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The Breast Cancer Resistance Protein (BCRP/ABCG2) influences the levels of enterolignans and their metabolites in plasma, milk and mammary gland

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Abbreviations: ABC, ATP-binding cassette; ABCG2, ATP-binding cassette subfamily G2; END, enterodiol; ENL, enterolactone; SDG, secoisolariciresinol diglucoside; SECO, secoisolariciresinol; UPLC-QTOF-MS, ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry

ABSTRACT

Lignans are phytoestrogens widely used in dietary supplements and functional foods. After oral ingestion, these polyphenols are metabolized to enterolignans, the main gut microbiota-derived metabolites with weak estrogenic/anti-estrogenic activities. The ABCG2 transporter is highly expressed in the mammary gland and could be responsible for enterolignan accumulation. We aimed here at evaluating the levels of enterolignans and their conjugates in plasma, milk and mammary tissue from wild-type and knockout Abcg2$^{-/-}$ female mice after a lignan-enriched diet for one week. In vitro transepithelial transport of enterolignans was also assayed with ABCG2-transduced cells. Enterolactone and enterodiol levels were higher in plasma and lower in milk from Abcg2$^{-/-}$ compared with wild-type mice. Both enterolactone and enterodiol were accumulated in the mammary gland but with significant differences only for enterolactone. Our results suggest that ABCG2 may be determinant for plasma and milk levels of enterolignans whose accumulation could exert chemopreventive effects against breast cancer.

Keywords: enterolactone, enterodiol, ABCG2, milk, mammary gland, gut microbiota

1. Introduction

Plant lignans and their derivatives have been reported to exert beneficial effects against prostate and breast cancer, cardiovascular and neurodegenerative diseases, osteoporosis, diabetes and menopausal symptoms (Figueiredo, de Albuquerque Maia, Guarda, Lisboa, & de Moura, 2017; Guglielmini, Rubagotti, & Boccardo, 2012; Högger, 2013; Huang et al., 2016; Teponno, Kusari, & Spiteller, 2016). These polyphenols are widely distributed in flaxseed, cereals, fruit and vegetables, olive oil, and beverages such as tea, coffee and wine (Kuijsten, Arts, Vree, & Hollman, 2005; Thompson, Boucher, Liu, Cotterchio, & Kreiger, 2006).

Due to its human health benefits, flaxseed is considered an emerging ingredient for functional foods (Kajla, Sharma, & Sood, 2015). Flaxseed contains significant levels of the lignan secoisolariciresinol diglucoside (SDG) (Thompson et al., 2006), which is transformed firstly in secoisolariciresinol (SECO) and undergoes further metabolism by the gut microbiota to produce enterodiol (END) and enterolactone (ENL). In general, under normal dietary conditions, END and ENL are the mammalian enterolignans that reach the highest concentration in plasma (Högger, 2013; Kuijsten, Arts, Hollman, van´t Veer, & Kampman, 2006), maintained by enterohepatic circulation. The intestinal epithelium contributes to the presystemic metabolism through conjugation by phase-II enzymes (Mukker, Michel, Muir, Krol, & Alcorn, 2014) so that enterolignans mainly occur as glucuronides in human serum and urine (Penttinen et al., 2007). This is consistent with extensive in vitro glucuronidation and sulfation of END and ENL previously reported by Jansen et al. (2005) in colon HT-29 and Caco-2 cell lines. Moreover, glucuronide and sulphate conjugates were found in the portal vein of rats following an oral administration of flaxseed (Axelson & Setchell, 1981).

Epidemiological studies correlate serum ENL concentration with lower incidence and mortality in breast cancer patients (Guglielmini et al., 2012; Seibold et al., 2014), suggesting that ENL might affect hormone-dependent tumour development and growth (Penttinen et al., 2007).

71 The majority of breast tumors are estrogen receptor (ER) positive and it has been suggested that
72 the estrogen-related effects of phytoestrogens are mediated via ERs (Albini et al., 2014). ENL
73 can bind ER in vitro (Mueller, Simon, Chae, Metzler, & Korach, 2004) as agonist and antagonist
74 for ERα. Instead of driving epithelial cells into proliferation, ENL and/or its conjugated
75 metabolites might compete for the receptor with endogenous estrogens and thereby can work as
76 antiestrogen. Alternatively, ENL and ENL metabolites may keep the cells in the differentiated
77 stage because these compounds may not exert the proliferative effects of estrogens (Penttinen et
78 al., 2007).
79 Human ABCG2 (Abcg2 in rodents), also known as Breast Cancer Resistance Protein, is a
80 member of the ATP-binding cassette (ABC) membrane transporters family that plays an
81 important role in limiting absorption (in the small intestine), mediating distribution (e.g. in the
82 blood–brain and blood–placental barriers) and facilitating elimination and excretion (in the liver
83 and kidney) of ABCG2 transport substrates (Horsey, Cox, Sarwat, & Kerr, 2016), such as
84 anticancer drugs, dietary compounds, food carcinogens and antibiotics (Jani et al., 2014; Merino
85 et al., 2005; van Herwaarden et al., 2003; Vlaming, Lagas, & Schinkel, 2009). Moreover,
86 ABCG2 has an important role in the active secretion of several compounds into milk (Jonker et
87 al., 2005). Its alternate name of Breast Cancer Resistance Protein comes from its notorious
88 function in extruding anticancer drugs from a variety of cancer cells, first observed in a breast
89 cancer cell line, which can result in multidrug resistance (Jani et al., 2014; Horsey, Cox, Sarwat,
90 & Kerr, 2016). In recent years, its role as a transporter of phytoestrogens and their conjugated
91 metabolites has gained special relevance (Alvarez et al., 2011; Bircsak & Aleksunes, 2015;
92 Quan, Wang, Dong, Zhang, & Wu, 2015; Tan, Li, Paxton, Birch, & Scheepens, 2013),
93 highlighting its interaction with the enterolignan ENL (Miguel et al., 2014).
94 The purpose of this work is to study the disposition of ENL, END and SECO and their
95 conjugated metabolites in plasma, milk and mammary gland from wild-type and Abcg2$^{-/-}$ mice

after the administration of a flaxseed-enriched diet. In addition, the role of Abcg2/ABCG2 in the transport of END and SECO will also be explored using parental and MDCKII cells transduced with Abcg2 murine and human ABCG2 transporters.

2. Materials and Methods

2.1. Chemicals/reagents and drugs

SDG, SECO, ENL, END, 6,7-dihydroxycoumarin (DHC), chrysin and the specific ABCG1 inhibitor PSC 833 were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). The specific ABCG2 inhibitor Ko143 used in cell cultures was obtained from Tocris (Bristol, U.K.). The flaxseed hull extract (LinumLife®EXTRA, Frutarom, Ltd., Herzeliya Israel) was provided by Tradichem S.L. (Madrid, Spain) and the mice diet was formulated by Research Diets, Inc (New Brunswick, NY, USA.). All the other chemicals were of analytical grade and obtained from commercial sources.

2.2. Cell cultures

Madin-Darby canine kidney epithelial cell (MDCKII parent cells) and their human ABCG2 and murine Abcg2 transduced sub-clones were kindly provided by Dr. A.H. Schinkel, Netherlands Cancer Institute (Amsterdam, The Netherlands). Culture conditions were as previously described (Merino et al., 2005). The cells were cultured in Dulbecco´s modified Eagle´s medium supplied with GlutaMAX™ (Life Technologies, Inc., Carlsbad, CA, USA) and supplemented with penicillin (50 units/mL), streptomycin (50 μg/mL), and 10% (v/v) fetal calf serum (Life Technologies) at 37 ºC in the presence of 5% CO_2. The cells were treated with trypsin every 3 to 4 days for sub-culturing.

2.3. Transport studies

Transport assays using Transwell plates were carried out as previously reported (Merino et al., 2005) with minor modifications. With this assay, the rate of basolaterally or apically directed translocation can be determined after adding the drug to the apical or basolateral side. ABCG2 transports its substrates in the apical direction (Fig. 1). Cells were seeded on microporous polycarbonate membrane filters (3.0 μm pore size, 24 mm diameter; Transwell 3414; Corning, NY, USA) at a density of 1.0×10^6 cells per well. Cells were grown for 3 d, and the medium was replaced each day. Transepithelial resistance was measured in each well using a Millicell ERS ohmmeter (Millipore, Bedford, MA, USA) before and after the experiment to check the tightness of the monolayer. Wells registering a resistance of 150 Ω or greater, after correcting for the resistance obtained in blank control wells, were used in the transport experiments. Before the start of the experiment, medium on both sides of the monolayer was replaced with 2 mL of OptiMEM (Life Technologies Inc.), free of serum, either with or without 1 μM Ko143, the ABCG2 inhibitor, for 2 h. After preincubation, the experiment was started (t=0) by replacing the medium in each apical and basolateral compartment with fresh medium containing 20 μM END or 50 μM SECO with or without Abcg2/ABCG2 inhibitor. The specific P-glycoprotein (P-gp) inhibitor PSC 833 (2.5 μM) was added to all compartments during SECO transepithelial transport. Aliquots of 100 μL were taken from the acceptor compartment at 2 and 4 hours, and stored at -20 ºC until high performance liquid chromatography analysis. The experiments were performed in triplicate.

2.4. HPLC analysis of SECO and END transports assays

The chromatographic system consisted of a Waters 2695 separation module and a Jasco 20200 Plus fluorescence detector. Integration was performed using Millenium 32 Chromatography manager software (Waters Corporation, Milford, MA, USA).

146 The conditions for HPLC analysis of SECO and END were based on a previously published
147 method by Mukker et al. (2010) with some modifications. Briefly, a reverse phase Waters X
148 Terra C18 5 μm 4.6 mm X 250 mm column was used and mobile phases consisted of 0.05%
149 formic acid + 0.1% trimethylamine in water, methanol and acetonitrile (62:32:6). The flow rate
150 was set to 1 mL/min. Fluorescence was measured with excitation and emission wavelengths at
151 277 and 617 nm, respectively. The column temperature was 40 ºC and the temperature of
152 samples was 4 ºC. Samples from the transport assays were not processed, and 50 μL of the
153 culture medium was injected directly onto the HPLC. Standard samples in OptiMEM medium
154 ranged from 0.156 to 10 ng/mL. Integration was performed using Millennium 32 software
155 (Waters).
156
157 *2.5. Animal experiment*
158 Mice (weighing 30 ± 2 g) were housed and handled in accordance with the European
159 guidelines described in EC Directive 2010/63/EU. The use of animals according to our
160 experimental design was approved by the Animal Care and Use Committee of the University of
161 Leon and Junta de Castilla y Leon (ULE_001_2015). The animals used in the experiments were
162 knockout Abcg2$^{-/-}$ and wild-type lactating female mice aged between 9 and 13 weeks, all of them
163 had > 99% FVB genetic background (n = 5-8). Animals were kindly provided by A. H. Schinkel
164 (The Netherlands Cancer Institute, Amsterdam, The Netherlands), and were kept in a
165 temperature-controlled environment with a 12-hour light/12-hour dark cycle. Mice were fed
166 normal nutritionally balanced rodent free of soybean meal feed, supplied by Research Diets, Inc.
167 This diet was supplemented with 1% of lignan-rich extract from flaxseed hulls
168 (LinumLife®EXTRA). The SDG content was 2 mg/g diet, and mean ingestion of SDG was
169 about 10 mg/mouse each day. The water was available *ad libitum*. All mice, wild-type and
170 Abcg2$^{-/-}$, were under this diet for seven days. After this period, blood, milk, and mammary gland

were obtained. Pups of approximately 10 days old were separated from their mothers approximately 4 hours before milk collection. Oxytocin (200 μL of 1 IU/mL solution) was administered subcutaneously to lactating mothers in order to stimulate milk secretion. Blood was collected by orbital bleeding and milk was obtained from the mammary glands by gentle vacuum suction after anaesthesia with isoflurane. Samples of mammary gland were obtained through a subcutaneous incision, tissue was removed and washed with PBS. At the end of the experiment, mice were subsequently killed by cervical dislocation. Heparinized blood samples were centrifuged immediately at 1000 × g for 10 min. Samples were stored at -20º C until their extraction and UPLC-QTOF-MS analysis.

2.6. UPLC-QTOF-MS analysis of in vivo sample

Mammary gland (0.3 g) was homogenized with 1.5 mL of sodium acetate buffer. Plasma and milk samples (200 μL) were mixed with 200 μL of the same buffer. These mixtures and 200 μL of homogenized mammary gland were treated with 200 mM of hydrochloric acid in methanol (800 μL), following the method of Bolca et al. (2010). After vortexing for 10 min and centrifugation at 15000 rpm (18000 g) for 10 min the supernatant was evaporated with N_2 and the dry residue re-dissolved in methanol (100 μL) and filtered (0.22 μm) prior to injection into the LC-MS system. Two internal standards (DHC and chrysin) were added to each sample (plasma, milk and mammary gland tissue). DHC (0.2 ppm) was added before sample preparation to control the extraction efficiency and chrysin (0.1 ppm) was added immediately prior to LC-MS analysis to monitor the signal variability of the equipment.

Analyses were performed using an Agilent 1290 Infinity LC system coupled to a 6550 Accurate-Mass qTOF (Agilent Technologies, Waldbronn, Germany) using an electrospray interface (Jet Stream Technology). The separation and detection was achieved with the conditions previously described by Miguel et al. (2014), with some modifications. Briefly, a

196 Poroshell 120 EC-C18 column (3 x 100 mm, 2.7 µm) (Agilent Technologies) was used for the
197 chromatographic separation operating at 30 ºC and 3 µL of injection volume. Water (A) and
198 acetonitrile (B), both with 0.1% formic acid were used as mobile phases with a flow rate of 0.4
199 mL/min and the following gradient: 5-25% B at 0-10 min, 25-40% B at 10-20 min; 40-90% B at
200 20-24 min; 90%-5% at 25-26 min and the column re-equilibrated for 4 min. Nitrogen was used
201 as nebulizing (9 L/min, 35 psi) and drying gas (280 ºC). Experiments were performed in negative
202 ion mode with a scan range 100-1100. Data were processed using the Mass Hunter Qualitative
203 Analysis software (B.06.00) which lists and rates possible molecular formulas consistent with
204 the accurate mass measurement and the true isotopic pattern. A target screening strategy was
205 applied to search for a list of potential metabolites taking into account information available in
206 the literature on absorption and metabolism of enterolignans (Quartieri et al., 2016). Screening
207 was based on mass filtering at the exact mass using narrow mass extraction window (0.01 m/z).
208 Reliable identification was deduced from molecular formulas, information reported in the
209 literature (Quartieri et al., 2016) and when possible, in comparison with authentic standards.
210 SDG, SECO, END and ENL were quantified by interpolation in the calibration curves of their
211 own standards. Calibration curves were linear over the concentration range LOQ- 0.5 µM for
212 END and ENL and LOQ-2µM for SDG and SECO. Limits of detection (LOD) and
213 quantification (LOQ) were calculated by following the criteria of S/N of 3 for the LOD and of 10
214 for the LOQ. LODs were 2.10, 2.80, 0.13 and 0.05 nM for SDG, SECO, END and ENL,
215 respectively. LOQs were 7.0, 9.3, 0.42 and 0.18 nM, for SDG, SECO, END and ENL,
216 respectively. These metabolites were quantified by peak area integration of their extracted ion
217 chromatograms (EIC) at m/z 685.2713, 361.161657, 301.1445 and 297.1132, respectively
218 (Supplemental Table 1). Regarding phase II metabolites, an accurate quantification was not
219 obtained due to the lack of available standards. However, their relative abundance was used to
220 compare the level of each compound among the different samples. The area under the curve

obtained using extracted ion chromatograms (EICs) was corrected taking into account the weight of the sample, extraction volume and dilution.

2.7. Statistical Analysis

Mann–Whitney U test was used to analyze the statistical significance of differences between mean values of variables. All analyses were carried out on the assumed significance level of $\alpha = 0.05$ using Statistica 10.0 software. Results are present as means and standard deviations (SDs).

3. Results

3.1. ABCG2 Transport in MDCKII cells

Active transport assays using parental and human ABCG2 and murine Abcg2 transduced MDCKII cells showed an increase in apically directed translocation of END and SECO in transduced cells (Tables 1 and 2). Efflux ratios basolateral-apical/apical-basolateral (BA/AB) for END (20 μM) of 13.1 and 4.1 were obtained in cells transduced cells with murine (MDCKII-Abcg2) and human (MDCKII-ABCG2), respectively. Selective involvement of the Abcg2/ABCG2 transporter was confirmed with the use of the Abcg2/ABCG2 inhibitor Ko143.

In the case of the SECO transport assay, the ABCB1 inhibitor PSC833 (2.5 μM) was present to suppress the moderate apically directed SECO transport possibly mediated by endogenous ABCB1 in the parental cell line. The efflux ratio of SECO (50 μM) in the presence of PSC 833 was 1.1 in active transport assays using parental cells, and 21.8 and 2.7 in MDCKII cells transduced with murine Abcg2 and human ABCG2, respectively. Again, the use of the Abcg2/ABCG2 inhibitor Ko143 confirmed the selective involvement of Abcg2/ABCG2.

246 *3.2. Effect of Abcg2 on plasma, milk, and mammary gland accumulation of ENL, END, SECO*
247 *and SDG*

248 The results obtained in wild-type and knockout Abcg2$^{-/-}$ mice showed differences in
249 enterolignan concentrations between both animal groups (Fig. 2). Plasma levels of ENL, END
250 and SECO were higher in Abcg2$^{-/-}$ than in wild-type mice, although significant differences ($P <$
251 0.05) were observed only for END levels. In addition, plasma levels of SECO were much lower
252 than those detected for END and ENL (Fig. 2).

253 Regarding milk disposition, END and ENL were readily excreted in milk and their levels
254 were opposite to those found in plasma levels, i.e. ENL, END and SECO concentrations were
255 lower in Abcg2$^{-/-}$ than in wild-type animals (Fig. 2), reaching statistical significance in the case
256 of ENL and END ($P < 0.01$).

257 In the case of the mammary gland, END and SECO concentrations were very similar in both
258 mice groups, with high levels in the case of END (Fig. 2). However, ENL concentration was
259 significantly higher in the wild-type group than in Abcg2$^{-/-}$ animals ($P < 0.05$).

260 SDG levels were insignificant in milk, very low in plasma and were not detected in the
261 mammary gland.

262

263 *3.3. Determination of the relative abundance of enterolignan glucuronides in plasma, milk and*
264 *mammary gland*

265 Target analyses of samples of plasma, milk and mammary gland by UPLC-QTOF-MS
266 allowed the identification of different enterolignan-derived metabolites. The most abundant
267 metabolites identified were END glucuronide, ENL glucuronide and SECO glucuronide
268 (Supplemental Table 1). Significant levels of enterofurans and their glucuronide metabolites
269 were also found (data not shown).

The levels of enterolignan glucuronides were not quantified due to the lack of available standards. However, their relative abundance was used to compare each compound between the different samples (Fig. 3) but not for comparison between the different metabolites. END glucuronide, ENL glucuronide and SECO glucuronide appeared in plasma and mammary gland and they were more abundant in Abcg2$^{-/-}$ than in wild-type mice. Differences were statistically significant in plasma for END glucuronide and SECO glucuronide, indicating that these compounds are Abcg2 substrates. In milk, only SECO glucuronide and END glucuronide were identified, although the levels were close to the detection limit.

4. Discussion and conclusion

Enterolignans have been reported to show a myriad of biological activities, including estrogenic/anti-estrogenic, antioxidant, anti-inflammatory, and anticancer effects in both cell models and in vivo studies (Crozier, Jaganath, & Clifford, 2009; Högger, 2013; Hu, Yuan, & Kitts, 2007). A reduction in mortality risk has been related with serum enterolactone levels > 10 nM (Guglielmini et al., 2012; Seibold et al., 2014). Factors involving enterolactone disposition include the intake of lignan precursors, intestinal bacterial activity and conjugating enzymes (Adlercreutz, 2002; Kuijsten et al., 2006; Lampe, Atkinson, & Hullar, 2006).

The results obtained in the present study give evidence for a role of ABCG2 in the transport of END and SECO. This finding adds a new factor that could affect the exposure of enterolignans, which is the activity of Abcg2/ABCG2. Plasma levels of ENL and END, and also SECO to a lesser extent, were higher in the Abcg2$^{-/-}$ mice, indicating that these compounds are substrates of the murine Abcg2 transporter, as was further confirmed in the in vitro assays. The transport of ENL by ABCG2-transduced subclones cells has been previously demonstrated (Miguel et al., 2014). An efflux ratio BA/AB of 1.2 in active transport assays using parental cells

295 and 16 and 3 in MDCKII-cells transduced with murine Abcg2 and human ABCG2, respectively,
296 were previously reported for ENL (Miguel et al., 2014), which was in agreement with our results
297 for END (Table 1).

298 In the case of females, these results are of vital relevance since urinary ENL and END levels
299 in women are positively and significantly associated with sex hormone binding globulin and
300 negatively with the plasma percentage of free estradiol, which is an additional risk factor for
301 breast cancer. In our experiment, dietary lignan content was 10 mg/d and the average value in
302 several women populations ranged from 0.4 to 13.8 mg/d. Caucasian women had the highest
303 intake of 13.8 mg/d (Nie et al., 2015). Enterolignans and derived conjugated metabolites reach
304 plasma concentrations in the range from nM to low μM. It has been calculated that the
305 concentrations of enterolignans achievable in the colon lumen in humans are 10-1000 μM
306 (Corsini et al., 2010). These levels of metabolites can show several effects on intracellular
307 signalling cascades, and are involved in several cellular functions including growth, proliferation
308 and apoptosis (Corsini et al., 2010; Crozier et al., 2009). Therefore, the increase of
309 approximately 60% of plasma concentrations of ENL and END in the Abcg2$^{-/-}$ mice obtained in
310 the present study could be physiologically relevant (Corsini et al., 2010).

311 Furthermore, the metabolism and tissue disposition of enterolignans (Högger, 2013) may be
312 directly affected by ABCG2 interaction. Intestine and liver are the main organs involved in the
313 conjugation of enterolignans with sulphate and glucuronic acid, the main conjugates being
314 glucuronides. Our results show that most of the identified metabolites were the glucoconjugates
315 (Fig. 3), in agreement with Jansen et al. (2005) who found that after 10 h more than 90% of ENL
316 appeared as glucoconjugates in intestinal cells. Both sulphate and glucuronide conjugates have
317 been described as ABCC2 and ABCG2 substrates (Krumpochova et al., 2012; Lampe et al.,
318 2006; van de Wetering & Sapthu, 2012). In fact, the transport of ENL sulphate, with low affinity,
319 was demonstrated in Abcg2$^{-/-}$ mice (van de Wetering & Sapthu, 2012). Our data for the

conjugated metabolites (Fig. 3) support also similar behaviour as that for END and ENL in plasma, where there was an increase in Abcg2$^{-/-}$mice, and thus the corresponding ENL glucuronide, END glucuronide and SECO glucuronide, also increased. On the other hand, several isozymes of UDP-glucuronyltransferase (UGT) catalyse the formation of these metabolites. Moreover, cellular conjugation could be altered when decreasing expression of efflux transporters or the activity of UGTs (Alvarez et al., 2011; Zhang et al., 2015; Wang et al., 2016). Therefore, impaired ABCG2-mediated efflux of enterolignans may increase their exposure to UGT and sulfatase and eventually produce greater amounts of metabolites, contributing to the higher levels of glucuronides in plasma of the Abcg2$^{-/-}$ mice (Fig. 3).

Our results support the notion that the interaction of ENL, END and SECO with Abcg2/ABCG2 explain the higher plasma levels detected in knockout mice (Fig. 2). However, significant differences were only obtained for END. Additional factors such as other transporters (P-glycoprotein in the case of SECO) or metabolism could also affect the systemic disposition of enterolignans. Nevertheless, the Abcg2/ABCG2 interaction was unequivocally demonstrated in the secretion into milk of these compounds. The presence of enterolignans in milk is of great importance since elevated ENL content might be interesting as a dietary source of ENL and could be an excellent strategy to increase the effects of lignans on human health. In fact, the effect of the bovine Y581S polymorphism of ABCG2 on milk secretion of ENL has recently been reported (Otero et al., 2016). Our results also show that END was actively secreted into milk by an Abcg2-dependent mechanism and that END reached high levels in milk after a chronic diet. Unfortunately, the high interindividual differences in the case of SECO precluded any conclusion.

Regarding the levels in the mammary gland, several studies have suggested the accumulation of END and ENL in certain tissues such as the liver, prostate and breast (Murray, Kang, Astheimer, & Price, 2007), which is consistent with the distribution characteristics of END

identified in the present study. Levels of lignans in the mammary gland are determinant for its morphogenesis (Rossini et al., 2010) and are involved in reducing risk of breast cancer incidence and related mortality (Guglielmini et al., 2012; Seibold et al., 2014). Whether phytoestrogens, including lignans, either increase or reduce the risk of breast cancer is proving to be one of the most challenging human health issues to be addressed (Nie et al., 2015). ENL has been found to exert biphasic effects on cell proliferation, with differential mechanisms of action on the proliferation of ERα positive cell lines at different concentrations (Nie et al., 2015). Indeed, the anti-estrogenic property of the lignans, which can be attributed to their weak antagonism to ER, might mitigate the estrogen-dependent aggressiveness in cancer cells (Dikshit, Gao, Small, Hales, & Hales, 2016). In this regard, there are multiple papers showing that ENL, SECO and flaxseed reduced tumor growth and tumor angiogenesis in athymic nude mice implanted with estrogen-dependent MCF-7 cells (Chen, Saggar, Corey, & Thompson, 2009; Lindahl, Saarinen, Abrahamsson, & Dabrosin, 2011; Saggar, Chen, Corey, & Thompson, 2010; Truan, Chen, & Thompson, 2012).

Overall, our results support an important interaction between the ABCG2 transporter and enterolignans after a repeated dietary administration that could exert important health benefits due to the relevant biological activities of these compounds.

Conflict of interest

Dr. José Angel Marañon is employed by Tradichem, SL. The other authors declare no conflict of interest.

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Figure captions

Fig 1. Transcellular transport assays. ABCG2 is expressed in the apical membrane of the cells and transports its substrates toward the apical direction. The rate of basolateral or apical translocation can be determined after adding the drug to the apical or basolateral side. Vectorial transport in the ABCG2- transduced cells is greater in the basolateral-to-apical direction than in the apical-to-basolateral direction. Different gray fading indicates different concentrations.

Fig. 2. Concentration of lignans (SDG and SECO) and enterolignans (END and ENL) in plasma, milk and mammary gland of wild-type and Abcg2$^{-/-}$ female mice (n = 7-8) fed with LinumLife®Extra 1% for 7 days. The samples were analyzed by UPLC-QTOF-MS. Enterolignan levels were quantified with their own standards. Data are presented as means ± SD. *P < 0.05.

Fig. 3. Determination of the peak areas (which is a measure of their relative abundance) of several glucuronide conjugated metabolites of enterolignans in plasma, milk and mammary gland of wild- type and Abcg2$^{-/-}$ female mice (n = 7-8) fed with LinumLife®Extra 1% for 7 days. The samples were analyzed by UPLC-QTOF-MS. Data are presented as means ± SD. *P < 0.05.

Table 1. Concentration of enterodiol (20 μM) permeated towards Apical (BL-AP transport) or Basolateral (AP-BL transport) compartments in cell cultures in the absence and in the presence of inhibitor (n = 3-4).

		Sampling time (h)	Concentration BL-AP (μM)	Concentration AP- BL (μM)	Ratio BL-AP/AP-BL
END	**Parental**	2 h	2.41 ± 0.47	1.28 ± 0.19	1.99 ± 0.54
		4 h	3.97 ± 0.81	2.26 ± 0.22	1.77 ± 0.37
	Abcg2 $^{-/-}$	2 h	5.88 ± 1.16	0.66 ± 0.18	9.05 ± 1.56
		4 h	11.53 ± 2.21	0.89 ± 0.26	13.14 ± 1.36*
	ABCG2	2 h	4.04 ± 0.20	1.13 ± 0.42	4.01 ± 1.60
		4 h	8.41 ± 0.89	2.11 ± 0.49	4.09 ± 0.68*
END + Ko 143 (1μM)	**Parental**	2 h	2.06 ± 0.25	1.23 ± 0.10	1.67 ± 0.17
		4 h	3.25 ± 0.68	1.95 ± 0.30	1.66 ± 0.10
	Abcg2 $^{-/-}$	2 h	2.17 ± 0.14	1.58 ± 0.12	1.37 ± 0.11
		4 h	3.93 ± 0.55	2.36 ± 0.32	1.67 ± 0.07
	ABCG2	2 h	2.56 ± 0.31	1.69 ± 0.39	1.61 ± 0.53
		4 h	4.54 ± 0.42	2.64 ± 0.30	1.74 ± 0.31

*P < 0.05 compared with parental cells.

Table 2. Concentration of SECO (50 μM) permeated towards Apical (BL-AP transport) or Basolateral (AP-BL transport) compartments in cell cultures in the presence of PSC 883 (2.5 μM), and in the absence and in the presence of inhibitor (n = 3-5).

		Sampling time (h)	Concentration BL-AP (μM)	Concentration AP- BL (μM)	Ratio BL-AP/AP-BL
SECO	**Parental**	2 h	6.94 ± 1.02	6.63 ± 1.46	1.08 ± 0.23
		4 h	11.64 ± 3.26	9.74 ± 2.90	1.08 ± 0.63
	Abcg2 $^{-/-}$	2 h	16.56 ± 1.38	1.42 ± 0.44	12.27 ± 2.86
		4 h	26.19 ± 2.27	1.21 ± 0.09	21.78 ± 2.32*
	ABCG2	2 h	10.07 ± 2.15	4.06 ± 1.42	2.67 ± 0.77
		4 h	19.50 ± 2.21	7.54 ± 2.25	2.71 ± 0.58*
SECO + Ko 143 (1μM)	**Parental**	2 h	7.92 ± 1.79	7.14 ± 1.20	1.14 ± 0.35
		4 h	15.59 ± 1.33	13.04 ± 2.13	1.21 ± 0.10
	Abcg2 $^{-/-}$	2 h	8.92 ± 0.72	6.22 ± 2.61	1.71 ± 0.90
		4 h	17.11 ± 1.84	13.21 ± 1.55	1.30 ± 0.09
	ABCG2	2 h	8.57 ± 0.37	8.38 ± 2.24	1.10 ± 0.39
		4 h	16.27 ± 1.46	13.67 ± 1.98	1.20 ± 0.07

*P < 0.05 compared to the parental cells.

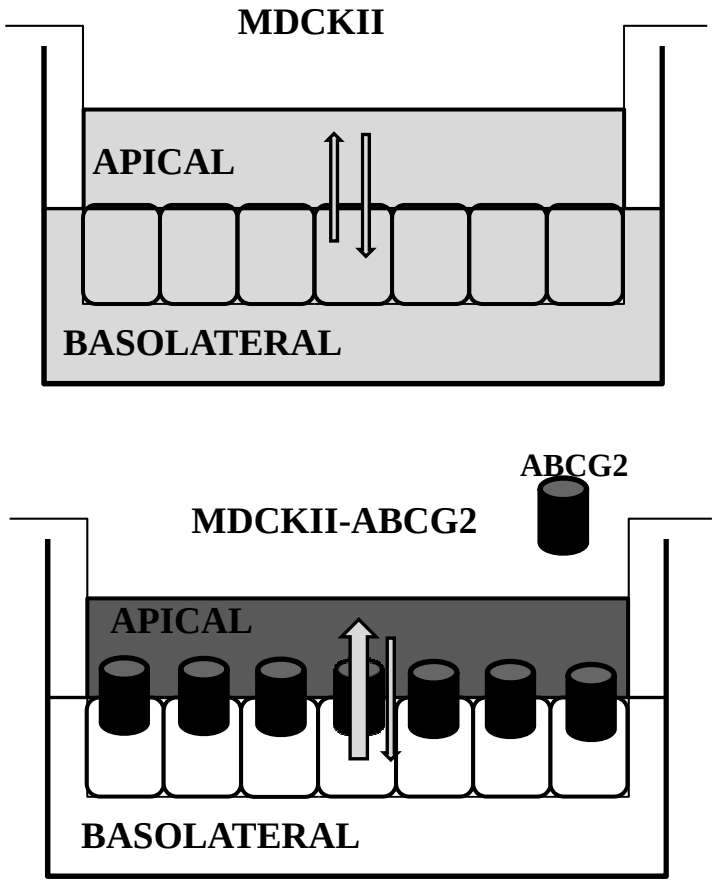
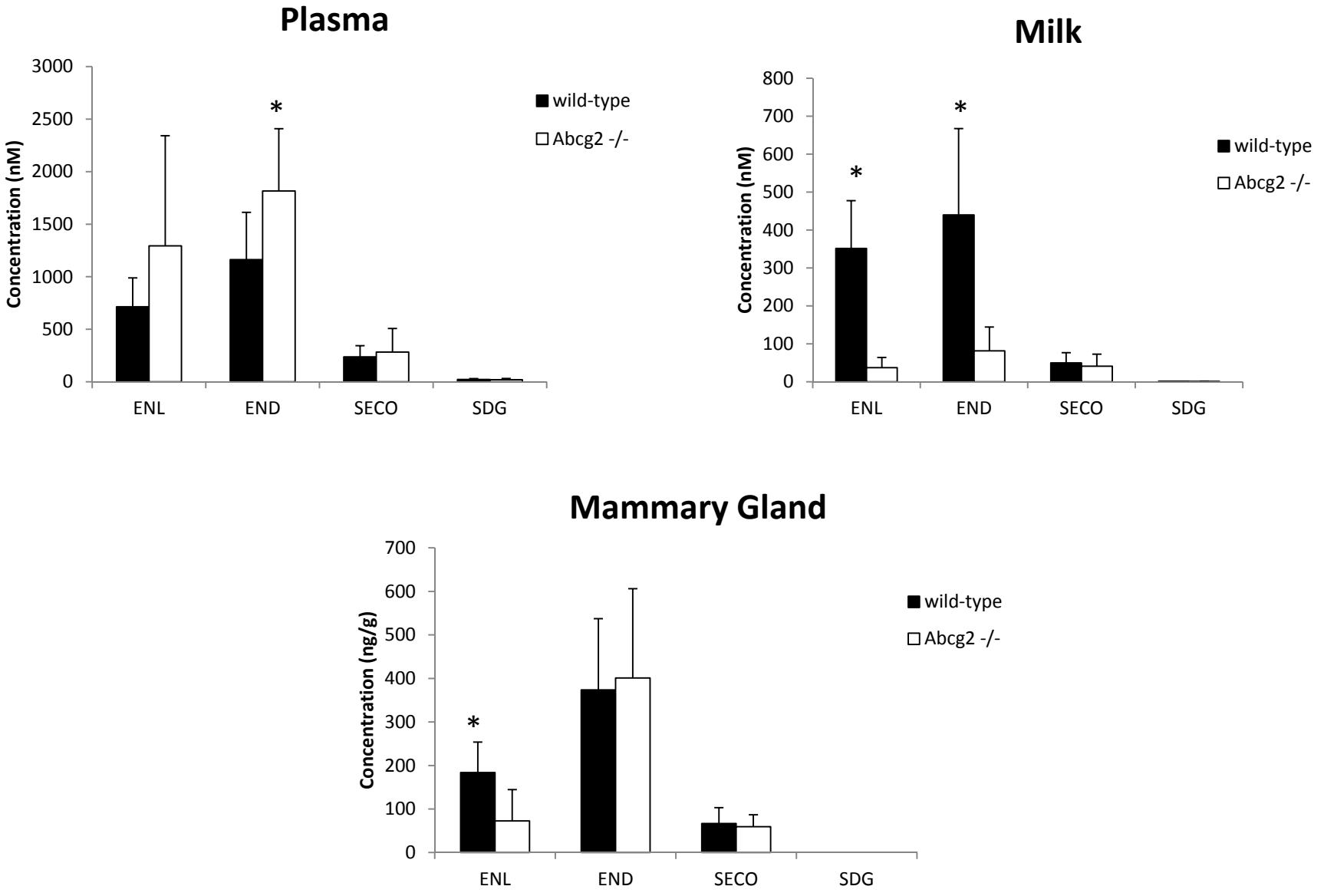


Fig. 1 (García-Mateos et al.)



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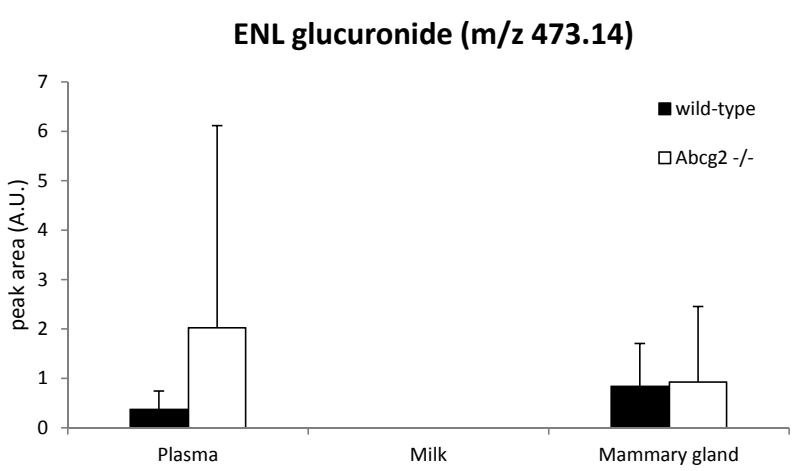

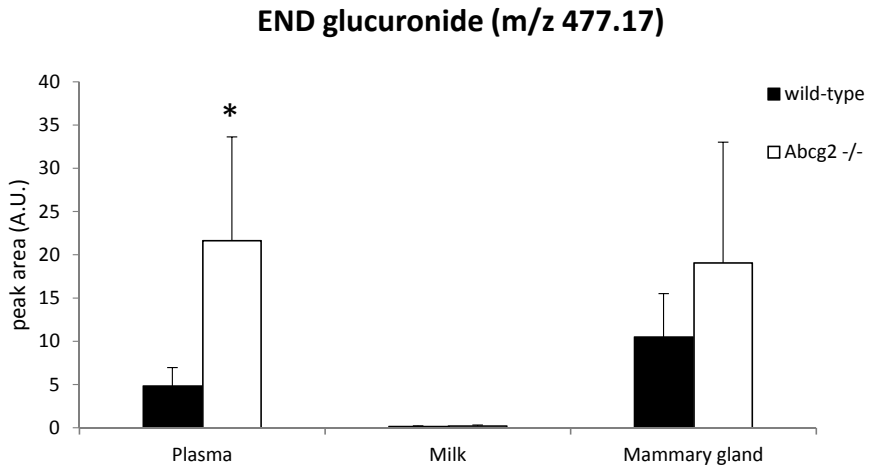

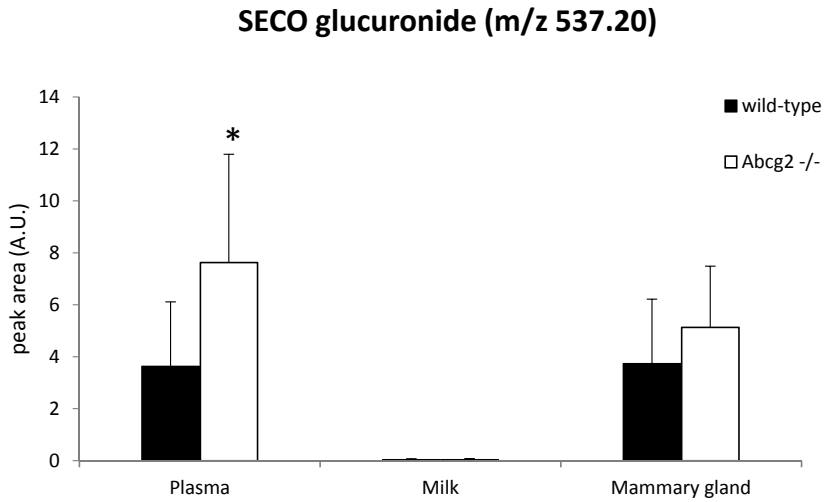

Fig. 3 (García-Mateos et al.)