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Analytical Methods

Analysis of the interaction between tryptophan-related compounds and ATP-binding cassette transporter G2 (ABCG2) using targeted metabolomics

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ABSTRACT

ATP-binding cassette transporter G2 (ABCG2) is involved in the secretion of several compounds in milk. The *in vitro* and *in vivo* interactions between tryptophan-related compounds and ABCG2 were investigated. The tryptophan metabolome was determined by liquid chromatography-tandem mass spectrometry in milk and plasma from wild-type and Abcg2$^{-/-}$ mice as well as dairy cows carrying the ABCG2 Y581S polymorphism (Y/S) and noncarrier animals (Y/Y). The milk-to-plasma ratios of tryptophan, kynurenic acid, kynurenine, anthranilic acid, and xanthurenic acid were higher in wild-type mice than in Abcg2$^{-/-}$ mice. The ratio was 2-fold higher in Y/S than in Y/Y cows for kynurenine. *In vitro* transport assays confirmed that some of these compounds were *in vitro* substrates of the transporter and validated the differences observed between the two variants of the bovine protein. These findings show that the secretion of metabolites belonging to the kynurenine pathway into milk is mediated by ABCG2.

1. Introduction

Tryptophan (Trp) is an aromatic amino acid critical for protein synthesis; in addition to this essential role, Trp is also the precursor of several bioactive compounds generated mainly through the kynurenine (KYN) and serotonin (5HT) pathways. Some of the Trp metabolites play important physiological roles (Cervenka, Agudelo, & Ruas, 2017). For example, 5HT has critical roles as a neurotransmitter, growth factor, and hormone (De Deurwaerdère & Di Giovanni, 2020); melatonin regulates the sleep-wake cycle and exhibits antioxidant properties (Boutin, Audinot, Ferry, & Delagrange, 2005); and KYN is involved in immune responses, inflammation, and neurotransmission (Stone, Stoy, & Darlington, 2013). The presence of some of these metabolites in the diet could have important effects on biological processes (Markus et al., 2000).

The transfer of a large number of metabolites and xenobiotics to milk is mediated by two transporter superfamilies: ATP-binding cassette (ABC) and solute carrier (SLC) transporters (García-Lino, Álvarez-Fernández, Blanco-Paniagua, Merino, & Álvarez, 2019). In particular, ABCG2 expression is induced during lactation in the mammary gland and represents the major route for active secretion of drugs and toxins, including some vitamins, into milk (van Herwaarden et al., 2007). ABCG2 is an efflux transporter expressed on the apical side of the cell membrane at anatomical sites important for xenobiotic disposition, such as the intestine, liver, and blood–brain barrier, playing major roles in different steps of pharmacokinetics. This protein transports drugs and environmental chemicals as well as endogenous and dietary compounds, such as flavonoids, porphyrins, estrone-3-sulphate, and uric acid (Safar, Kis, Erdo, Zolnerciks, & Krajcsi, 2019).

The function of ABCG2 in regulating milk content can be altered by the presence of several polymorphisms. In cattle, Cohen-Zinder et al. (2005) reported a single nucleotide polymorphism (SNP) encoding a substitution of a Ser with Tyr at amino acid position 581 (Y581S); this polymorphism was described as an *in vitro* and *in vivo* gain-of-function polymorphism (Otero, 2015; Real et al., 2011) and was shown to be directly involved in milk quality by affecting the presence of ABCG2 substrates in cow milk (García-Lino et al., 2019). In humans, several genetic analyses have demonstrated that SNPs leading to ABCG2

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deficiency are essential in the pathogenesis of hyperuricemia and gout (Woodward et al., 2009). The Q141K variant yields decreased ABCG2 protein expression and influences the risk of hyperuricemia and gout. Although serum Trp is a potential biomarker for gout (Liu et al., 2011), the role of ABCG2 in this relationship remains unexplored. Moreover, Dankers et al. (2013) suggested that the Trp metabolite kynurenic acid (KYNA) may interact with human ABCG2.

This study aimed to evaluate the interaction of Trp-related compounds with murine Abcg2 (mAbcg2) transporter through Trp metabolome analysis using plasma and milk samples from wild-type and Abcg2$^{-/-}$ mice. The outcomes were validated using *in vitro* transport studies with cells overexpressing murine Abcg2, and complementary Trp metabolome analysis was performed in plasma and milk samples from cows carrying or lacking the polymorphism Y581S in bovine ABCG2 (bABCG2).

2. Materials and methods

2.1. Standards and chemicals

Reference standards for tryptophan (Trp), melatonin, serotonin (5HT), 5-hydroxyindolacetic acid (5HIAA), kynurenine (KYN), kynurenic acid (KYNA), xanthurenic acid (XA), and anthranilic acid (AA) as well as the buffer 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Kynurenic acid-d5 (KYNA-d5) and serotonin-d5 (5HT-d5) were supplied by Toronto Research Chemicals (Toronto, Canada). Tryptophan-d5 (Trp-d5), 5-hydroxyindolacetic acid-d4 (5HIAA-d4), and kynurenine-13C6 (KYN-13C6) were from Alsachim (Illkirch-Graffenstaden, France). All other chemicals were of analytical grade and were obtained from commercial sources.

2.2. Animals

Animals were housed and handled according to institutional guidelines complying with European legislation (2010/63/EU). Experimental procedures were approved by the Animal Care and Use Committee of the University of León and the Junta de Castilla y León (ULE_011_2016 and ULE_002_2017).

Abcg2$^{-/-}$ (n = 9) and wild-type (n = 12) female mice 12–16 weeks of age (greater than 99% FVB genetic background) were kindly provided by Dr. A. H. Schinkel (The Netherlands Cancer Institute, Amsterdam, The Netherlands) and were kept in a temperature-controlled environment under a 12-h light/12-h dark cycle with *ad libitum* access to a standard diet (SAFE A04) and water. Pups (10 ± 2 days old) were separated from their mothers 4 h before milk collection. Oxytocin (200 μL of 1 IU/mL solution) was administered subcutaneously to lactating mothers to stimulate milk production 20 min before milk sampling. Milk samples were collected in the morning from the mammary glands by gentle pinching after anaesthesia with isoflurane. Blood samples were collected by cardiac puncture under anaesthesia with isoflurane and centrifuged immediately at 3000*g* for 15 min. One single milk sample (63–180 mg) and one single blood sample (300–700 μL) were collected from each mouse. At the end of the experiment, the mice were killed by cervical dislocation. Plasma and milk samples were stored at −20 °C (for <6 months) until analysis by liquid chromatography-tandem mass spectrometry (LC–MS/MS).

Lactating Holstein cows (n = 16; 2–5 years of age, weighing 630–1000 kg) were used. Animals were fed a standard diet consisting of maize silage (21.3% dry matter [DM]), dehydrate alfalfa hay (26.7% DM), oat-vetch hay (16.8% DM), and concentrate (36.8% DM; including rape, sunflower, and soy flours). The average daily milk yield was 42 ± 11 kg, and the milk contained 3.6 ± 1.2% fat and 3.1 ± 0.6% protein. The normal milking routine for all animals involved collection of milk three times per day. Samples were collected at a private farm located at Villalquite, Leon (Spain). The Y581S genotypes were determined in accordance with the procedure described by Komisarek & Dorynek (2009). Animals were divided into two groups of eight Y/S 581 heterozygous and eight Y/Y 581 homozygous cows. Individual milk samples were collected from the first morning milking by mechanical milking. Individual blood samples (4 mL) were collected from the tail vein, and plasma was separated by centrifugation at 3000*g* for 15 min. Plasma and milk aliquots (1 mL) were stored at −20 °C (for<6 months) until LC–MS/MS analysis.

2.3. Sample preparation for LC–MS/MS analysis

Samples from each animal were individually processed and analysed without pooling. For mouse samples, 70 μL plasma or the entire amount of collected milk (63–180 mg) was processed. For cow samples, 150 μL plasma or milk was used. Each sample was mixed with 300 μL acetonitrile to precipitate the proteins. After centrifugation, the supernatant was transferred to a clean tube, and 50 μL of the internal standard mixture (containing KYNA-d5, 5HT-d5, Trp-d5, 5HIAA-d4, and KYN-13C6) was added. The mixture was evaporated at room temperature under a nitrogen stream (<10 psi). After reconstitution with 150 μL water, 10 μL was injected into the system. The standards used for calibration were subjected to the same procedures.

2.4. Quantification of Trp-related compounds by LC–MS/MS

A previously described LC–MS/MS method (Marcos et al., 2016) was used for determination of Trp-related compounds in milk and plasma from mice and cows. The LC–MS/MS system consisted of an Acquity UPLC system (Waters, Milford, MA, USA) coupled to a triple quadrupole mass spectrometer (Quattro Premier for cow samples and TQS Micro for mouse samples; both from Waters) equipped with an electrospray ionisation interface. Chromatographic separation was achieved on an Acquity BEH C18 column (100 mm × 2.1 mm i.d., 1.7 μm; Waters) at a flow rate of 0.3 mL/min. Mobile Phase A consisted of 0.01% (v/v) formic acid and ammonium formate (1 mM) in ultra-pure water. Mobile Phase B consisted of 0.01% (v/v) formic acid and ammonium formate (1 mM) in HPLC grade methanol. A gradient elution was used for chromatographic separation of the analytes: 0 – 0.5 min: constant 1.0% B; 0.5–7.0 min: linearly increase from 1% B to 40% B; 7.0– 8.5 min: linearly increase from 40% B to 90.0% B; 8.5– 9.0 min: constant 90.0% B; 9.0–9.5 min: linearly decrease from 90% B to 1.0% B; 9.5– 12.0 min: constant 1.0% B. Analytes were determined in the selected reaction monitored mode including 2 ion transitions for each analyte.

2.5. Cell culture

Polarised Madin-Darby canine kidney epithelial cells (MDCKII cells) and mAbcg2-stably-transduced subclones were provided by Dr. A.H. Schinkel (Netherlands Cancer Institute, Amsterdam, The Netherlands) (Li et al., 2018). MDCKII cells stably transduced with both variants (S581 and Y581) of bABCG2 were previously generated by our group (Real et al., 2011). The transport proficiency of these cell lines (passages 20–30) was continually monitored by testing the transport of various established substrates. Culture conditions were as previously described (Perez et al., 2013; Otero, 2015).

2.6. Transport studies

Transepithelial transport assays using Transwell plates were carried out as described elsewhere (Perez et al., 2013; Otero, 2015), with minor modifications. Cells were grown for 3 days after seeding on microporous polycarbonate membrane filters at a density of 1.0 × 10^6 cells/well. To check the tightness of the monolayer, transepithelial resistance was measured in each well using a Millicell ERS ohmmeter (Millipore, Burlington, MA, USA). The transport medium consisted of Hanks' balanced salt solution (Sigma-Aldrich) supplemented with HEPES (25 mM). Two

Table 1
Levels of Trp-related compounds (ng/mL) in plasma and milk samples and milk-to-plasma ratios from wild-type and Abcg2$^{-/-}$ female mice (n = 9–12).

		Wild-type	Abcg2$^{-/-}$	*p* value
Plasma	Trp	118728 ± 29077	120223 ± 15268	0.378
	KYN	1628 ± 363	1451 ± 344	0.259
	KYNA	7.1 ± 2.5	6.6 ± 2.2	0.647
	XA	73 ± 37	69 ± 30	0.792
	AA	155 ± 98	124 ± 66	0.404
	5HT	11937 ± 5080	8643 ± 1959	0.079
	5HIAA	702 ± 138	581 ± 143	0.060
Milk	Trp	466 ± 249	225 ± 121	0.003*
	KYN	32 ± 17	15 ± 7	0.039*
	KYNA	42 ± 8	9.1 ± 3.0	< 0.001*
	XA	75 ± 40	7.8 ± 4.7	0.001*
	AA	36 ± 10	17 ± 7	0.01*
	5HT	41 ± 22	40 ± 19	0.585
	5HIAA	118 ± 24	84 ± 14	0.001*
Milk-to-plasma ratio	Trp	0.004 ± 0.002	0.002 ± 0.001	0.014*
	KYN	0.02 ± 0.01	0.01 ± 0.004	0.012*
	KYNA	6.9 ± 2.6	1.47 ± 0.52	< 0.001*
	XA	0.97 ± 0.48	0.22 ± 0.33	0.001*
	AA	0.23 ± 0.10	0.12 ± 0.04	0.008*
	5HT	0.004 ± 0.004	0.004 ± 0.003	0.794
	5HIAA	0.17 ± 0.04	0.15 ± 0.05	0.322

Results are expressed as mean concentrations ± SDs. *$p < 0.05$ versus the wild type.

hours before the start of the experiment, culture medium at both the apical (AP) and basolateral (BL) sides of the monolayer was replaced with 2 mL transport medium, with or without the specific ABCG2 inhibitor Ko143 (1 μM). The experiment started when the transport medium in the AP or BL compartment was replaced with fresh transport medium containing different compounds at a concentration of 10 μM. Cells were incubated at 37 °C in 5% CO_2, and 100-μL aliquots of medium were collected from the opposite compartment at 1, 2, 3, and 4 h; the collected medium was replaced with the same volume of fresh transport medium. The samples were stored at −20 °C. The concentrations of the studied compounds were subsequently determined by high-performance liquid chromatography (HPLC). Active transport across MDCKII monolayers was expressed as the relative transport ratio (R), defined as the apically directed transport percentage divided by the basolaterally directed translocation percentage, after 4 h.

2.7. HPLC analysis

HPLC analysis was used to determine the concentrations of the studied compounds in transepithelial transport assays. The chromatographic system consisted of a Waters 2695 separation module and a Waters 2998 ultraviolet (UV) photodiode array detector. The culture medium (50 μL) was injected directly into the HPLC system. Separation of the samples was achieved on a reverse-phase column (Atlantis T3 3 μm, 4.6 × 150 mm). The mobile phase consisted of 0.14% trifluoroacetic acid:acetonitrile (80:20). The mobile phase flow rate was set to 0.8 mL/min, and UV absorbance was measured at 238 nm. The temperature of the samples was 4 °C. Standard samples were prepared in the appropriate drug-free matrix, yielding a concentration range from 0.039 to 10 μg/mL, with coefficients of correlation greater than 0.99. The limit of quantification was in the range of 0.02–0.03 μg/mL, and the limit of detection was in the range of 0.005–0.014 μg/mL for all compounds.

2.8. Statistical analysis

Comparisons between groups were performed by Student's t-tests and Mann-Whitney U tests. All analyses were carried out at an assumed significance level of $p \leq 0.05$ using SPSS Statistics software v24 (IBM, Armonk, NY, USA). The results are shown as means ± standard deviations (SD).

3. Results and discussion

3.1. Determination of Trp-related compounds in milk from Abcg2$^{-/-}$ and wild-type mice and correlations with transport in cells transduced with mAbcg2

To elucidate the role of mAbcg2 transport in the active secretion of Trp-related compounds, a targeted metabolomic analysis was performed using plasma and milk samples from wild-type and Abcg2$^{-/-}$ female mice. The targeted LC–MS/MS method included eight analytes; all were detected in plasma and milk samples, except for melatonin (Table 1). There were no significant differences in plasma concentrations of the targeted metabolites between wild-type and Abcg2$^{-/-}$ mice. Nevertheless, milk concentrations of Trp, KYN, KYNA, XA, AA, and 5HIAA were higher in wild-type mice than in Abcg2$^{-/-}$ mice (Table 1). These differences were particularly high for XA and KYNA, whose concentrations were 5–10-fold higher in milk from wild-type mice than from Abcg2$^{-/-}$ mice. Higher milk-to-plasma ratios were also obtained for these six metabolites in wild-type mice compared with Abcg2$^{-/-}$ mice, except for 5HIAA. These data indicate that mAbcg2 plays a substantial role in the secretion of metabolites from the KYN pathway into milk.

To further verify the above-mentioned findings, Trp, KYN, KYNA, XA, and AA were tested *in vitro* using a transport assay with parental MDCKII and its mAbcg2-transduced subclones. In the parental MDCKII cell line, most of the molecules showed similar apically and basolaterally directed translocation (Fig. 1A). However, KYN and Trp displayed high basolaterally directed translocation, whereas apically directed translocation was very low, indicating the potential presence of an absorptive KYN and Trp transport process.

In mAbcg2-transduced cells (Fig. 1B), increased translocation from the BL to the AP compartment and reduced translocation from the AP to the BL compartment were observed compared with that in parental cells, and high relative transport ratios (AP/BL) were detected for KYN, KYNA, and XA. For AA and Trp, a low transport ratio similar to that of the parental cells was obtained. The apical transport of KYN, KYNA, and XA by mAbcg2 was completely inhibited by Ko143, a selective Abcg2 inhibitor (data not shown). These results indicate that KYN, KYNA, and XA are good *in vitro* substrates of mAbcg2. Only KYNA had been previously described as a potential ABCG2 substrate in humans (Dankers et al., 2013). Conversely, Trp and AA were not confirmed as *in vitro* substrates

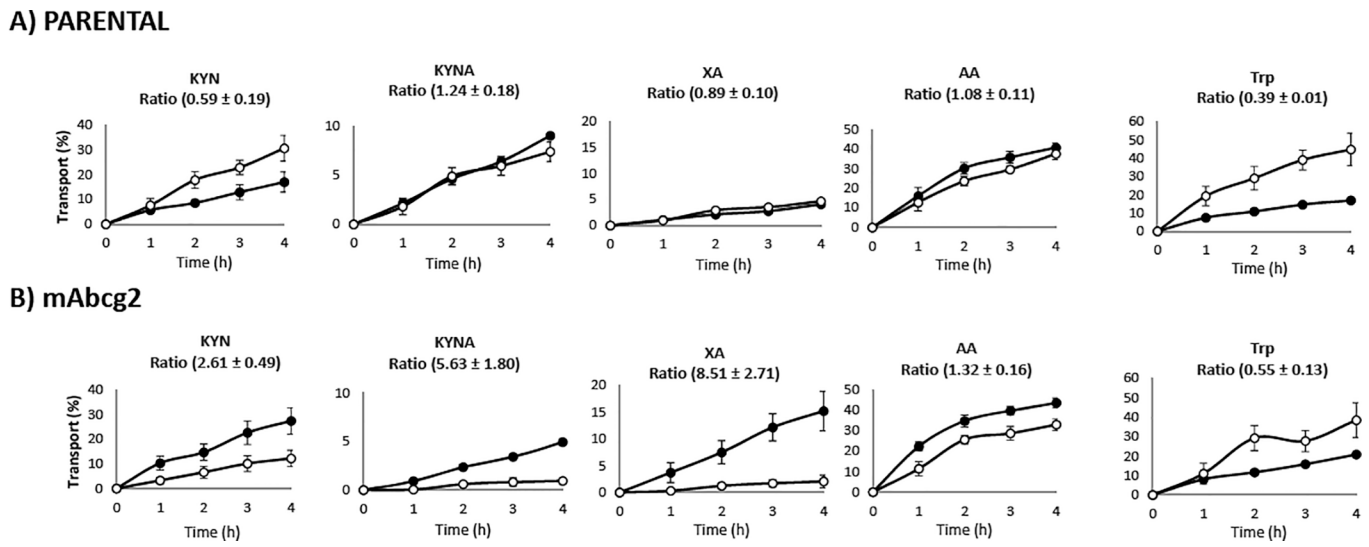

Fig. 1. Transepithelial transport of tested compounds (10 μM) in (A) parental MDCKII cells and (B) their mAbcg2-transduced derivatives. (○) translocation from the apical to the basolateral compartment; (●) translocation from the basolateral to the apical compartment. The vertical bars indicate the SDs (n = 3–8). Ratios are relative transport ratios (i.e. the apical directed translocation divided by the basolateral directed translocation) at 4 h.

Table 2
Levels of Trp-related compounds (ng/mL) in plasma and milk samples and milk-to-plasma ratios from noncarrier (Y/Y) and carrier (Y/S 581) cows (n = 8).

		Y/Y	Y/S	*p* value
Plasma	Trp	11283 ± 3053	12348 ± 2443	0.453
	KYN	1054 ± 381	1067 ± 256	0.935
	KYNA	8.1 ± 3.4	7.1 ± 0.5	0.407
	XA	95 ± 26	108 ± 18	0.263
	5HT	0.09 ± 0.07	0.14 ± 0.08	0.197
	5HIAA	0.011 ± 0.003	0.011 ± 0.003	0.724
	Melatonin	0.006 ± 0.004	0.005 ± 0.001	0.284
Milk	Trp	192 ± 74	252 ± 90	0.170
	KYN	24 ± 10	46 ± 18	0.012*
	KYNA	7.9 ± 3.8	8.2 ± 2.6	0.840
	XA	0.32 ± 0.12	0.31 ± 0.08	0.816
	5HT	< LOD	< LOD	–
	5HIAA	0.56 ± 0.27	0.81 ± 1.14	0.593
	Melatonin	0.003 ± 0.002	0.003 ± 0.001	0.713
Milk-to-plasma ratio	Trp	0.018 ± 0.008	0.020 ± 0.007	0.492
	KYN	0.02 ± 0.01	0.04 ± 0.02	0.012*
	KYNA	1.0 ± 0.4	1.2 ± 0.4	0.405
	XA	0.004 ± 0.001	0.003 ± 0.001	0.816
	5HT	<LOD	< LOD	–
	5HIAA	0.46 ± 0.17	0.67 ± 0.26	0.697
	Melatonin	0.46 ± 0.17	1.7 ± 0.8	0.157

Results are expressed as the mean concentrations ± SDs. *$p < 0.05$ versus wild type.

of mAbcg2; however, because of the positive results observed in the *in vivo* study, the *in vitro* interactions of these molecules with the mAbcg2 transporter cannot be excluded in other experimental conditions or models.

Among other physicochemical features, substrate binding to ABCG2 has been shown to be dependent on hydrophobic interactions, mainly those between hydrogen bond acceptors (HBAs) present in substrates and hydrogen bond donors (HBDs) present in the transmembrane region of the transporter (Matsson et al., 2007). In addition, Xu et al. (2015) demonstrated that substrate binding to the ABCG2 transporter increases with the number of HBAs present in the potential substrates. In our case, KYN, KYNA, and XA, identified as mAbcg2 substrates, yielded higher numbers of HBAs than did Trp, 5HIAA, 5HT, and AA (which are not *in vitro* mAbcg2 substrates; Supplementary Material, Table S1).

3.2. *Effects of the bovine ABCG2 Y581S SNP on secretion of Trp-related compounds into milk and correlations with their in vitro transport*

A similar metabolomic analysis was performed for milk and plasma samples from cows carrying the Y581S polymorphism and from noncarrier animals (Table 2). Targeted analytes were detected in plasma and milk samples, with the exception of AA, which was not detected in any sample, and 5HT, which was not detected in milk samples. There were no differences in plasma levels between Y/Y 581 and Y/S 581 cows for any compound tested. Significant differences were only found for KYN concentrations in milk (Table 2); which were 2-fold higher in Y/S cows (4.6 ± 1.8 ng/mL) than in Y/Y cows (2.4 ± 1.0 ng/mL). The milk-to-plasma ratio for KYN was also 2-fold higher in Y/S cows than in Y/Y cows (0.004 ± 0.002 versus 0.002 ± 0.001, respectively). This indicates that the polymorphism Y581S affects the *in vivo* active transport of KYN into cow milk. This is the first time that differences between both variants of cows (carriers and noncarriers of the Y581S polymorphism) have been observed for Trp bioactive metabolites. Similar differences were previously reported for milk secretion of fluoroquinolone drugs, anti-inflammatory drugs, and endogenous and dietary compounds (Otero, 2015; García-Lino et al., 2019). In fact, uric acid, which is related to Trp levels (Dankers et al., 2013), has been previously reported as an endogenous compound actively secreted into milk with a 2-fold increase in the milk-to-plasma ratio for carrier animals (Otero et al., 2016).

To confirm the role of the bovine Y581S polymorphism in the transport of KYN, transport assays were performed using polarised MDCKII parental cells and their subclones transduced with both bABCG2 variants (S581 and Y581; Fig. 2). The relative transport ratio (AP/BL) of KYN was significantly higher in S581-expressing cells than in parental cells (1.08 ± 0.25 versus 0.59 ± 0.19, respectively) because AP transport increased and BL transport decreased compared with that in parental cells. However, in the case of cells expressing Y581, no changes were observed in the transport ratio (AP/BL) compared with that in parental cells, indicating that KYN was not transported by this variant. Statistically significant differences were found between transport ratios (AP/BL) of both variants of bABCG2, Y581 and S581 (0.42 ± 0.25 versus 1.08 ± 0.25). Therefore, the differences between the two bovine variants indicate that the Y581S polymorphism affects the *in vitro* transport of KYN, with a higher *in vitro* transport capacity for the S581 variant, corroborating the differences found *in vivo* between carrier and

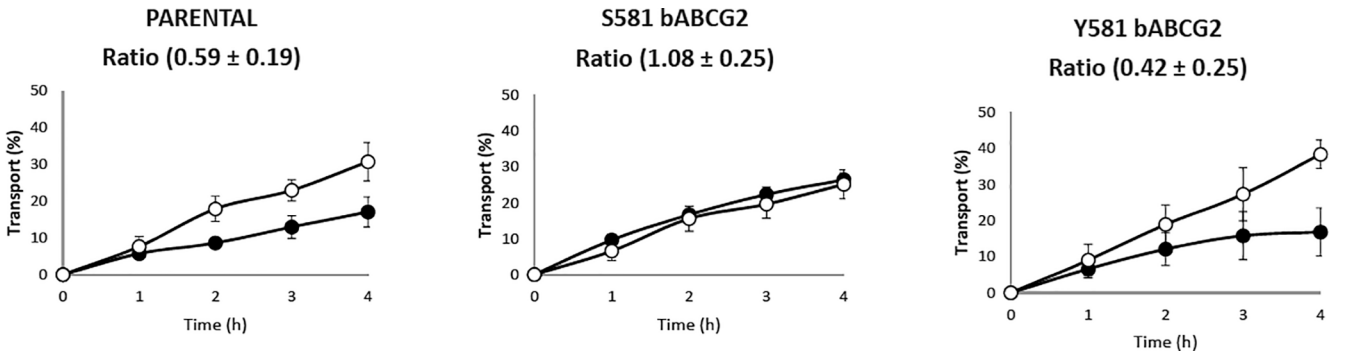

Fig. 2. Transepithelial transport of KYN (10 μM) in polarized MDCKII parental (non-transduced), MDCKII-S581-bABCG2, and MDCKII-Y581-bABCG2 monolayers. The vertical bars indicate the SDs (n = 5). (○) translocation from the apical to the basolateral compartment; (●) translocation from the basolateral to the apical compartment. Ratios are relative transport ratios (i.e., the apical directed translocation divided by the basolateral directed translocation) at 4 h.

noncarrier animals.

3.3. Potential relevance and limitations of the study

ABCG2 inhibitors, such as drugs (Barrera et al., 2013) and dietary compounds (Miguel et al., 2014), can alter the transfer of these Trp-related compounds into milk. Importantly, these interactions mediated by ABCG2 have been observed for other ABCG2 substrates. For example, consumption of a soy- or flaxseed-enriched diet modifies ABCG2-mediated *in vivo* milk secretion of the antimicrobial danofloxacin in sheep (Perez et al., 2013; Otero et al., 2018). Therefore, potentially different concentrations of Trp-related compounds in consumed milk owing to polymorphisms or inhibition of this transporter may affect the intake of these compounds by offspring or the dairy consumer. Nevertheless, further *in vivo* studies are needed to confirm this hypothesis.

In this LC–MS/MS metabolomic study, the number of cows carrying the Y581S polymorphism was limited, preventing us from determining the specific effects of the bovine Y581S polymorphism on more metabolites and from studying the effects of other variables, such as lactation stage or age. Future population studies will be needed to address these points. Despite this limitation, the study findings provide important insights into the roles of ABCG2 in Trp metabolite transport. Furthermore, correlations between the interactions of ABCG2 and Trp metabolites *in vivo* and *in vitro* were determined. Many compounds were identified, in contrast to other studies in which only a few metabolites were assessed (Cubero et al., 2005; Laeger, Görs, Metges, & Kuhla, 2012).

4. Conclusion

In this study, we evaluated the effects of ABCG2 in the presence of Trp-related bioactive metabolites in milk. ABCG2 was found involved in the transport of several Trp bioactive metabolites and relevant metabolites from the KYN pathway were secreted into milk by the mAbcg2 transporter. In addition, lactating dairy cows carrying the Y581S polymorphism produced milk with higher amounts of KYN compared with noncarriers.

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CRediT authorship contribution statement

Alba M. Garcia-Lino: Conceptualization, Methodology, Data curation, Formal analysis, Visualization, Investigation, Writing - original draft. **Alex Gomez-Gomez:** Conceptualization, Methodology, Data curation, Formal analysis, Visualization, Investigation. **Dafne Garcia-Mateos:** Methodology, Investigation. **Alvaro de la Fuente:** Methodology, Investigation. **Ana I. Alvarez:** Conceptualization, Methodology, Validation, Supervision. **Oscar J. Pozo:** Conceptualization, Methodology, Project administration, Resources, Supervision, Writing - Review & Editing. **Gracia Merino:** Conceptualization, Methodology, Project administration, Resources, Supervision, Writing - Review & Editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.128665.

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