells treated with transforming growth factor- β (TGF- β) in the absence or presence of extra virgin olive oil (EVOO)phenolics. This figure shows representative scatter plots of the difference in relative abundance of 84 mRNAs whose levels, in published results, are significantly altered during activation/deactivation of the EMT genetic program (≥ 3 -fold). (Green and red symbols) Downregulation and upregulation versus basal expression levels in untreated MCF-7 cells, respectively; (grey symbols) fold-change results that need to be validated with a sufficient number of biological replicates (*i.e.*, fold-change results may have greater variations of p value > 0.05 or the p value for the fold-change is either unavailable or relatively high [$p > 0.05$]) or they are uninterpretable because the gene's average threshold cycles were either not determined or greater than the define cut-off value (default 35) in both samples. (Color image available online at www.liebertpub.com/rej)

"oncogenic EMT," activation of EMT in response to short hairpin (sh)RNA-mediated knockdown of E-cadherin expression has been shown to increase the tumorigenic CD44^{high}/CD24^{low} mesenchymal population in transformed breast cancer cells. Indeed, combined immunohistochemical staining of positive (*e.g.*, SNAIL) and negative (*e.g.*, E-cadherin) EMT markers together with SMAD3/4 has been proven to be an efficient approach to identify CSC-like EMT phenotypic cells in samples of invasive human ductal breast carcinomas.³⁴ In these studies, loss of E-cadherin at intercellular junctions significantly correlated with nuclear expression of SNAIL and SMAD3/4 at invasive regions, thus supporting a role for SNAIL-SMAD3/4 complexes in the repression of junction proteins during breast cancer cell invasion. In our hands, TGF- β treatment of MCF-7 breast epithelial cancer cells *in vitro* notably recapitulated the ability of EMT to promote ontogenesis of the breast cancer stem cell molecular signature *in vivo* because TGF- β -promoted loss of

E-cadherin took place upon activation of critical mediators of TGF β signaling, such as SMAD4, and epithelial repressors, such as the SMAD transcriptional cofactor SNAIL2. Remarkably, EVOO phenolics largely nullified upstream activation of TGF- β signaling to impede downstream formation of EMT-promoting SMAD repressor complexes. By weakening the ability of TGF- β to fully induce mesenchymal cell states, our current findings illustrate for the first time that the anti-EMT activity *in vitro* of EVOO phenolics might translate into antifibrotic and anti-CSCs *in vivo*.

From a mechanistic perspective, and considering that EVOO phenolics remarkably repressed the ability of TGF- β ability to transcriptionally reprogram MCF-7 breast cancer epithelial cells to express pivotal drivers and effectors of the EMT genetic program including *SNAIL2*, *TCF4*, *VIM*, and *FN*, whereas they failed to fully prevent TGF- β -induced EMT, as determined by cellular morphology in MDCK cells, it might be reasonable to suggest that EVOO phenolics efficiently

impede an important subset of TGF- β -regulated EMT changes but fail to complete the EMT reversal program (*i.e.*, MET). This scenario agrees with the hypothesis that reversal of EMT not only requires re-establishment of epithelial gene transcription but further a regain of structural components of the epithelial cytoskeleton.⁶⁶

Our current description of a significant anti-EMT activity of EVOO phenolics supports and expands further earlier studies showing that the green tea polyphenol epigallocatechin-3 gallate (EGCG) can significantly reduce the invasive breast cancer phenotype by upregulating the epithelial marker E-cadherin while downregulating the proinvasive *SNAIL* gene.^{67,68} Indeed, the common ability of a naturally occurring complex polyphenols to alter the expression of key regulators in the EMT pathway may suggest that they share an identical target that activation/deactivation prevents or reverses TGF- β -driven EMT. Activation of the energy sensor adenosine monophosphate-activated protein kinase (AMPK) is a plausible mechanism through which polyphenolic phytochemicals may reduce the ability of TGF- β to activate and maintain the mesenchymal molecular signature. On the one hand, AMPK activation has been shown to abrogate TGF- β -induced SMAD3-dependent transcription in cultured human primary mesangial cells and adult mouse cardiac fibroblasts.^{69,70} On the other hand, naturally occurring polyphenols such as EGCG significantly induce increases in the phosphorylation status of AMPK and in AMPK activity.^{71–73} If EVOO complex polyphenols behave molecularly in an EGCG-like manner, it might be tempting to suggest that, by acting as AMPK agonists, they can blunt TGF- β -activated EMT-related events (*e.g.*, phosphorylation of signal transducer and activator of transcription 3 [STAT3], which upregulates expression of several factors important for EMT induction).^{74–76} Another plausible mechanism through which EVOO phenolics might inhibit EMT and reactivate an epithelial phenotype might relate to their ability to molecularly mimic the anti-EMT effects of the polyphenol resveratrol, which has recently been found to inhibit the expression of *SNAIL*, *SLUG*, and *ZEB1* in pancreatic CSCs.⁷⁷

Although it remains to be elucidated whether a resveratrol-like up-regulation of silent information regulator T1 (SIRT1) deacetylase activity may counterintuitively regulate EVOO phenolics-induced enhancement and/or maintenance of epithelial versus mesenchymal phenotypes (*i.e.*, inhibition of SIRT1 increases acetylation of histone H3 at the E-cadherin gene promoter, thus increasing E-cadherin expression,⁷⁸ whereas activation of SIRT1 reverses acetylation of SMAD3, thus inhibiting TGF- β -induced renal fibrosis),^{79,80} it should be noted that the dietary bioflavonoid quercetin also inhibits the expression of EMT markers (*e.g.*, vimentin) as well as the transcription factors *SNAIL* and *SLUG* to inhibit CSC characteristics, including invasion, migration, and self-renewal.^{81,82} Polyphenolic phytochemicals, therefore, could all cause inhibition of fibrogenic and oncogenic EMT-driven phenomena. It is noteworthy that all of them had been reported to be either inhibitors of the mitochondrial respiratory chain or inhibitors of the mitochondrial adenosine triphosphate (ATP) synthesis, thus suggesting the hypothesis that these compounds can inhibit mitochondrial ATP production and therefore activate AMPK by increasing the intracellular AMP/ATP ratio.^{83–85} In addition, numerous lines of evidence suggest that most dietary polyphenols, including EGCG, resveratrol, and

EVOO phenolics, have the capacity to mitigate cellular damage induced via metabolic production of reactive oxygen species (ROS) by mitochondrial complex I.^{86–90} Given that complex I can control the nicotinamide adenine dinucleotide (NAD⁺)-to-NADH ratio, thus allowing the activation of SIRT1,⁹¹ it might tempting to suggest that EVOO phenolics-targeted functioning of mitochondrial complex I might connect the master energy regulatory proteins AMPK (which senses the AMP/ATP ratio) and sirtuins (which require NAD to deacetylate protein substrates) molecularly. In this regard, it has been recently demonstrated that AMPK and SIRT1 can be connected in a linear pathway because AMPK activation upregulates the gene encoding the NAD synthetic enzyme Nampt, thus providing the crucial link to the downstream activation of SIRT1.^{92–94}

We should express caution, however, when interpreting *in vitro* data to actual actions of EVOO polyphenols in the body, especially if no data have been collected regarding the action of physiological metabolites of tested EVOO polyphenols on the same cell systems. To produce their beneficial effects, other than on the gastrointestinal (GI) tract itself, EVOO polyphenols must be absorbed into the body after oral ingestion and be carried by the bloodstream from the absorption site to target tissues and organs. Obviously, our *in vitro* assays do not take into account the *in vivo* bioavailability issue and can lead to false-positive interpretations (*i.e.*, the *in vitro* active compound within the EVOO phenolic extract is quickly metabolized or has limited bioavailability to the target organ). Thus, if there is no evidence for the absorption of a particular EVOO polyphenol (*e.g.*, oleuropein aglycon, decarboxymethyl oleuropein aglycon), it could make no biological sense to gain mechanistical insights (*e.g.*, anti-EMT effects) by exposing it to cultured cells obtained from the kidney and/or the breast. We should acknowledge that, similar to most pharmaceuticals, the body will treat consumed EVOO polyphenols as xenobiotics, or foreign substances, and EVOO polyphenols will be therefore subjected to the same protective xenobiotic-metabolizing and efflux mechanisms, which result not only in major changes in biological activity but also in increased rates of excretion from the body.^{95–97} Because parental EVOO polyphenols contain suitable functional groups (*e.g.*, hydroxyl groups), they can undergo conjugation reactions with endogenous compounds to yield more polar and water-soluble derivatives. The principal conjugation reaction is the formation of β -glucuronides catalyzed by uridine diphosphoglucuronosyl transferases (UGTs), but conjugation with a sulfo moiety or glutathione catalyzed by sulfotransferases (SULTs) and glutathione-S-transferases, respectively, also occurs. Less-polar conjugates may also be formed by methylation, catalyzed by catechol-O-methyltransferase (COMT).

Although absorption and bioavailability studies have revealed that single phenols tyrosol and hydroxytyrosol can be retrieved in plasma and urine after OO consumption,⁹⁸ there is an urgent need for data regarding the plasma/urine concentration of the free forms of various secoiridoid aglycones. For instance, after ingestion of flavonoids, conjugates of the aglycon, such as glucuronides, sulfates, and methylated metabolites, have been found to predominate in the blood circulation rather than the original plant glycoside or aglycon.⁹⁹ Indeed, all of the conjugation mechanisms are highly efficient, and free aglycones are either absent or present in low concentrations in plasma after consumption of nutritional doses. Because in studies with green tea polyphenols, the metabolites mostly had reduced biological activity, it might be tempting to suggest that limited

bioavailability of EVOO complex polyphenols and their conversion into less-active metabolites (e.g., glucuronidated or sulfated forms) could negatively affect an anti-EMT effect, if any, *in vivo*. In some systems, however, polyphenols-derived metabolites were found to have the equivalent or even greater activity than the parental polyphenols.¹⁰⁰ Thus, whereas some studies have suggested that methylations can increase the bioavailability of polyphenols, other studies have indicated a decrease in the anticancer benefits of methylated polyphenols.¹⁰¹

Kinetics of penetration and elimination of EVOO polyphenols in tissues is largely unknown and data on actual bioavailability are scarce, thus it is plausible that cellular metabolites may differ from those obtained in plasma. Whether EVOO polyphenols could accumulate in specific organs remains to be ascertained, and the presence of cellular-specific mechanisms to incorporate polyphenols is still highly controversial.^{102–105} Recently, we employed a nano liquid chromatography–electrospray ionization–time-of-flight mass spectrometry (nanoLC-ESI-TOF MS) method to evaluate both the cellular uptake and the metabolism of EVOO phenolics in human breast carcinomas cells highly sensitive to the growth inhibitory effects of secoiridoid-rich EVOO phenolic extracts (Rocío García-Villalba, Alegría Carrasco-Pancorbo, Javier A. Menéndez, Antonio Segura-Carretero, Alberto Fernández-Gutiérrez, submitted manuscript). Most of free EVOO phenolics disappeared from the culture medium (*i.e.*, the extracellular milieu) in different extents and at different times according to the type of phenolic we tested. When the time course of cellular uptake of parental phenolics was expressed as percent of the quantity originally supplemented in culture medium, we concluded that, as early as 15 min, oleuropein aglycone and decarboxymethyl oleuropein aglycon were rapidly incorporated into the cell. Secoiridoids were also the phenolic group most extensively metabolized, and methylation was the preferential pathway for conjugation of oleuropein aglycone and decarboxymethyl oleuropein aglycon, which was followed or preceded, in some cases, by hydrogenation reactions. We observed extremely low intracellular accumulations of parental secoiridoids, with only traces of some compounds being detected in the cytoplasm and membranous structures. In addition, the amounts of methylated forms increased with time strictly in parallel with the disappearance of parental secoiridoids in the extracellular milieu, a process that reached a maximum after 2 hr of incubation, thus suggesting a crucial role for COMT at metabolizing EVOO polyphenols at target tissues such as breast carcinomas.

These findings might be crucial when designing synergy strategies aimed to improve the bioavailability and hence bioactivity of EVOO polyphenols.^{95,96,106} First, because metabolism of dietary polyphenols by gut bacteria constitutes a significant barrier to their bioavailability, modulation of intestinal microflora population can be modulated by antibiotics or other natural products (e.g., catechins).¹⁰⁷ Second, efflux transporter inhibitors and metabolism enzyme modulators can significantly affect phenolic absorption and metabolism, which consequently can improve their bioavailabilities. Third, pharmacological inhibition of glucuronidation using piperine^{108,109} can be expected to increase bioavailability of EVOO phenolics. *In vivo* studies have revealed that curcumin bioavailability can be drastically increased in human volunteers (up to 2000%) when curcumin is administered together with piperine.¹¹⁰ Piperine has been found to also increase the bio-

availability of green tea polyphenols in mice.¹¹¹ Recently, Johnson et al.¹¹² examined the hypothesis that piperine will enhance the pharmacokinetic parameters of resveratrol via inhibiting its glucuronidation, thereby slowing its rapid metabolism and elimination. By using animal models, the degree of exposure to resveratrol was enhanced to 229% and the maximum serum concentration was increased to 1544% with the addition of piperine.¹¹² Fourth, several nutraceuticals, including curcumin, green tea polyphenols, coenzyme Q, quercetin, and others, have been packaged as nanoparticles and proven to be useful in “nanochemoprevention” and “nanochemotherapy.”¹¹³ Efforts are currently underway in our laboratories to develop biocompatible, biodegradable, and nontoxic nano-size liposomal formulations that offer the possibility of carrying and delivering EVOO polyphenols in the prevention or treatment of several pathological conditions.

In summary, excessive fibrosis due to a sustained and unresolved EMT occurs in many age-related human diseases, including heart failure, sclerosis, nonalcoholic steatohepatitis, and progressive renal fibrosis. CSCs generated from EMT induction provide a resource for cancer to recur and metastasize because EMT-phenotypic tumor cells acquire stem-like increased metastatic capacity and self-renewal ability. Our current findings reveal for the first time that naturally occurring phenolics in EVOO efficiently impede EMT, the common link in the life-threatening progression of organ fibrosis and cancer toward organ failure and metastasis, respectively. Forthcoming studies should definitely establish whether, by targeting mitochondrial complex I, EVOO-derived complex polyphenols such as secoiridoids can mimic an energy limitation situation that enhances the net effect of ATP and NAD to activate AMPK and SIRT1 and lastly restrain the EMT differentiation process.

Because poor *in vivo* bioavailability of EVOO phenolics is being considered a major obstacle in translating in humans their multitude of health-promoting properties found in preclinical studies, future studies should focus on how to increase the bioavailabilities of EVOO so we can rapidly accelerate the development of this class of compounds into effective chemopreventing, anticancer, and/or antiaging agents.^{53,54,56,114–118} Nevertheless, *Oleaceae* secoiridoids, such as oleuropein aglycone and decarboxymethyl oleuropein aglycon, certainly merit consideration for longevity studies and, perhaps, for ulterior design of more pharmacologically active second-generation anti-EMT molecules.

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Address correspondence to:

Javier A. Menendez, Ph.D.

Catalan Institute of Oncology, Girona (ICO-Girona)

Dr. Josep Trueta University Hospital

Avenida de França s/n

E-17007 Girona, Catalonia

Spain

E-mail: jmenendez@iconcologia.net; jmenendez@idibgi.org

Antonio Segura-Carretero, Ph.D.

Department of Analytical Chemistry

Faculty of Sciences, University of Granada

Avenida Fuentenueva s/n

E-18071 Granada, Andalusia

Spain

E-mail: ansegura@ugr.es

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